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Los Angeles

Natural Killer Cells as Immune Effectors for Selection, Differentiation and Resistance of Healthy and Transformed Stem Cells

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Oral Biology

by

Han-Ching Tseng Dick

2014

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ABSTRACT OF DISSERTATION

Natural Killer Cells as Immune Effectors for Selection, Differentiation and Resistance of Healthy and Transformed Stem Cells

By

Han-Ching Tseng Dick

Doctor of Philosophy in Oral Biology

University of California, Los Angeles 2014

Professor Anahid Jewett, Chair

Recent advancements in our understanding of anti-tumor immune responses and cancer biology have revealed an elaborate interaction between immune effectors as well as transformed cells in the tumor microenvironment. Effectors of the immune system are known to modulate the maturation of tumor cells and to select for cancer with reduced immunogenicity. The same effector mechanisms are likely responsible for shaping the development of healthy stem cells for the ultimate goal of repair and regeneration of damaged tissues and the discontinuation of chronic inflammation.

Natural Killer (NK) cells were first described based on their ability to mediate tumor cell lysis without prior sensitization, however much of their effector functions and dynamic interaction with cells in the microenvironment are still not completely understood. Many studies have demonstrated that NK cells may recognize and become activated by irradiated or stressed cells; yet, currently no studies have investigated the role of NK cells in selection and differentiation of stem cells and potential role of NK cells in the resolution of inflammation. The established paradigm suggests that NK cells are only capable of eliminating defective cells that arise in the body; but, data obtained in this study indicate that the functions of NK cells are not as limited as we once thought.

This study provides evidences that tumor cells and virally infected cells are not the only targets of NK cell-mediated cytotoxicity. Immune cells and healthy stem cells are also susceptible to lysis. NK cells obtained from the peripheral blood or those that have infiltrated into tumors have been shown to be cytotoxicity inactivated or also known as "anergized". In this study, an array of mediators that cause anergy in NK cells have been identified and extensively studied. Initiation of the anergic state in NK cells was once thought as an end-point, a last stop before apoptosis; however, presented data will demonstrate that anergy is a physiological stage that NK cells undergo to help support differentiation of stem cells. Taken together, all of the results provide a novel role for NK cells as effector cells in selection, differentiation and resistance of both transformed and healthy stem cells.

The dissertation of Han-Ching Tseng Dick is approved.

Nicholas Cacalano

Shen Hu

Ichiro Nishimura

Anahid Jewett, Committee Chair

University of California, Los Angeles

2014

DEDICATION

To write a dedication to my husband Jordan is impossible, even with the best writers in the world I cannot eloquently depict all that he has sacrificed and done for me. I wholeheartedly share this dissertation and degree with Jordan because I would not be where I am and who I am today without his unwavering love and support.

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I would like to acknowledge my fellow researchers and friends in the Weintraub Center for Reconstructive Biotechnology. I truly enjoyed working and learning from them. In addition, I would also like to thank Pauli Nuttle and Megan Scott for their continued assistance and efforts throughout the graduate program. You two are really the backbone of this department.

Last, but definitely not least, I would like to thank my father Hsieh-Kuo who gave up his own career and ambition for my sister and I and for that I am forever grateful. I also would like to thank my mother Tai-Li for teaching me that anything great in life is earned with diligence and perseverance and Lulu for being the annoying little sister that I love so much.

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1. **Tseng HC**, Inagaki A, Bui V, et al. (2014) Differential targeting of Glioblastoma cancer stem cells and their differentiated tumors by NKs and CTLs; continued cytokine and chemokine secretion and loss of NK cytotoxicity after tumor differentiation. Submitted.
2. **Tseng HC**, Cacalano N and Jewett A (2014) Natural Killer cells halt inflammation by inducing stem cell differentiation, resistance to NK cell cytotoxicity and prevention of cytokine and chemokine secretion. In press.
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4. **Tseng HC**, Bui V, Man, YG, et al. (2014) Induction of split anergy conditions natural killer cells to promote differentiation of stem cells through cell–cell contact and secreted factors. Frontiers in Immunology 5:269.
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7. Magister S, **Tseng HC**, Watts C, et al. (2014) Regulation of split anergy in natural killer cells through inhibition of cathepsin C by cystatin F. Submitted.
8. Park S, **Tseng HC**, Kanayama K, et al. (2014) Gammadelta T cells in the oral mucosa barrier immunity: Differential role in the development of ONJ-like lesions in zoledronate-treated mice. Submitted.
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15. **Tseng HC**, Arasteh A, Paranjpe A, et al. (2010) Increased Lysis of Stem Cells but Not Their Differentiated Cells by Natural Killer Cells; De-Differentiation or Reprogramming Activates NK Cells. PLoS ONE 5(7): e11590.
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INTRODUCTION

In the early 1970s a new subset of lymphocytes was discovered in mice based on their unique ability to mediate immediate cytotoxicity against tumor cells without prior sensitization [1-4]. The newly identified lymphocytes were called Natural Killer (NK) cells for their natural ability to eliminate targets and since its discovery the functions of NK cells have now expanded to encompass additional roles, such as modulation of cellular differentiation, tumor resistance, tissue repair and regeneration and resolution of inflammation.

NK cells are large granular lymphocytes that compose of 5-15% of all circulating lymphocytes within the human body with a half-life of 7 to 10 days in the periphery [5-7]. NK cells can be found within various parts of the body, such as gingival tissue [8] central nervous system [9, 10], blood, bone marrow, liver, lung, spleen and uterus [11]. NK cells are identified based on phenotypic and functional analyses. NK cells can be divided into two subsets based on surface marker expressions of CD16 and CD56, namely $CD16^{bright}CD56^{dim}$ and $CD16^{dim}CD56^{bright}$ [12-20]. $CD16^{bright}CD56^{dim}$ NK cell subset constitutes about 90% of the NK cell population and has high cytolytic capacity against tumor cells; meaning that this subtype of NK cells are extremely effective at identifying and eliminating tumors. Whereas, $CD16^{dim}CD56^{bright}$ subset is the minor subset of NK cells and functions as immunoregulatory cells that produce high levels of cytokines, such as IFN-γ and TNF-α [12, 18] (chapter 3 and 4) which may help with the repair process of damaged tissues.

Unlike T cells and B cells, which depend on somatically rearranged receptors, NK cells rely on a series of germline-encoded activation and inhibitory receptors to mediate lysis against

target cells [21-24] (Table 1). It is believed that the balance between activating and inhibitory signals, which NK cells receive from their surface receptors, determine their functional fate. Many of the receptors listed in Table 1 including CD16, killer immunoglobulin like receptors (KIR), NKG2 family of receptors that form a heterodimer with CD94, NKG2D, and natural cytotoxicity receptors (NCR) have all been subjects of many studies. Likewise, several key cytokines, chemokines, and adhesion molecules were found to have significant roles in maturation, differentiation, and effector function of NK cells. However, little is known regarding the function of Toll-Like Receptors (TLR), NOD-Like Receptors (NLR), and RIG like Receptors (RLR) in NK cell effector functions.

One of the identification markers for NK cells is CD16 (FcγRIII), which is a low affinity receptor for the Fc portion of immunoglobulin that is involved in antibody-dependent cell-mediated cytotoxicity (ADCC). CD16 can be found on NK cells, dendritic cells and macrophages [25], mast cells [26, 27], neutrophils [28]. NK cells bind to opsonized, or antibody coated, targets and signal through associated subunits which contains immunoreceptor tyrosine-based activation motif to mediate ADCC. In 1994, Klingemann's group isolated and characterized an immortalized NK cell line, NK92, which derived from a patient who had large granular lymphoma [29]. It is reported that the NK92 cell line, although lack CD16 expression, are capable of mediating cytotoxicity and can secrete IFN-γ and other cytokines. However, our studies have demonstrated that

Table 1- List of NK cell Activating and Inhibitory surface receptors and their ligands

Receptors	Ligands
Activating/inhibitory Receptors	
FcγRIII (CD16)	Fc of antibodies
CD2	CD58 (LFA-3)
LFA-1	ICAM-1
2B4	CD48
CD69	Unknown
DNAM-1 (CD226)	CD112, CD155
NKp80	AICL
Tactile (CD96)	CD155, CD111
TIGIT	CD112,CD113,CD155
CRTAM	TSLC1
C-type Lectin receptors –Activating/Inhibitory	
CD94/NKG2A/B	HLA-E
NKG2D	MICA, MICB, ULBP-1, ULBP -2, ULBP -3, ULBP -4, ULBP -5, ULBP -6
CD94/NKG2C	HLA-E
CD94/NKG2E/H	HLA-E, Qa-1b
Natural cytotoxicity receptors (NCR)	
NKp46 (NCR1)	Viral Hemagglutinin
NKp44 (NCR2)	Viral Hemagglutinin
NKp30 (NCR3)	B7h6, HCMV-pp65
Killer IG-like (KIR) – Activating/Inhibitory	
KIR2DLs, KIR3DLs, KIR2DS	HLA-A, HLA-B, HLA-C, HLA-G
Cytokines, growth factors, chemokines and other adhesion Receptors	Cytokines, growth factors, chemokines and other adhesion ligands
Toll-like receptors (TLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR)	Bacterial DNA, LPS, peptidoglycan, teichoic acids, flagellin, pilin, viral dsRNA and fungi zymosan

the extent of cytotoxicity mediated by NK92 cells pales in comparison to primary NK cells isolated from healthy patients (manuscript submitted). Several phase I clinical trials have been performed with NK92 and have received mixed responses. Klingemann et al. and colleagues were able to expand NK92 cells more than two hundred fold with medium supplemented with

IL-2 and human AB plasma and with a viability of more than 80%. Out of the twelve patients tested, only one survived and the remainder patients passed away due to progressive disease [30]. Another study performed by Drs. Tonn and Ottman demonstrated that even with the highest dose of NK92 ($9.4X10^9$) administered the patient still suffered from alveolar soft tissue sarcoma [31].

CD56 (Neural Cell Adhesion Molecule, NCAM) is another hallmark identification marker for NK cells. Three major isoforms exists for CD56 and each differs in their membrane association and their intracellular domains. CD56 is involved in a series of processes, such as cell-cell adhesion, proliferation, apoptosis, migration, synaptic plasticity and neurite development and outgrowth [32-35]. CD56 is also an established marker for many human cancer types, such as malignant NK/T-cell lymphomas [36, 37], medulloblastoma [38] and neuroendocrine carcinomas [39, 40]. The profiles of NK cells based on their ability to mediate cytotoxicity and secrete cytokines have been based on the expression of CD56 and CD16 [16, 41-46].

Other receptors that can greatly affect the effector functions of NK cells are Natural Cytotoxicity Receptors (NCRs) which include NKp30, NKp44, NKp46 and NKp80. The first identified NCR is NKp46 and it is the only NCR that is conserved in human and mice. The expression of NKp46 is constitutively, whether the cells are activated or not [47, 48]. Although it is mainly found on NK cells, it can also be found on intraepithelial T cells derived from patients with coeliac disease [49] and NKT cells [50]. Our laboratory has identified NKp46 as an inducer of split anergy in NK cells which causes a significant decrease in their ability to mediate cytotoxicity but increases their cytokine production, primary IFN-γ (please see below). In

contrast to NKp46, NKp44 is not expressed on resting NK cells, but only on activated cells. Long term activation of NK cells with IL-2, anti-CD16, monocytes and bacteria can induce its expression (manuscript in prep) [51]. Unlike NKp46 and NKp44 where the expression is mostly restricted to NK cells, NKp30 can be found on mature NK cells [52], cord blood T cells [53], γδ1+ T cells and endometrial epithelial cells [54].

The first ligands identified for the NCRs were viral proteins that include hemagglutinin of influenza and parainfluenza viruses [55, 56], hemagglutinin neuraminidases of the Newcastle disease virus and the Sendai virus [57]. NKp30 could bind to human cytomegalovirus tegument protein pp65, however this binding results in a decrease in the activation state of the NK cells [58]. NKp44 fusion proteins were shown to bind to envelope glycoproteins of the West Nile virus and of the dengue [59]. Aside from viral-derived ligands, bacterial- and parasite-derived ligands to NCRs have also been identified. NKp44 binds to Mycobacterium bovis, Mycobacterium tuberculosis, Nocardia farcinica and Pseudomonas aeruginosa [60-62]. Both NKp30 and NKp46 could bind to erythrocytes infected with Plasmodium falciparum, the malaria parasite [63].

Interleukin-2 (IL-2) is a leukocytotrophic cytokine that plays a crucial role in the development of T cells [64, 65] and the activation of NK cells [18, 19]. IL-2 receptor composes of three protein chains, α (CD25), β (CD122) and γ (CD132). The homodimeric α chains results in low-affinity receptor, the homodimeric β chains give rise to medium-affinity receptor, and the combination of α, β and γ constitute the high-affinity IL-2 receptor. The majority of the peripheral NK cells expresses only the intermediate affinity IL-2 receptors and thus stimulation

require high concentration of IL-2 [13]. Studies have evinced that the cytotoxic function of NK cells can be enhanced with IL-2 within 4 to 6 hours [13, 18, 19, 66]. Whiteside et al. demonstrated that IL-2 activated NK cells are as potent as $CD8^{+}$ T cells *in vivo* in head and neck squamous cell carcinoma [67]. However, its efficiency is heavily dependent on the dosage and the schedule of administration [68].

With the exception of malignant melanomas and renal cell carcinomas, immunotherapy of cancer has been unfavorable due to its toxicity [69, 70]. Systematic administration of IL-2 into patients causes numerous negative side effects, such activation of non-specific immune cells and inotropic properties [71]. However, few studies have shown that IL-2 activated NK cells are effective against NK resistant breast cancer and lymphoma cell lines [72, 73]. Overall, data from reports indicate that while there is a significant increase in the number of circulating NK cells, especially $CD56^{bright}$ subpopulation, the cytotoxicity of these NK cells remained unchanged.

NK cells mediate cytotoxicity against transformed and virus-infected cells via multiple cytolytic mechanisms. Through the granzyme and perforin dependent pathway, NK cells induce apoptosis in the target cell by releasing granules containing granzyme and perforin at the immunological junction between a NK cell and its intended target cell. Perforin, as the name suggests, actuates perforation in the cellular membrane and allow granzyme to enter into the cytosol of the target cell. Unlike cytotoxic T cells, the mRNA expression of perforin is constitutively expressed by NK cells and the addition of interleukin (IL)-2 cannot further upregulate its expression [74-76]. In absence of perforin, NK cells have been shown to be inadequate effectors of lysis since NK cells from perforin-deficient mice were unable to clear

Lymphocytic Choriomenningitis Virus (LCMV) [77, 78] and eliminate fibrosarcoma tumor cells [77]. Similarly, Symth et al. showed that perforin-knockout C57BL/6 mice were unable to control spontaneous lung metastasis in two separate animal models [79]. A rare and lethal human disorder known as familial haemophagocytic lymphohistiocytosis (FHL) highlights the importance of perforin dependent cytotoxicity [80]. The study done by Stepp et al. and colleagues utilized linkage analysis to demonstrate that either homozygous nonsense mutations or missense mutations in the coding regions of the perforin gene is responsible for FHL. Lymphocytes derived from patients have defective cytotoxic activity and the expression of both perforin and its commonly coupled serine protease partner, granzyme B, are significantly suppressed. Although it is conventionally believed that perforin-dependent cytotoxicity is the major mechanism in which NK cells use to mediated lysis, many studies provide counterevidence. For instance, Acha-Orbea et al. demonstrated that the inhibition of perforin expression by anti-sense oligonucleotide only resulted in a partial prevention of cytotoxicity which suggests that NK cells are able to utilize other cytolytic mechanism [81].

Perforins work synergistically with granzymes to induce apoptosis in target cells via both caspase dependent and independent pathways [82-85]. Granzymes (granules-associated enzymes) are serine proteases that can cleave and activate death proteins in tumor cells to initiate apoptosis. Pinkoski et al. and colleagues demonstrated that the primary mode of apoptotic action is through the release of mitochondrial cytochrome c [86]. Five granzymes have been identified in humans (A, B, H, K and M) with the most abundant subsets being granzyme A and B [87, 88]. It was initially believed that granzymes can only enter the cytosol of target cells via the pores created by perforin; however, recent studies show that mannose 6-phosphate receptor may be another

source of entry if the receptor is overexpressed on the cell surface [89, 90]. The expression of granzyme B is not restricted to only cytotoxic lymphocytes, as it is also present in mast cells [91], macrophages [92], dendritic cells [93], Kupffer cells [94], basophils [95], chondrocytes [96, 97], keratinocytes [98] and placental syncytial trophoblasts [99]. Granzyme B was also detected in several cancer types, such as human nasal-type NK/T cell lymphoma [100], lung carcinomas [101] and breast carcinoma [101, 102].

The activity of granzymes is regulated by cathepsins which govern many important cellular processes, such as apoptosis [103], protein processing[104], antigen presentation [105], cancer progression [106] and many more. Eleven cathepsins have been identified in humans: B, C, F, H, K, L, O, S, V, X and W [104, 107] each with its own unique functions and involvement in diseases. All cathepsins are synthesized as inactive zymogens and require autocatalysis or other active proteases in order to become catalytically active enzyme. Cathepsins C and H are ubiquitously distributed and are involved in the activation of granzyme A and B [108] which are utilized by both NK cells and cytotoxic T cells. Surprisingly, Sutton et al. and colleagues demonstrated that cytotoxic T cells derived from cathepsins C-null mice, which were granzyme A and B deficient, were still able to lyse MS9ll and mastocytoma tumor cells target cells. Although the effectiveness of tumor cell lysis was hindered slightly in cathepsins C-null animals, cytotoxic T cells were still able to induce same level of DNA fragmentation as wildtype controls.

The biosynthesis of cathepsins is regulated by their endogenous protein inhibitors, notably cystatins. Cystatins are tight-binding, and yet reversible, inhibitors of cysteine proteases. Within the three types of cystatins, cystatins C and F in the second group are most abundantly

present in immune cells, such as monocytes, dendritic cells, cytotoxic T cells and NK cells [109-113]. Cystatin F was discovered simultaneously by three independent groups in the late 1990s by cDNA cloning and mRNA overexpression in liver metastatic tumors [110, 111, 114]. Human cystatin F is secreted as an inactive disulfide-linked dimer that must be reduced to its monomeric form in order to be active [115, 116]. Cathepsin C is one of the protease targets of cystatin F [117] and cystatin regulation of cathepsins C results in inactivation of the granzymes present in cytotoxic T cells, NK cells and neutrophils [117-119].

Aside from utilizing perforin and granzymes, NK cells also confer cytotoxicity through death receptor pathways via FAS ligand, tumor necrosis factor (TNF) or TNF-related apoptosis inducing ligand (TRAIL), also known as Apo2 ligand. Five receptors for TRAIL have been identified in humans, however only two, TRAIL-R1 and TRAIL-R2, are capable of transducing apoptotic signals [120]. The expression of TRAIL can be induced by stimulation with IL-2, IL-15, interferon (IFN)-γ and αβ [121, 122]. Smyth et al. and Takeda et al. groups have shown in mouse that NK cells are capable of controlling the metastatic progression of tumors through TRAIL-dependent cytotoxicity [123, 124]. Another member of the TNF family is Fas ligand or CD95L, which NK cells use to suppress tumor growth [125]. Many tumors do not express Fas, however IFN-γ released by activated NK cells can upregulate the expression of Fas on tumor cell surface and mediate cytotoxicity through FasL pathway [126].

As direct killing of tumor cells or pathogen infected cells is critical and necessary, it is only one component of NK cells mediated cytotoxicity. NK cells can produce an array of cytokines and chemokines that are capable of activating other effector cells in the

microenvironment to contain and eradicate pathogens. IFN-γ, the only member of the type II class of interferons, is a key cytokine for both innate and adaptive immunity against viral and bacterial infections, as well as tumor control [13]. IFN-γ is produced by NK cells, Natural Killer T (NKT) cells and both CD4 and CD8 cytotoxic T lymphocytes [13]. Interferons have been implicated to play a role in embryonic development as well as cellular differentiation [127]. Due to its anti-angiogenic effects and its ability to active immune response, interferons have been the ideal candidate for cancer therapy [128]. Studies have reported that interferon treatment is beneficial, although not curative, in a group of patients with various tumor types, such as melanoma, renal cell carcinoma and AIDS-associated Kaposi's sarcoma [129-133]. This dissertation will also demonstrate that IFN-γ and TNF-α secreted by activated NK cells, primarily by anergized NK cells, promote differentiation of both transformed and healthy stem cells (please see chapters 3 and 4). Activated NK cells, T cells, B cells, mast cells and macrophages all produce TNF-α as soluble cytokine or membrane-bound protein. There are two TNF receptors that TNF-α can bind to, TNFR1 and TNFR2, and each are differentially expressed on cells. Under normal, healthy conditions, the concentration level of TNF-α is low and increases when inflammation or infectious agents are present [134, 135]. One of the key roles of TNF-α is the regulation of transcription factor NFκB (Nuclear Factor kappa-light-chain-enhancer of activated B cells).

NK cells are known for their innate ability to mediate cytotoxicity without prior sensitization; however, in the tumor microenvironment the effector functions of NK cells are significantly hindered. We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity while increasing IFN-γ secretion by the NK cells [136, 137]. On the other

hand NK resistant tumors such as RAJI cells do not induce loss of NK cell cytotoxicity nor do they induce IFN- γ secretion [136, 137]. Furthermore, following NK cell cultures with sensitive tumor-target cells, the target binding NK cells undergo phenotypic and functional changes expressing $CD16^{-}CD56^{+/dim/-}CD69^{+}$ phenotype and a small subpopulation of NK cells undergoes apoptosis [136, 137].

Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in oral and ovarian cancer patients [138, 139]. In addition, down-regulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [140]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [140]. Triggering of CD16 on NK cells by anti-CD16 antibody, which mimics the ligand binding effect, was found to down-modulate CD16 receptors. This causes a great loss of cytotoxicity and gain in cytokine secretion in NK cells which we have previously coined as "split anergy" [136, 137, 141-145]. In our studies, we have also identified other anergy inducing factors, such as monocytes, receptor triggering of NKp46, bacteria, and interaction with undifferentiated transformed and healthy cells (Fig. 1). In addition, a small subpopulation of $CD16^{+}$ $CD56^{dim}$ NK cells undergoes cell death similar to that seen during the interaction of NK cells with sensitive tumors. Loss of cytotoxicity in NK cells was significantly increased when NK cells were either treated with anti-MHC class I antibody [143] or treated with $F(ab)'_2$ fragment of anti-CD16 mAb [143, 145].

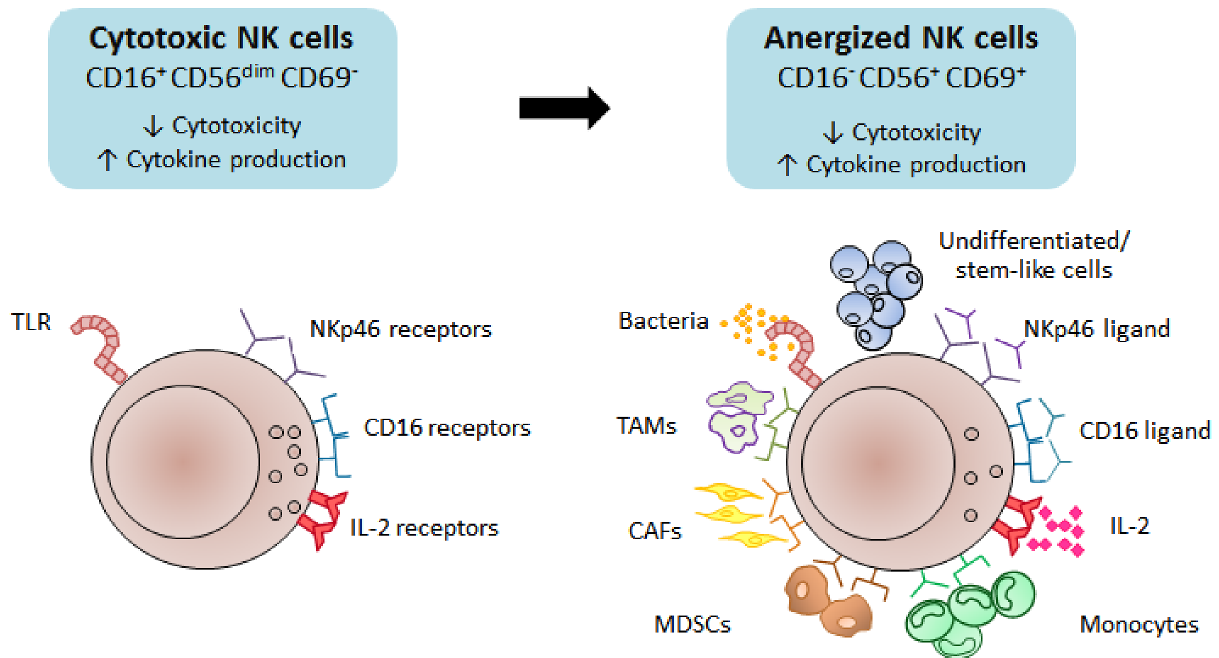

Fig. 1 Diagram of split anergy in NK cells

Immunosuppression and tumor escape from immune recognition are thought to be the two major factors responsible for the establishment and progression of cancer. It is shown that NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [146, 147]. However, the significance and the precise mechanism of NK cell function to become suppressed after their interaction with both tumor cells and healthy stem cells are not well understood.

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed when NK cells were co-incubated with OSCSCs which released significantly lower levels of GM-CSF, IL-6 and IL-8 and demonstrated decreased expression of phospho-Stat3, B7H1 and EGFR, and much lower constitutive NF-κB activity when compared to differentiated OSCCs [18]. More importantly, OSCSCs expressed CD133 and CD44^{bright} oral stem cell markers [18, 20], whereas differentiated OSCCs express lower CD44 surface receptors. To assess whether the stage of

differentiation of other tumor types also correlated with their sensitivity to NK cell mediated lysis we selected five pancreatic lines at different stages of differentiation based on a number of criteria including sphere formation and immunohistochemical analysis.

Panc-1 and MP-2, two poorly differentiated, BXPC3 and HPAF, two moderately differentiated and CAPAN-1, a well differentiated pancreatic tumors were co-cultured with the NK cells and NK cell mediated cytotoxicity were determined in a 4 h ^{51}Cr release assay. There was a significant correlation between the stage of differentiation of the tumors and the level of NK cell mediated lysis (Please see chapter 6). The highest NK cell cytotoxicity was obtained against poorly differentiated tumors Panc-1 and MP-2, intermediate lysis against moderately differentiated BXPC3 and HPAF and the lowest lysis were obtained against well differentiated CAPAN-1 cells (Please see chapter 6). Both untreated and IL-2 treated NK cells lysed poorly differentiated tumors much more than the differentiated tumors. Anti-CD16mAb treatment of NK cells abolished NK cell mediated cytotoxicity against all tumors and the combination of IL-2 and anti-CD16mAb significantly reduced IL-2 mediated lysis as expected.

In addition, two Glioblastoma Multiforme (GBM) stem-like tumors XO1GB and XO2GB, which were previously isolated and characterized [148-150], were found to be significantly more susceptible to NK cell mediated cytotoxicity when compared to differentiated U87 GBM tumors (Please see chapter 5). Since most stem-like tumors or poorly differentiated cells were significantly more susceptible to NK cell mediated cytotoxicity we reasoned that healthy, non-transformed primary stem cells may also be susceptible to NK cell mediated cytotoxicity.

We demonstrated previously that NK cells lysed hMSCs, hDPSCs and hESCs significantly [18]. All different types of stem cells became resistant to NK cell mediated cytotoxicity once they were differentiated [18]. In addition, higher sensitivity of hiPSCs to NK cell mediated lysis was also observed when compared to parental line from which they were derived. Differentiation of XO1GB and XO2GB also rendered them more resistant to NK cell mediated cytotoxicity. Increased lysis of cancer stem cells or non-transformed healthy stem cells may be attributed to the use of allogeneic NK cells, however, our previous work using autologous NK cells exhibited similar levels of cytotoxicity against hDPSCs when compared to lysis by allogeneic NK cells [18]. Taken together these results indicated that undifferentiated cells are targets of both allogeneic and autologous NK cells. Thus, the stage of differentiation of the cells is predictive of their susceptibility to NK cell mediated cytotoxicity.

Since the degree of differentiation in the cells is predictive of their sensitivity to NK cell mediated cytotoxicity, we reasoned that blocking NF-κB in the cells may de-differentiate and subsequently revert the cells to more of an undifferentiated phenotype, resulting in their increased susceptibility to NK cell mediated cytotoxicity. Indeed, blocking NF-κB in oral tumors was found to increase CD44 surface receptor expression, which is one of the hallmarks of stem. In addition, blocking of NF-κB nuclear function in a primary Oral tumor OSCCs and in a non-tumorigenic oral cells (HOK-16B) as well as in an established tumor line augmented cytotoxicity and the release of key cytokines such as IFN-γ from the NK cells [151, 152]. Similarly, inhibition of NF-κB by Sulindac increased the functional activation of NK cells and enhanced anti-tumor cytotoxic activity [151, 152].

In agreement with our studies, targeted deletion of IKK-b in epidermis of mice has previously been shown in one study to lead to inflammatory skin manifestations [153]. Elevated levels of cytokines and chemokines have also been demonstrated in the epidermis of patients and animals with Iκκb and Iκκb deletions [153, 154]. Mice with a keratinocyte- specific deletion of Iκκb demonstrated decreased proliferation of epidermal cells, and developed TNF-α dependent inflammatory skin disease [153]. In contrast, in other studies in which NF-κB function was blocked in dermal keratinocytes by a mutant IκB-a an increased proliferation and hyperplasia [155] and eventual development of cutaneous squamous cell carcinomas of skin were seen if mice were allowed to survive and reach adulthood [156, 157]. It is of interest to note that in these studies with diverse functional outcomes in keratinocytes, blocking TNF-α function resulted in the prevention of both the neoplastic transformation and the inflammatory skin disease. Elevated numbers of immune inflammatory cells recruited to the site of epidermis are likely responsible for the increased secretion of TNF-a. Indeed, we have demonstrated that synergistic induction of TNF-α could be observed when NF-κB knock down oral tumors were cultured with either PBMCs or NK cells [158].

Since tumorigenic and non-tumorigenic human oral keratinocytes acquire sensitivity to NK cell mediated lysis when NF-κB is inhibited, it is likely that this phenomenon is not specific to cancer or oral keratinocytes, and it may occur in other healthy non-transformed cell types. Indeed, when human primary monocytes were differentiated to dendritic cells they too became more resistant to NK cell mediated cytotoxicity [18]. Moreover, knock down of COX2 in primary mouse monocytes [18] or in mouse embryonic fibroblasts (Manuscript in prep), resulted

in the reversion or de-differentiation of the monocytes and fibroblasts respectively, and the activation of NK cell cytotoxicity.

Indeed, it is likely that any disturbance in cellular differentiation may predispose the cells to NK cell mediated cytotoxicity. Since STAT3 is an important factor increased during differentiation, blocking STAT3 is also critical in the activation of immune effectors [159]. In support of a critical role of STAT3 in immune evasion of tumor cells in humans, we and others have recently shown that GBM tumors display constitutive activation of STAT3 (Please see chapter 5) [160], and poorly induce activating cytokines and tumor-specific cytotoxicity in human peripheral blood mononuclear cells (PBMCs) and NK cells. Ectopic expression of dominant-negative STAT3 in the GBM tumors increased lysis of the tumor cells by the immune effectors and induced production of IFN-γ by the interacting immune effectors (Please see chapter 5).

Since NF-κB is shown to regulate IL-6 secretion in OSCCs, HOK-16B and HEp2 cells and secreted IL-6 in tumors is known to activate STAT3 expression and function, increase in NF-κB nuclear function could in turn induce STAT3 activation and result in a significant resistance of tumors to NK cell mediated cytotoxicity. Indeed, inhibition of NF-κB in oral tumors resulted in a significant decrease in IL-6 secretion by the tumor cells and the induction of IFN-γ secretion by the NK cells [18, 152]. Therefore, targeted knock down of STAT3 or signaling pathways upstream of STAT3, such as NF-κB, may de-differentiate the cells and predispose the cells to NK cell mediated cytotoxicity. Additional studies on gene knockouts were shown to greatly affect the effector functions of NK cells (Table 2).

Based on the accumulated work presented in this dissertation, we suggest that NK cells may have two significant functions; one that relates to the removal of excess proliferating stem cells and their selection. In this regard, NK cells could also lyse other effectors in the connective tissue area in order to not only decrease inflammation but also to be conditioned to promote differentiation and resistance of selected stem cells and eventual regeneration of the tissues (Figure 2). The second important task for NK cells is therefore, to support differentiation and promote tissue regeneration after altering their phenotype to cytokine secreting.

Table 2- Specific gene knock outs that activate the function of NK cells

Gene	References
NFkB	**[151, 158, 161]**
STAT3	**[162, 163]**
COX2	**Manuscript in prep**
CD44	**Manuscript in prep**
NEMO	**[164-166]**
MHC-1	**[167-172]**
TNF-α	**[173]**
DAP10/ DAP12	**[174]**

This dissertation has 9 chapters and demonstrates that:

Chapter 1: Increased lysis of stem cells but not their differentiated cells by Natural Killer cells; De-differentiation or reprogramming activates NK cells

Chapter 2: Strategies to rescue Mesenchymal Stem Cells (MSCs) and Dental Pulp Stem Cells (DPSCs) from NK Cell mediated Cytotoxicity

Chapter 3: Induction of split anergy conditions Natural Killer cells to promote differentiation of stem cells through cell-cell contact and secreted factors

Chapter 4: Natural Killer cells halt inflammation by inducing stem cell differentiation, resistance to NK cell cytotoxicity and prevention of cytokine and chemokine secretion

Chapter 5: Differential targeting of Glioblastoma cancer stem cells and their differentiated tumors by NK cells and CTLs

Chapter 6: NK differentiated stem-like pancreatic tumors while are resistant to NK mediated cytotoxicity become susceptible to chemo-drug induced cell death and do not metastasize

Chapter 7: Conditional COX2 knock out in mouse monocytes augments the cytotoxic and cytokine production functions of Natural Killer cells

Chapter 8: Osteoclasts as key subsets of immune effectors modulating the function of Natural Killer cells; Role in bisphosphonate driven differentiation

Chapter 9: Regulation of split anergy in natural killer cells through inhibition of cathepsin C by cystatin F

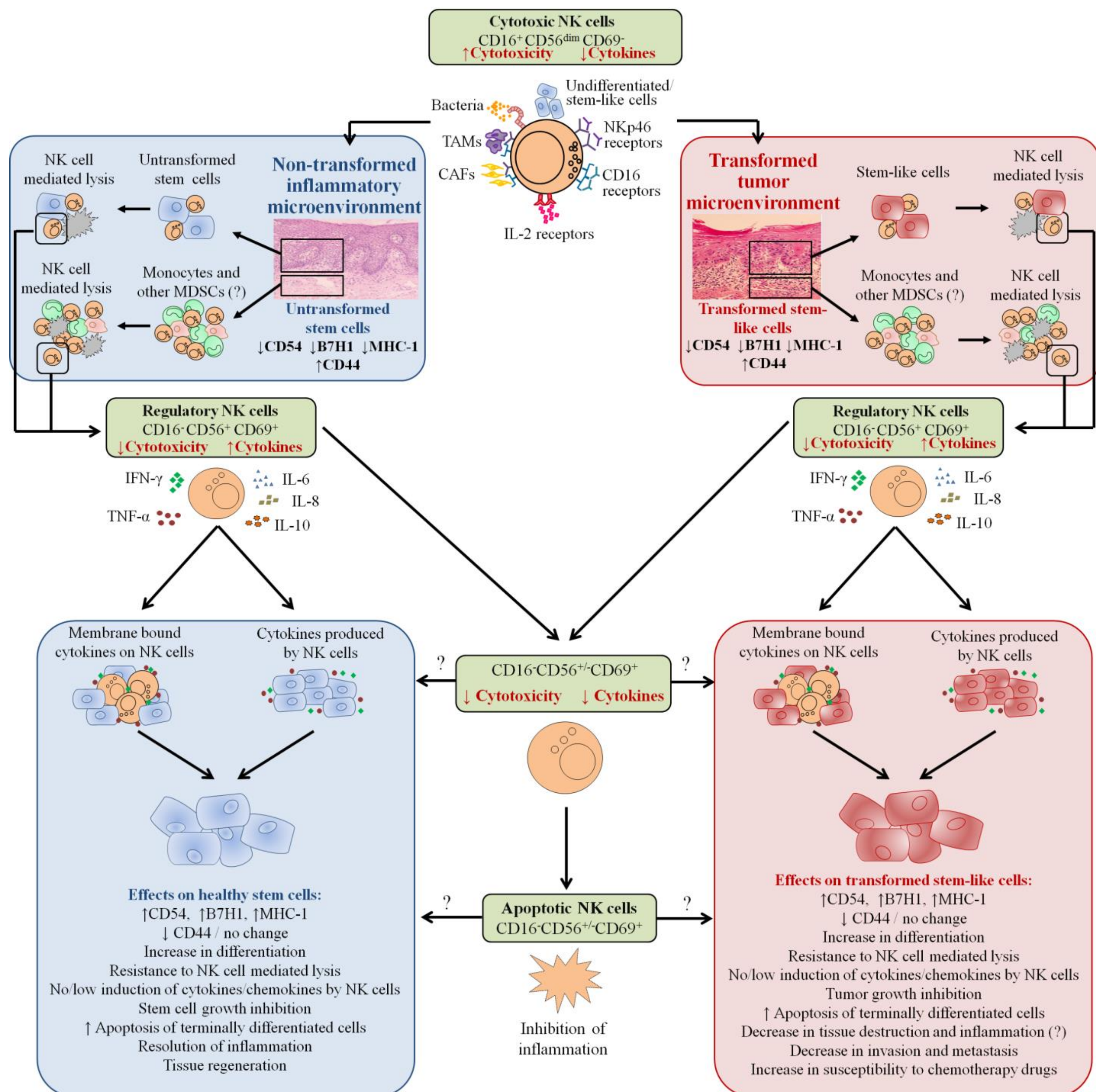

Figure 2. Hypothetical model of induction of regulatory NK cells by immune inflammatory cells and by the effectors of connective tissue to support differentiation of non-transformed stem cells and cancer stem cells.

CHAPTER 1

Increased lysis of stem cells but not their differentiated cells by Natural Killer cells; De-differentiation or reprogramming activates NK cell cytotoxicity

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Running Title: NK cells kill cancer stem cells

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Abstract

The aim of this study is to demonstrate the increased lysis of stem cells but not their differentiated counterparts by the NK cells, and to determine whether disturbance in cell differentiation is a cause for increased sensitivity to NK cell mediated cytotoxicity. Increased cytotoxicity and augmented secretion of IFN-γ were both observed when PBMCs or NK cells were co-incubated with primary UCLA oral squamous carcinoma stem cells (UCLA-OSCSCs) when compared to differentiated UCLA oral squamous carcinoma cells (UCLA-OSCCs). In addition, human embryonic stem cells (hESCs) were also lysed greatly by the NK cells. Moreover, NK cells were found to lyse human Mesenchymal Stem Cells (hMSCs), human dental pulp stem cells (hDPSCs) and human induced plueripotent stem cells (hiPSCs) significantly more than their differentiated counterparts or parental lines from which they were derived. It was also found that inhibition of differentiation or reversion of cells to a less differentiated phenotype by blocking NFκB or targeted knock down of COX2 in monocytes significantly augmented NK cell cytotoxicity and secretion of IFN-γ. Taken together these results suggested that stem cells are significant targets of the NK cell cytotoxicity, however, to support differentiation of tumor or healthy untransformed primary stem cells, NK cells may be required to lyse a number of stem cells and/or those which are either defective or incapable of full differentiation in order to lose their cytotoxic function and gain in the ability to secrete cytokines (split anergy). Therefore, patients with cancer may benefit from repeated allogeneic NK cell transplantation for specific elimination of cancer stem cells.

Introduction

Immunosuppression and tumor escape from immune recognition are thought to be the two major factors responsible for the establishment and progression of cancer. A number of factors responsible for the suppression of NK cell cytotoxicity in humans have been identified previously [175-180]. However, the significance and the precise mechanism of NK suppression induced during their interaction with either tumor cells or healthy primary cells are not well understood. It is shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [181-184]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [185-187]. In contrast the interaction of NK cells with the resistant tumors does not lead to suppression of NK cell cytotoxicity [137]. Many mechanisms have been proposed for the functional inactivation of tumor associated NK cells including the over-expression of Fas ligand, the loss of mRNA for granzyme B [176] and decreased CD16 and its associated zeta chain [188].

Many metastatic tumor cells exhibit constitutively elevated NFκB activity [189]. Increased NFκB activity is shown to have a causal relationship to neoplastic transformation, and uncontrolled cell growth in many cell types [189]. Human solid tumors exhibit constitutively activated NFκB [189].

We have previously shown that NK resistant primary oral keratinocyte tumors demonstrate higher nuclear NFkB activity and secrete significant levels of Granulocyte

Monocyte-Colony Stimulating Factor (GM-CSF), Interleukin(IL)-1β, IL-6 and IL-8 [190]. Moreover, the addition of Non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit NFκB have the ability to reverse immunosuppression induced by a tobacco-specific carcinogen in addition to their well-established ability to decrease oral dysplasia as well as induction of overt cancer in transgenic animals [191]. In agreement, we have previously demonstrated that inhibition of NFκB by Sulindac treatment of tumor cells increases functional activity of NK cells [192, 193]. Moreover, targeted inhibition of NFκB in skin epithelial cells resulted in the induction of auto-immunity and inflammation [153].

The exact mechanism by which NFκB nuclear function in oral keratinocytes modulate and shape the function of key interacting immune effectors is yet to be determined. We have previously shown that inhibition of NFκB by the IκB super-repressor in HEp2 tumors leads to significant increase in cytotoxicity and secretion of IFN-γ by the human NK cells [192, 193]. However, neither the underlying significance nor the physiological relevance of NFκB modulation in tumors or in primary cells responsible for the alteration of NK cell cytotoxic function have been studied previously. It is clear that the objective in cancer is to enhance the function of cytotoxic immune effectors to eliminate tumors and in auto-immunity and inflammation the aim is to inhibit immune effector function to prevent tissue damage. Therefore, dissection of the underlying mechanisms of immune activation when NFκB is modulated in the cells might help design strategies to target each disease accordingly. Indeed, targeted inhibition of NFκB function in both the intestinal epithelial cells and the myeloid cells was previously shown to result in a significant decrease in the size and the numbers of the tumor cells [194].

Here we have extended our previous results obtained by an established HEp2 oral tumor cells [192] to patient derived oral tumors demonstrating that blocking NFκB in these cells increases the activation of NK cell cytotoxicity. We have also used an immortalized but non tumorigenic oral keratinocytes HOK-16B since they were previously used as a model of dysplasia in a cancer progression model [195, 196].

In this report we demonstrate that the stage of differentiation of the cells is predictive of their sensitivity to NK cell lysis. Thus, UCLA-OSCSCs, which are less differentiated oral tumors are significantly more susceptible to NK cell mediated cytotoxicity; however, their differentiated counterparts UCLA-OSCCs are significantly more resistant. In addition, both hESCs and iPSCs as well as a number of other stem cells such as hMSCs and hDPSCs were found to be significantly more susceptible to NK cell mediated cytotoxicity. Based on these results, we propose that NK cells may play a significant role in differentiation of the cells by providing critical cytokines. However, to drive differentiation, NK cells will have to first receive signals from undifferentiated stem cells or those which have disturbed or defective capabilities to differentiate in order to lose cytotoxicity and gain in cytokine producing phenotype. These alterations in NK cell effector function will ultimately aid in driving differentiation of a minor population of surviving healthy as well as transformed cells. In cancer patients since the majority of NK cells have lost their cytotoxic activity, they may eventually contribute rather than halt the progression of cancer by not only driving the differentiation of tumor cells but more importantly, by allowing the growth and expansion of the pool of cancer stem cells.

Materials and Methods

Cell Lines, Reagents, and Antibodies

RPMI 1640 supplemented with 10% FBS was used for the cultures of human and mouse NK cells and human PBMCs. UCLA-OSCCs and UCLA-OSCSCs were isolated from freshly resected tongue tumors, and were cultured in RPMI 1640 supplemented with 10% FCS. The immortalized human oral keratinocytes with type 16 human papillomavirus DNA (HOK-16B) were cultured as described previously [197] in keratinocytes growth medium (KGM) supplemented with reagents supplied in the bullet kit (Clonetics Corp., San Diego, CA). The mouse and human NK and monocyte purification kits were obtained from Stem Cell Technologies (Vancouver, Canada). Recombinant IL-2 was obtained from NIH- BRB. The anti-CD133 and CD44 were obtained from Miltenyi biotec (Auburn, CA). Antibody to CD90 was purchased from Pharmingen/BD (San Diego, CA). Antibodies for CD16 and B7H1 were purchased from ebiosciences (San Diego, CA). EGFR antibody (Erbitox) was purchased from UCLA pharmacy. The antibodies against p65 subunit of NF□B and pSTAT3 were purchased from Santa Cruz (Santa Cruz, CA). Blocking antibodies against CD126 were purchased from Biosource (Camarillo, CA).

Human Mesenchymal stem cells (hMSCs), human Embryonic Stem cells (hESCs), human Dental Pulp Stem cells (hDPSCs), human induced pluripotent stem cells (hiPSCs)

hMSCs were obtained from Poietics, Cambrex Bio Science (Walkerville, MD) and they were cultured in Mesenchymal Stem Cell Basal Medium (MSCBM) supplemented with Mesenchymal Cell Growth Supplement (MCGS) (Cambrex Bio Science Walkerville ,MD). The

MSCs were differentiated into osteoblasts using Osteogenic differentiation media which comprises of Osteogenic Differentiation BulletKit® that contains Basal Medium and one Osteogenic SingleQuot Kit® also purchased from Cambrex Bio Science.(Walkerville, MD). Human Mesenchymal stem cells were cultured in Mesenchymal Stem Cell Basal Medium (MSCBM) with the growth supplements according to the manufacturer's recommendations. For the induction of osteogenesis, MSC were seeded at a density of ($1X10^4$ cells/well) in Osteogenic media with the recommended supplements. Media was replaced every three days and the cells were used in the experiments when they were 80% confluent.

hDPSCs were isolated as described previously [198] and they were cultured in complete DMEM supplemented with 10% FBS. DPSCs were differentiated using b-glycerophosphate, ascorbic acid and dexamethasone as reported previously [198].

hESC line H9 and hiPSC line hiPSC18 [199] were used in this study. H9 and hiPSC18 were used at passages 45-50. hESC and hiPSC were grown on irradiated mouse embryonic fibroblasts (MEFs) in DMEM/F12 supplemented with 20% Knockout serum replacement (Invitrogen), 1mM glutamine, 1 X nonessential amino acids (NEAA), and 4 ng/ml of bFGF as previously described [200]. 2-mercaptoethanol (1 mM Sigma) and penicillin/streptomycin (Hyclone) were added to growing cultures. For coculture assays, cells were seeded at a density of 10^5 cells/well on Matrigel (BD Sciences) in conditioned media plus HA-1077, as previously described [200]. Neonatal human dermal fibroblasts (NHDF-iPSC parental fibroblast line from ATCC) [199] were cultured in DMEM supplemented with 10% FBS, 1mM glutamine, 1X NEAA and penicillin/streptomycin.

Purification of human and mouse NK cells and monocytes

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors and all the procedures were approved by the UCLA-IRB. PBMCs and NK cells from healthy donors were isolated as described before [137]. Briefly, peripheral blood lymphocytes were obtained after Ficoll-hypaque centrifugation and purified NK cells were negatively selected by using an NK cell isolation kit (Stem Cell Technologies, Vancouver, Canada). The purity of NK cell population was found to be greater than 90% based on flow cytometric analysis of anti-CD16 antibody stained cells. The levels of contaminating CD3+ T cells remained low, at 2.4%±1%, similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures. The adherent subpopulation of PBMCs was detached from the tissue culture plates and monocytes were purified using isolation kit obtained from Stem Cell Technologies (Vancouver, Canada). Greater than 95% purity was achieved based on flow cytometric analysis of CD14 antibody stained monocytes.

All animal work performed was based on the guidelines established and approved by UCLA-IACUC (2006-074-12). Single cell preparations of mouse splenocytes were used to negatively select for mouse NK cells using mouse NK isolation kit purchased from Stem Cell Technologies (Vancouver, Canada). The purity of mouse NK cells were greater than 90% based on staining with NK1.1 and DX5 antibodies. Murine monocytes were purified from bone marrow using monocyte isolation kit obtained from Stem Cell Technologies (Vancouver, Canada). The purity of monocytes was greater than 90% based on staining with anti-CD14

antibody.

ELISA and Multiplex Cytokine Array kit

Single ELISAs were performed as described previously [137]. Fluorokine MAP cytokine multiplex kits were purchased from R&D Systems (Minneapolis, MN) and the procedures were conducted as suggested by the manufacturer. To analyze and obtain the cytokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines provided by the manufacturer. Analysis was performed using the Star Station software.

Surface and DNA Staining and apoptosis assay

Staining was performed by labeling the cells with antibodies as described previously [142] [137, 201].

Western Blot

Treated and untreated cells were lysed in a lysis buffer containing 50mM Tris-HCL (pH 7.4), 150mM NaCl, 1% Nonidet P-40 (v/v), 1mM sodium orthovanadate, 0.5mM EDTA, 10mM NaF, 2mM PMSF, 10μg/mL leupeptin, and 2U/mL aprotinin for 15 minutes on ice. The samples were then sonicated for 3 seconds. The cell lysates were centrifuged at 14,000 rpm for 10 minutes and the supernatants were removed and the levels of protein were quantified by the Bradford method. The cell lysates were denatured by boiling in 5x SDS sample buffer. Equal amounts of cell lysates were loaded onto 10% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Billerica MA). The membranes were blocked with 5% non-fat milk in PBS plus 0.1% Tween-20 for 1 hour. Primary antibodies at the predetermined dilution were

added for 1 hour at room temperature. Membranes were then incubated with 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody. Blots were developed by enhanced chemiluminescence (ECL- purchased from Pierce Biotechnology, Rockford, IL).

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [193]. Briefly, different numbers of purified NK cells were incubated with ^{51}Cr–labeled tumor target cells. After a 4 hour incubation period the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}}$$

LU 30/10^6 is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X100.

Retroviral and lentiviral transduction

UCLA-OSCCs were infected with culture supernatants of NIH 3T3 packaging cells transfected with either $\text{IkB}_{(S32AS36A)}$super-repressor or mutant IkBα (IkBαM) or their EGFP control vectors. The retroviral vectors were generated in Dr. Nicholas Cacalano's laboratory. Forty eight hours after infection the UCLA-OSCCs or HOK-16B cells were sorted for high expressing GFP cells and were grown and used in the experiments.

NFkB-Luciferase lentiviral reporter vector was produced by co-transfection of the packaging cell line 293T [198] using Calcium Phosphate precipitation. UCLA-OSCCs and UCLA-OSCSCs were seeded at a density of $2x10^5$ cells per well in a 6-well culture plate 24hrs before transduction. The following day, cells were transduced with the NFkB-Luciferase lentiviral reporter vector. To enhance transduction efficiency, the cationic polymer Polybrene was used at a final concentration of 8µg/ml. After six hours of incubation, medium was re-freshed and transduced cells were incubated for an additional 42 hours. Cells were then harvested, lysed and luciferase activity was measured [RLU/s] using a luminometer. An internal lentiviral vector control constitutively expressing Luciferase was used to normalize values.

Luciferase reporter assay

Transfections were also performed using NFkB Luciferase reporter vector [202] and Lipofectamine 2000 reagent (Invitrogen, CA) in Opti-MEM media (Invitrogen, CA) for 18 hours after which they were adhered to the plate overnight before different immune effectors at 1:1 Effector to target ratios were added. The cells were then lysed with lysis buffer and the relative Luciferase activity was measured using the Luciferase assay reagent kit obtained from Promega (Madison, WI)

Alkaline Phosphatase (ALP) staining

Human MSCs were co-cultured with and without untreated and IL-2 treated PBMCs as indicated in the result section. Cells were then washed twice with PBS and incubated with 120mM of Tris buffer (pH = 8.4) containing 0.9mM Napthol AS-M Phosphate and 1.8mM Fast Red TR (both purchased from Sigma, MO) for 30 minutes at 37˚C. After 30 minute incubation,

cells were washed three times with PBS and then fixed with 1ml cold ethanol (100%) for 30 minutes. The stained cultures were scanned using an Epson scanner 1250.

Statistical analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

Results

Identification and characterization of patient-derived primary oral squamous cancer stem cells (UCLA-OSCSCs)

We screened a number of different primary oral squamous cell carcinomas (OSCC) derived from patients at UCLA, and selected to concentrate on two specific primary tumors based on their phenotypic characteristics and sensitivity to NK cell mediated cytotoxicity. UCLA-OSCCs were found to have higher surface expression of B7H1 and EGF-R and moderate expression of CD44 and no surface expression of CD133 whereas UCLA-OSCSCs expressed no or very low expression of B7H1, EGF-R and very high expression of CD133 and CD44^{bright} (please see supplemental Fig.1). No surface expression of MHC-Class II (data not shown) or CD90 could be seen on either tumor type. In addition, UCLA-OSCSCs secreted no or very low levels of IL-6, IL-8 and GM-CSF whereas they secreted higher levels of VEGF when compared to UCLA-OSCCs (Tables 1 and 2). Moreover, they did not express phospho-Stat3 when cultured in the presence and absence of EGF (please see supplemental Fig.1). More importantly, no or very low NFkB activity could be detected in UCLA-OSCSCs when compared to UCLA-OSCCs (please see supplemental Fig.1). Therefore, the profiles of cytokines secreted by UCLA-OSCCs and UCLA-OSCSCs resembled those of vector alone and $I\kappa B_{(S32AS36A)}$ super-repressor transfected HEp2 cells respectively (Table 2) [192, 193]. Thus, UCLA-OSCSCs express phenotypic characteristics of oral cancer stem cells [203, 204]. Furthermore, they were smaller in size and proliferated at a much higher rate when compared to UCLA-OSCCs cells (data not shown). We used these two primary oral tumors to study NK cell activation.

Increased NK cell cytotoxicity against UCLA-OSCSCs but not those of UCLA-OSCCs

We have previously shown that blocking NFkB in HEp2 tumor cells decreased IL-6 and IL-8 secretion substantially and resulted in an increased sensitivity of HEp2 tumor cells to NK cell mediated cytotoxicity [192, 193]. Therefore, using the levels of cytotoxicity, IFN-γ and IL-6 secretion, we could demonstrate a direct correlation between decreased IL-6 and increased IFN-γ secretion in the co-cultures of NK cells with NFkB knock down HEp2 cells and increased susceptibility to IL-2 activated NK cell killing. Induction of NK cell anergy by anti-CD16 antibody even though abrogated the ability of IL-2 treated NK cells to lyse HEp2 cells the same treatment resulted in a significant induction of IFN-γ secretion in the co-cultures of NK cells with HEp2 cell transfectants [144, 145]. To extend our findings to patient derived oral tumors, UCLA-OSCC and UCLA-OSCSCs were tested for their sensitivity or resistance to NK cell mediated cytotoxicity. The cytotoxic activities of IL-2 treated PBMCs (Fig.1A) and NK cells (Fig.1B) were significantly higher against UCLA-OSCSCs cells when compared to UCLA-OSCCs. Untreated PBMCs or NK cells lysed UCLA-OSCSCs tumors significantly more than UCLA-OSCCs (Fig.1B). However, the levels of lysis by untreated NK cells were considerably lower than that obtained by IL-2 treated PBMCs or NK cells (Fig 1A and 1B). Treatment of PBMCs or NK cells with anti-CD16 mAb decreased cytotoxicity significantly against both tumor types, however, the levels of lysis by the NK cells remained higher against UCLA-OSCSCs in all the NK samples tested (Fig. 1). IL-2 treated NK cells co-cultured with UCLA-OSCSCs oral tumor cells exhibited higher expression of CD69 activation antigen when compared to those co-cultured with UCLA-OSCC oral tumors (data not shown).

Increased induction of IFN-γ was paralleled with a decreased secretion of IL-6 in co-cultures of NK cells with UCLA-OSCSCs oral tumors

Untreated and IL-2 treated NK cells were co-cultured with UCLA-OSCC and UCLA-OSCSCs and the induction of a number of key cytokines, including those which were correlated with NK resistant tumor phenotype, were determined in the supernatants recovered from the co-cultures of the immune effectors with oral tumors after an overnight incubation. In the presence of untreated NK cells co-cultured with UCLA-OSCC, synergistic induction of GM-CSF, IL-6 and IL-8 could be observed since much lower levels of these cytokines were induced either in the presence of immune effectors alone or tumor cells alone (Table 2). The levels of above-mentioned cytokines were considerably lower in the co-cultures of untreated NK cells with UCLA-OSCSCs (Table 2). Even though VEGF secretion was significantly higher in UCLA-OSCSCs, the levels exceeded that of the baseline levels produced by the tumor cells alone when untreated NK cells were co-cultured with UCLA-OSCC cells and not that of UCLA-OSCSCs (Table 2). Increased GM-CSF secretion in the presence of UCLA-OSCCs as compared to UCLA-OSCSCs was more evident in untreated NK cells (Table 2).

NK cell sensitivity of tumors correlated with an increased IFN-γ secretion in the presence of lower IL-6 and IL-8 secretion in IL-2 activated NK cells co-cultured with UCLA-OSCSCs (Table 2). Indeed, when ratios of IL-6 to IFN-γ was considered a direct correlation between sensitivity to NK cell mediated killing and decreased ratios of IL-6 to IFN-γ could be seen (Table 2). Finally, both cell lines exhibited lower amounts of VEGF secretion in the presence of IL-2 treated NK cells, indicating the ability of IL-2 treated NK cells to exert significant inhibitory effect on VEGF secretion. However, the residual levels remained higher in the co-cultures of IL-2 treated NK cells with UCLA-OSCC than UCLA-OSCSCs when compared to the baseline

secretion by the tumors alone (Table 2). Thus, several important cytokine profiles were identified for NK sensitive and resistant oral tumors after their co-culture with NK cells.

Blocking NFkB in UCLA-OSCCs and HOK-16B oral epithelial cells lowered IL-6 to IFN-γ ratios and increased their sensitivity to NK cell mediated cytotoxicity

As indicated previously UCLA-OSCCs and HOK-16B oral keratinocytes represent oral cancer progression model since HOK-16B are immortalized but non tumorigenic thus could represent a model of dysplastic keratinocytes [144, 190, 195, 196]. HOK-16B and UCLA-OSCCs were transduced with EGFP alone or IκBαM or $I\kappa B_{(S32AS36A)}$super-repressor retroviral constructs and sorted for high GFP expressing cells using flow cytometry (data not shown). The inhibition of NFκB by the IκBαM or $I\kappa B_{(S32AS36A)}$super-repressor retroviral vector in UCLA-OSCC and HOK-16B was confirmed by measuring NFκB activity using luciferase reporter assay (Figs. 2A and 2B). IκBαM or $I\kappa B_{(S32AS36A)}$super-repressor transduced UCLA-OSCC (Fig. 2C) and HOK-16B (Fig. 2D) tumor cells secreted substantially lower levels of IL-6 when compared to EGFP transduced UCLA-OSCCs and HOK-16B cells. Thus, transduction of UCLA-OSCCs and HOK-16B with IκBαM or $I\kappa B_{(S32AS36A)}$super-repressor constructs exhibited the same functional profiles as those observed in transfected HEp2 oral tumor cells with $I\kappa B_{(S32AS36A)}$ construct [192, 193]. Similar to HEp 2-cell transfectants, UCLA-OSCCs and HOK-16B cells transduced with IκBαM or $I\kappa B_{(S32AS36A)}$super-repressor constructs did not exhibit elevated levels of cell death when assessed by flow cytometric analysis of Annexin V and PI stained cells (data not shown). In addition, there was a significant decrease in the surface expression of ICAM-1 in TNF-α and IFN-γ treated $I\kappa B_{(S32AS36A)}$ transduced UCLA-OSCCs (83% decrease) and HOK-

16B cells (78% decrease) when compared to EGFP alone transduced cells. These results also indicated that IL-6 secretion in oral tumor cells is regulated by the function of NFκB.

Untreated or IL-2 treated NK cells were added to EGFP or $I\kappa B_{(S32AS36A)}$ transduced UCLA-OSCCs and IκBαM transduced HOK-16B oral keratinocytes and the levels of IL-6 and IFN-γ secretion were determined in the co-cultures with the NK cells after an overnight incubation. IL-2 activated NK cells secreted lower levels of IL-6 when co-cultured with $I\kappa B_{(S32AS36A)}$ transduced UCLA-OSCCs (Fig. 2E) and IκBαM HOK-16B (Fig. 2F) cells as compared to EGFP transduced oral keratinocytes. In contrast, higher induction of IFN-γ secretion could be observed in supernatants recovered from the co-cultures of NK cells with $I\kappa B_{(S32AS36A)}$ transduced UCLA-OSCCs (Fig. 2G) and IκBαM transduced HOK-16B (Fig. 2H) oral keratinocytes as compared to EGFP transduced oral tumors. Similar NK cell response patterns were obtained when NFκB was inhibited in HEp2 cells [192, 193]. Finally, IL-2 treated NK cells lysed NFkB knock down OSCCs (Fig. 2I) and HOK-16B (Fig. 2J) cells significantly more than EGFP transfected cells.

Significant lysis of Embryonic Stem Cells (hESCs), Induced Pluripotent Stem Cells (iPS), Dental Pulp Stem Cells (DPSCs), and Mesenchymal Stem Cells (MSCs) by untreated or IL-2 treated NK cells

Highly purified human NK cells were cultured with and without IL-2 for 12-24 hours before they were added to ^{51}Cr labeled hESCs (Fig. 3A), iPSCs (Fig. 3D), DPSCs (Fig. 3G) and MSCs (Fig. 3J). Addition of untreated NK cells had lower cytotoxicity against different populations of stem cells whereas activation with IL-2 increased cytotoxicity against all stem cell

populations significantly ($p<0.05$) (Fig. 3). Therefore, human stem cells are greatly lysed by the NK cells.

Lysis of hESCs, iPSCs, DPSCs, and MSCs by untreated and IL-2 treated NK cells is inhibited by anti-CD16 antibody treatment, however, the same treatment induced significant secretion of IFN-γ by the NK cells in the presence and absence of stem cells.

As shown in a number of previous studies anti-CD16 mAb treatment induced anergy in a great majority of the NK cells as well as it induced death in a subset of NK cells, thereby inhibiting NK cell cytotoxicity against different populations of stem cells ($p<0.05$) (Figs. 3). Addition of the combination of IL-2 and anti-CD16 treatment also induced anergy and NK cell death (data not shown) and inhibited NK cell cytotoxicity against stem cells when compared to IL-2 activated NK cells ($p<0.05$) (Fig. 3). Untreated or anti-CD16 mAb treated NK cells did not secrete IFN-γ when co-cultured with any of the stem cell populations, however, both IL-2 treated and IL-2 in combination with anti-CD16 mAb treated NK cells in the presence and absence of stem cells secreted significant levels of IFN-γ ($p<0.05$) (Fig. 3B,3E,3H,3K). Indeed, stem cells triggered significant secretion of IFN-γ from IL-2 treated NK cells when compared to IL-2 treated NK cells in the absence of stem cells. In addition, there was a synergistic induction of IFN-γ secretion in IL-2 and anti-CD16 mAb treated NK cells in the absence of stem cells, and the levels either remained the same or exceeded those in the absence of stem cells when IL-2 and anti-CD16mAb treated NK cells were cultured with stem cells (Fig. 3). There was a direct correlation between secretion of bFGF by stem cells and cytotoxicity by IL-2 and IL-2+anti-CD16 treated NK cells (Fig. 3C,3F,3I,3L).

Lysis of MSCs by untreated and IL-2 treated NK cells is inhibited by monocytes, however, the addition of monocytes induced significant secretion of IFN-γ by the NK cells in the presence and absence of stem cells.

Monocytes were purified from PBMCs and irradiated as indicated in the Material and Methods section. MSCs were co-cultured with irradiated monocytes for 24-48 hours before they were labeled with ^{51}Cr and used in the cytotoxicity assays against NK cells. NK cells were left untreated or pre-treated with anti-CD16 antibody and/or IL-2 for 24-48 hours before they were used in the cytotoxicity assays against MSCs. The addition of monocytes to MSCs significantly protected the MSCs (Fig. 4A) from NK cell mediated cytotoxicity ($p<0.05$). Significant inhibition of NK cell cytotoxicity by monocytes could be observed against untreated and IL-2 treated NK samples ($p<0.05$) (Fig 4A). Monocytes also increased the levels of alkaline phosphatase staining in MSCs and prevented decrease in alkaline phosphatase expression induced by IL-2 activated NK cells (data not shown). Untreated or anti-CD16 antibody treated irradiated monocytes did not mediate cytotoxicity against MSCs, (data not shown). Overall, these experiments indicated that monocytes protect MSCs against NK cell mediated lysis.

As expected IL-2 treated NK cells secreted moderate amounts of IFN-γ which were synergistically increased when co-cultured in the presence of MSCs ($p<0.05$) (Fig. 4B). The addition of anti-CD16 mAb in combination with IL-2 to NK cells in the absence of MSCs increased secretion of IFN-γ when compared to IL-2 alone treated NK cells in the absence of MSCs. IFN-γ secreted levels remained similar between IL-2 alone and IL-2 and anti-CD16 mAb treated NK cells cultured with MSCs (Fig. 4B). Monocytes added to IL-2 or IL-2 and anti-CD16 antibody treated NK cells in the absence of MSCs or those in the presence of MSCs,

synergistically increased the levels of secreted IFN- γ ($p<0.05$) (Fig. 4B). However, the highest increase in IFN- γ release was seen when monocytes were added to IL-2 or IL-2 and anti-CD16 mAb treated NK cells with MSCs (Fig. 4B). These results indicated that monocytes increased IFN-γ in co-cultures with MSCs, and further synergized with IL-2 or IL-2 and anti-CD16 mAb treated NK samples to increase the release of IFN-γ in the co-cultures of NKs and MSCs. Similar results were obtained when NK cells were co-cultured with monocytes and DPSCs [205].

MSCs are significantly more sensitive to lysis by IL-2 treated NK cells than their differentiated counterparts and they trigger significant release of IFN-γ by IL-2 activated NKs.

To determine whether differentiation decreases sensitivity of stem cells to NK cell mediated cytotoxicity we first chose to concentrate on MSCs. To assess whether differentiation of MSCs similar to oral tumors decreases sensitivity of these cells to NK cell mediated cytotoxicity we determined NK cell cytotoxicity against MSCs and their differentiated osteoblasts using un-fractionated PBMCs as well as NK cells. MSCs were cultured in the absence and presence of untreated and IL-2 treated PBMCs at 10:1 PBMC to MSC ratio and the levels of ALP staining were determined after 2 days of incubation. The addition of untreated PBMCs to MSCs triggered some differentiation of MSCs as assessed by Alkaline Phosphatase (ALP) staining (Figs. 5A and 5B). No significant staining with ALP can be seen by either the PBMCs or MSCs alone (Figs. 5A and 5B). Treatment of PBMCs with IL-2 and their subsequent co-culture with MSCs lysed the cells and prevented induction of ALP, therefore, no or very low detection of ALP could be observed (Figs. 5A and 5B). The co-culture of differentiated osteoblasts with PBMCs was performed as described above with MSCs. As shown in Figs. 5C

and 5D both the untreated and IL-2 treated PBMCs triggered significant increase in ALP staining in osteoblasts. IL-2 treated PBMCs triggered much higher levels of ALP staining when compared to untreated PBMCs (Figs. 5C and 5D). The levels of ALP staining in osteoblasts were substantially lower in the absence of PBMCs and no significant ALP staining can be seen in untreated or IL-2 treated PBMCs in the absence of osteoblasts (Figs. 5C and 5D). These results indicated that stem cells were sensitive to lysis by IL-2 treated PBMCs whereas their differentiated counterparts were more resistant and unlike stem cells they were able to resist death and further upregulate ALP when cultured with IL-2 treated PBMCs. In addition, when the levels of VEGF secretion were determined higher induction of VEGF secretion by MSCs could be observed when compared to osteoblasts (Fig. 5E).

To further demonstrate the resistance of osteoblasts to cell death, MSCs and those differentiated to osteoblasts were also cultured in the absence and presence of different concentrations of HEMA and the levels of cell death were determined after an overnight incubation. Shown in Fig. 5F, HEMA induced significant cell death in undifferentiated MSCs when compared to differentiated osteoblasts. In addition, undifferentiated MSCs were significantly more sensitive to lysis by IL-2 treated NK cells when compared to their differentiated counterparts (Fig. 5G), and triggered significant secretion of IFN-γ in co-cultures with IL-2 treated NK cells (Fig. 5H). Moreover, when MSCs were cultured with NK cells alone or NK cells with monocytes significant induction of B7H1 surface expression could be observed in surviving MSCs (Fig. 5I). Since monocytes increase survival of MSCs, accordingly, more surviving MSCs was observed in co-cultures with NK cells and monocytes than with NK cells alone [205]. Monocytes alone were not able to elevate B7H1 expression on the surface of MSCs

(data not shown). The intensity of NK cell induced B7H1 expression on MSCs were similar to that induced by treatment of MSCs with IFN-γ (Fig. 5J). Thus, these results suggested that sensitivity of MSCs to cell death and to NK cell mediated cytotoxicity correlated with the degree of differentiation of these cells. Moreover, NK cells may contribute to differentiation and resistance of MSCs by increased induction of key resistance factors such as B7H1.

Differentiated DPSCs are more resistant to NK cell mediated cytotoxicity

DPSCs were differentiated to odontoblasts by the addition of b-glycerophosphate, ascorbic acid and dexamethasone as reported previously [198], and NK cell cytotoxicity were determined against both the differentiated and undifferentiated DPSCs. As shown in Fig. 6 significantly less NK cell cytotoxicity as well as IFN-γ secretion could be obtained against differentiated DPSCs by untreated, IL-2 treated and IL-2 plus anti-CD16 mAb treated NK cells when compared to undifferentiated DPSCs. In addition, significantly less NK cell cytotoxicity could be seen against passage 10 undifferentiated DPSCs when compared to passage 3 undifferentiated DPSCs (data not shown). Therefore, a stepwise decrease in NK cell cytotoxicity could be observed depending on the stage of the differentiation of DPSCs.

Decreased sensitivity of dendritic cells to NK cell mediated lysis

To demonstrate that resistance of NK cell mediated cytotoxicity by increased differentiation of stem cells is not restricted to only certain types of cells, we used monocytes and their differentiated counterpart dendritic cells to determine sensitivity to NK cell mediated lysis. As shown in Fig. 7 monocytes were significantly more sensitive to NK cell mediated cytotoxicity than DCs.

IPS cells are more susceptible to NK cell mediated cytotoxicity than their parental line

Since more differentiated cells were less sensitive to NK cell mediated lysis, we aimed at characterizing the sensitivity of iPS cells as well as the parental line from which they were derived to NK cell mediated lysis. As shown in Fig. 8 untreated or IL-2 treated NK cells lysed iPS cells significantly more than the parental line. Treatment of NK cells with anti-CD16 mAb or a combination of IL-2 and anti-CD16 mAb decreased cytotoxicity mediated by the NK cells (Fig. 8). Therefore taken together the results shown thus far suggest that any attempt in re-programming or de-differentiating the cells may result in increased sensitivity of the cells to NK cell mediated lysis. We, therefore, performed additional experiments using mice which had targeted knock down of COX2 gene in myeloid subsets to determine whether blocking COX2 which is shown to be elevated in many tumors and is important in differentiation of the cells can elevate sensitivity to NK cell mediated lysis.

Targeted inhibition of COX2 in bone marrow derived monocytes from *LysMCre+/-* mice increased cytotoxicity and secretion of IFN-γ by IL-2 treated NK cells

Purified NK cells obtained from spleens of control mice and those with targeted knock down of COX2 gene in myeloid cells [206] were cultured with and without bone marrow derived purified monocytes for 6 days before they were added to ^{51}Cr YAC cells and cytotoxicity were determined in 4 hours ^{51}Cr release assay. As shown in Fig. 9A NK cells purified from *Cox-2flox/flox LysMCre/+* mice and cultured with autologous COX2-/- monocytes lysed YAC cells significantly more, whereas NK cells from control mice (*Cox-2flox/flox LysM+/+)* cultured with autologous COX2+/+ monocytes had very little cytotoxicity. Similarly, NK cells purified from

Cox-2flox/flox LysMCre/+ mice and cultured with autologous COX2-/- monocytes secreted higher levels of IFN-γ when compared to NK cells from control mice (*Cox-2flox/flox LysM+/+)* cultured with autologous COX2+/+ monocytes (Fig. 9B).

Discussion

We have characterized the interaction of two primary oral tumors and a transformed but non-tumorigenic oral keratinocyte line with NK cells and identified several important profiles which could distinguish between differentiated NK resistant oral tumors from undifferentiated NK sensitive tumor stem cells. The results also indicated that the levels of NK cell cytotoxicity may vary depending on the expression and function of NFκB in tumors. Thus, increased NFκB appears to be an important factor of differentiation, survival and function of primary oral tumors during their interaction with NK cells.

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed when NK cells were co-incubated with UCLA-OSCSCs which released significantly lower levels of GM-CSF, IL-6 and IL-8 (Tables 1 and 2) and demonstrated decreased expression of phospho-Stat3, B7H1 and EGFR, and much lower constitutive NFκB activity when compared to differentiated UCLA-OSCCs (please see the supplemental data). More importantly, UCLA-OSCSCs expressed CD133 and $CD44^{bright}$ oral stem cell markers (please see the supplemental data). Addition of untreated fresh NK cells to UCLA-OSCCs, which were unable to lyse the tumor cells, synergistically contributed to the elevation of the above mentioned cytokines in the co-cultures of NK cells with UCLA-OSCCs. In contrast, untreated NK cells, which lysed UCLA-OSCSCs, were either unable to increase or moderately increased the secretion of resistant factors in the co-cultures of NK cells with UCLA-OSCSCs. Untreated NK cells increased the secretion of VEGF in NK-UCLA-OSCC co-cultures whereas a decrease in VEGF secretion was observed in NK- UCLA-OSCSCs co-cultures when compared to those secreted by the tumors alone.

Although the majority of secreted cytokines were elevated in UCLA-OSCCs when compared to UCLA-OSCSCs, the levels of VEGF secretion were higher in UCLA-OSCSCs when compared to UCLA-OSCCs. This observation is in agreement with the previously published results demonstrating decreased secretion of VEGF during the progression of head and neck tumors [207].

Increase in IFN-γ secretion was correlated with a decrease in secretion of IL-6 in the co-cultures of NK cells with UCLA-OSCSCs when compared to UCLA-OSCCs. Furthermore, secretion of VEGF by tumor cells was significantly suppressed by the potent cytotoxic function of IL-2 activated NK cells. Therefore, from these results a specific profile for NK resistant oral tumors emerged which demonstrated increased GM-CSF, IL-6 and IL-8 secretion in the context of decreased IFN-γ secretion during their interaction with the NK cells. In contrast, co-cultures of cancer stem cells with NK cells demonstrated increased IFN-γ in the context of lower GM-CSF, IL-6 and IL-8 secretion.

Many aggressive and metastatic tumor cells exhibit constitutively elevated NFκB activity [189]. Similar to HEp2 cells [192] blocking NFκB in UCLA-OSCCs and HOK-16B cells increased IFN-γ secretion and augmented the cytotoxic function of IL-2 activated NK cells against these cells (Fig. 2). Inhibition of NFκB in UCLA-OSCCs and HOK-16B was confirmed by several observations. First, the synergistic induction of ICAM-1 by TNF-α and IFN-γ treatment, which was previously shown to be due to increased function of NFκB [193], was greatly abrogated when UCLA-OSCCs and HOK-16B cells were transduced with IκB super-repressor (data not shown). Second, significant decrease in IL-6 secretion could be observed in

both cells and in the co-cultures of immune effectors with UCLA-OSCCs and HOK-16B cells transduced with IκB super-repressor. Lastly, decreased binding of NFκB was observed using luciferase reporter assay in NFκB knock down cells. Therefore, the profiles of NFκB knock down cells resembled those of undifferentiated UCLA-OSCSCs cells based on a number of parameters tested.

It appears that NFκB in primary oral keratinocytes may serve as the master molecular switch between IL-6 and IFN-γ secretion in the co-cultures of NK cells with tumors. IL-6 is secreted constitutively by oral squamous cell carcinomas [208, 209] and it is found to be elevated in oral cancer patients [208, 210]. IL-6 is known to interfere with IFN-γ signaling by the induction of Th2 differentiation via activation of NFAT which subsequently inhibits Th1 polarization [210, 211]. IL-6 is also known to induce Stat3 activation. Since blocking Stat3 function in tumor cells is also known to activate adaptive immunity [211, 212] it may be that IL-6 induced Stat3 is in part responsible for no/low activation of NK cells and cell death in the co-cultures of NK cells and either HEp2 cells or UCLA-1 or HOK-16B tumors. These possibilities are currently under investigation in our laboratory.

Since UCLA-OSCSCs were significantly more susceptible to NK cell mediated cytotoxicity we hypothesized that healthy, untransformed primary stem cells may in general be more susceptible to NK cell mediated cytotoxicity. We show in this paper that NK cells lyse hMSCs, hDPSCs, hESCs and iPS cells significantly. Taken together these results indicated that undifferentiated cells are targets of NK cell cytotoxicity. However, once NK cells lyse a proportion of sensitive targets they lose their cytotoxic function and gain in the ability to secrete

cytokines (split anergy) that could support differentiation of the cells not lysed by the NK cells. Indeed, similar to NK cells cultured with undifferentiated sensitive tumor stem cells or primary untransformed stem cells, the treatment of NK cells with IL-2 and anti-CD16 mAb resulted in the loss of cytotoxicity, gain in IFN-γ secretion and down modulation of CD16 surface receptors [137, 142]. Loss of cytotoxicity and gain in cytokine secretion was also seen when NK cells were cultured with MSCs and DPSCs in the presence of monocytes (Fig. 4) and [205].

In vivo physiological relevance of above-mentioned observations could be seen in a subpopulation of NK cells in peripheral blood, uterine and liver NK cells which express low or no CD16 receptors, and have decreased capacity to mediate cytotoxicity and is capable of secreting significant amounts of cytokines [213, 214]. In addition, 70% of NK cells become CD16 dim or negative immediately after an allogeneic or autologous bone marrow transplantation [213]. Since NK cells lose their cytotoxic function and gain in cytokine secretion phenotype and down modulate CD16 receptors after their interaction with tumor cells or healthy primary stem cells [137, 142], it is tempting to speculate that in vivo identified CD16- NK cells and in vitro tumor induced CD16- NK cells may have similar developmental pathways since they have similar if not identical functional properties.

Since undifferentiated cells are targets of NK cells, it is logical that NFκB knock down cells are found to be more susceptible to NK cell mediated cytotoxicity since this process may revert the cells to a relatively less differentiated state and be the cause of activation of NK cells. Indeed, any disturbance in the process of differentiation should in theory result in an increase in the sensitivity of the targets to NK cell mediated cytotoxicity since this process is important for

modifying the phenotype of NK cells to cytokine secreting cells in order to support differentiation of the remaining viable competent cells. In this regard knocking down COX2 in monocytes may also result in reversion or de-differentiation of the monocytes and the activation of NK cell cytotoxicity. Thus, the stage of differentiation of the cells is predictive of the susceptibility of the cells to NK cell mediated cytotoxicity. In this regard we have also found higher sensitivity of hiPSCs to NK cell mediated lysis when compared to parental line from which they were derived. In addition, MSCs not only become resistant to NK cell mediated cytotoxicity after differentiation, but also their level of differentiation increases when they are cultured with the NK cells. As shown here co-culture of NK, monocytes and stem cells are found to result in decreased lysis of stem cells, increased secretion of IFN-γ by the NK cells and elevation of B7H1 surface expression on the stem cells (Figs. 4 and 5). Thus, stem cells which survive should exhibit differentiation markers such as increase in NFκB and STAT3 and augmented secretion of GM-CSF, IL-6 and IL-8 after interaction with NK cells and monocytes (Fig. 10).

Based on the results presented in this paper it is tempting to speculate that NK cells may have two significant functions; one that relates to the removal of stem cells that are either defective or disturbed or in general more in numbers than are required for the regeneration of damaged tissue. Therefore, the first task of NK cells is to select stem cells that are competent and are able to fully differentiate to required tissues. The second important task of NK cells is to support the differentiation of the selected cells after altering the NK phenotype to cytokine secreting cells. This process will not only remove cells that are either infected or transformed, but also it will ensure the regeneration of damaged or defective tissues. Therefore, processes in

which suboptimal differentiation and regeneration of the tissues are obtained, a chronic inflammatory process may be established causing continual tissue damage and recruitment of stem cells and NK cells. Indeed, a generalized inflammatory condition in patients with Nemo mutations has already been described previously, and mice with the knockdown of NFκB develop skin pathologies similar to that of inflammatory skin disease [153, 215].

The inability of patient NK cells to kill cancer stem cells due to flooding of NK cells by proliferating cancer stem cells and conversion of NK cells to cytokine secreting cells may likely be a mechanism by which cancer cells remain viable and metastasize. Therefore, there should be two distinct strategies by the NK cells to eliminate tumors, one which targets stem cells and the other which targets differentiated cells. In theory this should be achieved in oral cancer patients by the use of EGFR antibody since this antibody should target the differentiated oral tumors whereas stem cells should be eliminated by the activated NK cells. However, since the great majority of patient NK cells have modified their phenotype to support differentiation of the cells, they may not be effective in eliminating cancer stem cells. Therefore, cancer stem cells may accumulate and eventually result in the demise of the patient. These patients may therefore, benefit from the repeated allogeneic NK cell transplantation for elimination of cancer stem cells.

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Table 1. UCLA-OSCSCs similar to HEp2-IκB$_{(S32AS36A)}$ tumor cells secreted no or lower levels of GM-CSF, IL-6 and IL-8.

	GM-CSF pg/ml (MFI*)	IL-6 pg/ml (MFI)	IL-8 pg/ml (MFI)
HEp2-vec	0±0 (30)	20.6±1 (565)	685±20 (1390)
HEp2-IκB$_{(S32AS36A)}$	0±0 (29)	1.5±0 (67)	17±0 (453)
UCLA-OSCCs	19.8±2 (79)	58.4±3 (1554)	906.3±50 (7583)
UCLA-OSCSCs	0±0 (32)	0±0 (11)	245.2±12 (3247)

HEp2-vec, HEp2-IκB$_{(S32AS36A)}$, UCLA-OSCCs, and UCLA-OSCSCs were cultured at 1X10^5 cells/ ml and the constitutive levels of secreted GM-CSF, IL-6, and IL-8 were determined using multiplex ELISA array kit. The concentrations of secreted cytokines were determined using the standard curve for each cytokine. * Mean fluorescence intensity (MFI). One of three representative experiments is shown.

Table 2. Increased ratios of IL-6 to IFN-γ secretion in NK resistant UCLA-OSCCs when compared to NK sensitive UCLA-OSCSCs

Tumor cells	+/- Immune cells	GM-CSF pg/ml	IL-8 pg/ml	VEGF pg/ml	IL-6 pg/ml	IFN-γ pg/ml	Ratio IL-6/IFN-γ
UCLA-OSCCs	- NK	20	438.4	620.4	126	0.8	-
UCLA-OSCCs	+ NK (-IL-2)	148.8	723.2	784	215	1	215
UCLA-OSCCs	+ NK (+IL-2)	565.8	282.2	145.5	179	820	0.22
UCLA-OSCSCs	- NK	0.1	23.3	1745	13	1	-
UCLA-OSCSCs	+ NK (-IL-2)	25	66.7	1256	65	1	12.5
UCLA-OSCSCs	+ NK (+IL-2)	1068.9	12.5	158	12	1730.6	0.007
No tumors	+ NK (-IL-2)	0.8	0	0.4	11	0.6	18
No tumors	+ NK (+IL-2)	403.2	3.14	8.6	13	290	0.44

NK cells ($1X10^6$/ml) were left untreated or treated with IL-2 (1000 units/ml) for 12-24 hours before NK cells ($1X10^5$/ml) were added to primary oral tumors at an effector to target ratio of 1:1. Tumor cells were each cultured alone or in combination with NK cells as indicated in the table and the supernatants were removed from the cultures after an overnight incubation. The levels of cytokine secretion were determined using antibody coated multiplex microbead immunoassay. For simplification of the table standard

deviations are not included and they ranged from 0% to a maximum of 5% of the amount obtained for each cytokine. One of three representative experiments is shown.

Figure Legends

Fig. 1. Increased NK cell cytotoxicity against UCLA-OSCSCs

PBMCs and NK cells were left untreated or treated with IL-2 (1000 units/ml) or anti-CD16 mAb (3µg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3µg/ml) for 12-24 hours before they were added to ^{51}Cr labeled primary oral tumors. PBMC (A) and NK cell (B) cytotoxicity was determined using a standard ^{51}Cr release assay and the lytic units $30/10^6$ were calculated using inverse number of effectors required to lyse 30% of the tumor cells X 100. Differences between untreated, anti-CD16 mAb treated or IL-2 and/or anti-CD16 mAb treated NK cell killing between UCLA-OSCCs and UCLA-OSCSCs were significant at a p value of <0.05. One of four representative experiments is shown in this figure.

Fig. 2. Increased cytotoxicity, decreased secretion of IL-6 and increased secretion of IFN-γ in co-cultures of NK cells with NFκB knock down UCLA-OSCCs and HOK-16B cells

IκB $_{(S32AS36A)}$ transduced UCLA-OSCCs (A) and IκBαM transduced HOK-16B cells (B) and their EGFP transduced controls were transfected with 8 µg of NFκB Luciferase reporter vector and treated with and without TNF-α (20ng/ml) for 18 hours. The relative Luciferase activity was then determined in the lysates according to the manufacturer's recommendation and fold induction in luciferase activity was determined relative to untreated cells. IκB $_{(S32AS36A)}$ transduced UCLA-OSCCs (C) and IκBαM transduced HOK-16B cells (D) and their EGFP transduced controls were cultured at

$2X10^5$ cells/ml, and after an overnight incubation the supernatants were collected and the levels of secreted IL-6 were determined using ELISA specific for IL-6. IκB $_{(S32AS36A)}$ transduced UCLA-OSCCs and IκBαM transduced HOK-16B cells and their EGFP transduced controls were co-cultured with untreated or IL-2 (1000 u/ml) treated NK cells at 1:1 effector to target ratio. After an overnight incubation the supernatants from the co-cultures of UCLA-OSCCs and HOK-16B cells with NK cells were collected and the levels of secreted IL-6 (E and F), and IFN-γ (G and H) were determined by specific ELISAs for each cytokine. NK cells were left untreated or treated with IL-2 for 12-24 hours before they were added to IκB $_{(S32AS36A)}$ transduced UCLA-OSCCs and IκBαM transduced HOK-16B cells and their EGFP transduced controls. Differences between EGFP transduced and those with either IκB $_{(S32AS36A)}$ transduced UCLA-OSCCs or IκBαM transduced HOK-16B cells were significant for IL-2 treated NK cells at a p value of <0.05. IκB $_{(S32AS36A)}$ transduced UCLA-OSCCs and IκBαM transduced HOK-16B cells and their EGFP transduced controls were ^{51}Cr labeled before they were co-cultured with untreated or IL-2 (1000 u/ml) treated NK cells. After 4 hours of incubation at 37C cytotoxicity of NK cells were assessed using a standard ^{51}Cr release assay (I and J). lytic unit $30/10^6$ cells were determined using inverse number of effectors required to lyse 30% of the tumor cells X 100. Differences between IκB $_{(S32AS36A)}$ transduced UCLA-OSCCs or IκBαM transduced HOK-16B cells and those with EGFP transduced were significant in IL-2 treated PBMCs at a p value of <0.05. One of three representative experiments is shown in this figure.

Fig. 3. Lysis of hESCs, hiPSCs, hDPSCs and hMSCs by untreated and IL-2 treated NK cells is inhibited by anti-CD16 antibody treatment, however, the same treatment induced significant secretion of IFN-γ by the NK cells

NK cells ($1X10^6$/ml) were left untreated or treated with IL-2 (1000 units/ml), or anti-CD16 mAb (3μg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3μg/ml) for 12-24 hours before they were added to ^{51}Cr labeled hESCs, hiPSCs, hDPSCs and hMSCs. NK cell cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay, and the lytic units $30/10^6$ were determined using inverse number of NK cells required to lyse 30% of hESCs (A), hiPSCs (D), hDPSCs (G) and hMSCs (J) X 100. NK cells were treated as described above and each NK sample at ($1X10^5$/ml) were either cultured in the absence or presence of hESCs, hiPSCs, hDPSCs and hMSCs at an NK to stem cell ratio of 1:1. After an overnight culture, supernatants were removed from the co-cultures and the levels of IFN-γ (B,E,H,K), and bFGF (C,F,I,L) secretion were determined using specific ELISAs. One of a minimum three representative experiments for each stem cell population is shown in this figure.

Fig. 4. Monocytes decrease the lysis of hMSCs by the NK cells, but significantly augment the secretion of IFN-γ in the co-cultures of NK, monocyte and hMSCs

MSCs ($1X10^6$ cells/plate) were cultured with the irradiated monocytes (10 Gy) (monocyte: MSC ratio of 1:1) for 24-48 hours before they were removed from the plates, washed and labeled with ^{51}Cr and used as targets in the cytotoxicity assays against NK cells. The NK samples were either left untreated or treated with anti-CD16 mAb (3μg/ml), IL-2 (1000 u/ml), or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb

(3μg/ml) for 24-48 hours before they were added to ^{51}Cr labeled MSCs at different effector to target (E:T) ratios. Supernatants were removed after 4 hours of incubation and the released radioactivity counted by a γ counter. % cytotoxicity was determined at different E:T ratio, and $LU_{30}/10^6$ cells were calculated using the inverse of the number of effectors needed to lyse 30% of the MSCs X100.. One of three representative experiments is shown in this figure (A). MSCs ($1X10^5$ cells/well) were co-cultured with and without irradiated Monocytes at 1:1 MSCs to monocytes for 24-48 hours before untreated or IL-2 (1000 u/ml) pre-treated or anti-CD16 mAb (3μg/ml) pre-treated, or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3μg/ml) pre-treated NK cells at 1:1:1 NK:monocyte:MSC ratios were added. NK cells were pre-treated as indicated for 24-48 hours before they were added to the co-cultures of monocytes and MSCs. NK samples were also cultured in the absence of monocytes and MSCs. After 24-48 hours of the addition of NK cells the supernatants were removed from the cultures and the levels IFN-γ were determined using ELISA. One of five representative experiments is shown in this figure (B).

Fig. 5. MSCs are significantly more sensitive to lysis by IL-2 treated NK cells than their differentiated counterparts and they trigger significant release of IFN-γ by IL-2 activated NK cells

hMSCs were seeded at 3 to $4X10^5$ cells per well in Stem cell medium in the presence and absence of untreated PBMCs or IL-2 (1000u/ml) treated PBMCs (PBMC to Stem cell ratio 10:1). After 2 days of co-cultures, Alkaline Phosphatase staining was performed. A1 to C1 (triplicates of hMSCs in the absence of PBMCs), A2 to C2 (hMSC

in the presence of untreated PBMCs), A3 to C3 (MSC in the presence of IL-2 treated PBMCs), A4 (naïve PBMCs alone), B4 (IL-2 treated PBMCs alone) (A). The ALP stain densities for each well were determined using photoshop software (B). hMSCs were cultured in differentiation medium for 1 week and differentiated Osteoblasts were then seeded at 3 to $4X10^5$ cells per well in the presence and absence of untreated PBMCs and IL-2 (1000u/ml) treated PBMCs (PBMC to Stem cell ratio 10:1). After 2 days of co-cultures Alkaline Phosphatase staining was performed. A1 to C1 (triplicates of Ostoblastic cells in the absence of PBMCs), A2 to C2 (Ostoblastic cells in the presence of untreated PBMCs), A3 to C3 (Ostoblastic cells in the presence of IL-2 treated PBMCs), A4 (untreated PBMCs alone), B4 (IL-2 treated PBMCs alone) (C). The ALP stain densities for each well were determined using photoshop software (D). hMSCs and Osteoblasts were cultured with and without untreated PBMCs as described above and after two days of incubation the supernatants were removed and subjected to specific ELISA for VEGF (E). NK cells ($1X10^6$/ml) were left untreated or treated with IL-2 (1000 units/ml), or anti-CD16 mAb (3μg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3μg/ml) for 12-24 hours before they were added to ^{51}Cr labeled MSCs or osteoblasts, and NK cell cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay, and the lytic units $30/10^6$ were determined using inverse number of NK cells required to lyse 30% of the MSCs or osteoblasts X 100 (F). Undifferentiated MSCs and those differentiated to osteoblasts at ($1X10^5$/ml) were cultured in the absence and presence of untreated NK cells or IL-2 treated NK cells at 1:1 ratio, and after two days of incubation the supernatants were removed and subjected to specific ELISA for IFN-γ (G). MSCs at ($1X10^5$/ml) were either cultured with untreated NK cells or IL-2 treated NK

cells alone (1:1; MSC:NK) or with untreated NK and IL-2 treated NK cells with monocytes at (1:1:1; MSC:NK:monocytes). After an overnight incubation, the cells were washed and B7H1 surface expression was determined on MSC gated populations. Isotype control antibodies were used as controls (H). MSCs were left untreated or treated with IFN-γ (500 u/ml). After an overnight incubation, MSCs were washed and the B7H1 surface expression was determined on MSC (I).

Fig. 6. Undifferentiated DPSCs are significantly more sensitive to lysis by IL-2 treated NK cells and trigger increased secretion of IFN-γ from the NK cells than their differentiated counterparts

NK cells ($1X10^6$/ml) were left untreated or treated with IL-2 (1000 units/ml), or anti-CD16 mAb (3μg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3μg/ml) for 12-24 hours before they were added to ^{51}Cr labeled undifferentiated and differentiated DPSCs, and NK cell cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay. Lytic units $30/10^6$ were determined using inverse number of NK cells required to lyse 30% of the DPSCs X 100. Passage 8 differentiated and undifferentiated DPSCs were used (A). Undifferentiated DPSCs and those differentiated to odontoblasts at ($1X10^5$/ml) were cultured with and without untreated NK cells or IL-2 (1000 units/ml), or anti-CD16 mAb (3μg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3μg/ml) treated NK cells at 1:1 ratio and after two days of incubation the supernatants were removed and subjected to specific ELISA for IFN-γ (B).

Fig. 7. Monocytes are significantly more sensitive to NK cell mediated cytotoxicity than DCs

NK cells ($1X10^6$/ml) were left untreated or treated with IL-2 (1000 units/ml), or anti-CD16 mAb (3μg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3μg/ml) for 12-24 hours before they were added to ^{51}Cr labeled autologous monocytes or ^{51}Cr labeled autologous DCs, and NK cell cytotoxicity were determined using a standard 4 hour ^{51}Cr release assay and the lytic units $30/10^6$ were determined using inverse number of NK cells required to lyse 30% of the monocytes or DCs X 100. One of four representative experiments is shown in this figure.

Fig. 8. iPSCs are more susceptible to NK cell mediated cytotoxicity than their parental line

NK cells ($1X10^6$/ml) were left untreated or treated with IL-2 (1000 units/ml), or anti-CD16 mAb (3μg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3μg/ml) for 12-24 hours before they were added to ^{51}Cr labeled iPSCs or ^{51}Cr labeled parental cells from which the iPSCs were derived, and NK cell cytotoxicity were determined using a standard 4 hour ^{51}Cr release assay and the lytic units $30/10^6$ were determined using inverse number of NK cells required to lyse 30% of the iPSCs or parental cells X 100.

Fig. 9. Targeted inhibition of COX2 in bone marrow monocytes increased NK cell cytotoxicity and secretion of IFN-γ by IL-2 treated NK cells

Purified NK cells and monocytes were obtained from spleens and bone marrows of 3 pooled control mice and those with targeted knock down of COX2 gene in myeloid

cells respectively (n=3). Purified NK cells and monocytes from control mice and those with targeted knock down of COX2 gene in myeloid cells were then cultured with IL-2 (1000u/ml) at 1:1 NK: monocyte ratios for 6 days before they were added to ^{51}Cr labeled YAC cells, and NK cell cytotoxicity was determined in 4 hour ^{51}Cr release assay. The lytic units $30/10^6$ were determined using inverse number of NK cells required to lyse 30% of the YAC cells X 100 (A). NK cells were cultured as described in Fig. 9A and after 6 days of incubation the supernatants were removed and IFN-γ secretion were measured in the supernatants using a specific ELISA (B). One of five representative experiments is shown in this figure.

Fig. 10. Schematic representation of hypothetical model of oral cancer stem cell differentiation by NK cells and monocytes

Interaction of cancer stem cells or primary stem cells with monocytes and NK cells results in the loss of NK cell cytotoxicity due partly to the induction of resistance of cancer stem cells by monocytes and indirectly by monocytes serving as targets of NK cells [205], thus serving as a shield which protects the stem cells from lysis by the NK cells. Loss of NK cell cytotoxicity by monocytes and gain in secretion of IFN-γ results in a significant induction of transcription factors, cytokines and growth factors in stem cells and differentiation of stem cells.

Fig. 1

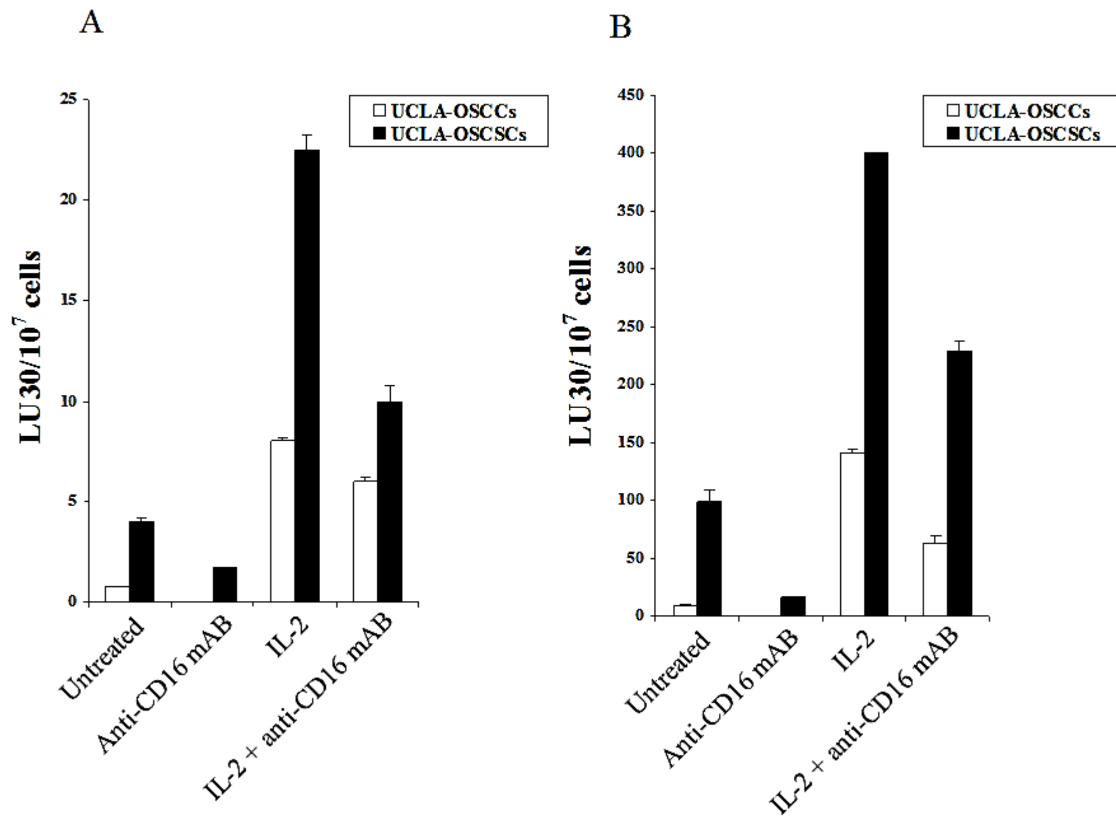
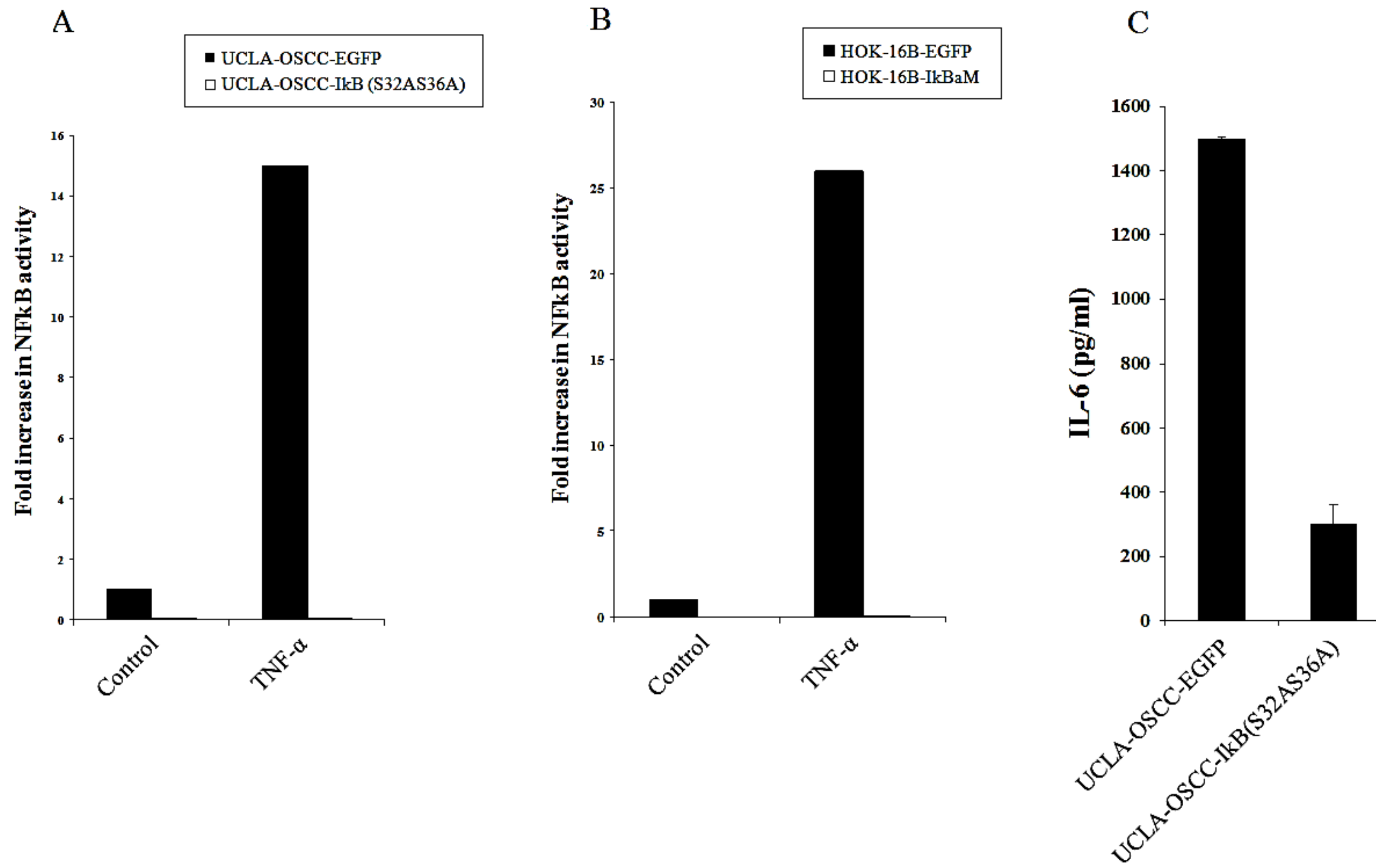
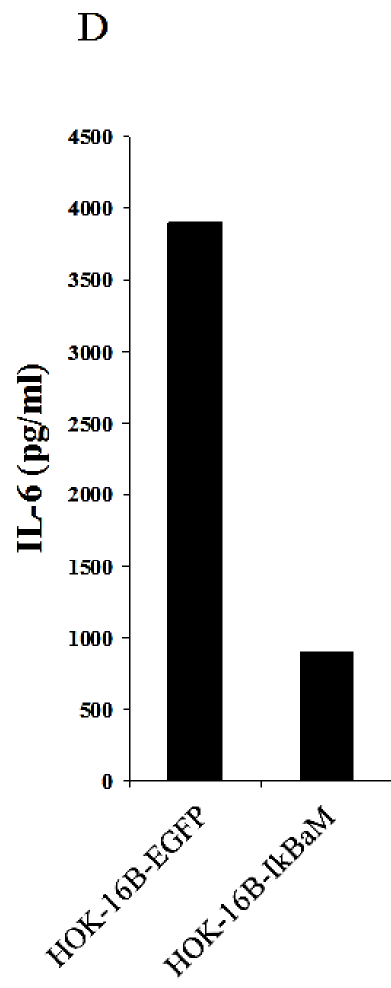


Fig. 2

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3500
3000
2500
2000
1500
1000
500
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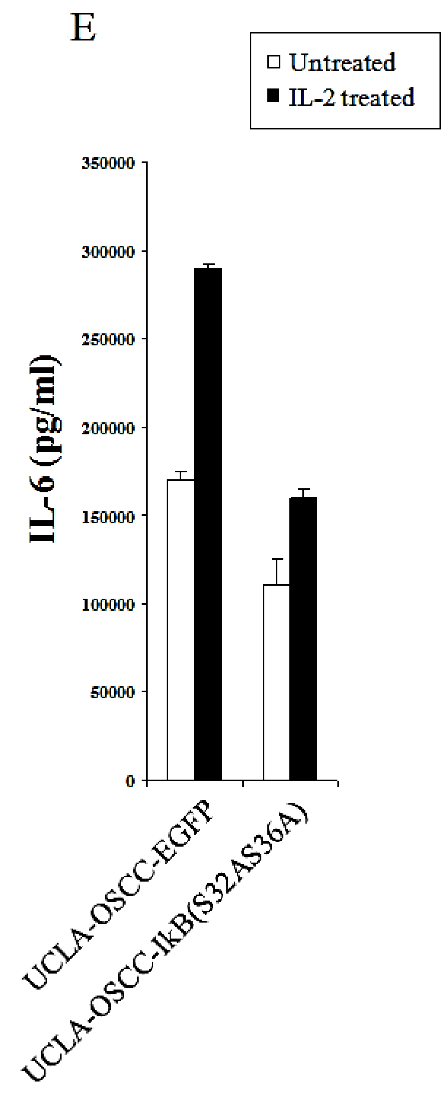
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IL-2 treated
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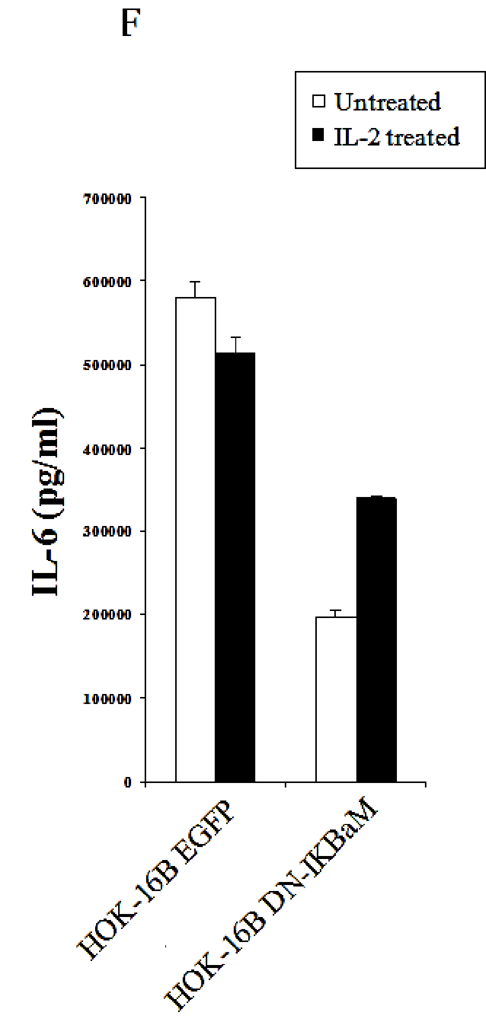
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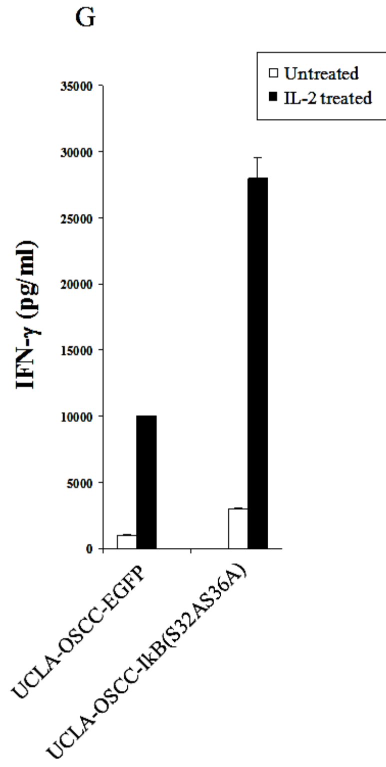

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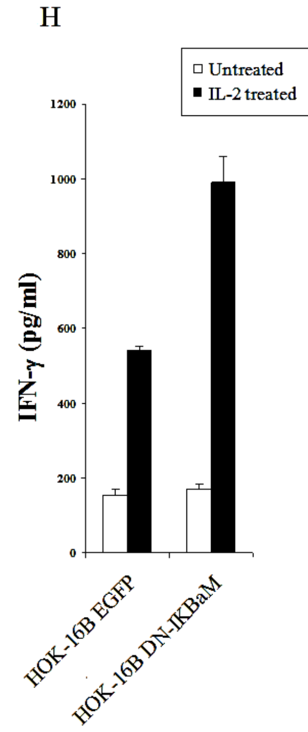

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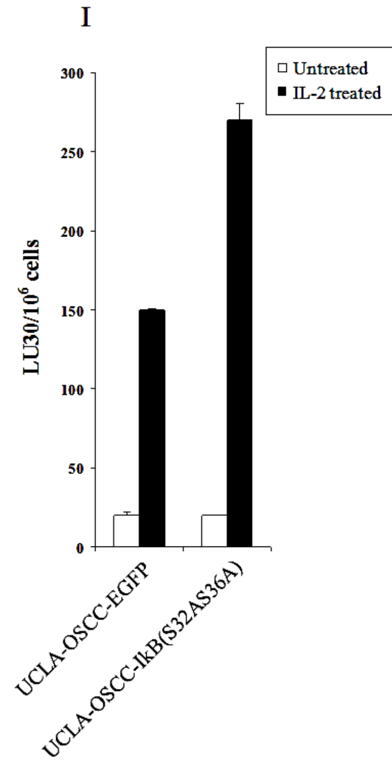

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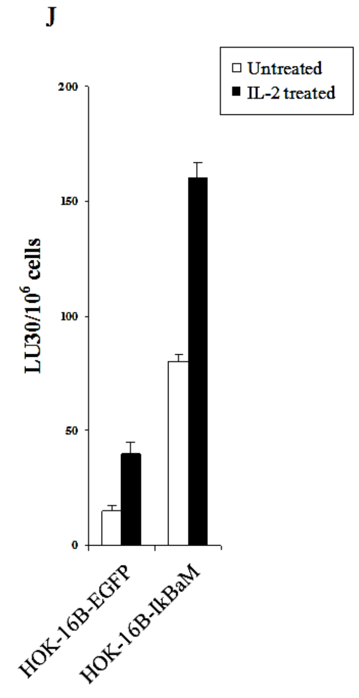
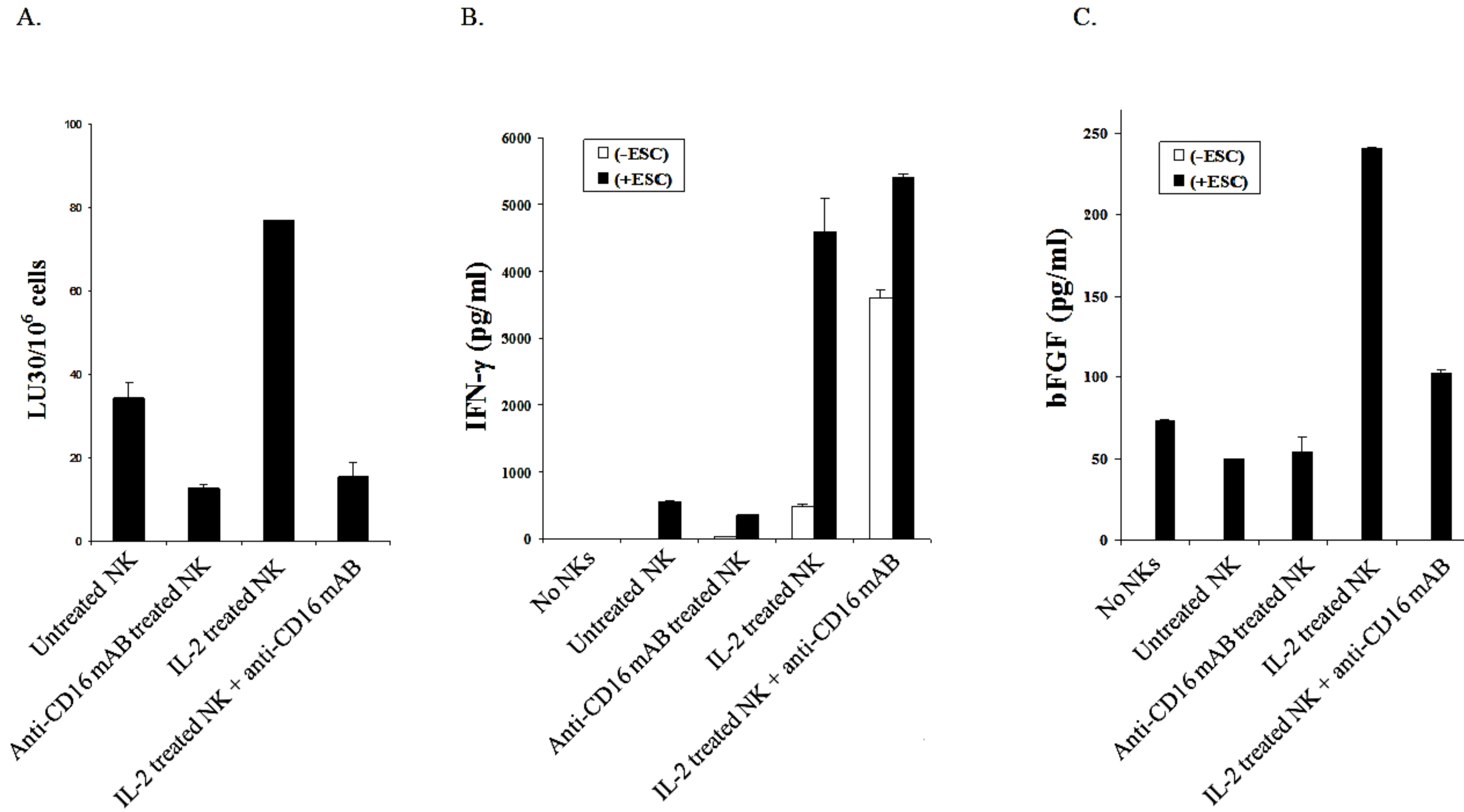


Fig. 3



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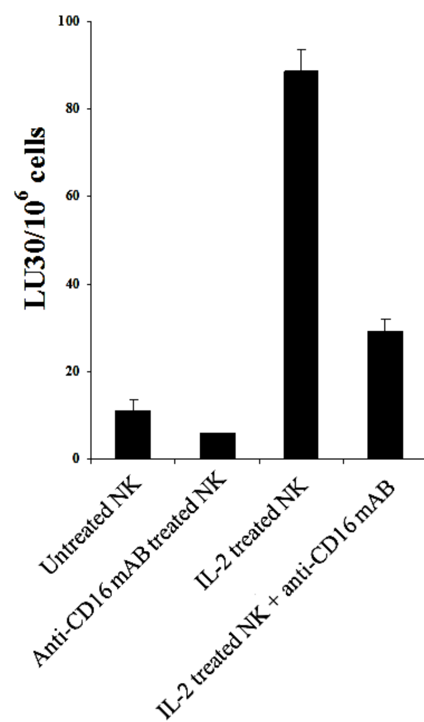

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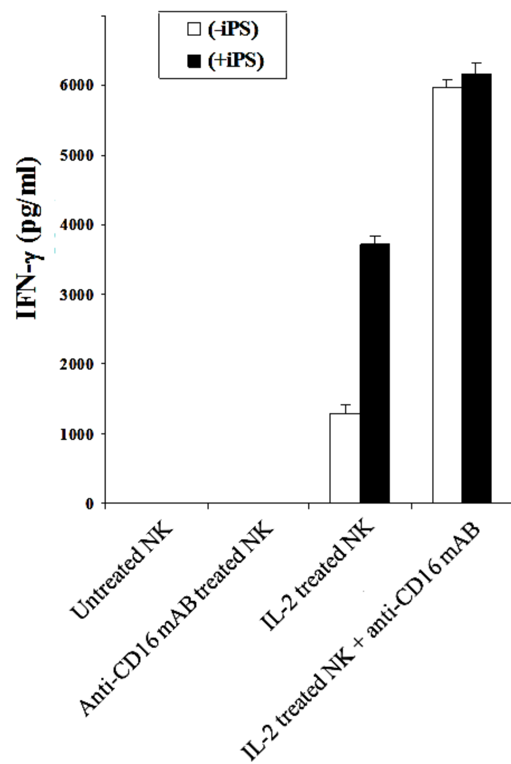

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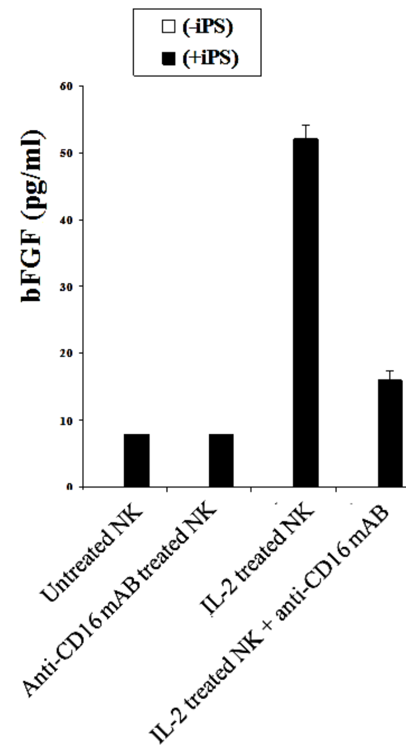

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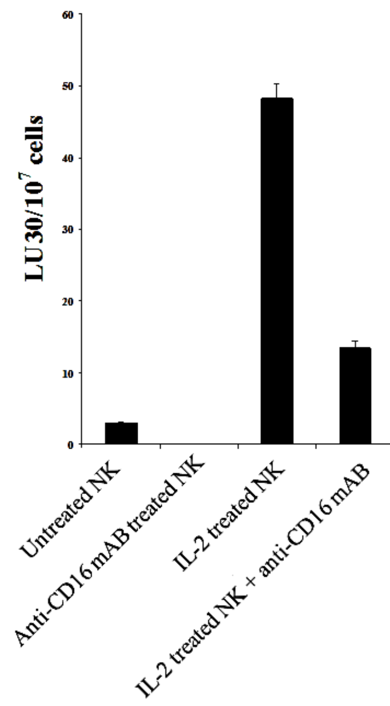

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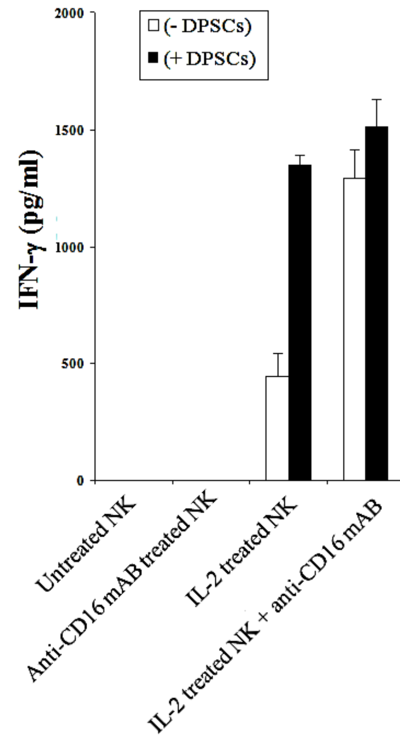

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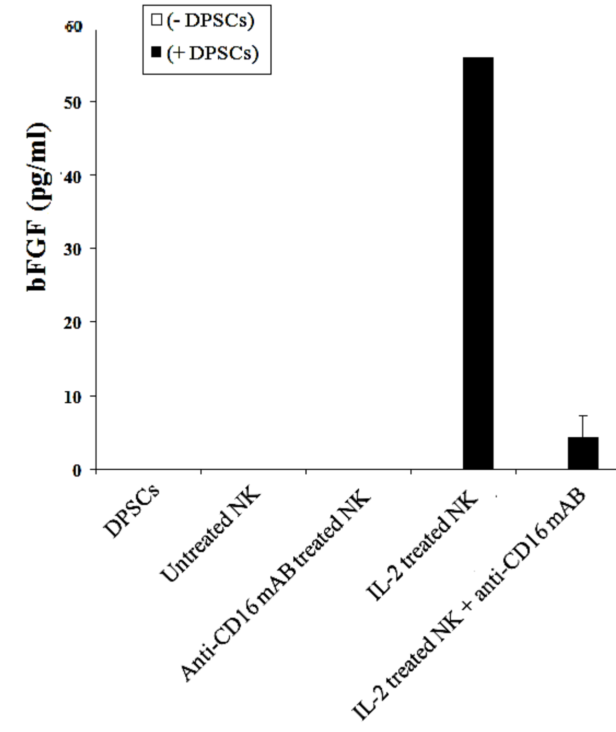

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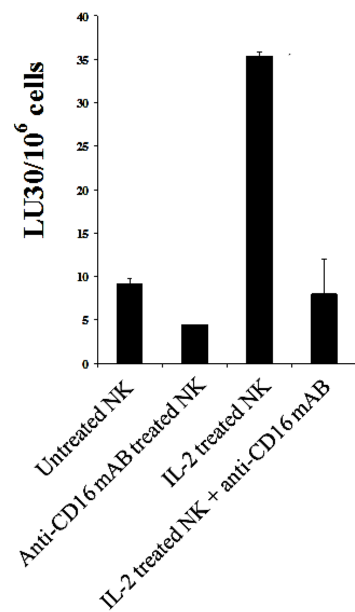

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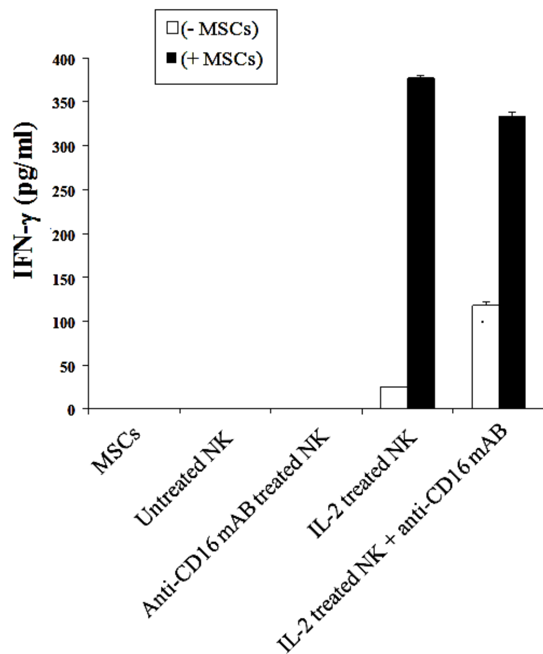

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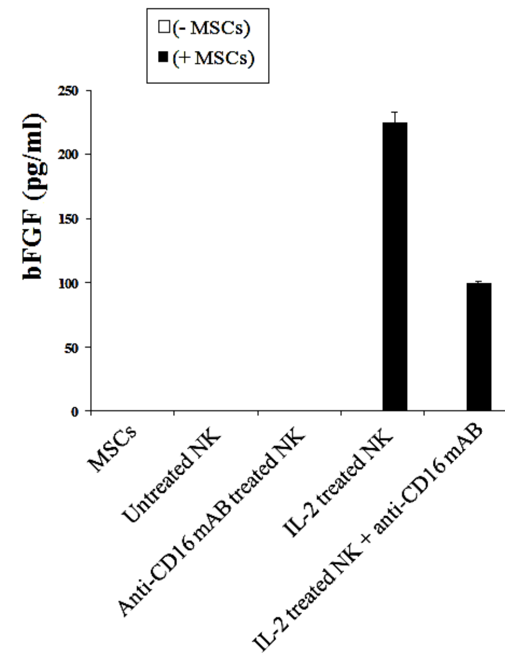


Fig. 4

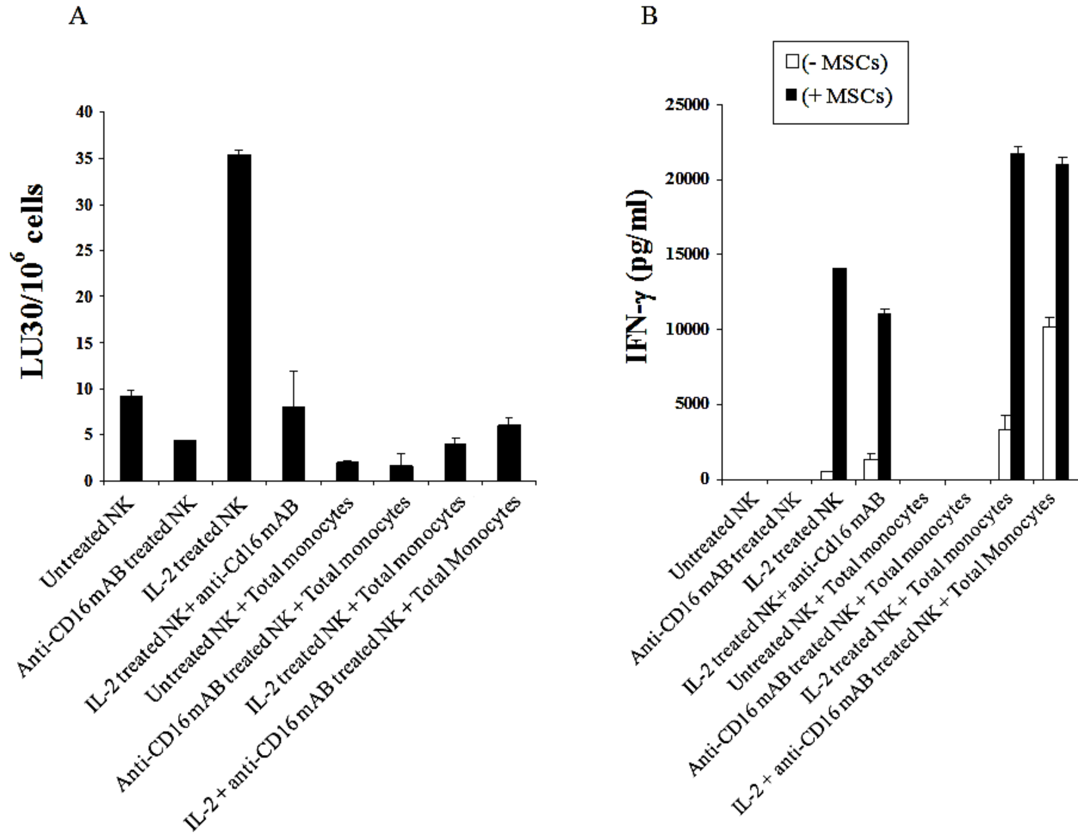
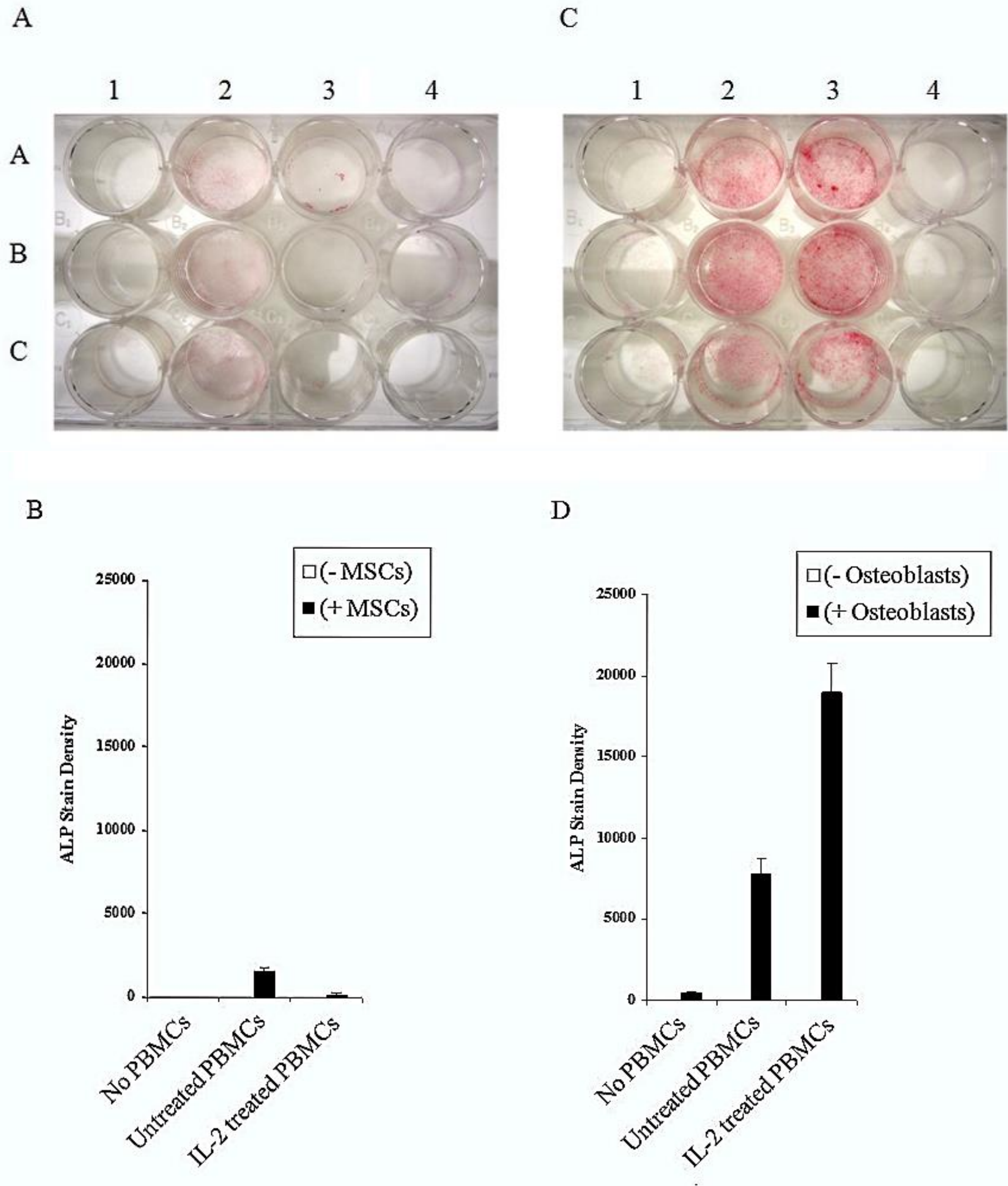
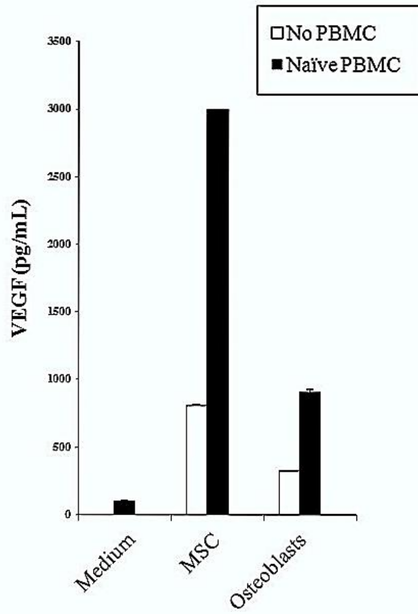


Fig. 5

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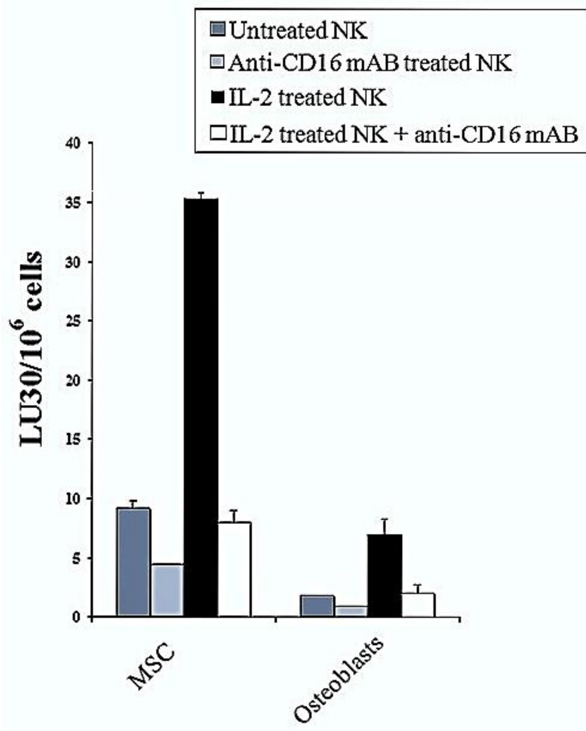
2500
2000
1500
1000
500
0
Medium
MSC
Osteoblasts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Anti-CD16 mAB treated NK
IL-2 treated NK
IL-2 treated NK + anti-CD16 mAB
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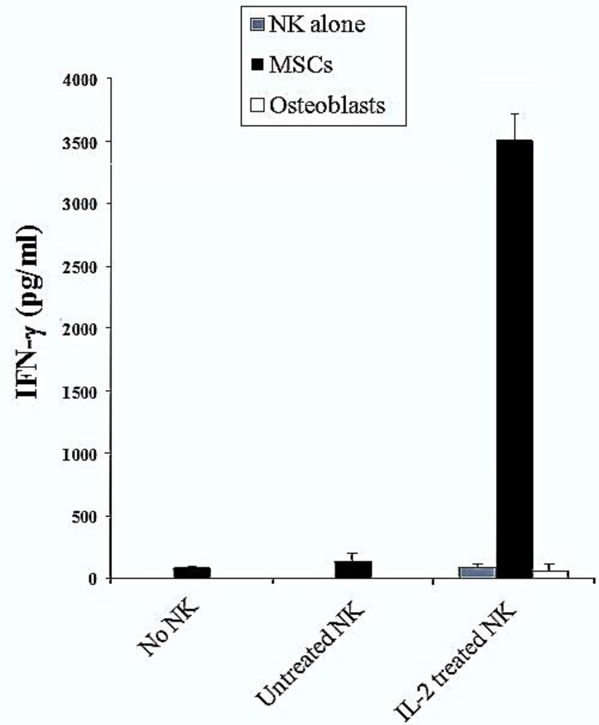
35
30
25
20
15
10
5
0
MSC
Osteoblasts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3000
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1000
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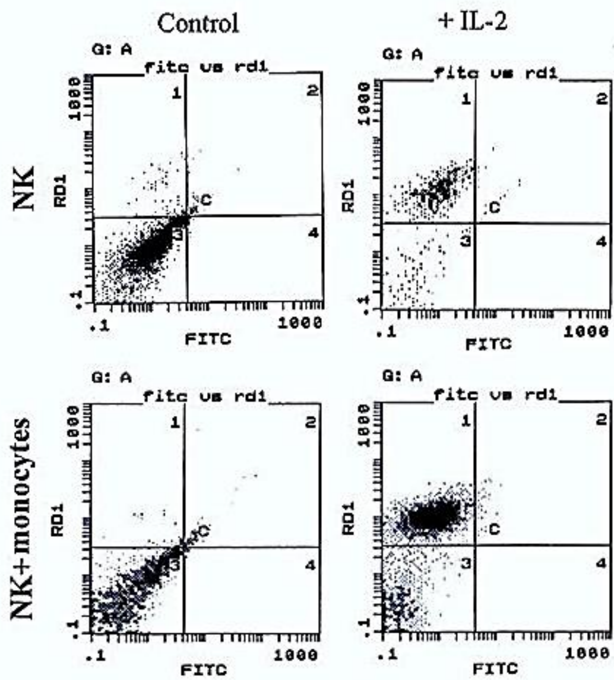
Untreated NK
IL-2 treated NK

H



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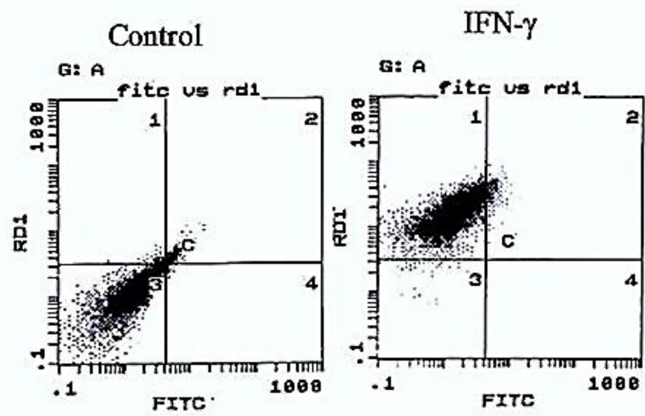


Fig. 6

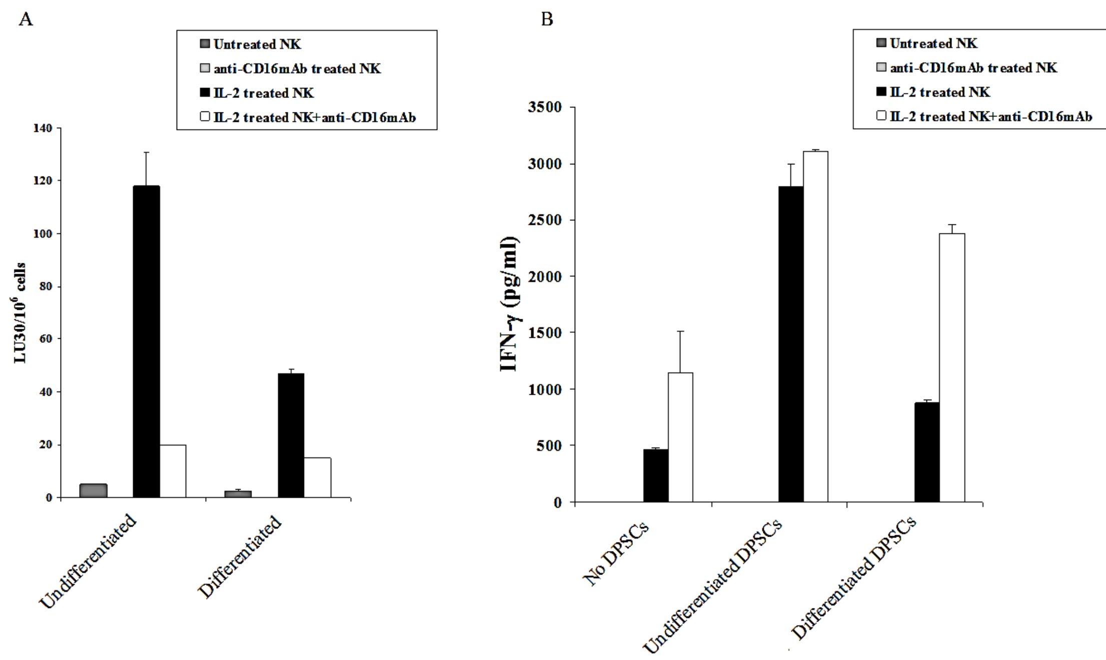


Fig. 7

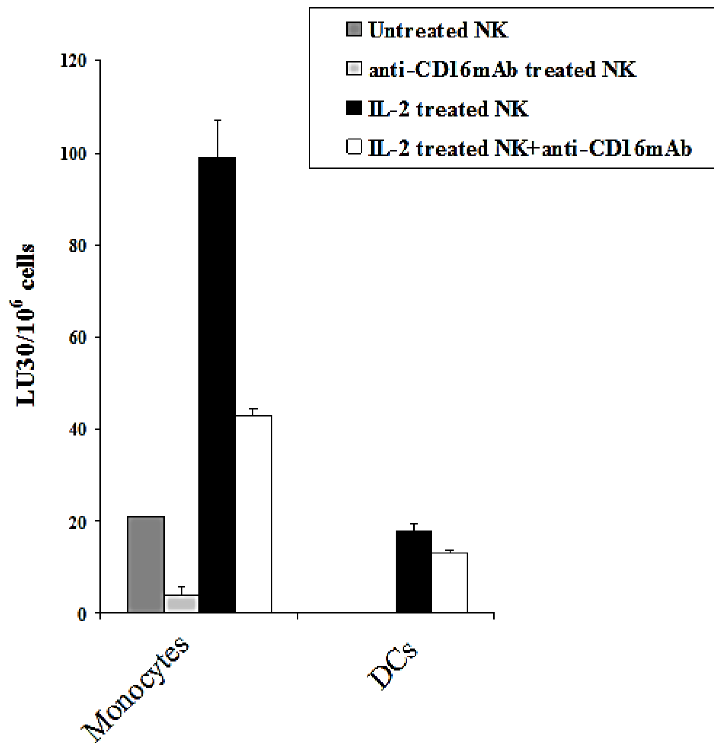


Fig. 8

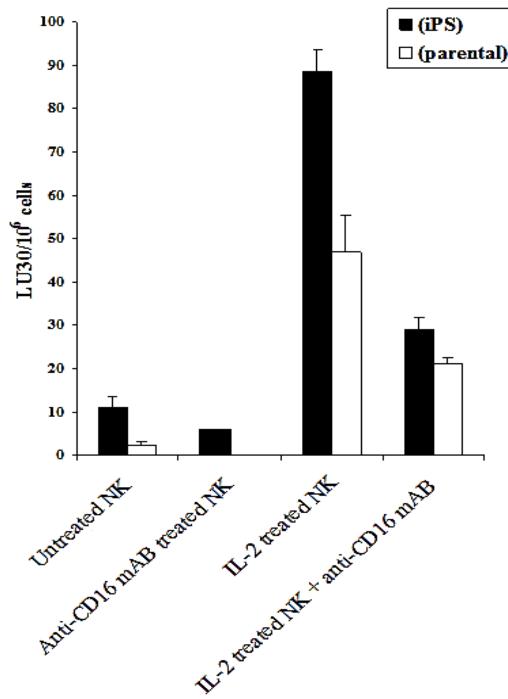


Fig. 9

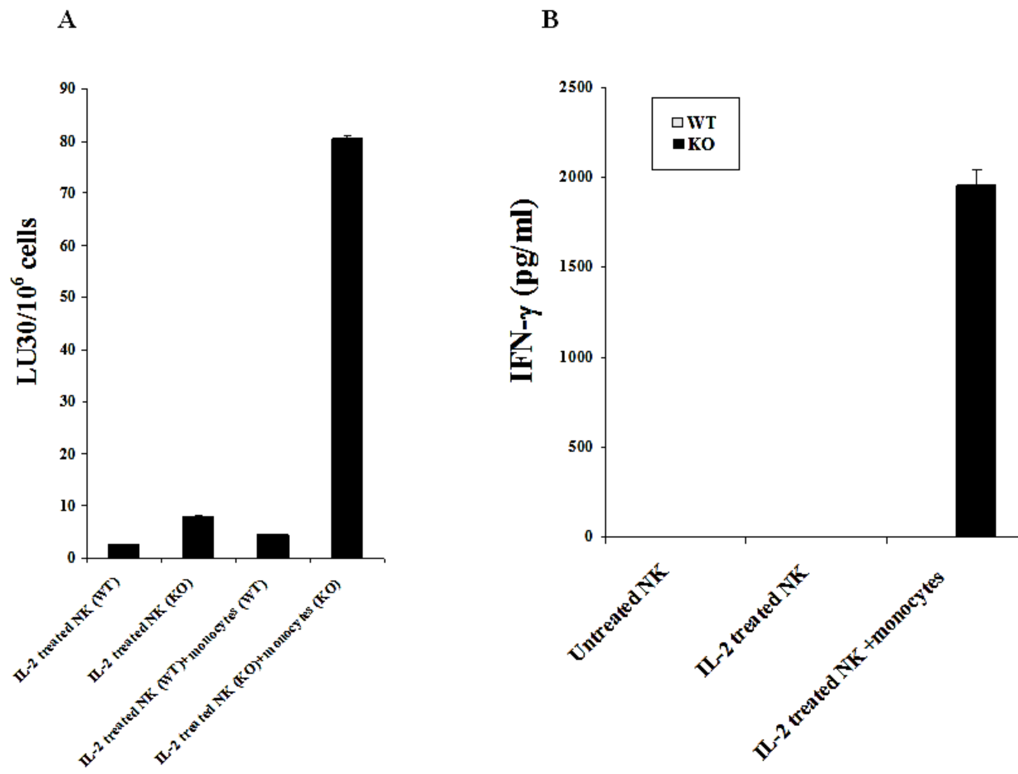
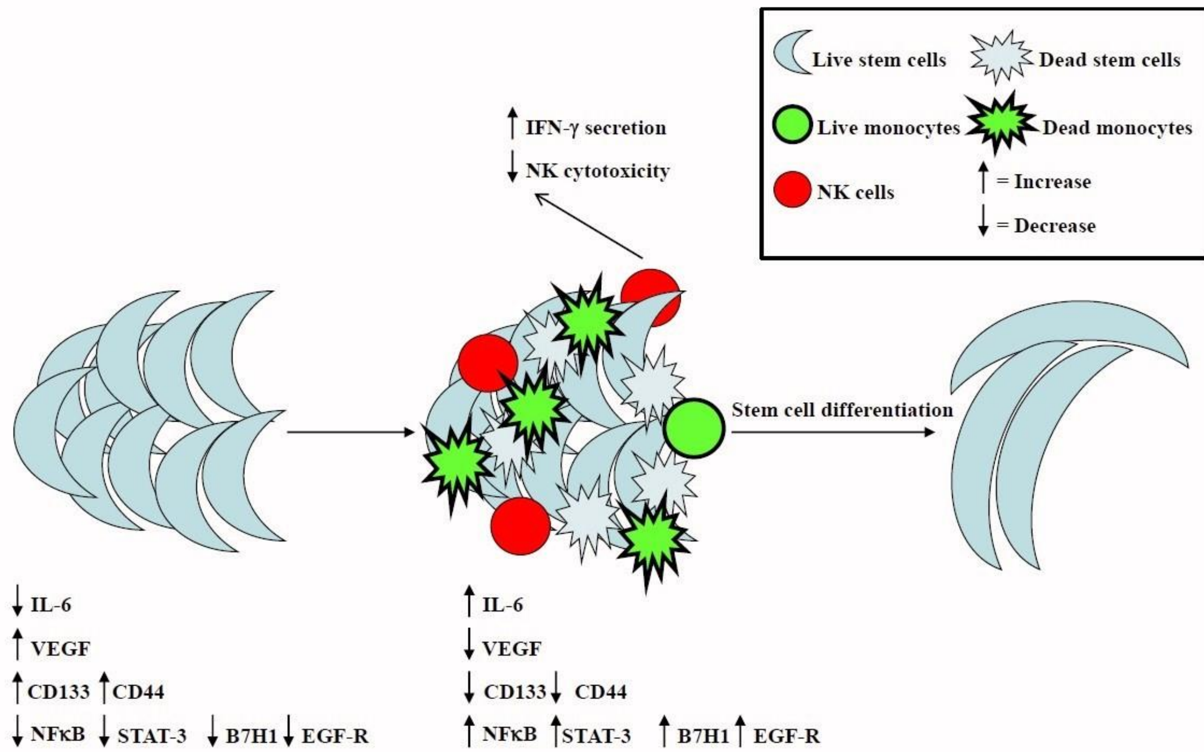


Fig. 10



CHAPTER 2

Strategies to rescue Mesenchymal Stem Cells (MSCs) and Dental Pulp Stem Cells (DPSCs) from NK cell mediated cytotoxicity

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Running title: Lysis of stem cells by NK cells

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Abstract

Background: The aim of this paper is to study the function of allogeneic and autologous NK cells against Dental Pulp Stem Cells (DPSCs) and Mesenchymal Stem Cells (MSCs) and to determine the function of NK cells in a three way interaction with monocytes and stem cells.

Methodology/Principal Findings: We demonstrate here that freshly isolated untreated or IL-2 treated NK cells are potent inducers of cell death in DPSCs and MSCs, and that anti-CD16 antibody which induces functional split anergy and apoptosis in NK cells inhibits NK cell mediated lysis of DPSCs and MSCs. Monocytes co-cultured with either DPSCs or MSCs decrease lysis of stem cells by untreated or IL-2 treated NK cells. Monocytes also prevent NK cell apoptosis thereby raising the overall survival and function of NK cells, DPSCs or MSCs. Both total population of monocytes and those depleted of $CD16^+$ subsets were able to prevent NK cell mediated lysis of MSCs and DPSCs, and to trigger an increased secretion of IFN-γ by IL-2 treated NK cells. Protection of stem cells from NK cell mediated lysis was also seen when monocytes were sorted out from stem cells before they were added to NK cells. However, this effect was not specific to monocytes since the addition of T and B cells to stem cells also protected stem cells from NK cell mediated lysis. NK cells were found to lyse monocytes, as well as T and B cells.

Conclusion/Significance: By increasing the release of IFN-γ and decreasing the cytotoxic function of NK cells monocytes are able to shield stem cells from killing by the NK cells, resulting in an increased protection and differentiation of stem cells. More importantly studies

reported in this paper indicate that anti-CD16 antibody can be used to prevent NK cell induced rejection of stem cells.

Introduction

Previous reports demonstrated significant immunomodulatory effect of Mesenchymal Stem Cells (MSCs) on different immune effector subsets such as cytotoxic T cells and Natural Killer (NK) cells [185, 186, 216-218]. Several reports have also indicated the immunosuppressive nature of MSCs on NK cells [186, 187, 219]. Moreover, activated NK cells were shown to lyse MSCs significantly. In particular, both allogeneic and autologous MSCs were shown to be targets of NK cell mediated lysis [220, 221]. Therefore, on one hand the MSCs were shown to be immunosuppressive and on the other they were found to be sensitive to lysis by the NK cells. Thus, these diverse results prompted us to evaluate the function of NK cells against two different types of stem cells, namely DPSCs and MSCs. Although some information is known about the function of NK cells against MSCs, no studies have been performed thus far to determine the effect of NK cells on DPSCs. DPSCs were shown to form colonies in vitro, and they were capable of osteogenic, chondrogenic and adipogenic differentiation [222]. In order to find ways to protect stem cells from lysis by the NK cells we need to first determine the magnitude and the mechanisms by which NK cells lyse stem cells and second to implement strategies based on those findings to protect stem cells from NK cell lysis.

Many diverse mechanisms were hypothesized to mediate the immunosuppressive effect of MSCs. TGFβ and Hepatocyte Growth Factor (HGF) were both shown to be the mediator of T cell suppression by MSCs [223]. Leukemia Inhibitory Factor induced in co-cultures of MSCs and lymphocytes was shown to mediate suppression of T cell proliferation [224]. IFN-γ induced by T cells increased B7H1 inhibitory co-stimulatory receptors on MSCs and resulted in the suppression of T cells [225]. Immunosuppressive function of MSCs is elicited by the

combination of IFN-γ, TNF-α, IL-1α and IL-1β cytokines and caused elevation in the levels of chemokines and inducible nitric oxide (iNOS) [226]. Finally, indoleamine 2,3-dioxygenase (IDO) and Prostaglandin E2 (PGE2) were also shown to be the key mediators of MSC inhibition of NK cells [187]. Overall, these studies indicated the inhibitory role of key factors induced during the interaction of MSCs with the immune effectors.

We have previously coined the term of "split anergy" for the responses observed by NK_{DC} (NK cells dissociated from tumor conjugates) and NK_{C} (NK cells not dissociated from the tumor conjugate). Whereas NK_{DC} responded to IL-2 activation for cytotoxicity, they were unresponsive to IL-2 mediated induction of proliferation or secretion of cytokines. In contrast, NK_{C} showed an inverse response namely, they did not respond to IL-2 activation for cytotoxicity, but proliferated and secreted cytokines [136, 137]. Treatment of NK cells with IL-2 and anti-CD16mAb also induced split anergy by significantly decreasing the NK cell cytotoxicity while increasing the cytokine secretion capabilities of NK cells [142-145]. Furthermore, IL-2 rescued anti-CD16 mAb mediated apoptosis induced in a subset of NK cells [142]. Loss of cytotoxicity in NK cells was exacerbated when NK cells were either treated with $F(ab)'_2$ fragment of anti-CD16 mAb or treated with a combination of MHC-Class I and anti-CD16 mAb while the same treatments resulted in an increased secretion of cytokines [143, 145]. These results suggested that increase in signaling load on NK cells is likely to result in a decrease in cytotoxicity while increasing secretion of cytokines by the NK cells. Therefore, three distinct functional outcomes could be observed in NK cells which have either interacted with sensitive tumor-target cells or treated with anti-CD16 mAb in the presence of IL-2 treatment, namely; 1-Loss of cytotoxicity, 2-gain in the ability to secrete cytokines and 3- death in a subset

of NK cells. Whether these functions are carried out by the same NK cell subsets or there are distinct subsets of NK cells with distinct functional capabilities require further studies. However, similar to T cells, NK cells can also be functionally anergized or induced to undergo cell death during increased signaling load.

Increased activity of NFκB in tumor cells was found to be inhibitory for the function of NK cells since blocking NFκB in tumor cells made these cells susceptible to lysis by the NK cells [192, 193]. Therefore, to determine which subpopulations of immune effectors were able to protect DPSCs/MSCs against NK cell mediated lysis, we undertook studies to first identify the subset of immune effectors, which was able to increase NFκB in stem cells substantially, and to determine their role in the overall increase in the survival and function of stem cells.

It is quite well established in tumor immunotherapy that certain effectors of the myeloid arm of the immune system induce resistance in tumor cells and cause suppression of NK cell mediated cytotoxicity [227]. Monocytes, an important subset of the myeloid arm of the immune effectors are known to be the major inducers of resistance in tumor cells and are shown to significantly aid in the progression of cancer in tumor-bearing hosts [227-229]. However, the mechanisms by which monocytes contribute to the progression of cancer have not been fully elucidated yet. Therefore, studies reported in this paper may also have the potential to shed light on this very important question. Our recent findings indicated that monocytes protect oral tumors via NFκB dependent and independent pathways (manuscript submitted). In addition, the role of monocytes in suppression of NK cell mediated cytotoxicity against MSCs/DPSCs has not been explored yet. Furthermore, whether a unique subset or all monocytes mediate suppression of NK

cell mediated cytotoxicity against MSCs needs to be explored. Studies reported in this paper are significant in several ways. First, we demonstrate that both allogeneic and autologous NK cells are capable of lysing stem cells significantly and that lysis can be blocked by the treatment of NK cells with anti-CD16 antibody. Second, either the total populations of monocytes or those depleted of $CD16^{+}$ subsets are capable of increasing survival of DPSCs/MSCs against NK cells in three way interactions, raising the overall secretion of key cytokines responsible for the resistance and differentiation of stem cells and protection against NK cell mediated cytotoxicity. Therefore, the effect of monocytes on NK cells are reminiscent of the effect of anti-CD16 mAb or target mediated induction of split anergy in NK cells [137, 142]. Thus, the protection of stem cells against NK cell lysis is partly due to the direct induction of resistance of stem cells by monocytes and indirectly by serving as a shield to protect lysis of stem cells by the NK cells since they too are greatly susceptible to NK cell mediated lysis in a three way interaction.

Finally, by delineating the optimal cell-cell interaction required for the protection of stem cells from NK cell mediated cytotoxicity, we can design strategies to successfully implant either allogeneic or autologous stem cells in different tissue engineering applications.

Overall, our studies demonstrate that monocytes are important catalysts of survival for different cellular subsets, and protect stem cells from NK cell mediated lysis, thereby elevating the collective functions of the cells.

Materials and Methods

Cell Lines, Reagents, and Antibodies

HEp2 cells were obtained from ATCC and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. MSCs were purchased from Clonetics and cultured with the basal medium provided by the manufacturer. DPSCs were isolated as described previously [198] and they were cultured in complete DMEM supplemented with 10% FBS. Recombinant IL-2 was obtained from the NIH repository. The NK, $CD16^{+}$ and $CD16^{-}$ monocyte purification kits were obtained from Stem Cell technologies (Stem Cell, Vancouver, Canada). The ELISA kits for IFN-γ, VEGF and IL-6 were purchased from R&D Systems (Minneapolis, MN). The TNF-α ELISA was developed in our laboratory and reported previously [142]. The multiplex cytokine array kit was purchased from R&D Systems. The Fluorescein Isothiocyanate (FITC) conjugated Annexin V/ Propidium Iodide (PI) kit was purchased from Coulter Immunotech (Miami, FL).

Purification of NK cells, Total monocytes and $CD16^{-}$ Monocytes

PBMCs from healthy donors were isolated as described before [137]. Briefly, peripheral blood lymphocytes were obtained after Ficoll-hypaque centrifugation and adherence to the plate for 1 hour. Purified NK cells were negatively selected by using an NK cell isolation kit. The purity of NK cell population was found to be greater than 90% based on flow cytometric analysis of anti-CD16 antibody stained cells. The levels of $CD3^{+}$ T cell staining in purified population of NK cells remained low at 2.4%±1%, similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures. The adherent subpopulation of

PBMCs was detached from the tissue culture plates and the total population of monocytes and those depleted of $CD16^+$ subsets ($CD16^-$) were purified using isolation kits obtained from Stem Cell Technologies (Vancouver, Canada). Greater than 95% purity was achieved for each subset based on flow cytometric analysis of CD14 and CD16 antibody stained monocytes. Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors and all the procedures were approved by the UCLA-IRB.

ELISA and Multiplex Cytokine Array kit

Single ELISAs were performed as described previously [13]. Fluorokine MAP cytokine multiplex kits were purchased from R&D Systems (Minneapolis, MN) and the procedures were conducted as suggested by the manufacturer. To analyze and obtain the cytokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines provided by the manufacturer. Analysis was performed using the Star Station software.

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [193]. Briefly, MSCs or DPSCs were co-cultured with irradiated (10 Gy) subsets of monocytes for 24-48 hours before they were labeled with ^{51}Cr for 1 hour, after which they were washed and added to NK samples. In several experiments monocytes were sorted out from the stem cell co-cultures before they were labeled with ^{51}Cr and added to the NK cells. 100% removal of monocytes from the stem cells was achieved when the sorted samples were checked either by microscopy or by flow cytometric analysis of stained cells. After a 4 hour incubation period, the supernatants were

harvested and counted for released radioactivity. The percentage of cytotoxicity was calculated as follows;

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm - spontaneous cpm}}{\text{Total cpm – spontaneous cpm}}$$

LU $30/10^7$ cells were calculated using the inverse of the number of effector cells needed to lyse 30% of tumor target cells X100.

Surface and DNA Staining and apoptosis assay

Staining was performed by labeling the cells with antibodies or propidium iodide and Annexin V as described previously [142] [137, 201].

Luciferase reporter assay

Transfections were performed using NFκB Luciferase reporter vector [202] and Lipofectamine 2000 reagent (Invitrogen, CA) in Opti-MEM media (Invitrogen, CA) for 18 hours after which they were adhered to the plate overnight before different immune effectors at 1:1 Effector to target ratios were added. The cells were then lysed with lysis buffer and the relative Luciferase activity was measured using the Luciferase assay reagent kit obtained from Promega (Madison, WI).

Statistical analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post test was used to compare the different groups.

Results

MSCs and DPSCs were selected based on their phenotypic and functional properties

MSCs were $CD166^{+}CD105^{+}CD99^{+}CD34^{-}CD45^{-}$ and $CD14^{-}$ based on the flow cytometric analysis (data not shown). In addition, MSCs were capable of differentiating to osteogenic, chondrogenic and adipogenic lineages (data not shown, www.clonetics.com). DPSCs were isolated as described previously [198] and is shown by us and by others [222] to have osteogenic, chondrogenic and adipogenic properties.

IL-2 activated NK cells are potent inducers of cell death in MSCs

Highly purified human NK cells were treated with and without IL-2 for 8-12 hours before they were added to ^{51}Cr labeled MSCs (Fig. 1A) or DPSCs (Fig. 1B) or NK sensitive K562s (Fig. 1C). The addition of freshly isolated untreated NK cells had lower cytotoxic activity against MSCs (Fig. 1A), DPSCs (Fig. 1B) and K562s (Fig. 1C). However, activation with IL-2 increased cytotoxicity substantially and resulted in a significant lysis of stem cells ($p<0.05$) (Fig. 1).

Anti-CD16 antibody induces death in a subset of untreated and IL-2 treated NK cells and inhibits NK cell mediated lysis of MSCs and DPSCs

NK cells were left untreated or treated with anti-CD16 antibody and/or IL-2 for 8-12 hours before they were added to ^{51}Cr labeled MSCs or DPSCs. As shown in a number of previous studies [142, 144, 145] and figure 4 below, anti-CD16 mAb treatment induced death in a subset of NK cells and inhibited NK cell cytotoxicity against MSCs and DPSCs ($p<0.05$) (Fig. 1). The addition of the combination of IL-2 and anti-CD16 mAb treatment also induced death in

a subset of NK cells and inhibited NK cell cytotoxicity against MSCs and DPSCs when compared to IL-2 activated NK cells ($p<0.05$) (Fig. 1).

Monocytes prevent NK cell mediated lysis of MSCs and DPSCs

Monocytes were purified from PBMCs and irradiated as indicated in the Material and Methods section. MSCs and DPSCs were co-cultured with allogeneic irradiated monocytes for 24-48 hours before they were labeled with ^{51}Cr and used in the cytotoxicity assays against NK cells. NK cells were left untreated or pre-treated with anti-CD16 antibody and/or IL-2 for 24-48 hours before they were used in the cytotoxicity assays against MSCs and DPSCs. The addition of monocytes to MSCs significantly protected the MSCs (Fig. 2 and 3A) and DPSCs (Fig. 3B) from NK cell mediated cytotoxicity ($p<0.05$). Significant inhibition of NK cell cytotoxicity by monocytes could be observed against untreated and IL-2 treated NK samples ($p<0.05$) (Figs. 2 and 3). Monocytes also increased the levels of alkaline phosphatase staining in MSCs and prevented decrease in alkaline phosphatase expression induced by IL-2 activated NK cells (data not shown). Untreated or anti-CD16 antibody treated live or irradiated monocytes were not able to mediate cytotoxicity against MSCs, DPSCs or K562 cells (data not shown). Overall, these experiments indicated that monocytes protect MSCs and DPSCs against NK cell mediated lysis. The reason cytotoxicity was shown both as percentage of killing over different E:T ratios (Figs. 1 and 2) and as Lu30/10^7 cells (Fig. 3) is to demonstrate the clear relationship between cytotoxicity and calculated lytic units which will be used from here on after.

Both the total populations of monocytes and those depleted of CD16$^+$ subsets prevented NK cell mediated cytotoxicity against MSCs and DPSCs.

Since unbound anti-CD16 antibody or shed antibody from the NK cells could affect the small subpopulation of $CD16^{+}$ monocytes by modulating their function, we opted to sort out this small subset of monocytes from the total population of monocytes (16^{-}) and compared their function to the activity of monocytes containing both the $CD16^{+}$ and $CD16^{-}$ subpopulations (total population) for the prevention of NK cell mediated cytotoxicity against MSCs and DPSCs. Both total population and $CD16^{+}$ depleted subsets of monocytes could significantly prevent NK cell mediated lysis of MSCs (Fig. 3A) and DPSCs (Fig. 3B and 3C). Similar results were obtained in regards to the protection of DPSCs by monocytes against NK cell mediated lysis when the function of NK cells and monocytes was assessed with both allogeneic and autologous DPSCs (Fig. 3C). Furthermore, the removal of CD16 antibody by extensive washings of NK cells before their culture with monocytes and stem cells had similar results to those which were left unwashed and used in co-cultures with monocytes and stem cells (data not shown).

Monocytes prevent anti-CD16 antibody mediated NK cell death

Since monocytes were capable of protecting MSCs and DPSCs from NK cell mediated lysis we reasoned that they may also protect NK cells from undergoing cell death. Therefore, to investigate the protection of NK cells by monocytes, untreated or IL-2 treated and/or anti-CD16mAb treated NK cells were co-cultured with monocytes immediately after purification and treatment, and their viability was determined after an overnight incubation. As shown in Fig. 4 monocytes prevented NK cell death induced by anti-CD16 antibody in the presence and absence of IL-2 treatment ($p<0.001$). Therefore, monocytes are potent inducers of NK cell survival.

Monocytes increase IFN-γ, IL-6, TNF-α and VEGF secretion substantially when cultured with and without NK cells and MSCs or NK cells and DPSCs.

Irradiated monocytes were added to MSCs for 24-48 hours before the addition of untreated or IL-2 pre-treated and /or anti-CD16 antibody pre-treated NK cells. After 24-48 hours of incubation with NK cells the supernatants were removed, and the levels of IFN-γ, IL-6, TNF-α and VEGF secretion were determined using both multiplex cytokine array assay and single ELISAs (Fig. 5). Monocytes increased the secretion of IL-6 and TNF-α in co-cultures with either NK cells or with MSCs ($p<0.05$) (Figs. 5A and 5B). However, the highest increase was obtained when monocytes were added to NK and MSCs ($p<0.05$) (Figs. 5A and 5B). As expected, IL-2 increased the secretion of TNF-α and IL-6 moderately in NK cells and the combination of IL-2 and anti-CD16 mAb had synergistic effect on the release of TNF-α by the NK cells (Figs. 5A and 5B). The addition of monocytes increased synergistically the secretion of IL-6 and TNF-α in all treated NK samples ($p<0.05$) (Figs. 5A and 5B). MSCs induced synergistically the secretion of IL-6 when cultured with NK samples ($p<0.05$) (Fig. 5A). Secretion of IL-6 and TNF-α were significantly augmented when monocytes were added to MSCs and NK cells when compared to those in the absence of monocytes ($p<0.05$) (Figs. 5A and 5B). However, the levels of IL-6 and TNF-α reach plateau when monocytes were added to untreated or anti-CD16 mAb and/or IL-2 treated NK cells and MSCs (Figs. 5A and 5B).

Secretion of VEGF was only observed in samples containing MSCs, and monocytes further increased secreted VEGF by MSCs ($p<0.05$) (Fig. 5C). Finally, as expected IL-2 treated NK cells secreted moderate amounts of IFN-γ which were synergistically increased when co-

cultured in the presence of MSCs ($p<0.05$) (Fig. 5D). The addition of anti-CD16 mAb in combination with IL-2 to NK cells in the absence of MSCs increased secretion of IFN-γ when compared to IL-2 alone treated NK cells in the absence of MSCs. IFN-γ secreted levels remained similar between IL-2 alone and IL-2 and anti-CD16 mAb treated NK cells cultured with MSCs (Fig. 5D). Monocytes added to IL-2 or IL-2 and anti-CD16 antibody treated NK cells in the absence of MSCs or those in the presence of MSCs, synergistically increased the levels of secreted IFN-γ ($p<0.05$) (Fig. 5D). However, the highest increase in IFN-γ release was seen when monocytes were added to IL-2 or IL-2 and anti-CD16 mAb treated NK cells with MSCs (Fig. 5D). These results indicated that monocytes increased all of the above-mentioned cytokines in co-cultures with MSCs, and further synergized with IL-2 or IL-2 and anti-CD16 mAb treated NK samples to increase the release of cytokines in the co-cultures of NKs and MSCs. Similar results were obtained when NK cells were co-cultured with monocytes and DPSCs (Fig. 6).

Both the total population of monocytes or those depleted of $CD16^+$ subset trigger significant release of IFN-γ by NK cells in a three way interaction with MSCs or DPSCs

Purified irradiated total monocytes or those depleted of $CD16^+$ subsets were each co-cultured with MSCs or DPSCs for 24-48 hours before untreated or IL-2 and/or anti-CD16 mAb pre-treated NK cells were added. The incubation was then continued for another 24-48 hours before the supernatants were removed and subjected to specific ELISAs for IFN-γ (Fig. 6). The differences in secretion of IFN-γ between the two monocyte samples were either moderate or very slight when NK, monocytes and MSCs were all present in the co-cultures (Figs. 6A and 6B). Monocytes cultured with or without MSCs (Fig. 6A) and DPSCs (Fig. 6B) increased secretion of IFN-γ substantially when added to IL-2 or IL-2 and anti-CD16 antibody treated NK

cells. Since monocytes alone or monocytes cultured with MSCs or DPSCs did not have any effect on IFN-γ secretion in all the experiments tested, we did not include them in Fig. 6 (data not shown).

Protection of DPSCs from NK cell mediated cytotoxicity could still be seen when monocytes were sorted out from DPSCs before they were exposed to NK cells

Purified total population of monocytes or those depleted of $CD16^{+}$ subsets were each co-cultured with DPSCs for 24-48 hours, before they were sorted out and removed from DPSCs. DPSCs were then labeled with ^{51}Cr and cultured with untreated or IL-2 and/or anti-CD16 mAb pre-treated NK cells. As shown in Fig 7A, protection from NK cell mediated lysis could still be seen when monocytes were removed from DPSCs before their exposure to NK cells. However, this effect was not specific to monocytes alone since the addition of either T cells (Fig. 7B) or B cells (Fig. 7C) to DPSCs and their removal after 24-48 hours were also protective against NK cell mediated lysis of DPSCs.

Monocytes and T cells are targets for NK cell mediated lysis

Since significant IFN-γ secretion could also be obtained in the co-cultures of IL-2 treated NK cells with monocytes we determined whether they were also targets of NK cell mediated lysis. As shown in Fig. 8 both monocytes and T cells were targets of NK cell mediated lysis. In accordance, significant IFN-γ secretion could also be obtained in supernatants removed from co-cultures of NK cells with monocytes (Fig. 9A) or T cells (Fig. 9A) or B cells (Fig. 9B). One distinction was in the magnitude of secreted IFN-γ by the NK cells treated with IL-2 and anti-

CD16 mAb in which those cultured with monocytes secreted higher levels than those cultured with either T cells or B cells (Fig. 9).

Monocytes are major inducers of NFκB activity in epithelial cells

Purified subpopulations of the immune effectors, namely, highly purified monocytes, Natural Killer cells, PMNs and total populations of peripheral blood mononuclear cells (PBMCs) and peripheral blood lymphocytes (PBLs) were each co-cultured with NFκB reporter transfected HEp2 cells (Fig. 10), and the levels of NFκB activity were determined after 4 hours of incubation. As shown in Fig. 10 monocytes induced the highest increase in NFκB activity whereas all the other populations including NK cells had minimal or no effect on NFκB activity ($p<0.05$).

Discussion

Successful transplantation of stem cells in an immunocompetent host will eventually depend on the ability of transplanted stem cells to survive during their interaction with the host immune effectors. Although a number of previous studies have attributed a lack of immunogenicity to stem cells, others have shown significant lysis of these cells by the cytotoxic immune effectors [186, 187, 219]. Therefore, detailed analysis of immune cell interaction with stem cells is important, and should provide the basis for designing novel strategies to increase the viability and function of stem cells in different tissue engineering applications. It is therefore, important to determine the potential mechanisms by which stem cells can be protected from the lysis by cytotoxic immune effectors.

To study the detailed function of cytotoxic immune effectors against stem cells we purified human NK cells and studied their effect on MSCs and DPSCs. In addition, we determined the interaction of NK cells with other subsets of immune effectors during their interaction with stem cells. In agreement with previous studies we also show in this report that NK cells can target and kill MSCs significantly. Indeed, when compared to K562 lysis, NK cells can also mediate significant lysis of MSCs (Figs 1 and 2) and DPSCs. Moreover, we demonstrate for the first time that DPSCs are also targets of NK cell mediated killing, and that stem cells in general have exquisite sensitivity to NK cell mediated lysis. In support of this notion, we have recently determined that human embryonic stem cells are the most sensitive stem cells to NK cell mediated cytotoxicity when compared to DPSCs (manuscript in

preparation). In addition, autologous DPSCs are as good of a target for NK cell mediated lysis as the allogeneic DPSCs.

As expected, IL-2 treated NK cells lysed a much higher percentage of MSCs and DPSCs when compared to untreated NK cells, although a range of 20% to 70% cytotoxicity could be observed by untreated NK cells depending on the donor NK cells used to lyse stem cells (data not shown). In addition, anti CD16 antibody treated NK cells lost their cytotoxic function against MSCs and DPSCs since this treatment resulted in anergy as well as the death of a subset of NK cells and prevention of cytotoxicity against MSCs and DPSCs. Moreover, significant induction of IFN-γ secretion could be observed in supernatants removed from the co-cultures of either IL-2 or IL-2 and anti-CD16 mAb treated NK cells with MSCs and DPSCs.

When MSCs and DPSCs were cultured with either viable or irradiated monocytes before they were exposed to IL-2 treated NK cells a significant decrease in NK cell mediated cytotoxicity could be observed. Interestingly, significant lysis of MSCs and DPSCs by untreated NK cells was also significantly and reproducibly blocked by the addition of monocytes (Fig. 2). To determine whether decreased lysis of stem cells by NK cells was due to competitive lysis of monocytes by NK cells we performed several experiments. Indeed, we confirmed that monocytes were also lysed by NK cells significantly. In addition, we found not only monocytes but also T and B cells to be significant targets of NK cell mediated killing. Furthermore, when we co-cultured stem cells with monocyte subsets and sorted to remove the monocytes from the stem cells we could still observe significant inhibition of NK cell mediated lysis, arguing against the protection of stem cell lysis by NK cells being solely on the bases of competitive lysis of

monocytes. Therefore, even though lysis of monocytes by the NK cells may in part contribute to the prevention of NK cell lysis of stem cells, interaction of monocytes with stem cells can also provide resistance of stem cells against NK cell cytotoxicity. However, this function was not specific to monocytes since the addition of T and B cells to stem cells and their removal by sorting before their culture with NK cells could also provide resistance of stem cells against NK cell mediated cytotoxicity. Therefore, it is likely that interaction of NK cells with stem cells in the presence of other immune effectors is complex and it is governed by not only the ability of NK cells to lyse other immune effectors, but also due to increased resistance of stem cells to NK cell lysis after interaction with subsets of immune effectors.

Decrease in NK cell lysis of MSCs and DPSCs was paralleled with a significant induction of IFN-γ in a stepwise manner. Indeed, when MSCs or DPSCs were cultured with IL-2 treated NK cells alone we could observe significant induction of IFN-γ secretion. However, the highest increase was seen when NK cells were cultured with MSCs or DPSCs in the presence of monocytes. Therefore, although decreased killing of stem cells by the NK cells could be observed in the presence of monocytes, synergistic secretion of IFN-γ by the NK cells in the presence of monocytes and stem cells could be observed, indicating a discordant relationship between cytotoxicity and IFN-γ secretion. This was similar to the profiles which we had seen when NK cells were treated with IL-2 and anti-CD16 antibody in which significant decrease in cytotoxicity of NK cells could be observed in parallel with increased secretion of IFN-γ (Fig. 5, 6 and 9). We have previously shown that IL-2 rescues NK cells from anti-CD16 mAb mediated apoptosis [142], therefore, it is possible that monocytes also contribute to rescue from apoptosis and increase in secretion of IFN-γ by the NK cells. Indeed, monocytes prevent anti-CD16

mediated NK cell apoptosis (Fig. 4). Rescue of NK cells from apoptosis could be an important function of monocytes since 1- monocytes are known to increase NFκB activity in the cells, and 2-lysis of monocytes by the NK cells could result in activation and increased resistance of NK cells to cell death. In this respect T and B cells can also provide increased survival of NK cells since they too are targets of NK cell killing, but they are not capable of inducing NFκB in epithelial cells to the levels which were induced by monocytes. In addition, even though T and B cells could cause resistance of stem cells to NK cell mediated cytotoxicity, they could not raise the levels of IFN-γ secretion by the IL-2 and anti-CD16 mAb treated NK cells to the levels which were observed when these cells were cultured with monocytes and stem cells (Fig. 9).

These observations prompted us to speculate regarding the significance of interaction of monocytes with NK cells and stem cells. It is plausible that monocytes serve as not only effectors which provide survival for NK cells and stem cells but also they may serve as shields against NK cell lysis of stem cells. Similar to anti-CD16 antibody mediated effect on IL-2 treated NK cells, monocytes too can shield stem cells from killing by the NK cells by increasing the total IFN-γ release by the NK cells while decreasing the cytotoxic function of NK cells (split anergy), resulting in an increased protection and differentiation of stem cells. Indeed, monocytes also increased TNF-α, IL-6 and VEGF secretion in the co-cultures of stem cells with NK cells which could further augment induction of NFκB and increased differentiation of stem cells. The shielding effect of monocytes could be a more generalized function of immune effectors since NK cells can also target T and B cells. This may have significant implications regarding the role of NK cells in not only limiting inflammation, but also the significance of other immune effectors in shielding and limiting the cytotoxic function of NK cells against stem cells in order

to raise maximally the secretion of key cytokines for speedy and optimal differentiation of stem cells during inflammation. This is precisely what is observed in cancer patients in whom global decrease in NK, cytotoxic T cells and monocytes have all been reported [180]. In addition, the increase in IFN-γ secretion by NK cells should provide the initial steps in increasing antigen specific functions of T and B cells and initiation of adaptive immunity.

We have recently observed an NFκB independent rescue of oral epithelial cells by monocytes (manuscript in prep). Therefore, it is likely that monocytes elevate survival of MSCs and DPSCs through NFκB dependent and independent pathways. In addition, both the total population of monocytes and those depleted of $CD16^+$ subsets were able to increase NFκB and survival of oral epithelial cells (manuscript in prep) and MSCs resulting in an increase in cytokine secretion.

We have previously reported that rescue from cell death is governed by inverse induction of NFκB and JNK and decreased secretion of IgFbp6 in epithelial cells [198, 230]. [231]. Whether monocytes prevent stem cell death through similar mechanisms should await future investigation.

Our previous work demonstrated the decoupling of NK cell cytotoxicity from cytokine secretion [137]. The cytotoxic function of non-dissociated NK-tumor cell conjugates remained very low and did not increase when supplemented with IL-2 although they proliferated and secreted higher amounts of TNF-α and IFN-γ in the supernatants [137]. In contrast, NK cells dissociated from the target conjugates exhibited more cytotoxic activity, but they proliferated

poorly and secreted lower levels of TNF-α or IFN-γ following IL-2 treatment. More importantly, a fraction of NK cells in non-dissociated NK-tumor conjugates were programmed for cell death by apoptosis [137]. Furthermore, the addition of intact and F(ab)'[2] fragment of anti-CD16 antibodies in combination with IL-2 significantly and reproducibly inhibited the cytotoxic function of NK cells, but the NK cells secreted relatively higher amounts of IFN-γ [142, 144, 145]. As mentioned above, we observed a similar trend when monocytes were added to MSCs/DPSCs and the IL-2 treated NK cells. This function of anti-CD16 receptor antibody and monocytes therefore can be exploited to decrease rejection of stem cells by the NK cells. Indeed, for optimal engraftment of stem cells one may require to target both cytotoxic NK and T cells and in this regard the use of the combination of anti-CD16 antibody and OKT3 antibody should theoretically achieve this objective. We are currently using this strategy to improve engraftment of stem cells in pulp tissues.

Given the high sensitivity of stem cells to NK cell cytotoxicity, it is possible that significant elevation in NK cell signaling by MSCs and subsequent initiation of signaling for anergy and cell death of a subset of NK cells contribute to the observed suppression of NK cell cytotoxicity. Indeed, significant down modulation of NK cell receptors NKp30, NKp44 and NKG2D was also observed after interaction of NK cells with MSCs [187]. Receptor down-modulation could likely be a mechanism to limit the levels of signaling for the survival of the NK cells. Therefore, the suppression of NK cell cytotoxic function observed by MSCs could well be due to over-stimulation of NK cells by the stem cells and induction of cell death in NK cells. Therefore, the reason MSCs were considered to prevent auto-immunity may be due to their

competitive binding and inactivation and removal of killer NK cells. These possibilities are currently under investigation in our laboratory.

Overall, studies reported in this paper provide evidence for an important function of monocytes in protection of MSCs and DPSCs from NK cell mediated lysis resulting in an increased secretion of key cytokines for optimal survival, proliferation and differentiation of stem cells in a three way interaction.

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Figure legends

Fig. 1. Potent lysis of MSCs and DPSCs by the Natural Killer cells: Inhibition by anti-CD16 antibodies

Highly purified NK cells were left untreated or treated with anti-CD16 mAb (3 µg/ml), IL-2 (1000 u/ml), or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3 µg/ml) for 8-12 hours before they were added to the ^{51}Cr labeled MSCs (Fig. 1A) or ^{51}Cr labeled DPSCs (Fig. 1B) or ^{51}Cr labeled K562 (Fig. 1C) at different effector to target (E:T) ratios. After 4 hours of incubation the supernatants were removed and the released radioactivity was counted by a γ counter. % cytotoxicity was determined as indicated in the Materials and Methods section. One of five representative experiments is shown in this figure.

Fig. 2. Decreased killing of MSCs by NK cells when MSCs were co-cultured with monocytes prior to the addition of NK cells. Monocytes were purified from PBMCs and irradiated as indicated in the Material and Methods section

MSCs ($1X10^6$ cells/plate) were cultured with the irradiated monocytes (monocyte: MSC ratio of 1:1) for 24-48 hours before they were removed from the plates, washed and labeled with ^{51}Cr and used as targets in the cytotoxicity assays against NK cells. The NK samples were either left untreated (Fig. 2A) or treated with anti-CD16 mAb (3µg/ml) (Fig. 2B) , IL-2 (1000 u/ml) (Fig. 2C), or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3µg/ml) (Fig. 2D) for 24-48 hours before they were added to ^{51}Cr labeled MSCs at different effector to target (E:T) ratios. Supernatants were removed after 4 hours of incubation and the released radioactivity counted by

a γ counter. % cytotoxicity was determined as indicated in the Material and Methods section. One of three representative experiments is shown in this figure.

Fig. 3. Total monocytes or those depleted of $CD16^+$ subsets of monocytes protect MSCs and DPSCs against NK cell mediated cytotoxicity

MSCs (Fig. 3A) or DPSCs (Fig. 3B) each at $1X10^6$ cells/plate were co-cultured with highly purified and irradiated total monocytes or $CD16^-$ subsets of monocytes at 1:1 MSCs to monocyte ratio for 24-48 hours before they were detached, washed and labeled with ^{51}Cr and added to untreated or IL-2 (1000 u/ml) pre-treated or anti-CD16mAb (3μg/ml) pre-treated, or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3μg/ml) pre-treated NK cells at different E:T ratios. NK cells were pre-treated as indicated for 24-48 hours before they were added to the co-cultures of monocytes with MSCs. After 4 hours of incubation the supernatants were removed and the released radioactivity counted by a γ counter. % cytotoxicity was determined at different E:T ratio, and $LU_{30}/10^7$ cells were calculated using the inverse of the number of effectors needed to lyse 30% of the MSCs or DPSCs X100. Both autologous and allogeneic DPSCs (Fig. 3C) at $1X10^6$ cells/plate were cultured with highly purified and irradiated total monocytes or CD16- subsets of monocytes at 1:1 DPSCs to monocytes for 24-48 hours before they were detached, washed and labeled with ^{51}Cr and added to untreated or IL-2 (1000 u/ml) pre-treated or anti-CD16mAb (3μg/ml) pre-treated, or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3μg/ml) pre-treated NK cells at different E:T ratios. NK cells were pre-treated as indicated for 24-48 hours before they were added to the co-cultures of monocytes with DPSCs. After 4 hours of incubation the supernatants were removed and the released radioactivity counted by a γ

counter. % cytotoxicity was determined, and $LU_{30}/10^7$ cells were calculated using the inverse of the number of effectors needed to lyse 30% of DPSCs X100.

Fig. 4. Monocytes prevent NK cell death induced by anti-CD16mAb

NK cells were labeled with FITC before they were either left untreated or treated with anti-CD16 mAb (3μg/ml), IL-2 (1000 u/ml), or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3μg/ml) and immediately added to monocytes at 1:1 (NK : monocyte) ratios. After an overnight incubation the levels of cell death in NK cells were determined by flow cytometric analysis of Propidium Iodide stained cells. FITC labeled NK cells were gated to determine the levels of PI stained NK cells. 10,000 events were analyzed for each sample. One of three representative experiments is shown in this figure.

Fig. 5. Monocytes synergize with NK cells and MSCs to induce IL-6, TNF-α, VEGF and IFN-γ

MSCs ($1X10^5$ cells/well) were co-cultured with and without irradiated Monocytes at 1:1 MSCs to monocytes for 24-48 hours before untreated or IL-2 (1000 u/ml) pre-treated or anti-CD16mAb (3μg/ml) pre-treated, or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3μg/ml) pre-treated NK cells at 1:1 NK: monocyte ratios were added to the MSC: moncyte co-cultures. NK cells were pre-treated as indicated for 24-48 hours before they were added to the co-cultures of monocytes and MSCs. NK samples were also cultured in the absence of monocytes and MSCs. After 24-48 hours of the addition of NK cells the supernatants were removed from the cultures and the levels of IL-6 (Fig. 5A), TNF-α (Fig. 5B), VEGF (Fig. 5C),

and IFN-γ (Fig. 5D) were determined using multiplex cytokine array kit. The results were also confirmed for each individual cytokine using single ELISAs (data not shown). One of three representative experiments is shown in this figure.

Fig. 6. Total monocytes or CD16^{-} subsets of monocytes synergize with NK cells and MSCs or DPSCs to induce IFN-γ secretion

MSCs (Fig. 6A) or DPSCs (Fig. 6B) at $1X10^{5}$ cells/well were co-cultured with highly purified and irradiated total monocytes or CD16^{-} subsets of monocytes at 1:1 MSCs or DPSCs to monocyte ratios for 24-48 hours before untreated or IL-2 (1000 u/ml) pre-treated or anti-CD16mAb (3μg/ml) pre-treated, or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3μg/ml) pre-treated NK cells at 1:1 MSCs or DPSCs to NK cells were added. NK cells were pre-treated as indicated for 24-48 hours before they were added to the co-cultures of monocytes and MSCs or DPSCs. After 24-48 hours of the addition of NK cells the supernatants were removed and subjected to specific ELISA for IFN–γ. One of four representative experiments is shown in this figure.

Fig. 7. Decreased lysis of DPSCs by NK cells were seen when monocytes, T cells and B cells were sorted out from co-cultures of DPSCs with these cells before the addition of NK cells

Purified total population of monocytes or those depleted of CD16^{+} subsets (Fig. 7A), purified T cells (Fig. 7B) and purified B cells (Fig. 7C) were each co-cultured with $1X10^{6}$ DPSCs per plate at 1:1 ratios for 24-48 hours, before the subsets of monocytes or T cells or B cells were sorted out and removed from DPSCs. Sorted DPSCs from monocytes, T cells and B

cells were then labeled with 51Cr, washed and added to untreated or IL-2 (1000 u/ml) pre-treated or anti-CD16mAb (3μg/ml) pre-treated, or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3μg/ml) pre-treated NK cells. After 4 hours of incubation the supernatants were removed and the released radioactivity counted by a γ counter. % cytotoxicity was determined at different E:T ratio, and $LU_{30}/10^7$ cells were calculated using the inverse of the number of effectors needed to lyse 30% of the DPSCs X100.

Fig. 8. Monocytes and T cells are targets for NK cell mediated lysis

Highly purified NK cells were left untreated or treated with anti-CD16 mAb (3μg/ml), IL-2 (1000 u/ml), or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3μg/ml) for 8-12 hours before they were added to the ^{51}Cr labeled DPSCs, or ^{51}Cr labeled monocyts or ^{51}Cr labeled T cells at different effector to target (E:T) ratios. After 4 hours of incubation the supernatants were removed and the released radioactivity counted by a γ counter. % cytotoxicity was determined at different E:T ratio, and $LU_{30}/10^7$ cells was calculated using the inverse of the number of effectors needed to lyse 30% of either DPSCs, monocytes or T cells X100. One of three representative experiments is shown in this figure.

Fig. 9. Total monocytes or T cells or B cells synergize with NK cells and DPSCs to induce IFN-γ secretion

DPSCs ($1X10^5$ cells/well) were co-cultured with highly purified total monocytes (Fig. 9A and 9B) or T cells (Fig. 9A) or B cells (Fig. 9B) at 1:1 DPSCs to monocyte or T cell or B cell ratios for 24-48 hours before untreated or IL-2 (1000 u/ml) pre-treated or anti-CD16mAb (3μg/ml) pre-treated, or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3μg/ml) pre-

treated NK cells at 1:1 DPSCs to NK cells were added. NK cells were pre-treated as indicated for 24-48 hours before they were added to the co-cultures of monocytes or T cells or B cells and DPSCs. After 24-48 hours of the addition of NK cells the supernatants were removed and subjected to specific ELISAs for IFN−γ. One of four representative experiments is shown in this figure.

Fig. 10. Monocytes are potent inducers of NFκB activity in HEp2 cells

HEp2 cells were transfected with NFκB luciferase reporter vector before they were co-cultured with monocytes, purified NK cells, peripheral blood lymphocytes (PBLs), polymorphonuclear (PMNs) and peripheral blood mononuclear cells (PBMCs) at 1:1 ratio. After 4 hours of incubation the fold induction of NFκB activity in the samples was determined over the control HEp2 cells in the absence of immune effectors.

Fig. 1A

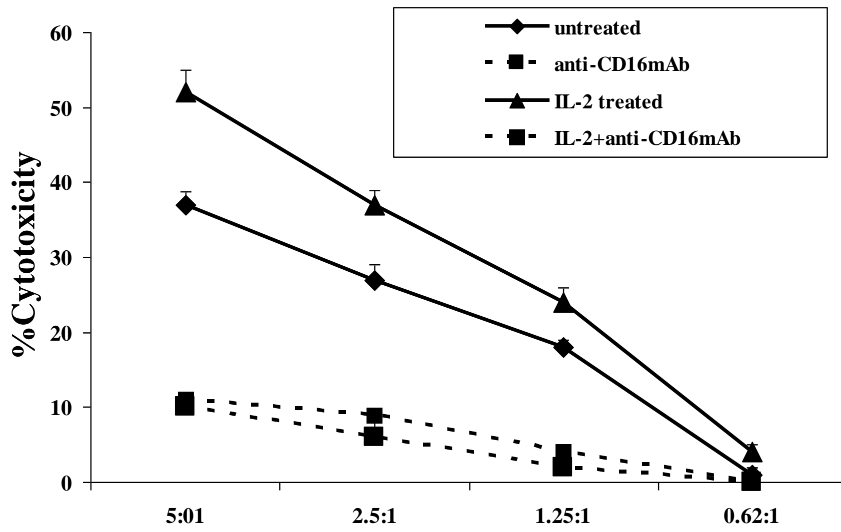


Fig. 1B

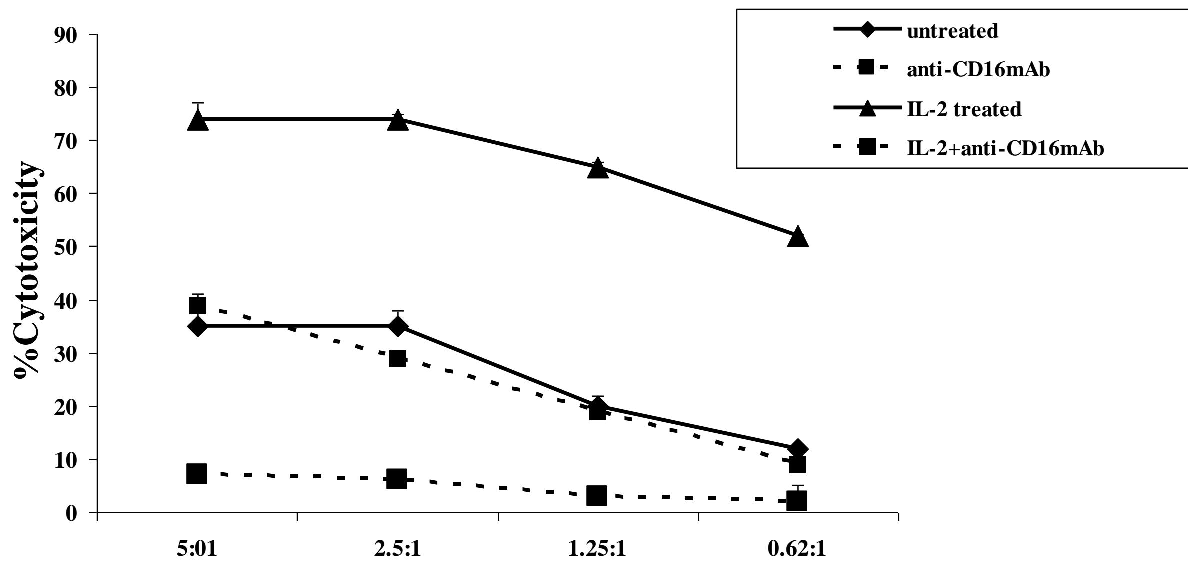


Fig. 1C

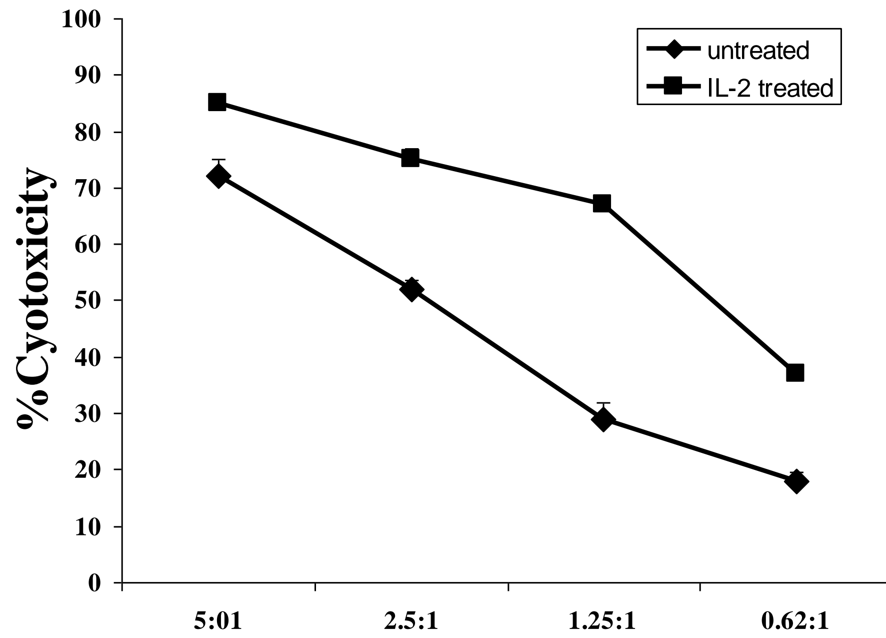
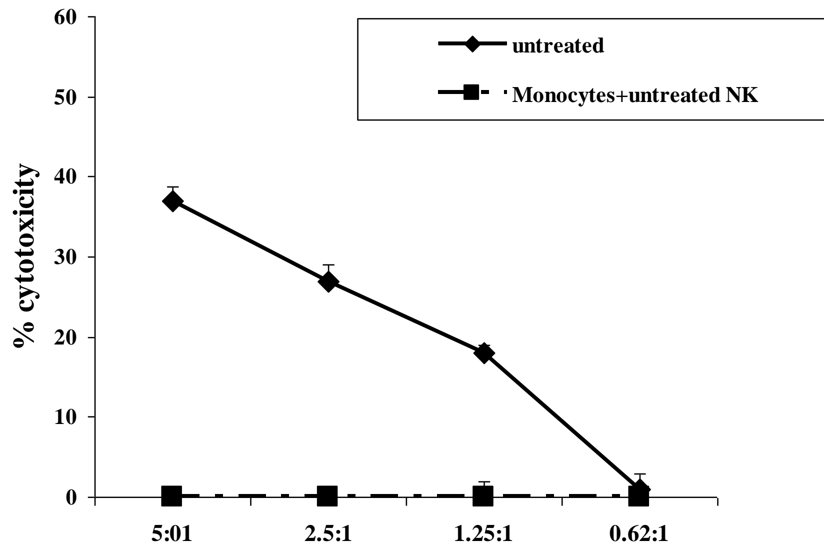


Fig. 2A



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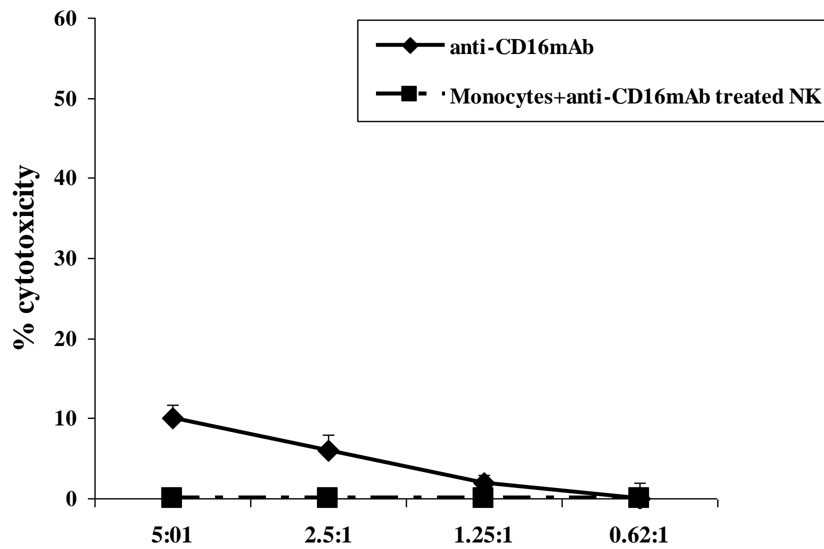

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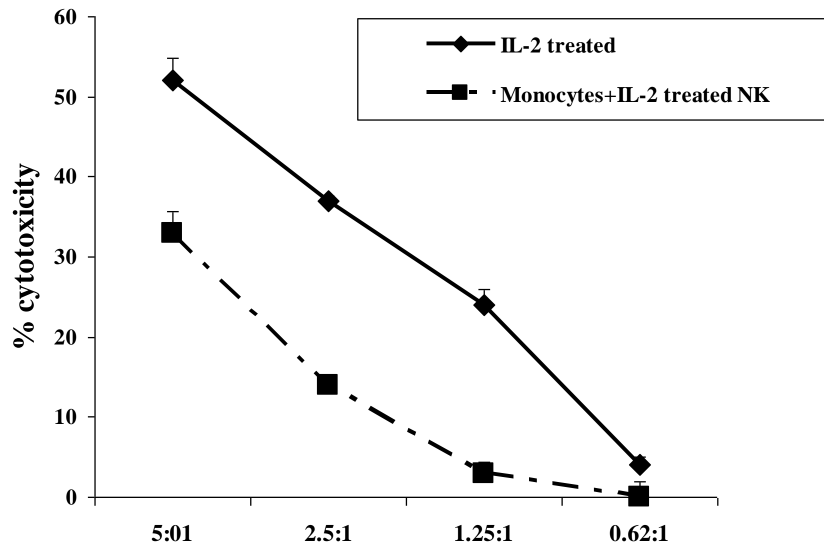


Fig. 2D

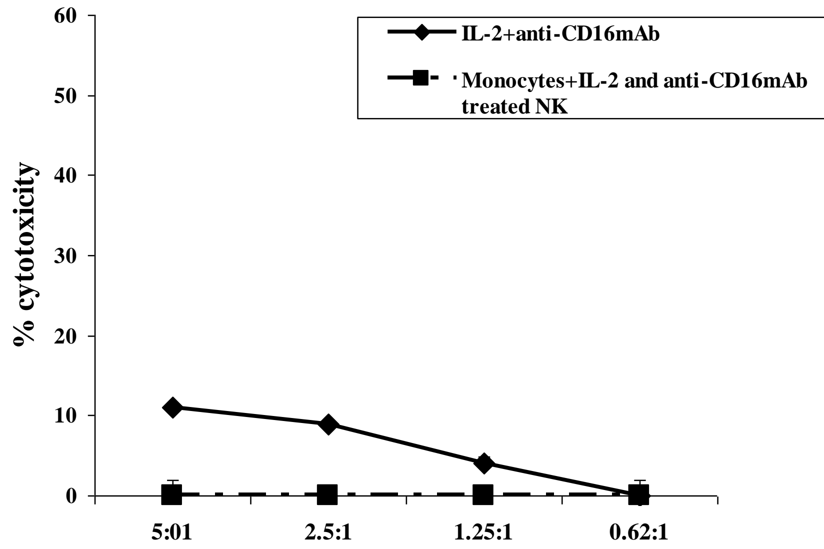
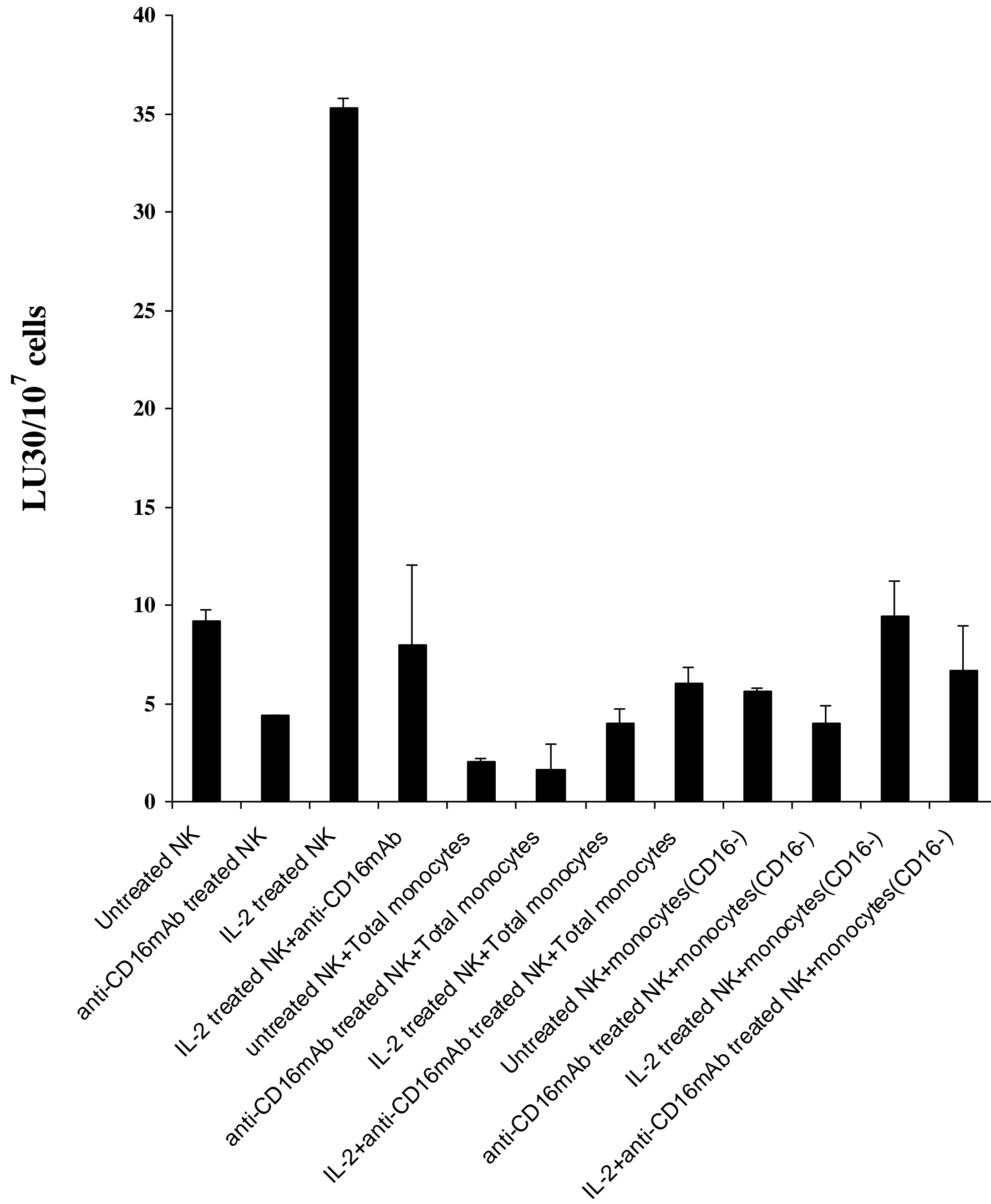


Fig. 3A



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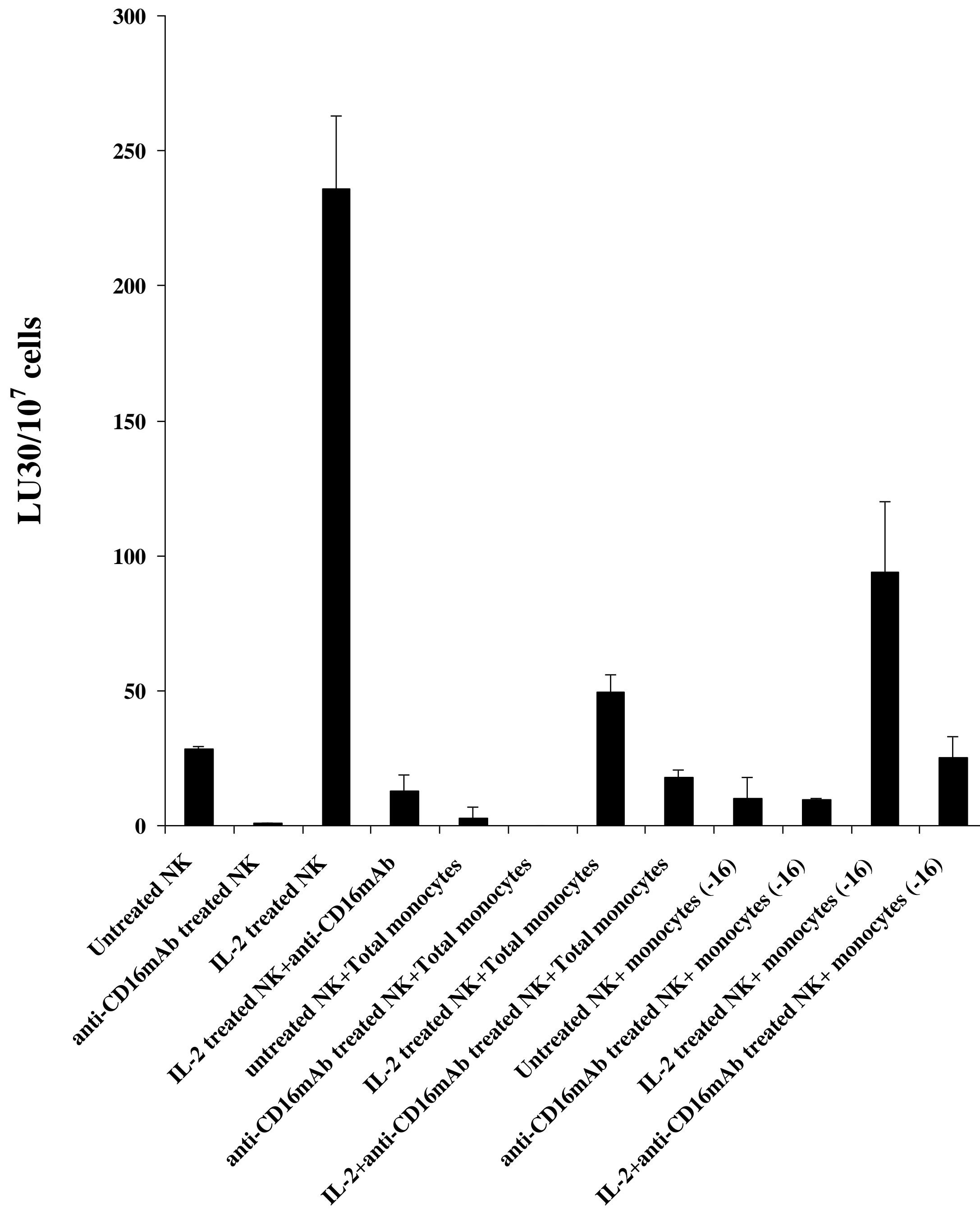


Fig. 3C

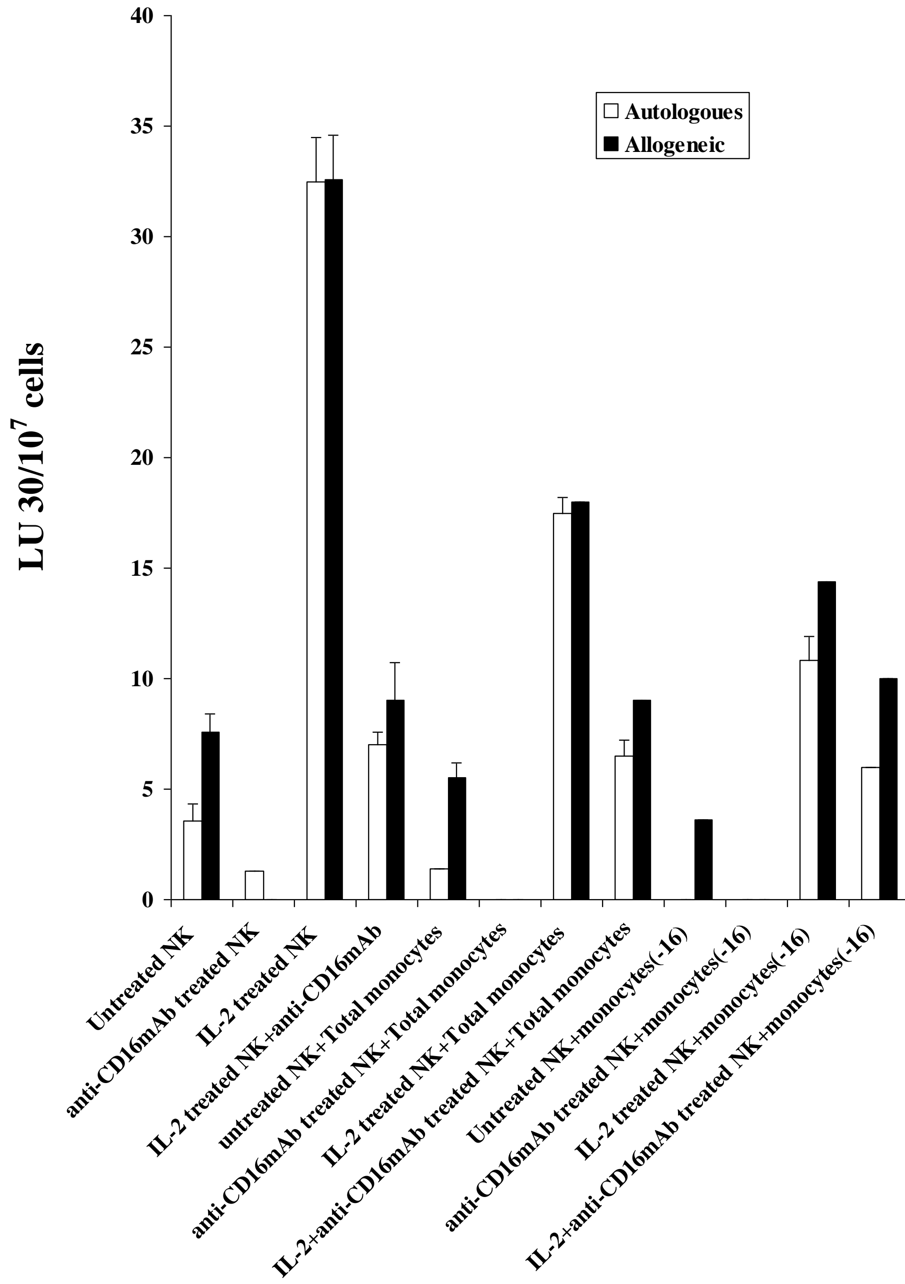


Fig. 4

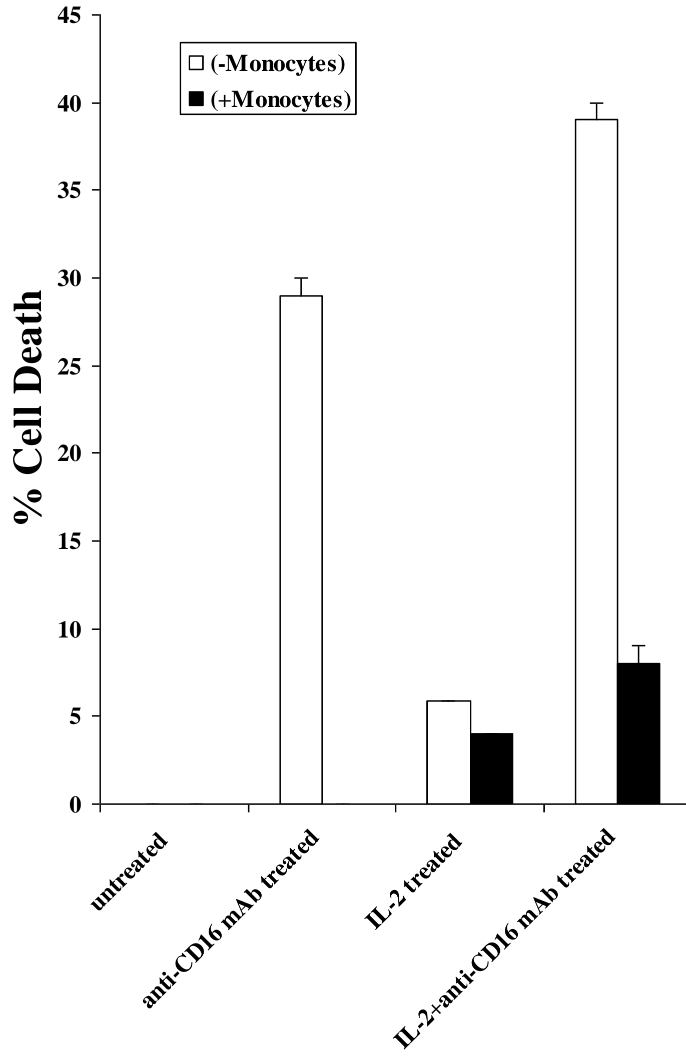


Fig. 5A

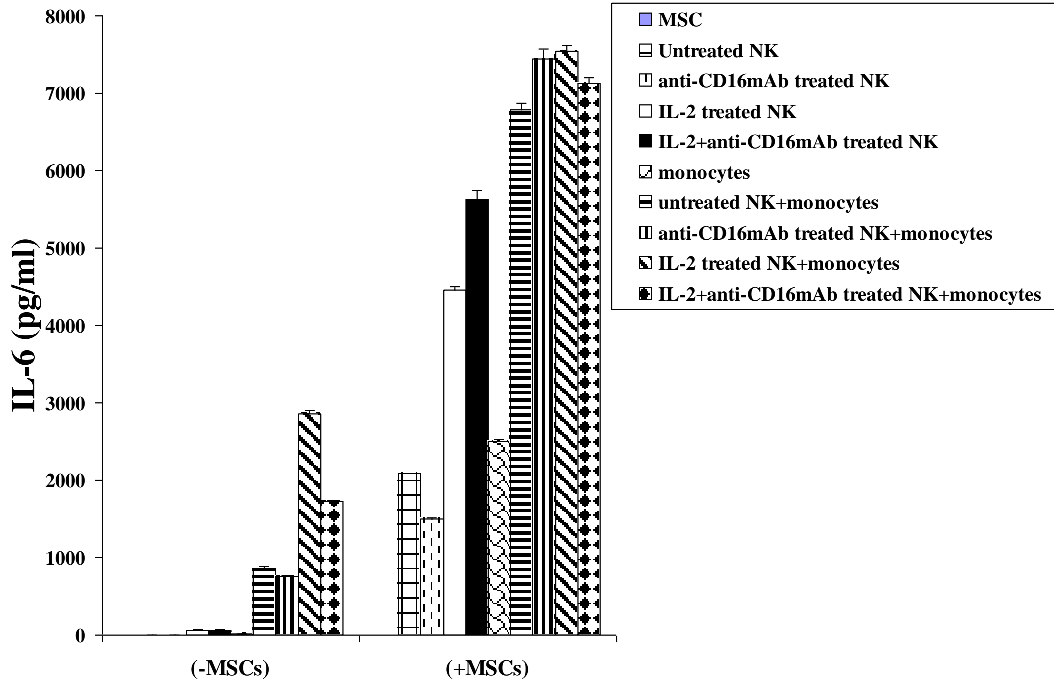


Fig. 5B

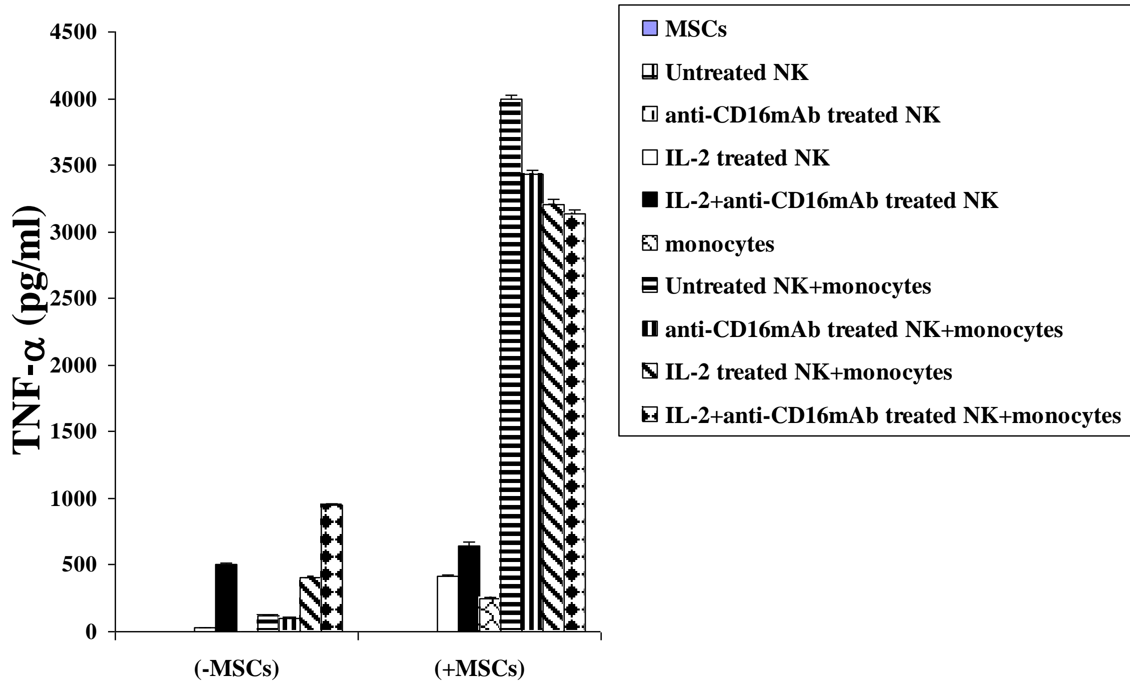


Fig. 5C

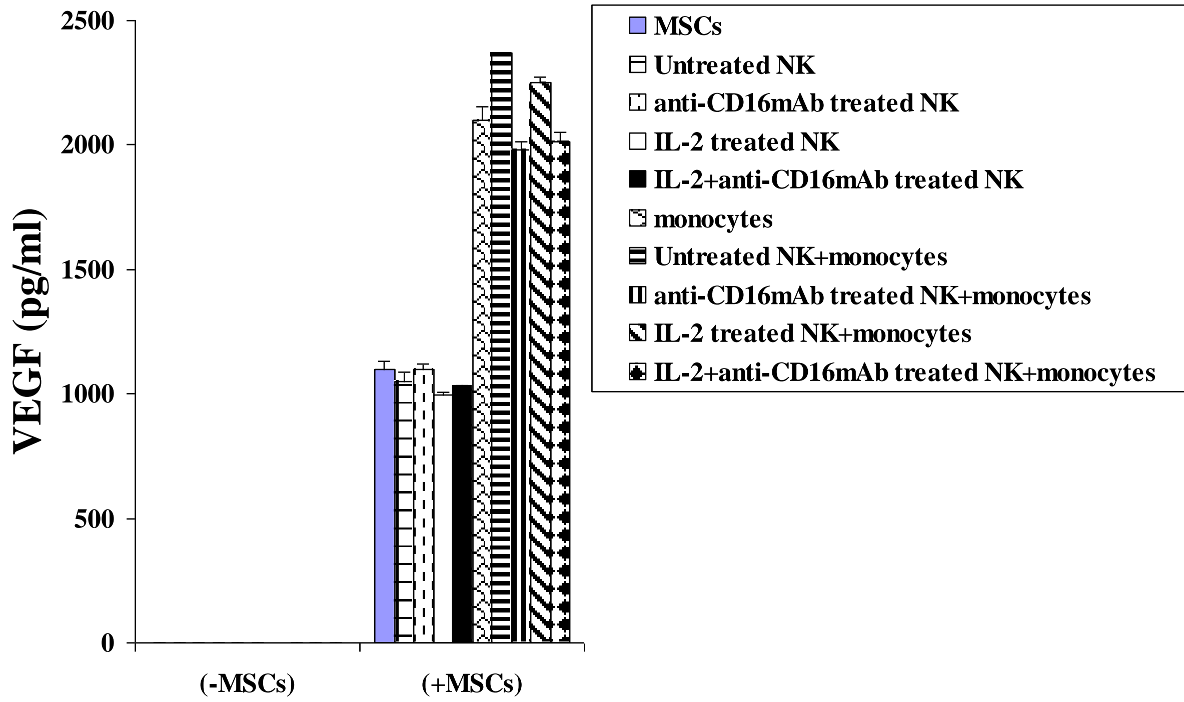


Fig. 5D

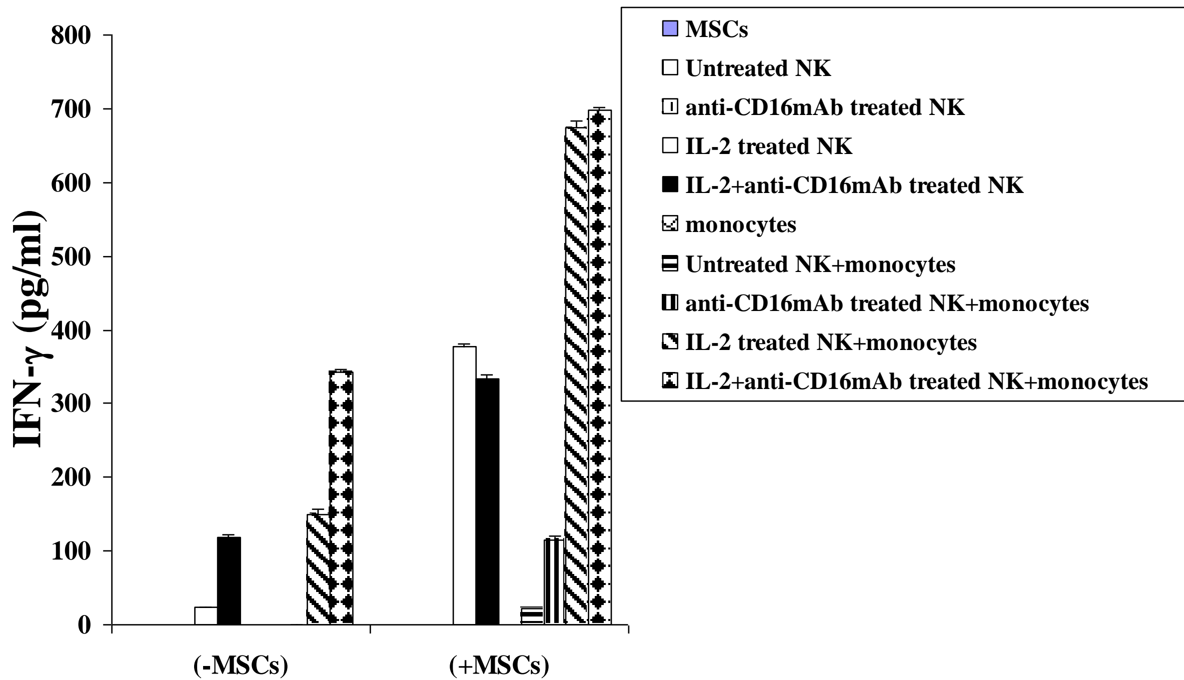
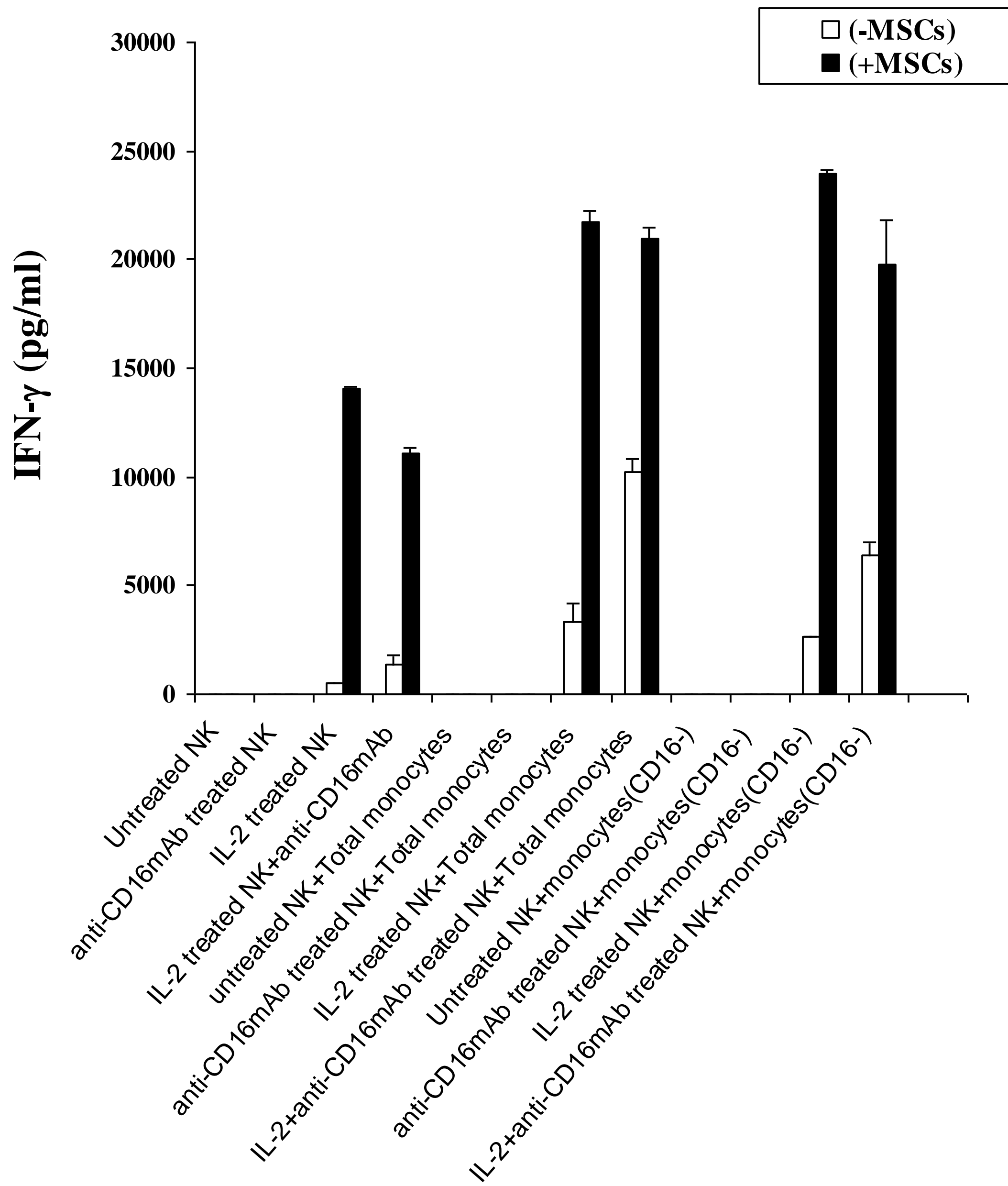


Fig. 6A



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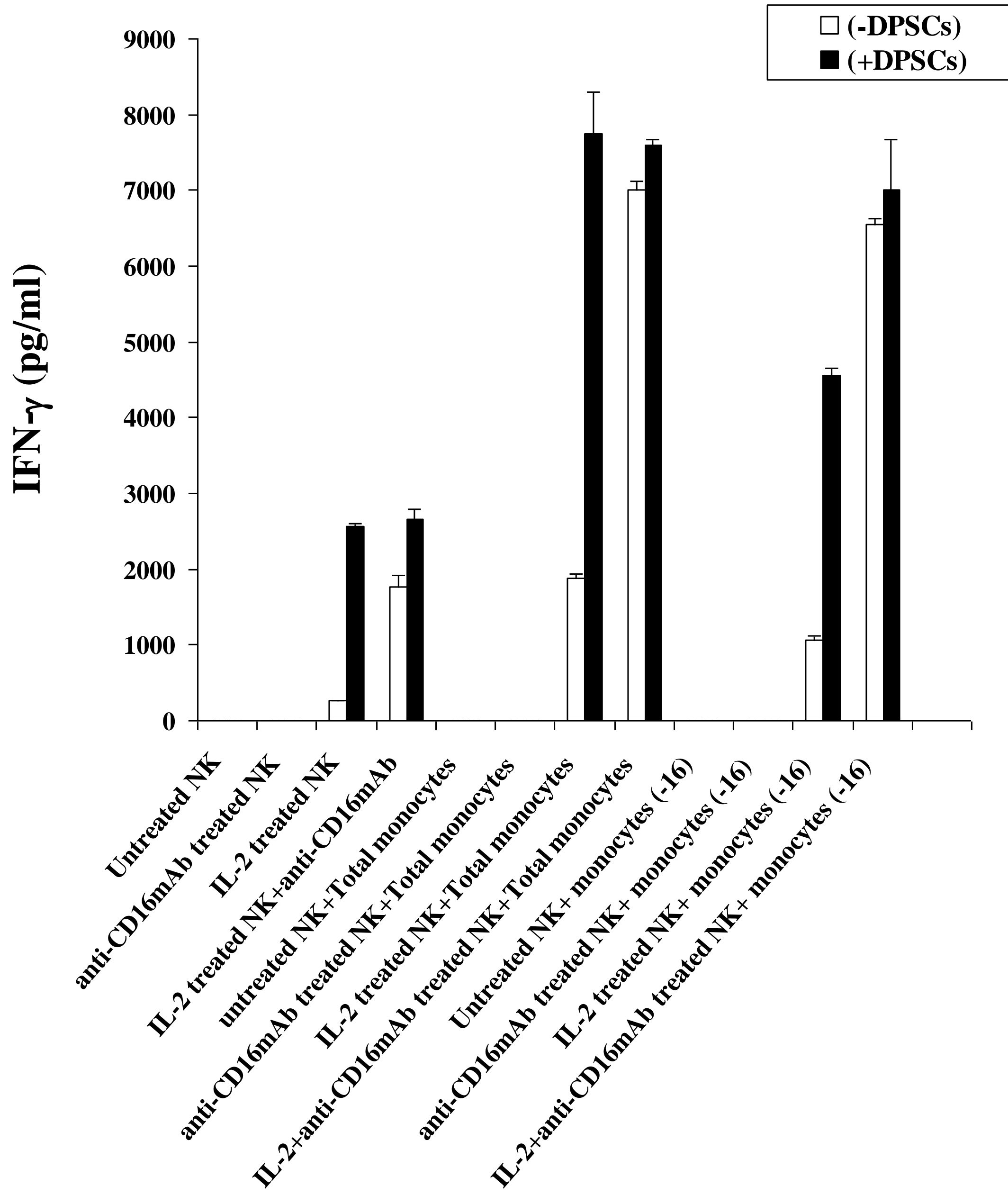


Fig. 7A

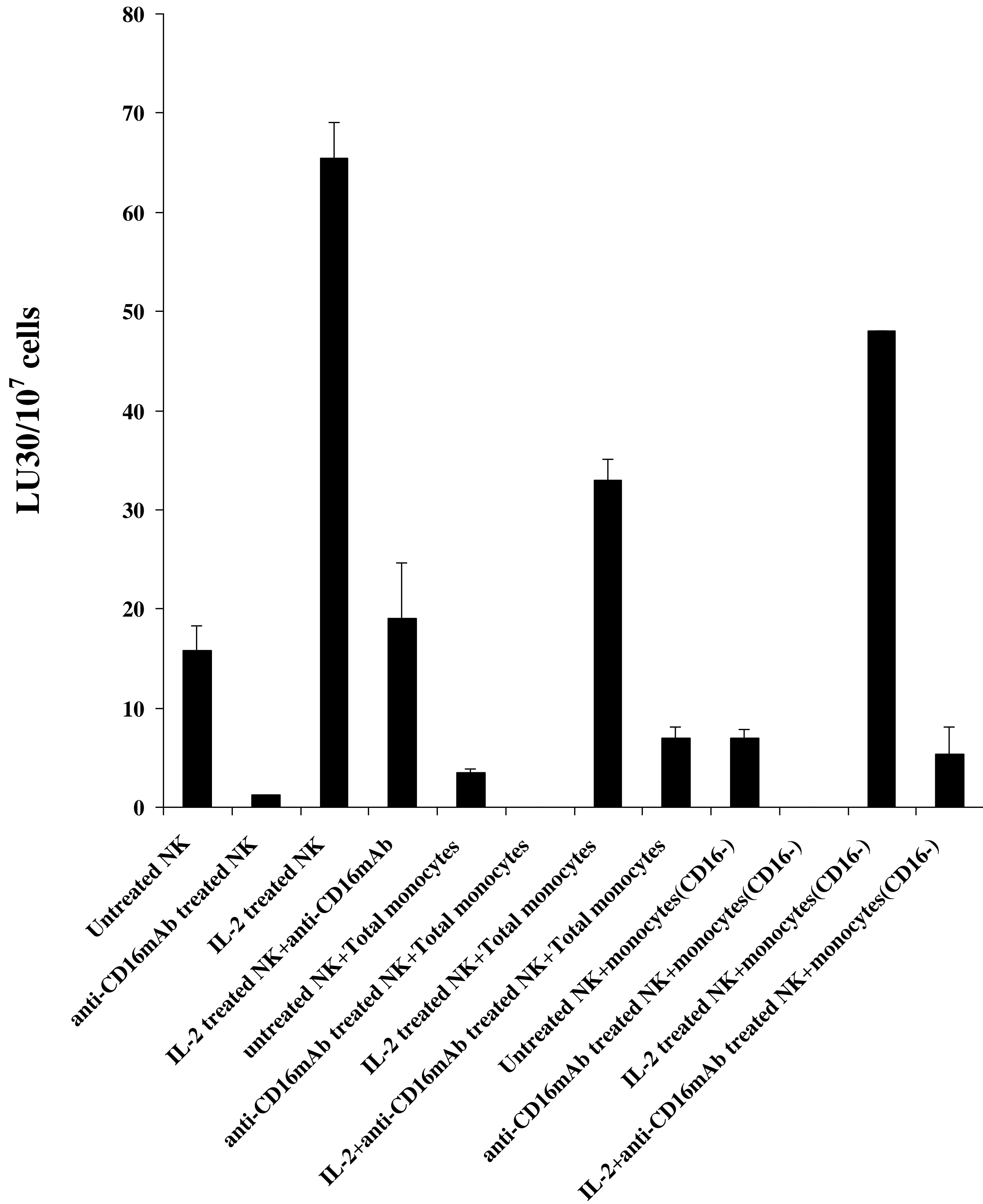


Fig. 7B

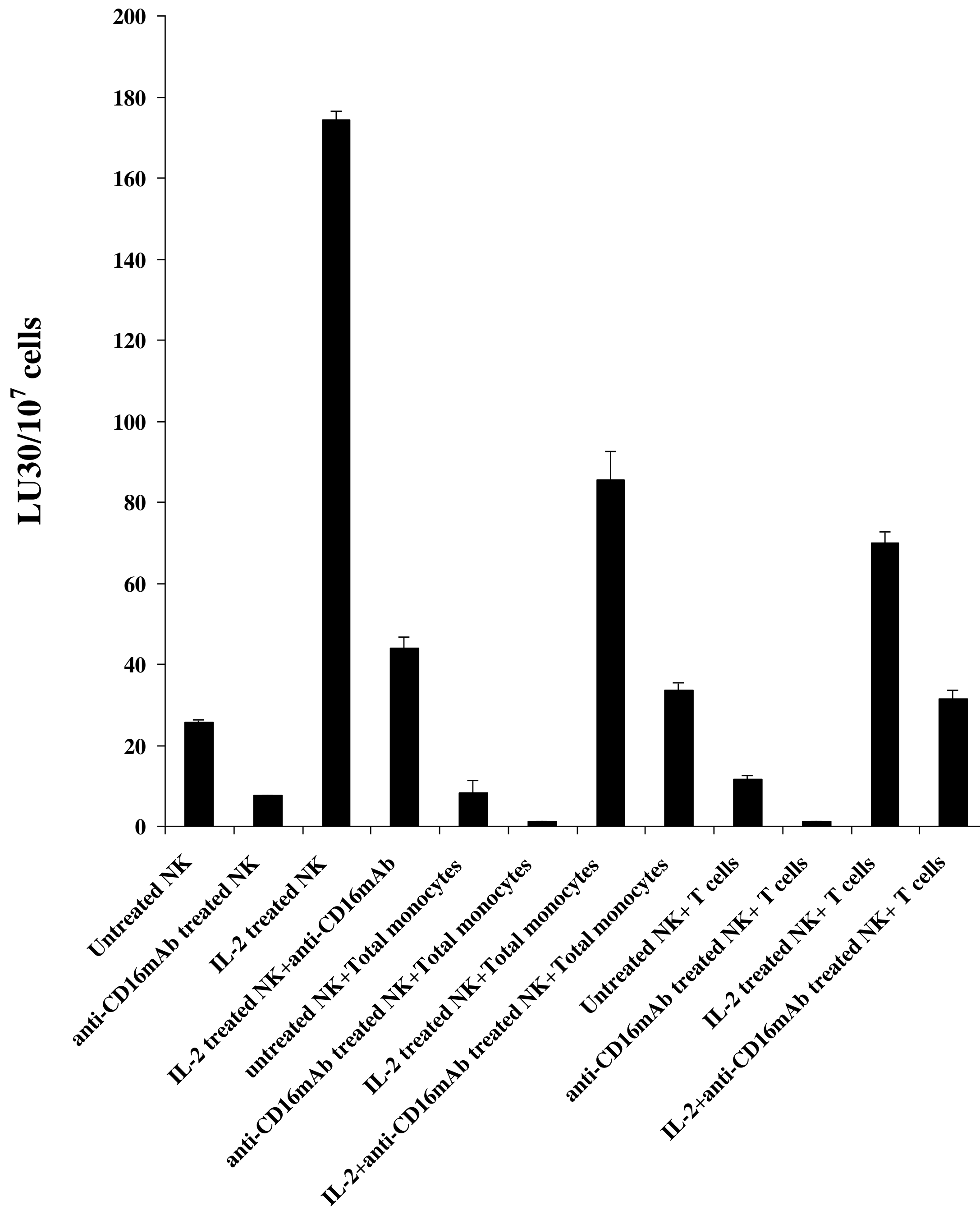


Fig. 7C

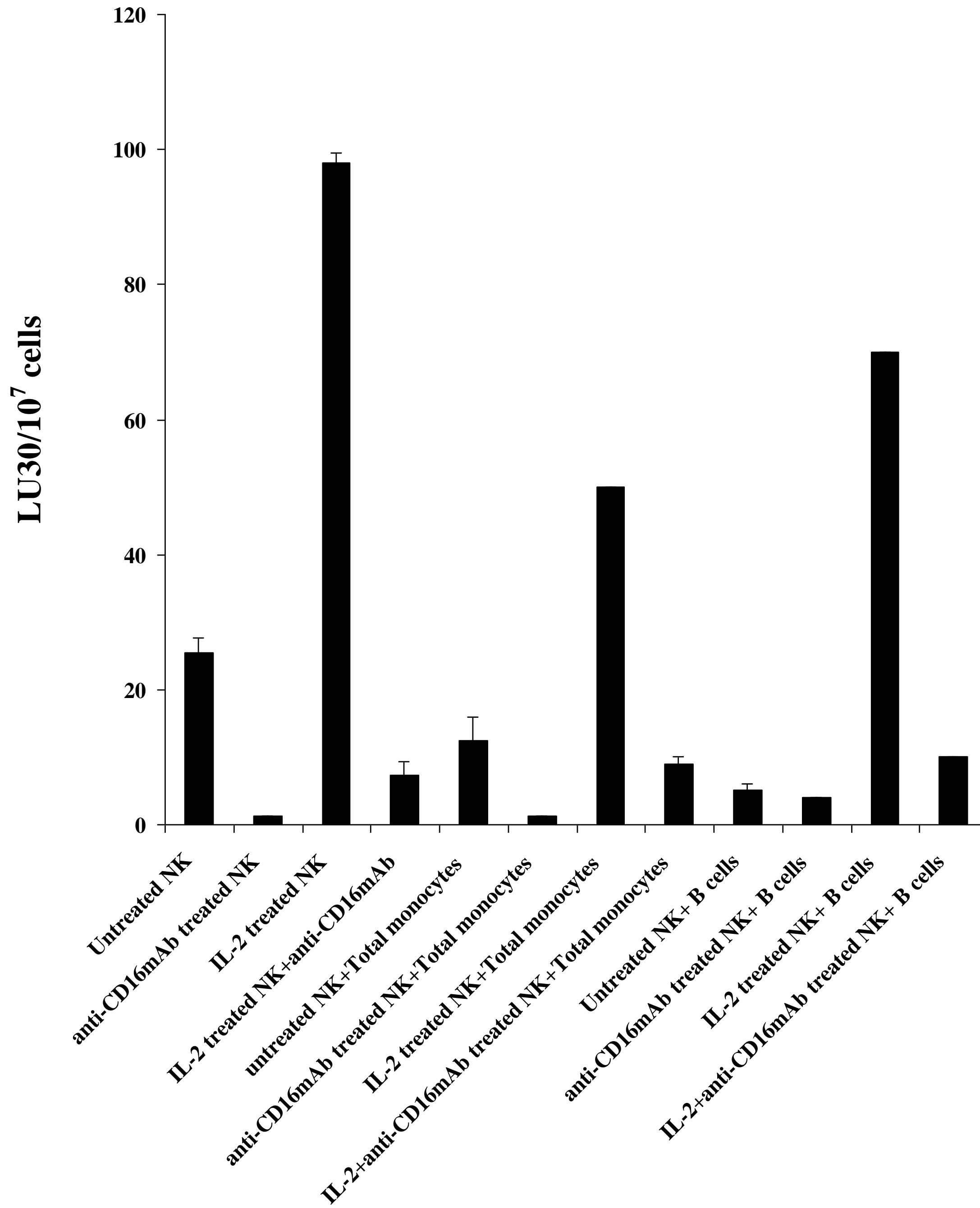


Fig. 8

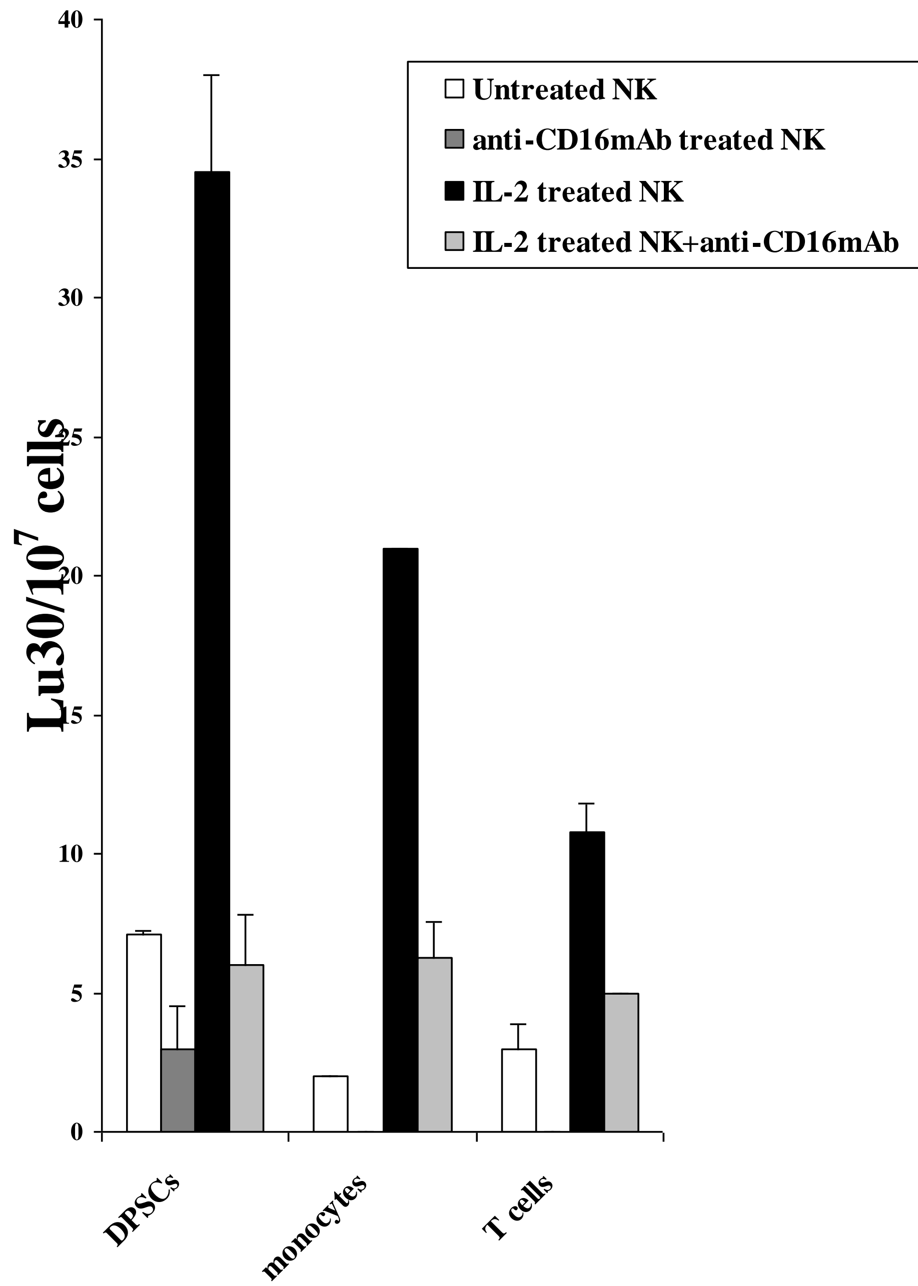


Fig. 9A

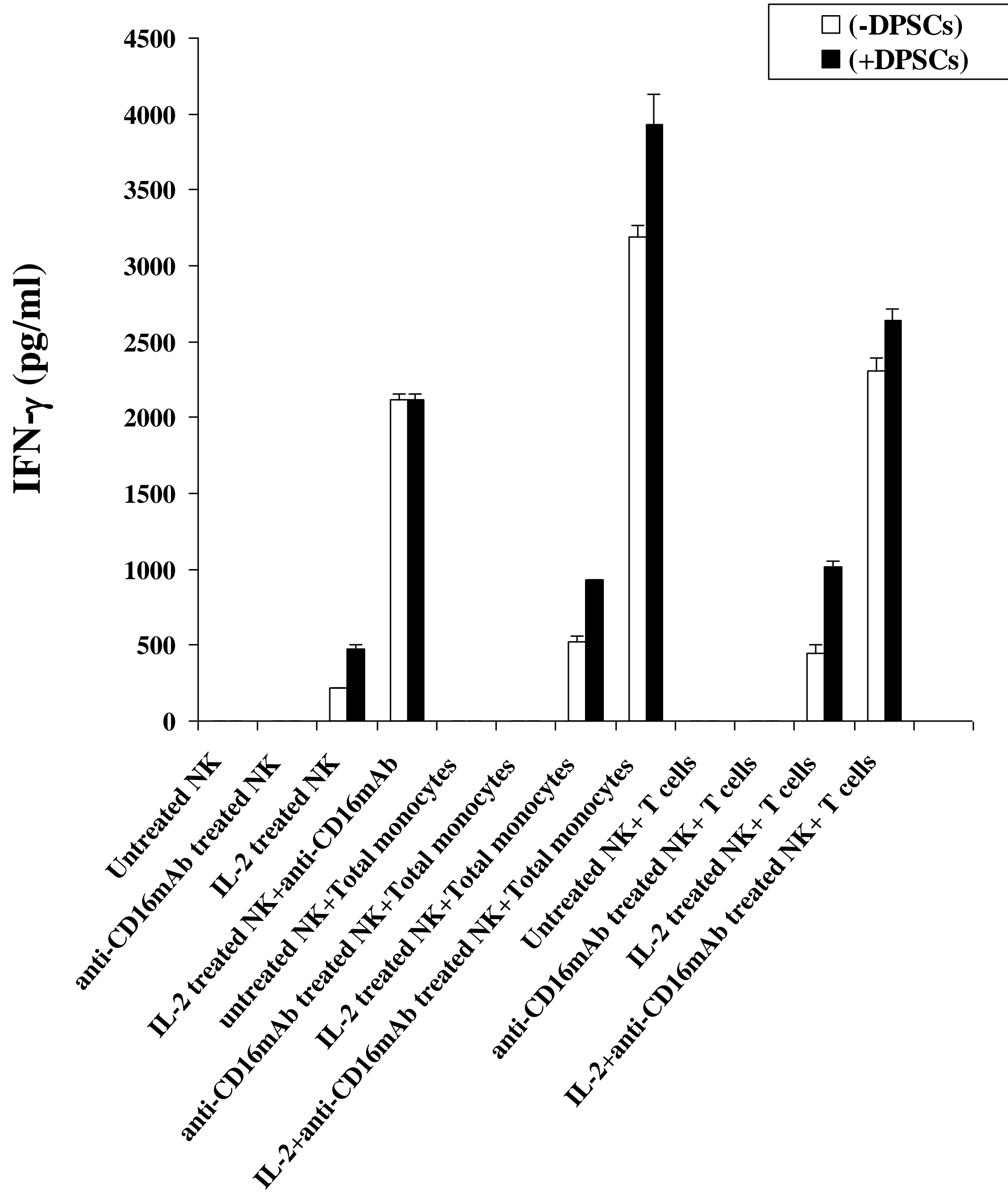


Fig. 9B

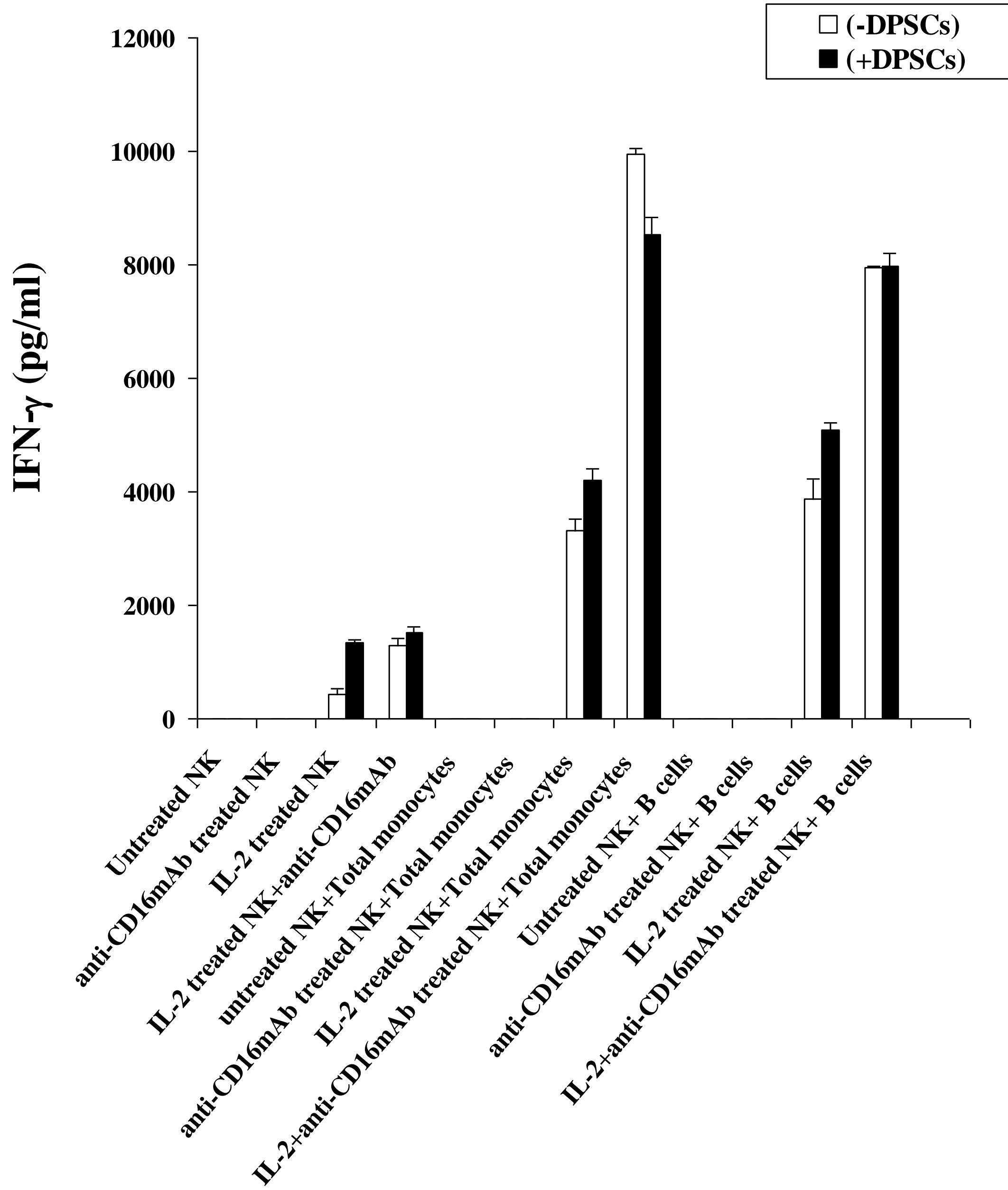
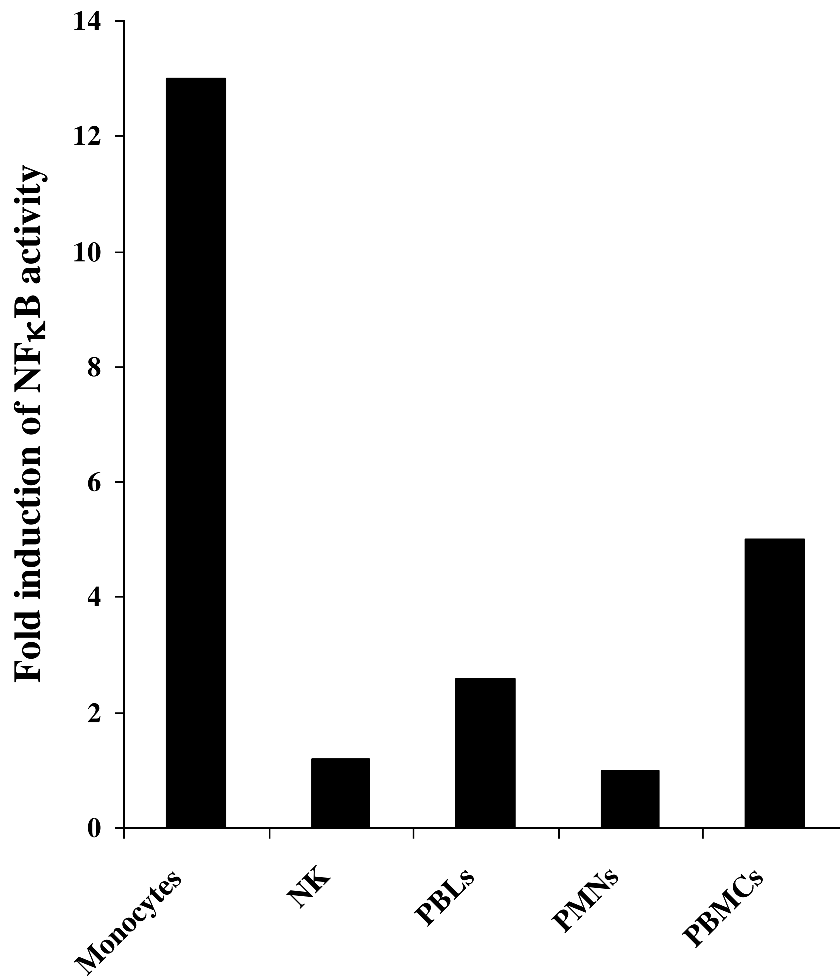


Fig. 10



CHAPTER 3

Induction of split anergy conditions Natural Killer cells to promote differentiation of stem cells through cell-cell contact and secreted factors

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Running Title: anergized NK cells induce differentiation of stem cells

Keywords: IFN-γ, NK, OSCSCs, OSCCs, MP2, cytotoxicity, regulatory NK

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Abstract

In this paper we provide evidence that anergized NK cells through secreted factors and direct cell-cell contact have the ability to induce differentiation of healthy Dental pulp Stem Cells (DPSCs) and Stem Cell of Apical Papillae (SCAP) as well as transformed Oral Squamous Cancer Stem Cell (OSCSCs) and Mia-Paca-2 (MP2), poorly differentiated stem-like pancreatic tumors, resulting in their resistance to NK cell mediated cytotoxicity. Induction of NK cell resistance and differentiation in the stem cells correlated with the increased expression of CD54, B7H1 and MHC class I, and mediated by the combination of membrane bound or secreted IFN-γ and TNF- α from the NK cells since antibodies to both cytokines and not each one alone were able to inhibit differentiation or resistance to NK cells. Similarly, antibodies to both TNF-α and IFN-γ were required to prevent NK mediated inhibition of cell growth, and restored the numbers of the stem cells to the levels obtained when stem cells were cultured in the absence of anergized NK cells. Interestingly, the effect of anti-IFN-γ antibody in the absence of anti-TNF-α antibody was more dominant for the prevention of increase in surface receptor expression since its addition abrogated the increase in CD54, B7H1 and MHC class I surface expression. Antibodies to CD54 or LFA-1 was unable to inhibit differentiation whereas antibodies to MHC class I but not B7H1 increased cytotoxicity of well-differentiated Oral Squamous Carcinoma Cells (OSCCs) as well as OSCSCs differentiated by the IL-2+anti-CD16mAb treated NK cells whereas it inhibited the cytotoxicity of NK cells against OSCSCs. Thus, NK cells may inhibit the progression of cancer by killing and/or differentiation of cancer stem cells which severely halt cancer growth, invasion and metastasis.

Introduction

Recent advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between the immune effectors and the tumor cells. Effectors of the immune system are known to shape tumor cells (immune-editing) and select for cancers with reduced immunogenicity and enhanced capacity to actively induce immunosuppression. However, the same effector mechanisms are likely responsible for the selection of healthy stem cells with enhanced capacity to induce immunosuppression for the ultimate goal of wound healing, tissue regeneration and cessation of inflammation. Much work has been done to identify strategies by which tumor cells evade the immune system. Altered expression of MHC class I molecules which modulate the function of T and NK cells are one of the examples of such mechanism. In addition, tumor cells induce T and NK cell apoptosis, block lymphocyte homing and activation, and dampen macrophage and dendritic cell function by releasing immunosuppressive factors such as Fas, VEGF, IL-6, IL-10, TNF-α, GM-CSF and IL-1β. Many factors responsible for the suppression of NK cell cytotoxicity in humans have been previously identified [175-180]. It has been shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK and T cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [181-184, 232-235]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [185-187].

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity while increasing IFN-γ secretion by the NK cells, and induces cell death in a small

subset of NK cells [136, 137]. On the other hand NK resistant tumors such as RAJI cells do not induce loss of NK cell cytotoxicity nor IFN-γ secretion [136, 137]. Furthermore, following NK cell cultures with sensitive tumor-target cells overnight, the target binding NK cells undergo phenotypic and functional changes expressing CD16-CD56+/dim/- CD69+ phenotype [136, 137]. Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in cancer patients including those who suffer from oral and ovarian cancers [138, 139]. In addition, down-regulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [140]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [140]. These observations suggested that CD16 receptors may play an important role in target cell induced loss of NK cell cytotoxicity. Furthermore, we have previously shown that triggering of CD16 on NK cells was found to result in down-modulation of CD16 receptors and in a great loss of cytotoxicity and augmented secretion of IFN-γ which we have previously coined as "split anergy" [136, 137, 141-145]. Loss of cytotoxicity in NK cells was significantly increased when NK cells were either treated with anti-MHC class I antibody [143] or treated with F(ab)'$_2$ fragment of anti-CD16 mAb [143, 145].

Progress has been made in identification of the upstream mechanisms which control the expression of immunosuppressive factors in tumor cells. Two key control elements, NFκB and STAT3 were shown to regulate coordinately the production of multiple tumor-derived immunosuppressive molecules and play a pivotal role in tumor cell immune suppression.

The significance and exact mechanisms by which NFκB nuclear function in oral tumors modulate and shape the function of key interacting immune effectors is starting to unravel. We have previously shown that differentiated primary oral epithelial tumors, unlike their cancer initiating cells, demonstrate higher nuclear NFκB activity and secrete significant levels of cytokines and chemokines, and are resistant to NK cell mediated cytotoxicity [18, 190]. Moreover, inhibition of NFκB in differentiated OSCCs, or in non-tumorigenic oral keratinocytes (HOK-16B) leads to a significant increase in NK cell mediated cytotoxicity and secretion of IFN-γ [192, 193]. In addition, targeted inhibition of NFκB in skin epithelial cells resulted in the induction of auto-immunity and inflammation [153]. Also, blocking of NFκB function in both the intestinal epithelial cells and myeloid cells was previously shown to result in a significant decrease in size and numbers of the tumor cells [194].

Our previous studies indicated that the stage of maturation and differentiation of healthy untransformed stem cells, as well as transformed tumorigenic cancer stem cells, is predictive of their sensitivity to NK cell lysis. In this regard we have shown that OSCSCs, which are stem-like oral tumors, are significantly more susceptible to NK cell mediated cytotoxicity; whereas, their differentiated counterpart OSCCs is significantly more resistant [18]. In addition, hESCs and hiPSCs, as well as a number of other healthy normal stem cells such as hMSCs and hDPSCs, were found to be significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts [18]. Based on these results, we proposed that NK cells may play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact. In addition, we have shown previously that CD14+HLADR- monocytes can condition NK cells to lose cytotoxicity and secrete inflammatory cytokines [19,

236-239]. The signals received from the stem cells or monocytes alter the phenotype of NK cells and cause NK cells to lose cytotoxicity and change into cytokine producing cells. These alterations in NK cell effector function are thought to ultimately aid in driving differentiation of a minor population of surviving, healthy, as well as transformed stem cells. In this paper we demonstrate that anergized NK cells contribute to differentiation and subsequent resistance of stem cells to NK cell mediated cytotoxicity through cell-cell contact and secreted cytokines.

Materials and Methods

Cell Lines, Reagents, and Antibodies

RPMI 1640 supplemented with 10% FBS (Gemini Bio-Products, CA) was used for the cultures of human NK cells and monocytes. OSCCs and stem-like OSCSCs were isolated from oral cancer patient tongue tumors at UCLA, and cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% antibiotic antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine, 0.2% gentamicin (Gemini Bio-Products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, PA). Mia Paca 2 (MP2) were cultured in DMEM with 10% FBS and 1% penicillin and streptomycin (Gemini Bio-Products, CA). DPSCs and SCAPs were cultured in DMEM complete medium supplemented with 2% FBS and 1% penicillin and streptomycin (Gemini Bio-Products, CA). To induce differentiation of DPSCs, they were cultured with DMEM in the presence of ascorbic acid (50ug/mL), Na-β-glycerophosphate (10mM) (Sigma Aldrich, MO) and dexamethasone (10^{-8}M).

Recombinant IL-2 was obtained from NIH- BRB. Recombinant TNF-α and IFN-γ were obtained from Biolegend (San Diego, CA). The anti- B7H1 was a generous gift from Dr. Liping Chen. Antibodies to CD16, CD54 and LFA-1 were purchased from Biolegend (San Diego, CA). Anti-MHC class I were prepared in our laboratory and 1:100 dilution was found to be the optimal concentration to use. PE – anti-CD26, anti-CD54, anti-CD44, anti-B7H1, anti-CD166, anti-CD326 and anti-CD338 were obtained from Biolegend (San Diego, CA). Antibodies to TNF-α and IFN-γ were prepared in our laboratory and 1:100 dilution was found to be the optimal concentration to use. The human NK and monocyte purification kits were obtained from Stem

Cell Technologies (Vancouver, Canada). Cisplatin was obtained through Ronald Reagan Pharmacy at UCLA. Monensin was purchased through Biolegend (San Diego, CA). Propidium iodide is purchased from Sigma Aldrich (Buffalo, NY).

Purification of NK cells and monocytes

PBMCs from healthy donors were isolated as described before [137]. Briefly, peripheral blood lymphocytes were obtained after Ficoll-hypaque centrifugation and purified NK cells were negatively selected by using an NK cell isolation kit (Stem Cell Technologies, Vancouver, Canada). The purity of NK cell population was found to be greater than 90% based on flow cytometric analysis of anti-CD16 antibody stained cells. The levels of contaminating CD3+ T cells remained low, at 2.4%±1%, similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures. The adherent subpopulation of PBMCs was detached from the tissue culture plates and the total population of monocytes was purified using isolation kit obtained from Stem Cell Technologies (Vancouver, Canada). Greater than 95% purity was achieved based on flow cytometric analysis of CD14 and CD16 antibody stained monocytes. Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors and all the procedures were approved by the UCLA-IRB.

ELISA and Multiplex Cytokine Array kit

Single ELISAs were performed as described previously [137]. Fluorokine MAP cytokine multiplex kits were purchased from R&D Systems (Minneapolis, MN) and the procedures were conducted as suggested by the manufacturer. To analyze and obtain the cytokine concentration, a

standard curve was generated by either two or three fold dilution of recombinant cytokines provided by the manufacturer. Analysis was performed using the Star Station software.

Surface Staining and cell death assays

Staining was performed by labeling the cells with antibodies or propidium iodide as described previously [142] [137, 201].

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [193]. Briefly, different numbers of purified NK cells were incubated with ^{51}Cr–labeled tumor target cells. After a 4 hour incubation period the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm - spontaneous cpm}}{\text{Total cpm – spontaneous cpm}}$$

LU 30/10^6 is calculated by using the inverse of the number of effector cells needed to lyse 30% of tumor target cells X100.

Stem cell differentiation with NK cell supernatant

Human NK cells were purified from healthy donor's PBMCs as described above. NK cells were left untreated or treated with anti-CD16mAb (3ug/ml), IL-2 (1000 units/ml) or a combination or IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18- 24 hours before the

supernatants were removed and used in differentiation experiments. The amounts of IFN-γ produced by activated NK cells were assessed with IFN-γ ELISA (Biolegend, CA). Differentiation of OSCSCs was conducted with gradual daily addition of increasing amounts of NK cell supernatant. On average a total of 1500 pg of IFN-γ containing supernatants obtained from IL-2+anti-CD16mAb treated NK cells was added for 5 days to induce differentiation and resistance of OSCSCs to NK cell mediated cytotoxicity. DPSCs and SCAP cells required on average a total of 3600pg of IFN-γ containing supernatants obtained from IL-2+anti-CD16mAb treated NK cells during a 7 day treatment, whereas MP2 tumors required a total of 7000pg of IFN-γ containing supernatants from IL-2+anti-CD16mAb treated NK cells for 7 days to promote differentiation and resistance to NK cell mediated cytotoxicity. Afterwards, target cells were rinsed with 1X PBS, detached and used for experiments.

Stem cell differentiation with 2% paraformaldehyde fixed NK cells

Human NK cells were purified as described above. NK cells were left untreated or treated with anti-CD16mAb (3ug/ml), IL-2 (1000 units/ml) or a combination or IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18-24 hours. Afterwards, supernatants were removed and the NK cells were fixed with freshly prepared 2% paraformaldehyde for 15 minutes. NK cells were then rinsed three times with 1X PBS and added to tumor cultures. Differentiation of OSCSCs was conducted on average in 5 days with daily and gradual addition of increasing amounts of fixed NK cells. During the differentiation process of OSCSCs, NK cells were added to tumor cells at an effector to target ratio of 0.75 to 1 for 5 days. For the differentiation of DPSCs, an effector to target ratio of 2:1 was added to culture for 7 days. After the treatment period, NK cells were removed from the tumors and the target cells were used for experiments.

Treatment of NK cells with monensin

NK cells were purified from healthy donor's PBMCs as described above. NK cells were left untreated or treated with IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) with or without monensin (1:1300) for 24 hours as per manufacturer's recommendation. Afterwards, the supernatants were harvested from NK cells and the amounts of IFN- γ produced were measured with IFN-γ ELISA (Biolegend, CA). NK cells were then fixed with freshly prepared 2% paraformaldehyde for 15 minutes and then washed three times with 1X PBS. Thereafter, fixed NK cells were added to OSCSCs at an effector to target ratio of 0.75 to 1. After 5 days of treatment, NK cells were removed from culture and OSCSCs were used for experiments.

Statistical analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

Results

Resistance of differentiated OSCCs but not stem-like OSCSCs and DPSCs to NK cell mediated cytotoxicity; Loss of NK cell cytotoxicity and gain in secretion of IFN-γ after NK cell receptor signaling

NK cells were left untreated or treated with anti-CD16 antibody and/or IL-2 for 18-24 hours before they were used with OSCSCs or their differentiated counterparts OSCCs. As shown previously and in here, using time lapse microscopic analysis NK cells mediated much higher lysis of stem like OSCSCs when compared to differentiated OSCCs (Fig. 1A). We had previously characterized stem-like OSCSCs and differentiated OSCCs based on their surface expression [18], and in this report we extended the characterization of stem-like OSCSCs to include other stem cell markers of CD26, CD326 (EpCam), CD166 and CD338 (Fig. 1B-1C). As shown in Fig. 1B and 1C OSCSCs expressed significantly higher amounts of CD26, CD326 and CD338 and lower amounts of CD166 similar to those reported previously [240-243]. In agreement with the increased expression of CD338 (Fig. 1C), OSCSCs were significantly more resistant to cisplatin (Fig. 1D) and Paclitaxel (manuscript in prep) when compared to differentiated counterparts. In accordance to the data obtained with the time lapse microscopy both untreated and IL-2 treated NK cells mediated higher lysis of OSCSCs when compared to OSCCs in a 4 hour ^{51}Cr release assay ($P<0.05$), and IL-2 treated NK cells secreted significant levels of IFN-γ in co-culture with OSCSCs when compared to OSCCs (Fig. 1E, 1F). Anti-CD16 mAb treatment inhibited NK cell cytotoxicity against both OSCSCs and OSCCs ($p<0.05$), however it did not induce any appreciable secretion of IFN-γ (Fig. 1E, 1F). The addition of the combination of IL-2 and anti-CD16 mAb treatment although inhibited NK cell cytotoxicity

significantly against OSCSCs and OSCCs when compared to IL-2 activated NK cells alone ($p<0.05$), it induced higher release of IFN-γ when cultured in the presence and absence of OSCSCs (Fig. 1E, 1F). The levels of IFN-γ secretion remained much less in the co-cultures of IL-2 and/or anti-CD16 mAb treated NK cells with OSCCs when compared to those cultured with OSCSCs ($P<0.05$) correlating with the decreased cytotoxicity by IL-2 treated NK cells against OSCCs (Fig. 1E, 1F). Therefore, anti-CD16 mAb in combination with IL-2 induced split anergy in NK cells resulting in a great loss of cytotoxicity but significant gain in secretion of IFN-γ against oral stem-like tumors (Fig. 1E, 1F).

To determine whether similar results to those demonstrated above can be obtained by healthy untransformed primary stem cells and their differentiated counterparts we performed the following experiments. NK cells were treated as described in Fig. 1A before they were added to ^{51}Cr labeled DPSCs and their differentiated counterparts. As shown in Fig. 1G IL-2 treated NK cells mediated much higher lysis of undifferentiated DPSCs when compared to differentiated DPSCs ($P<0.05$). Anti-CD16 mAb treatment inhibited NK cell cytotoxicity against both undifferentiated and differentiated DPSCs ($p<0.05$), however it did not induce any appreciable secretion of IFN-γ (Fig. 1H). The addition of the combination of IL-2 and anti-CD16 mAb treatment although inhibited NK cell cytotoxicity against undifferentiated and differentiated DPSCs significantly, when compared to IL-2 activated NK cells alone ($p<0.05$) it induced higher release of IFN-γ when cultured in the presence and absence of DPSCs (Fig. 1H). The levels of IFN-γ secretion remained much less in the co-cultures of IL-2 or IL-2 and anti-CD16 mAb treated NK cells with differentiated DPSCs when compared to those cultured with undifferentiated DPSCs ($P<0.05$) correlating with the decreased cytotoxicity by IL-2 treated NK

cells against differentiated DPSCs (Fig. 1G and 1H). The levels of IFN-γ secretion in the co-cultures of undifferentiated DPSCs with IL-2 alone or IL-2 in combination with anti-CD16 mAb treated NK cells plateaued (Fig. 1H).

Monocytes induce split anergy in NK cells resulting in a significant loss of NK cell cytotoxicity against OSCSCs and DPSCs and increased secretion of IFN-γ by the anergized NK cells

Monocytes were purified from PBMCs and irradiated at 20 Gy. OSCSCs were co-cultured with allogeneic irradiated monocytes before they were labeled with ^{51}Cr and used in the cytotoxicity assays against NK cells. NK cells were treated as indicated in Fig. 1 and they were used in the cytotoxicity assays against OSCSCs and DPSCs. The addition of irradiated monocytes to OSCSCs significantly protected the OSCSCs (Fig. 2A) from NK cell mediated cytotoxicity ($p<0.05$). Significant inhibition of NK cell cytotoxicity by monocytes could be observed against untreated and IL-2 treated NK samples ($p<0.05$) (Fig. 2A). Purified irradiated monocytes were also co-cultured with OSCSCs and untreated or IL-2 and/or anti-CD16 mAb pre-treated NK cells were added before the supernatants were removed and subjected to specific ELISAs for IFN-γ (Fig. 2B). Irradiated monocytes cultured with OSCSCs increased secretion of IFN-γ substantially when added to IL-2 or IL-2 and anti-CD16 antibody treated NK cells. Since monocytes alone or monocytes cultured with OSCSCs did not have any effect on IFN-γ secretion in all the experiments tested, we did not include them in Fig. 2 (data not shown). In addition, when secretion of IFN-γ were determined in 8 days, IL-2+anti-CD16mAb treated NK cells continuously secreted higher levels of IFN-γ only in the presence of monocytes at days 4 and 6, whereas IL-2+anti-CD16 mAb treated NK cells in the absence of monocytes had very low levels

of secretion at days 4 and 6 ($P<0.05$) (Fig. 2C). No significant secretion could be observed in any of the samples at day 8 (Fig.2C). At day one IL-2+anti-CD16 mAb treated NK cells secreted high levels of IFN-γ and the levels increased in the presence of monocytes (Fig. 2C). Monocytes in the absence of NK cells were unable to secrete IFN-γ (data not shown). Overall, these experiments indicated that monocytes induce split anergy in NK cells resulting in protection of OSCSCs from NK cell mediated lysis while substantially increasing the secretion of IFN-γ.

Similar to OSCSCs, DPSCs were co-cultured with and without irradiated allogeneic and autologous monocytes before they were labeled with ^{51}Cr and used in the cytotoxicity assays against allogeneic and autologous NK cells. NK cells were treated as indicated in Fig. 1 before they were used in the cytotoxicity assays against DPSCs. As shown in Fig. 2D untreated, IL-2 treated and IL-2+ anti-CD16mAb treated autologous NK cells lysed DPSCs to similar extents to those obtained with allogeneic NK cells. Addition of irradiated monocytes to DPSCs inhibited NK cell mediated cytotoxicity in all treated NK samples and the levels of decrease were similar between autologous and allogeneic NK cells (Fig. 2D). Significant inhibition of NK cell cytotoxicity could be obtained by treating autologous and allogeneic NK cells with IL-2 and anti-CD16 mAb when compared to IL-2 treated NK cells. In contrast IL-2 and anti-CD16 mAb treated NK cells secreted large amounts of IFN-γ and the levels substantially increased when added to monocyte treated DPSCs (Fig. 2E). Similar levels of IFN-γ secretion was obtained by both autologous and allogeneic NK cells when these cells were treated with IL-2 alone or IL-2+anti-CD16 mAb in the presence and absence of DPSCs (Fig. 2E).

Supernatants from the combination of IL-2 and anti-CD16 mAb treated NK cells induced resistance of OSCSCs and DPSCs to NK cell mediated cytotoxicity and increased differentiation antigens on the surface of OSCSCs

NK cells were treated as described in Fig. 1A before their supernatants were removed and added to OSCSCs as described in the materials and methods section. Treatment of OSCSCs with IL-2+anti-CD16mAb treated NK cell supernatants decreased NK cell mediated cytotoxicity significantly by untreated and IL-2 treated NK cells ($P<0.05$) (Fig. 3A). Resistance of OSCSCs to NK cell mediated cytotoxicity could also be observed after their treatment with IL-2 and much less with anti-CD16mAb treated NK supernatants, but the levels of NK resistance were significantly less when compared to those induced by IL-2+anti-CD16mAb treated NK cell supernatants ($P<0.05$) correlating with the degree of differentiation based on the surface receptors (please see below). Supernatants harvested from irradiated monocytes with IL-2+anti-CD16 treated NK cells and added to OSCSCs resulted in a further increase in the resistance of OSCSCs to NK cell mediated cytotoxicity (Fig. 3A). Supernatants from irradiated monocytes and IL-2 treated NK cells were also able to induce resistance of OSCSCs ($P<0.05$) but the levels of resistance were less when compared to that induced by supernatants obtained from the combination of IL-2 and anti-CD16 mAb treated NK cells in the presence of irradiated monocytes (Fig. 3A).

Similarly, treatment of DPSCs with IL-2+anti-CD16mAb treated NK cell supernatants significantly decreased cytotoxicity mediated by IL-2 treated NK cells ($P<0.05$) (Fig. 3B). Addition of supernatants removed from the co-cultures of IL-2 treated NK cells with monocytes to DPSCs increased resistance of these cells against IL-2 treated NK cells, however, the highest

resistance was observed in DPSCs treated with supernatants removed from cultures of monocytes with the combination of IL-2 and anti-CD16 mAb treated NK cells (Fig. 3B).

Addition of paraformaldehyde fixed IL-2+anti-CD16 mAb treated NK cells with and without irradiated monocytes to OSCSCs and autologous DPSCs mediated resistance of OSCSCs and DPSCs against NK cell mediated cytotoxicity

Similar to the treatment of OSCSCs with the NK cell supernatants, OSCSCs became resistant to NK cell mediated cytotoxicity after their co-culture with IL-2+anti-CD16mAb treated paraformaldehyde fixed NK cells ($P<0.05$) when compared to the addition of untreated or anti-CD16 mAb treated or IL-2 treated fixed NK cells (Fig. 3C). The complete removal of Fixed NK cells from the OSCSCs prior to the cytotoxicity assays were determined by microscopic assessment. Co-cultures of IL-2 and IL-2+anti-CD16mAb treated NK cells with monocytes before their fixation and addition to OSCSCs increased resistance of these cells against untreated and IL-2 treated NK cells and the highest resistance was observed in OSCSCs which were cultured with monocytes and the combination of IL-2 and anti-CD16 mAb treated NK cells although monocytes cultured with IL-2 treated NK cells were also able to induce resistance in OSCSCs (Fig. 3C).

Similarly, cultures of DPSCs with paraformaldehyde fixed autologous IL-2+anti-CD16 mAb pre-treated NK cells decreased NK cell mediated cytotoxicity by untreated and IL-2 treated NK cells significantly ($P<0.05$) (Fig. 3D). Cultures of irradiated monocytes with the IL-2 treated NK cells before their addition to DPSCs increased resistance of these cells against IL-2 treated NK cells moderately, however, the highest resistance was observed in DPSCs which were

cultured with monocytes and the combination of IL-2 and anti-CD16 mAb treated NK cells (Fig. 3D).

Induction of NK resistance in OSCSCs correlated with the increased expression of CD54, B7H1, MHC class I and decreased expression of CD44

We then compared NK cell resistance induced by the NK cell supernatant in OSCSCs to expression of key cell surface receptors. Among many surface receptors tested, CD44, CD54, B7H1 and MHC class I expression was found to correlate significantly with the differentiation and resistance of OSCSCs to NK cell mediated cytotoxicity (Fig. 4A). As shown in Fig. 4A the levels of CD54, MHC class I and B7H1 increased substantially on OSCSCs whereas the levels of CD44 decreased in the presence of IL-2+anti-CD16mAb treated NK cell supernatants (Fig. 4A). Treatment of OSCSCs with IL-2 alone treated NK supernatants modulated the above mentioned surface receptors moderately, and the levels were less when compared to those modulated by the supernatants obtained from IL-2+anti-CD16mAb treated NK cells (Fig. 4A). As expected supernatants obtained from the monocytes with IL-2+anti-CD16mAb treated NK cells up-regulated both CD54 and MHC-class I substantially more when compared to either monocytes alone or IL-2+anti-CD16mAb treated NK cells in the absence of monocytes (Fig. 4B). Since the highest increase in resistance to NK cell cytotoxicity and augmented expression of CD54, B7H1 and MHC class I were found in the presence of the treatment of OSCSCs with supernatants from IL-2+anti-CD16mAb treated NK cells; i.e. anergized NK cells, we used this treatment hereafter for the remaining of the studies.

To determine growth dynamics of OSCSCs after treatment with the NK supernatants the number of attached and detached OSCSCs were counted after treatment with the supernatants by microscopic evaluation and the levels of cell death were determined by staining with Propidium iodide followed by flow cytometric analysis. As shown in Fig. 4C there was a decrease in the numbers of OSCSCs after their treatment with IL-2 and anti-CD16mAb treated NK cell supernatants when compared to untreated OSCSCs or those treated with untreated NK cell supernatants (Fig. 4C). However, the highest decrease was observed when OSCSCs were cultured with the supernatants obtained from the combination of IL-2+anti-CD16mAb treated NK cells with monocytes (Fig. 4C). Interestingly, monocytes alone increased growth of the OSCSCs when compared to untreated OSCSCs (Fig. 4C). When the numbers of detached OSCSCs were determined, no significant differences could be observed in those treated with NK supernatants alone and the highest increase was seen when OSCSCs were cultured with the supernatants obtained from the combination of IL-2+anti-CD16 mAb treated NK cells with monocytes (Fig. 4C). Similarly, 12% increase in cell death could be seen when OSCSCs were treated with the combination of IL-2+anti-CD16mAb treated NK cells with monocytes, whereas no cell death in attached OSCSCs could be observed when they were treated with the IL-2+anti-CD16mAb treated NK cells in the absence of monocytes (Fig. 4D). Although slight increases in cell death of detached OSCSCs were seen when these cells were treated with IL-2+anti-CD16 mAb treated NK cell supernatants (10%), the levels increased substantially in detached cells when OSCSCs were treated with the supernatants from the combination of IL-2+anti-CD16mAb treated NK cells and monocytes (Fig. 4D).

Induction of resistance in OSCSCs to NK cell mediated cytotoxicity by anergized NK cells is mediated by the combination of IFN-γ and TNF-α and not each cytokine alone

To examine the mechanisms by which OSCSCs become resistant by anergized NK cells, we determined NK cell mediated cytotoxicity when OSCSCs were treated with supernatants of NK cells treated with anti-CD16mAb and IL-2 in the presence and absence of each of IFN-γ and TNF-α antibodies alone or their combination. As shown in Fig. 5A-5C the addition of each of the IFN-γ and TNF-α antibody alone had a slight inhibitory effect on the induction of resistance of OSCSCs by the supernatants of NK cells treated with IL-2+anti-CD16mAb, however, the combination of anti-IFN-γ and anti-TNF-α abrogated the resistance of treated OSCSCs completely (Fig. 5A-5C). The inhibition of OSCSCs resistance to NK cell mediated cytotoxicity by the combination of anti-IFN-γ and anti-TNF-α antibodies could be observed when either untreated (Fig. 5A), IL-2 treated (Fig. 5B) or IL-2+anti-CD16mAb treated NK cells (Fig. 5C) were used to assess cytotoxicity. Addition of isotype control antibodies to IL-2+anti-CD16mAb treated NK cell supernatants did not change the resistance of OSCSCs to NK cell mediated cytotoxicity (Fig. 5). Resistance of OSCSCs induced by supernatants from IL2+anti-CD16 mAb treated NK cells correlated with increased expression of CD54, B7H1 and MHC class I as shown above and the addition of a combination of anti-TNF-α and anti-IFN-γ antibodies prevented the up-regulation of these receptors (Fig. 5D). The effect of anti-IFN-γ mAb in the absence of anti-TNF-α antibody, however, was more dominant for surface receptor modulation than cytotoxicity or cell growth (please see below) since its addition abrogated the increase in surface receptor expression substantially (Fig. 5D). In addition, the rate of OSCSC cell growth was decreased when supernatants obtained from IL-2+anti-CD16 treated NK cells were added, and this decrease was completely inhibited in the presence of the combination of anti-IFN-γ and anti-

TNF-α antibodies and not each antibody alone (Fig. 5E). Moreover, when the numbers (Fig. 5E) and viability (Fig. 5F) of OSCSCs attached or detached from the culture plates were determined after the addition of supernatants from the IL-2+anti-CD16 mAb treated NK cells, decreased growth rate with equal numbers of attached and detached OSCSCs were seen and the addition of the combination of anti-TNF-α and anti-IFN-γ restored the growth rate and eliminated the increase in detached OSCSCs (Fig. 5E, 5F). No or slight decreases in the viability of attached OSCSCs could be seen after their treatment with the supernatants from IL-2+anti-CD16 mAb treated NK cells. In contrast, significant increases in cell death of detached OSCSCs could be seen (Fig. 5F). Interestingly, treatment of OSCSCs with supernatants from IL-2+anti-CD16mAb treated NK cells in the presence of anti-IFN-γ alone demonstrated reduced death of detached OSCSCs (Fig. 5F).

Similar results to those obtained with OSCSCs were also observed when undifferentiated or stem-like Mia Paca (MP2) pancreatic tumors or Stem Cell of Apical Papillae (SCAP) were used to treat with the supernatants of NK cells treated with IL-2+anti-CD16mAb in the presence and absence of anti-TNF-α and/or anti-IFN-γ (Fig. 1 supplemental).

Induction of differentiation and resistance to NK cell mediated cytotoxicity of OSCSCs by anergized paraformaldehyde fixed NK cells is mediated by the combination of IFN-γ and TNF-α and not each cytokine alone

Similar to the data shown above, the addition of each of the IFN-γ and TNF-α antibody alone had a slight inhibitory effect on the resistance of OSCSCs induced by the paraformaldehyde fixed NK cells treated with IL-2+anti-CD16mAb, however, the combination

of anti-IFN-γ and anti-TNF-α abrogated the resistance of treated OSCSCs completely (Fig. 6A). IL-2+anti-CD16 mAb pre-treated and paraformaldehyde fixed NK cells neither were able to mediate cytotoxicity (Fig. 6B) nor secrete IFN-γ (Fig. 6C) or TNF-α (data not show) after fixation, and they expressed membrane bound TNF-α and IFN-γ when assessed by flow cytometric analysis using antibodies to TNF-α and IFN-γ (Fig. 6D). The inhibition of OSCSCs resistance to NK cell mediated cytotoxicity by the combination of anti-IFN-γ and anti-TNF-α antibodies could be observed when IL-2 treated NK cells were used to assess cytotoxicity (Fig. 6A). Resistance of OSCSCs by IL2+anti-CD16 mAb treated fixed NK cells correlated with increased expression of CD54, B7H1 and MHC class I and the addition of a combination of anti-TNF-α and anti-IFN-γ antibodies prevented up-regulation of these receptors (Fig. 6E). Similarly, the effect of anti-IFN-γ mAb in the absence of anti-TNF-α antibody was more dominant for surface receptor expression than cytotoxicity or cell growth since its addition abrogated the increase in surface receptor expression substantially (Fig. 6A and Fig. 6E and Fig. 6F). The rate of OSCSCs cell growth was decreased when IL-2+anti-CD16 treated fixed NK cells were added, and this decrease was completely inhibited in the presence of the combination of anti-IFN-γ and anti-TNF-α antibodies and not each antibody alone (Fig. 6F). Moreover, when the numbers (Fig. 6F) and viability (Fig. 6G) of attached OSCSCs from the cultured plates were determined after the addition of IL-2+anti-CD16 mAb treated fixed NK cells, decreased growth rate of OSCSCs were seen and the addition of the combination of anti-TNF-α and anti-IFN-γ restored the growth rate (Fig. 6F). No or slight decreases in the viability of attached OSCSCs could be seen after their treatment with the IL-2+anti-CD16 mAb treated fixed NK cells (Fig. 6G). Although, increased numbers of detached OSCSCs could be observed in the presence of IL-2+anti-

CD16mAb treated fixed NK cells, accurate numbers of detached OSCSCs and their viability could not be established because of contamination with fixed NK cells.

Similar results to those obtained with OSCSCs were also observed when undifferentiated or stem-like MP2 or SCAPs were used to treat with the IL-2+anti-CD16mAb treated fixed NK cells in the presence and absence of anti-TNF-α and/or anti-IFN-γ (data not shown).

To determine whether monensin treated fixed anergized NK cells lose the ability to induce resistance of OSCSCs against NK cell mediated cytotoxicity, first we established that treatment of NK cells with IL-2+anti-CD16mAb in the presence of monensin blocked substantially the release of IFN-γ in the supernatants (Fig. 6H). Addition of monensin in combination with IL-2+anti-CD16 treated and fixed NK cells to OSCSCs inhibited resistance of OSCSC against untreated (Fig. 6I), IL-2 (Fig. 6J) or IL-2+anti-CD16mAb (Fig. 6K) treated NK cells when compared to IL-2+anti-CD16mAb treated fixed NK cells in the absence of monensin treatment. Treatment of NK cells with the combination of IL-2+anti-CD16mAb and monensin inhibited the increase in CD54 and MHC class I expression on OSCSCs when compared to those induced by IL-2+anti-CD16mAb treated and fixed NK cells (Fig. 6L). No cell death could be observed in attached OSCSCs with any of treatments (Fig. 6M).

Combination of rTNF-α and rIFN-γ induce differentiation and resistance of OSCSCs to NK cell mediated cytotoxicity

OSCSCs were treated with rTNF-α and/or rIFN-γ in the presence and absence of anti-TNF-α mAb or anti-IFN-γ antiserum. As shown in Fig. 7A addition of rTNF-α to OSCSCs was able to induce some resistance against IL-2 treated NK cell mediated cytotoxicity and this

resistance was moderately blocked by the addition of anti-TNF-α antibody. rIFN-γ treated OSCSCs were significantly more resistant to IL-2 treated NK cells when compared to rTNF-α treated OSCSCs and the addition of anti-IFN-γ blocked resistance ($p<0.05$) (Fig. 7A). Since the addition of rIFN-γ had reduced the NK cytotoxicity substantially, further decrease with the combination of rTNF-α and rIFN-γ was difficult to demonstrate in this experiment (Fig. 7A). No significant cell death could be observed in all treated samples at the concentrations of rTNF-α and rIFN-γ tested (Fig. 7B). However, at the higher concentration of rTNF-α and rIFN-γ, when the combination of rTNF-α and rIFN-γ were used, an increase in the numbers of detached OSCSCs and cell death in both the attached and detached OSCSCs but not MP2 or SCAPs could be observed (data not shown). Both rTNF-α and rIFN-γ were able to upregulate CD54 and MHC class I expression significantly, however, only rIFN-γ was able to upregulate B7H1 expression moderately on OSCSCs (Fig. 7C). Addition of a combination of rTNF-α and rIFN-γ synergistically induced the expression of CD54 and B7H1, but not MHC-class I (Fig. 7C). Similar results to those obtained with OSCSCs were also obtained with SCAPs and DPSCs. RTNF-α induced resistance to NK cell mediated cytotoxicity less than rIFN-γ and the combination of rIFN-γ and rTNF-α further reduced IL-2 mediated cytotoxicity (Fig. 2A supplemental). Unexpectedly, the level of CD44 was augmented by the treatment of rTNF-α and rIFN-γ and their combination, and the addition of anti-TNF-α and anti-IFN-γ antibodies blocked the increase substantially on DPSCs whereas it inhibited the increase in CD54, B7H1 and MHC class I expression by the combination of rTNF-α and rIFN-γ (Fig. 2B supplemental).

Antibodies to CD54 or LFA-1 was unable to inhibit differentiation in OSCSCs

Addition of antibodies to CD54 (Fig. 8A) or its ligand LFA-1 (Fig. 8B) in the initiation of differentiation of OSCSCs with supernatants from IL-2+anti-CD16mAb treated NK cells for the duration of differentiation and their subsequent removal by extensive washing before labeling of OSCSCs with 51 Cr and addition to freshly isolated IL-2 treated NK cells did not reverse differentiation in supernatant differentiated OSCSCs, however, it did inhibit cytotoxicity moderately in IL-2+anti-CD16mAb supernatant differentiated OSCSCs. In addition, unlike the effect of antibodies to TNF-α and IFN-γ, addition of anti-CD54 or anti-LFA-1 did not restore the numbers of OSCSCs to the levels seen with the untreated OSCSCs as seen in Fig 5E (data not shown). Moreover, the addition of CD54 and LFA-1 antibodies to untreated OSCSCs and those treated with untreated NK supernatants for the duration of differentiation and extensive washing to remove remaining antibodies before 51Cr labeling and culture with freshly isolated NK cells in 51Cr release assay did not change NK cell cytotoxicity (Fig. 8A and 8B).

Antibodies to B7H1 and MHC-class I did not change or increased cytotoxicity of OSCSCs differentiated by IL-2+anti-CD16mAb treated NK supernatants respectively

In contrast to anti-CD54 or anti-LFA-1 which were added at the initiation of differentiation and were present for the entire differentiation period, antibodies to B7H1 and MHC class I were added after the differentiation in the cultures of NK cells with the 51Cr labeled differentiated targets only for 4 hours to assess their significance in cytotoxicity and not differentiation since B7H1 and MHC class I were shown previously to regulate cytotoxicity of NK cells and were found to be elevated on the surface of OSCSCs differentiated with the IL-2+anti-CD16mAb treated NK supernatants. As shown in Fig. 9A the addition of B7H1 antibody even though moderately increased cytotoxicity when added to untreated OSCSCs, it had no

effect when added to IL-2+anti-CD16mAb NK supernatant differentiated OSCSCs even though the surface expression of B7H1 was significantly elevated on differentiated OSCSCs. In contrast, the addition of anti-MHC class I significantly increased cytotoxicity when added to IL-2+anti-CD16mAb NK supernatant differentiated OSCSCs, whereas it had inhibitory effect when added to untreated OSCSCs (Fig. 9B). Addition of anti-MHC class I to well-differentiated OSCCs also increased cytotoxicity significantly (Fig. 9B).

Discussion

In this paper we provide evidence that conditioned or anergized NK cells have the ability to induce resistance and differentiation of stem cells through secreted factors and direct cell-cell contact. Both secreted and membrane bound IFN-γ and TNF-α were found to be important for the induction of resistance of OSCSCs and DPSCs to NK cell mediated cytotoxicity, and inhibition of cell growth and proliferation, since combination of antibodies to TNF-α and IFN-γ and not each one alone was able to restore susceptibility to NK mediated cytotoxicity and increase the number of tumor cells. On the other hand, IFN-γ appears to be more dominant in increasing cell surface receptors on differentiated OSCSCs and DPSCs since the addition of anti-IFN-γ antibody was able to block the expression of CD54, MHC-class I and B7H1 largely, even though the addition of the combination of rIFN-γ and rTNF-α was found to induce CD54, B7H1 and MHC class I expression synergistically. IL-2 and anti-CD16mAb activated fixed NK cells expressed membrane bound TNF-α and IFN-γ and mediated differentiation and resistance of OSCSCs and DPSCs. Indeed, both soluble and immobilized IFN-γ was shown to mediate neuronal differentiation in a dose dependent manner [244], and membrane bound IFN-γ has previously been shown to protect mice from metastasis of lewis lung carcinomas to a comparable extent to that mediated by the secreted cytokines [245]. In addition, membrane bound TNF-α confers protection against Mycobacterial infection [246]. These studies underscore the significance of both secreted and membrane bound forms of TNF-α and IFN-γ in tumor therapy and infections. Moreover, the addition of monensin to IL-2+anti-CD16mAb pre- treated fixed NK cells was also able to prevent differentiation, and increase the numbers, viability and susceptibility of stem cells to NK cell mediated cytotoxicity in the presence of lower expression

of CD54, MHC class I and B7H1 surface receptors. These studies indicated that both membrane bound as well as secreted forms of TNF-α and IFN-γ are important in differentiation of stem cells.

When the extent of resistance to NK cell mediated cytotoxicity, cell growth, attachment to culture plates and viability were determined in OSCSCs, MP2 and DPSCs after differentiation with the supernatants or fixed NK cells treated with IL-2+anti-CD16mAb, distinct cell signatures in respect to the outcome of differentiation were obtained for each cell type. In addition, the amount of supernatants used, and the length of treatment time were two important factors influencing the outcome of differentiation. While OSCSCs were sensitive to the differentiation effect of NK cell supernatants and exhibited detachment and cell death at relatively lower amounts of supernatant treatment, and in shorter period of treatment time, MP2, SCAPs and DPSCs were significantly more resistant, and they neither detached nor underwent cell death in the detached population at higher amounts of NK supernatants and in longer period of treatment. These differences could be due to the levels of TNF-α and IFN-γ receptor expression on each cell type and is the subject of our future studies. In addition, such differences may be the basis for why certain tumors are better controlled by the NK cells than the others, and provide the rationale for designing effective strategies to eliminate tumors. In agreement, differential effects of IFN-γ and TNF-α in either causing resistance in tumors or sensitizing them to NK cell mediated cytotoxicity has also been reported previously [247-249].

Since CD54 was strongly elevated during differentiation we aimed at understanding its role in differentiation. Addition of anti-CD54 or anti-LFA-1 antibodies at the initiation of

differentiation of OSCSCs with IL-2+anti-CD16mAb treated NK supernatants, did not change differentiation of OSCSCs since no restoration of cell number could be observed (data not shown), and their addition were moderately inhibitory for the cytotoxicity of NK cells in IL-2+anti-CD16 mAb supernatant differentiated OSCSCs suggesting that cell contact through CD54 and LFA-1 may not be important for the differentiation of OSCSCs by the NK supernatants. In addition, both B7H1 and MHC class I are shown to regulate cytotoxicity of NK cells and are elevated on the surface of NK differentiated OSCSCs. Addition of antibodies to B7H1 did not increase cytotoxicity of NK cells against differentiated OSCSCs even though the surface expression of B7H1 was significantly elevated on differentiated OSCSCs. In contrast, addition of antibody to MHC class I significantly elevated cytotoxicity of NK cells against NK differentiated OSCSCs and this correlated with significant augmentation of MHC class I on NK differentiated OSCSCs. Similarly, the addition of anti-MHC class I antibody to the co-cultures of NK cells with differentiated primary OSCCs, which naturally express high surface MHC class I expression, mediated increased cytotoxicity by the NK cells. In contrast, addition of antibodies to MHC class I in the co-cultures of NK cells with stem-like OSCSCs was inhibitory for NK cell cytotoxicity. This inhibition could be due to the effect of anti-MHC class I antibody on NK cells and not on the tumor cells since we have previously shown that the addition of anti-MHC class I antibody to CD16 mAb activated NK cells mediated significant inhibition of NK cell cytotoxicity [143]. Whether increase in NK cell cytotoxicity against differentiated tumors by anti-MHC class I is due to ADCC or blocking of inhibitory ligands on NK cells awaits future investigation.

It is likely that other immune effectors such as T cells and NKT cells may also contribute to the pool of IFN-γ and TNF-α when they are activated, and thus they may be important for driving differentiation of tumors. In this regard CD4+ Th1 cells may play a crucial role. Future studies should be able to establish the significance of other cell types in differentiation and resistance of tumor cells.

Our results suggest two very important functions for the NK cells. One potential function of NK cells is to limit the number of stem cells, and second to support differentiation of the stem cells and their progeny. In respect to the oral squamous cell carcinomas since the majority of immune effectors are found at the connective tissue area (Fig. 10) [236-239, 250], it is likely that NK cells may first encounter and interact with either the other immune effectors or the effectors of connective tissue such as fibroblasts. However, there is also the possibility that NK cells may first encounter the basal epithelial stem cells in which case by eliminating the stem cells, they too can become anergized (Fig. 10). By eliminating a subset of stem cells or after their interaction with other immune inflammatory cells or effectors of connective tissue NK cells could then be in a position to support differentiation of remaining stem cells since they will be conditioned to lose cytotoxicity and induce cytokine and growth factor secretion. It is interesting to note that all of the immune effectors isolated from oral gingival tissues of healthy as well as diseased gingiva have CD69+ phenotype, with the exception that the numbers of immune effectors are much less in the healthy oral gingival tissues when compared to diseased tissues [236-239, 250] (manuscript in prep). In addition, lack of significant infiltration of NK cells in the tumor nest and the localization of NK cells in the immune rich compartment which surrounds the tumor in other tumor types, also provides the rationale and the means for the induction of split anergy in NK

cells primarily by other immune effectors in the tumor microenvironment [250]. Such mechanisms of NK cell conditioning by the other immune effectors such as Myeloid Derived Suppressor Cells (MDSCs) explain why the cytotoxic function of NK cells are greatly reduced in the tumor microenvironment as well as in circulating NK cells.

There should be two distinct strategies to eliminate tumors, one which targets stem cells and the other which targets differentiated cells. Since cancer stem cells are found to be more resistant to chemotherapeutic drugs but sensitive to NK cell mediated killing while differentiated oral tumors are more resistant to NK cell mediated killing but relatively more sensitive to chemotherapeutic drugs (Fig. 1), combination therapy should be considered for the elimination of both undifferentiated and differentiated tumors. In addition, since a great majority of patient NK cells have modified their phenotype to support differentiation of the cells, they may not be effective in eliminating cancer stem cells. Therefore, these patients may benefit from repeated allogeneic NK cell transplantation for elimination of cancer stem cells. In this regard depletion of NK anergizing effectors such as monocytes in the tumor microenvironment which condition NK cells to lose cytotoxicity, via radiation or chemotherapeutic drugs before allogeneic NK cell transplantation should in theory provide such strategy for targeting the cancer stem cells by the NK cells. However, this strategy may also halt or decrease the ability of NK cells to drive optimal differentiation of the tumors and tilt the balance towards a more inflammatory tumor microenvironment which may run the risk of fueling the growth and expansion of more cancer stem cells. Indeed, NK supernatant differentiated tumors were unable to trigger secretion of cytokines and chemokines, providing a less inflammatory tumor microenvironment which may limit the growth and expansion of tumor cells (manuscript submitted).

Alternatively, a strong tumor differentiating microenvironment may be induced by the balanced release and membrane expression of key cytokines by the NK cells anergized with effectors such as monocytes or healthy stem cells in hope that most if not all of the newly arising cancer stem cells are induced to differentiate in order to become less aggressive. The benefit of such approach will be the ability of chemotherapeutic drugs to target the differentiated tumors in addition to the lack of differentiated tumors to metastasize (data not shown). Indeed, our recent in vivo data indicated that cancer stem cells have the ability to grow faster and metastasize, whereas the differentiated tumors grew slower and remained localized for a long period of time without metastasizing (manuscript in prep). It is possible that the successful cancer therapy may lie between a balance in the two abovementioned approaches depending on the type of the tumor and the status of patients' immune system. The most dangerous and devastating outcome of the cancer is its ability to deplete NK cells and other immune inflammatory cells. In this case, not only cancer stem cells will be surviving but they will also remain poorly differentiated which may establish a vicious cycle of tumor growth and loss of immune effectors in the tumor microenvironment and in the periphery. NK cell immunotherapy in these patients should be highly beneficial.

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Figure legends

Fig. 1. Resistance of differentiated DPSCs and OSCCs but not stem-like OSCSCs or DPSCs to untreated, IL-2 treated and IL-2+anti-CD16 treated NK cell cytotoxicity; Loss of NK cell cytotoxicity and gain in secretion of IFN-γ after NK cell receptor signaling

OSCCs or OSCSCs were seeded at $1X10^5$ cells/well in 24-well plate for 24 hours prior to the addition of highly purified NK cells pre-treated with IL-2 (1000 units/ml) for 24 hours. NK cells were added to tumor cells at 2:1 effector to target ratio. At time zero when NK cells were added to the tumor culture, a final concentration of 10ug/mL of propidium iodide (PI) was also added. The cells were then subsequently tracked for over 72 hours using time-lapse microscopy with Nikon Eclipse Ti-E inverted microscope fitted with a culture chamber to provide cells with a stable temperature of 37°C with 5% CO_2. An image was taken every 15 minutes and a representation is shown at day 1, day 1 ½ and day 3. OSCSCs but not OSCCs which are lysed take up PI and appear orange in the time lapse (A). The surface expression of CD26, CD44, CD166 and CD326 on OSCCs and OSCSCs were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies. Isotype control antibodies were used as control. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities for each histogram (B). The surface expression of CD338 on OSCCs and OSCSCs was assessed by flow cytometric analysis after staining with PE conjugated CD338 (right graphs in the histogram). Isotype control antibodies were used as control (left graphs in the histograms) (C). OSCCs and OSCSCs were left untreated or treated with 10-80 μg/mL of Cisplatin for 18 hours, after which the tumor cells were washed with 1XPBS, detached, and stained with propidium iodide (PI) and percent cell death was determined using flow cytometric analysis (D). NK cells were left untreated or treated with IL-2 (1000 units/ml), anti-CD16mAb (3

μg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 μg/ml) for 18 hours before they were added to ^{51}Cr labeled OSCSCs and OSCCs (E) or undifferentiated and differentiated DPSCs (G). NK cell mediated cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100. NK cells were treated as described in Fig. 1E and each NK sample was either cultured in the absence or presence of OSCSCs and OSCCs (F) or undifferentiated and differentiated DPSCs (H) at an NK cell to target cell ratio of 0.5:1. After an overnight incubation, the supernatant was removed from the co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs. One of minimum three representative experiments is shown in each of Fig. 1B-1H.

Fig. 2. Monocytes induce split anergy in NK cells resulting in a significant loss of NK cell cytotoxicity against OSCSCs and DPSCs and increased secretion of IFN-γ by the NK cells.

NK cells were purified from healthy donors and left untreated or treated with IL-2 (1000 units/ml), anti-CD16 mAb (3 μg/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) in the presence or absence of autologous monocytes (1:1 ratio of NK : Monocytes) for 24 hours. After which they were washed and added to 51Cr labeled OSCSCs in a 4 hour chromium release assay. Percent cytotoxicity was obtained at different effector to target ratio, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100 (A). NK cells were treated as described in Fig. 2A and either cultured in the absence or presence of OSCSCs. After an overnight incubation, supernatants were removed from co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs (B). Highly purified NK cells were left untreated or treated as described

in Fig. 2A. After 1, 4, 6 and 8 days post treatment, supernatants were removed from NK cell cultures and the levels of IFN- γ (C) release were determined using specific ELISAs. NK cells were purified from healthy donors and left untreated or treated with IL-2 (1000 units/ml), anti-CD16 mAb (3 μg/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) in the presence or absence of autologous and allogeneic monocytes (1:1 ratio of NK : Monocytes) for 24 hours. After which they were washed and added to 51Cr labeled autologous and allogeneic DPSCs in a 4 hour chromium release assay (D). Percent cytotoxicity was obtained at different effector to target ratio, and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. NK cells were treated as described in Fig. 2E and either cultured in the absence or presence of autologous and allogeneic DPSCs (NK: DPSC of 0.2:1). After an overnight incubation, supernatants were removed from the co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs (E). One of minimum three representative experiments is shown in each of Fig. 2A-2E.

Fig. 3. Supernatants and paraformaldehyde fixed NK cells treated with IL-2 in combination with anti-CD16mAb with and without monocytes increased resistance of OSCSCs and DPSCs to NK cell mediated cytotoxicity

NK cells were purified from healthy donors and left untreated or treated with IL-2 (1000 units/ml), anti-CD16 mAb (3 μg/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) in the presence or absence of autologous monocytes (1:1 ratio of NK : Monocytes) for 24 hours. After which, the same amounts of supernatants from different treatments of NK cells were removed and added to OSCSCs for a period of 5 days (A) or DPSCs for a period of 7 days (B). NK supernatant treated OSCSCs or DPSCs were then washed with 1X

PBS, detached and labeled with ^{51}Cr and used in the cytotoxicity assay against freshly isolated NK cells. Untreated or IL-2 (1000 u/ml) treated NK cells were used to assess cytotoxicity against NK supernatant treated target cells using a standard 4 hour ^{51}Cr release assay. Percent cytotoxicity was determined at different effector to target ratio, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. NK cells were purified from healthy donors and left untreated or treated with IL-2 (1000 units/ml), anti-CD16 mAb (3 μg/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) in the presence or absence of monocytes (1:1 ratio of NK: monocytes) for 24 hours. Treated NK samples were then washed and fixed with 2% paraformaldehyde for 15 minutes before they were either added to OSCSCs at 0.75:1 for 5 days (C) or DPSCs at 2:1 for 7 days (D). The fixed NK cells were then completely removed from OSCSCs and DPSCs and the target cell sensitivity to NK cell mediated lysis were determined using a standard 4 hour ^{51}Cr release assay using freshly isolated, and untreated or IL-2 treated (1000u/ml) NK cells. Removal of fixed NK cells from stem cell cultures were assessed using microscopic observation. Percent cytotoxicity was determined at different effector to target ratio, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. One of minimum three representative experiments is shown in each of Fig. 3A-3D.

Fig. 4 Induction of resistance to NK cell mediated cytotoxicity and inhibition of growth in OSCSCs correlated with the increased expression of CD54, B7H1, MHC class I and decreased expression of CD44 on OSCSCs treated with supernatants from IL-2+anti-CD16mAb treated NK cells with and without monocytes

NK cells were purified from healthy donors and left untreated or treated with IL-2 (1000 units/ml), anti-CD16 mAb (3 μg/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours. Thereafter, the same amounts of supernatants from different treatments of NK cells were removed and added to OSCSCs for 5 days. OSCSCs were then washed, and the expression of CD54, CD44, B7H1 and MHC Class 1 were assessed after staining with the PE conjugated antibodies using flow cytometry (A). NK cells were purified from healthy donors and left untreated or treated with IL-2 (1000 units/ml), anti-CD16 mAb (3 μg/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) in the presence or absence of autologous monocytes (1:1 ratio of NK : Monocytes) for 24 hours. Thereafter, the same amounts of supernatants from different treatments of NK cells were removed and added to OSCSCs. After 5 days of incubation with the NK cell supernatants, OSCSCs were washed with 1X PBS, and the expression of CD54 and MHC-1 were assessed after staining with the PE conjugated antibodies using flow cytometry (B). Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities for each histogram. At the end of the incubation of OSCSCs with NK cell supernatants, OSCSCs which were remained attached to the plate and those which detached during the incubation period were collected separately, and the number of cells (C) and their viability (D) were assessed using microscopy and propidium iodide staining followed by flow cytometric analysis respectively. One of minimum three representative experiments is shown in each of Fig. 4A-4D.

Fig. 5. Induction of resistance of OSCSCs to NK cell mediated cytotoxicity and inhibition of growth in OSCSCs by IL-2+anti-CD16mAb treated NK cells is mediated by the combination of IFN-γ and TNF-α and not each cytokine alone

Highly purified NK cells were treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours, after which the same amounts of supernatants from different treatments were removed and added to OSCSCs in the presence and absence of anti-TNF-α (1:100) and/or anti-IFN-γ (1:100) or isotype control antibodies for a period of 5 days. The cytotoxicity against untreated and NK supernatant treated OSCSCs in the presence of antibodies to untreated NK cells (A), IL-2 treated NK cells (B) or IL-2 and anti-CD16mAb treated NK cells (C) were assessed using a standard 4 hour ^{51}Cr release assay. Percent cytotoxicity was obtained at different effector to target ratio, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. The surface expression of CD54, CD44, B7H1 and MHC Class 1 on untreated and NK supernatant treated OSCSCs as described above were assessed after PE conjugated antibody staining using flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities in each historgram (D). At the end of the incubation of OSCSCs with NK cell supernatants, OSCSCs which were remained attached to the plate and those which detached during the incubation period were collected separately, and the number of cells (E) and their viability (F) were assessed using microscopy, and propidium iodide staining followed by flow cytometric analysis respectively. One of minimum three representative experiments is shown in each of Fig. 5A-5F.

Fig. 6. Induction of differentiation, inhibition of cell growth and resistance to NK cell mediated cytotoxicity in OSCSCs induced by IL-2+anti-CD16mAb treated and paraformaldehyde fixed NK cells is mediated by the combination of IFN-γ and TNF-α and not each cytokine alone

Highly purified NK cells were left untreated or treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours, after which the NK cells were fixed with 2% paraformaldehyde and added to OSCSCs (0.75:1 NK:OSCSC ratio) in the presence and absence of anti-TNF-α (1:100) and/or anti-IFN-γ (1:100) or isotype control antibodies for a period of 5 days. The media containing the fixed NK cells were removed and extensively washed from each treated OSCSCs and the cytotoxicity against OSCSCs were assessed using freshly isolated untreated NK cells or those treated with IL-2 using a standard 4 hour ^{51}Cr release assay. The complete removal of fixed NK cells from OSCSCs was determined by microscopy. Percent cytotoxicity was obtained at different effector to target ratio, and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100 (A). The lack of cytotoxic function of 2% paraformaldehyde fixed untreated and IL-2 treated NK cells were assessed against OSCSCs and OSCCs. NK cells were left untreated or treated with IL-2 for 24 hours before they were fixed with 2% paraformaldehyde (B). NK cells were purified from healthy donor and left untreated or treated with IL-2 (1000 units/ml) and the combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml). After an overnight treatment, NK cells were fixed with freshly prepared 2% paraformaldehyde and washed twice with 1X PBS. After 24 hours post fixation, supernatants were then collected and the levels of IFN-γ were measured with specific ELISA (C). NK cells purified from healthy donors were left untreated or treated with a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 24 hours, after which the

NK cells were washed extensively and stained with PE conjugated anti-TNF-a mAb followed by flow cytometric analysis. IFN-γ expression was assessed using purified mouse anti-Human IFN-γ antibody followed by PE conjugated goat anti-mouse IgG. Isotype control antibodies were used as controls. The numbers on the right hand corner are percentage positive for each histogram. The histogram overlay for the anti-TNF-a or anti-IFN-g stained untreated (left) and IL-2+anti-CD16mAb treated NK cells (right) are also shown in this figure. (D). The surface expression of CD54, CD44, B7H1 and MHC Class 1 on untreated and fixed NK treated OSCSCs as described above were assessed after PE conjugated antibody staining using flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities for each histogram (E). At the end of the incubation of OSCSCs with fixed NK cells, OSCSCs which were remained attached to the plate were collected, and the number of cells (F) and their viability (G) were assessed using microscopy, and propidium iodide staining followed by flow cytometric analysis respectively.

Highly purified NK cells were left untreated or treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) in the presence and absence of Monensin (1:1300) for 24 hours, after which each sample of NK cells were extensively washed and fixed with 2% paraformaldehyde and added to OSCSCs (0.75:1 NK:OSCSC) for a period of 5 days. Inhibition of IFN-γ release in Monensin treated NK cells before fixation with 2% paraformaldehyde were assessed by ELISA (H). The results were compared to OSCSCs in the absence of fixed NK cells. The media containing the fixed NK cells were removed and extensively washed from each treated OSCSCs and the cytotoxicity against each condition of OSCSCs were assessed using freshly isolated untreated NK cells (I), those treated with IL-2 (J)

or treated with the combination of IL-2+anti-CD16mAb (K) using a standard 4 hour ^{51}Cr release assay. The complete removal of fixed NK cells from OSCSCs was determined by microscopy. Differentiated OSCCs were used as control for the differentiation of OSCSCs. Percent cytotoxicity was obtained at different effector to target ratio, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. The surface expression of CD54, CD44, and MHC Class 1 on untreated and fixed NK treated OSCSCs as described above were assessed after PE conjugated antibody staining followed by flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities for each histogram (L). At the end of the incubation of OSCSCs with fixed NK cells, the media containing the fixed NK samples were removed from OSCSCs and the viability of the cells were assessed using propidium iodide staining followed by flow cytometric analysis (M). One of minimum three representative experiments is shown in each of Fig. 6A-6M.

Fig. 7. Combination of rTNF-α and rIFN-γ induce differentiation and resistance of OSCSCs and SCAPs to NK cell mediated cytotoxicity

OSCSCs (A) were left untreated or treated with recombinant human TNF-α (2 ng/ml), recombinant human IFN-γ (6 units/ml) or the combination of human TNF-α (2ng/ml) and recombinant human IFN-γ (5 units/ml) in the presence or absence of antibodies against TNF-α (1:100) and/or IFN-γ (1:100) for 24 hours. Afterwards, the cells were detached from the tissue culture plates and labeled with ^{51}Cr and used in a standard 4 hour chromium release assay against untreated and IL-2 (1000u/ml) treated NK cells. Pretreatment of NK cells with IL-2 were carried out for 18-24 hours. Percent cytotoxicity was determined at different effector to target ratio, and

the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. OSCSCs were treated as described in figure 7A and their viability was assessed using propidium iodide staining followed by the flow cytometric analysis (C). Surface expressions of CD54, CD44, B7H1 and MHC-1 on OSCSCs (C) treated as described in Fig. 2A were determined using staining with PE conjugated antibodies followed by flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentage and the mean channel fluorescence intensities in each histogram. One of minimum three representative experiments is shown in each of Fig. 7A-7C.

Fig. 8. Antibodies to CD54 or LFA-1 was unable to reverse differentiation but inhibited moderately the cytotoxicity of NKs against OSCSCs

Highly purified NK cells were left untreated or treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours after which the same amounts of supernatants were removed and added to the OSCSCs in the presence and absence of anti-CD54mAb (10μg/ml) (A) and anti-LFA-1 (1:100) (B) for 5 days. Treated OSCSCs were then washed extensively and used in a standard 4 hour ^{51}Cr release assay against IL-2 (1000u/ml) activated NK cells. Pretreatment of NK cells with IL-2 were carried out for 18-24 hours. Percent cytotoxicity was determined at different effector to target ratio, and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100.

Fig. 9. Antibodies to MHC-class I increased cytotoxicity of OSCSCs differentiated by IL-2+anti-CD16mAb treated NK supernatants, whereas antibodies to B7H1 did not change cytotoxicity

OSCSCs were treated with supernatants harvested from untreated or IL-2 + anti-CD16mAb treated NK cells for 5 days. Treatment of NK cells with the combination of IL-2+anti-CD16mAb were carried out for 18-24 hours before the supernatants were removed and added to OSCSCs. Thereafter, treated OSCSCs were washed and labeled with 51Cr and used in the cytotoxicity assay in the presence and absence of anti-B7H1 (5μg/ml) before their addition to NK cells. Percent cytotoxicity was determined at different effector to target ratio, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100 (A). OSCSCs were left untreated or treated with the supernatants prepared from the IL-2+anti-CD16mAb treated NK cells for 5 days before they were washed , ^{51}Cr labeled, and treated with either isotype control antibody or anti-MHC class I antibody (1:100) for 10 minutes prior to their use in the cytotoxicity assay against IL-2 (1000u/ml) treated NK cells. OSCCs were also ^{51}Cr labeled and treated with and without isotype control or anti-MHC class I (1:100) antibodies before they were used in the cytotoxicity assay against IL-2 treated NK cells. To prepare NK cell supernatant for the treatment of OSCSCs, NK cells were treated with the combination of IL-2 (1000 u/ml) and anti-CD16mAb (3 μg/ml) for 18-24 hours before the supernatants were harvested and added to OSCSCs. The treatment of IL-2 (1000u/ml) was also carried out for 18-24 hours before their use in the cytotoxicity assay. Percent cytotoxicity was determined at different effector to target ratio, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100 (B).

Figure 10. Hypothetical model of induction of anergized NK cells by immune inflammatory cells and by the effectors of connective tissue to support differentiation of non-transformed

stem cells and cancer stem cells resulting in their resistance to NK cell mediated cytotoxicity.

NK cell anergy in tumor microenvironment, as well as in non-transformed immune inflammatory microenvironment, is shown. Significant infiltration of immune effectors right beneath the epithelial layer can be seen in a connective tissue area where immune inflammatory cells are likely to anergize NK cells to lose cytotoxicity and gain the ability to secrete cytokines, a term which we previously coined 'split anergy' in NK cells, and to support differentiation of the basal epithelial layer containing stem cells. NK cells are likely to encounter and interact with other immune effectors such as monocytes/macrophages or other myeloid-derived suppressor cells (MDSC), or with connective tissue-associated fibroblasts (CAF), in order to be conditioned to form anergized/regulatory NK (NKreg) cells. NK cells may also directly interact with stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they can become conditioned to support differentiation of other stem cells. NK cell-differentiated epithelial cells will no longer be killed or induce cytokine secretion by the NK cells, resulting in the resolution of inflammation.

Fig. 1A

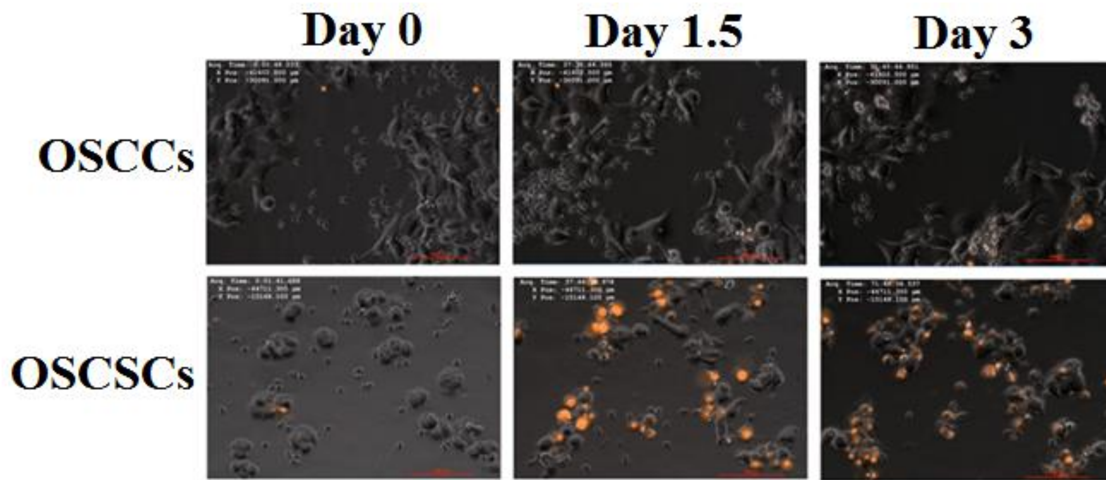


Fig. 1B

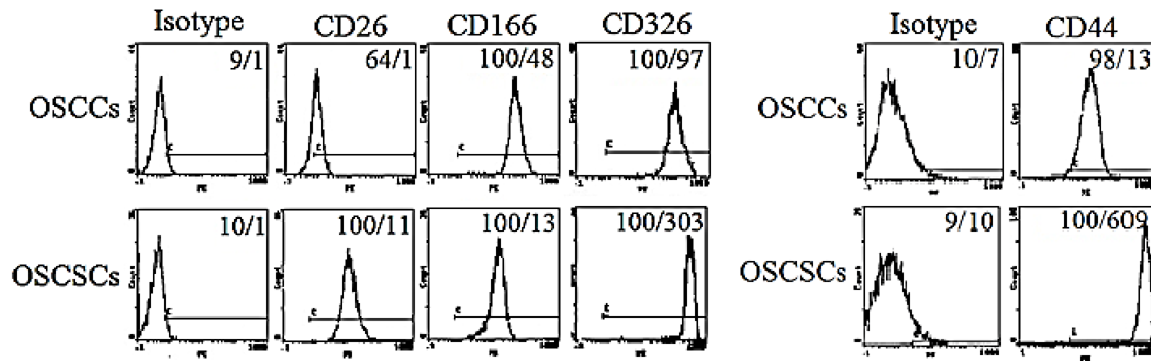


Fig. 1C

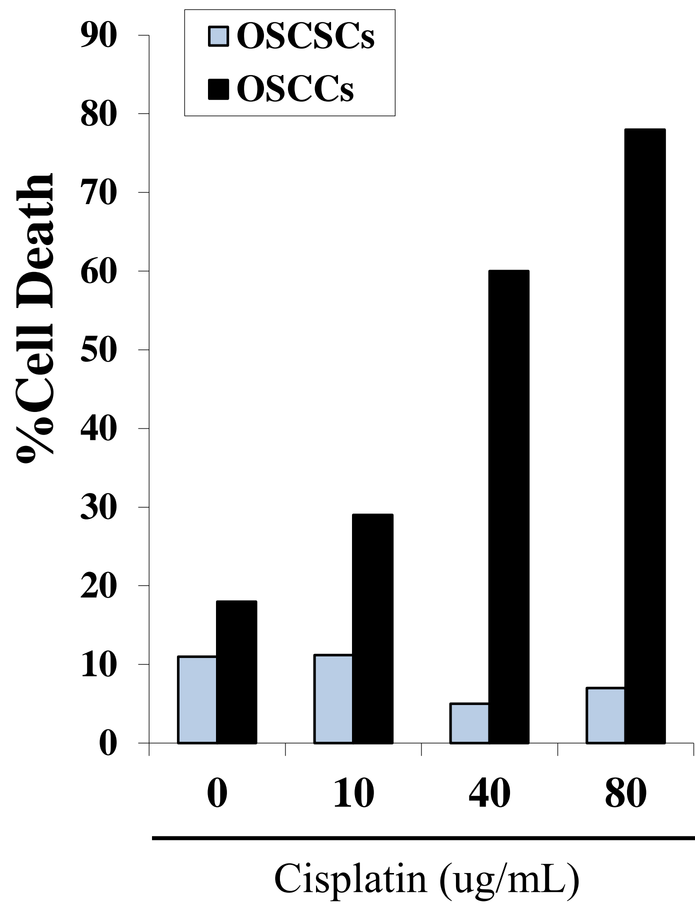


Fig. 1D

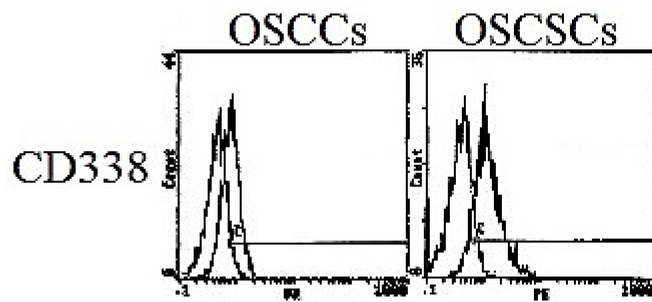


Fig. 1E

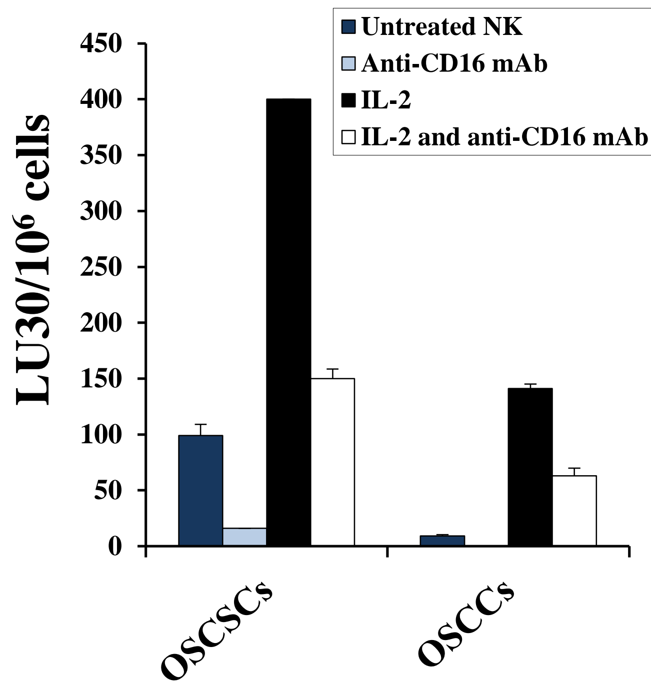
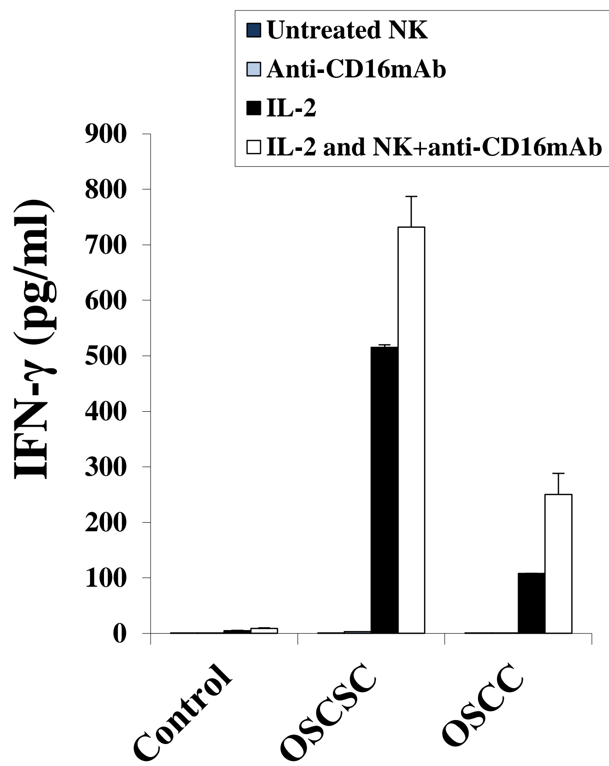


Fig. 1F



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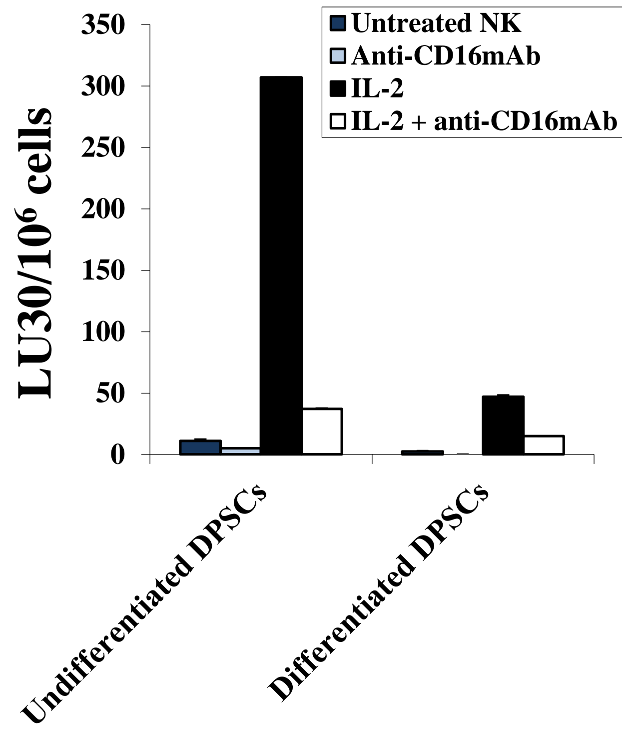


Fig. 1H

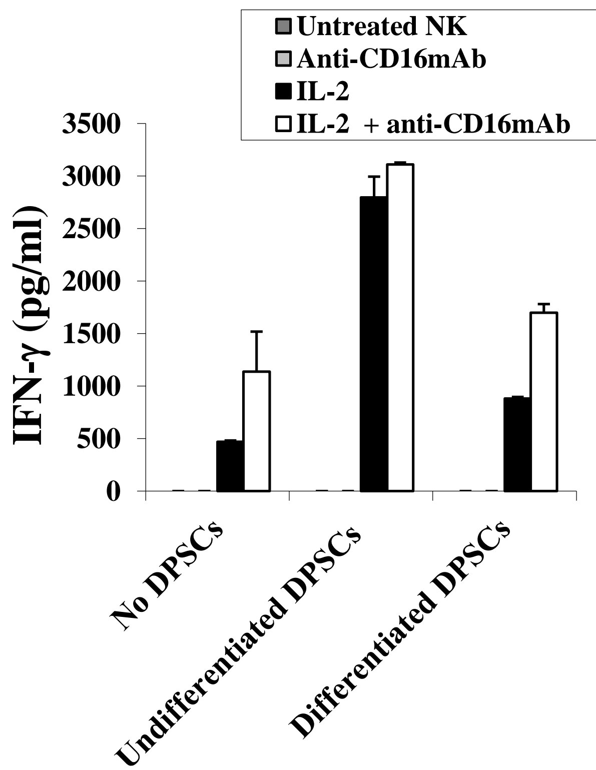
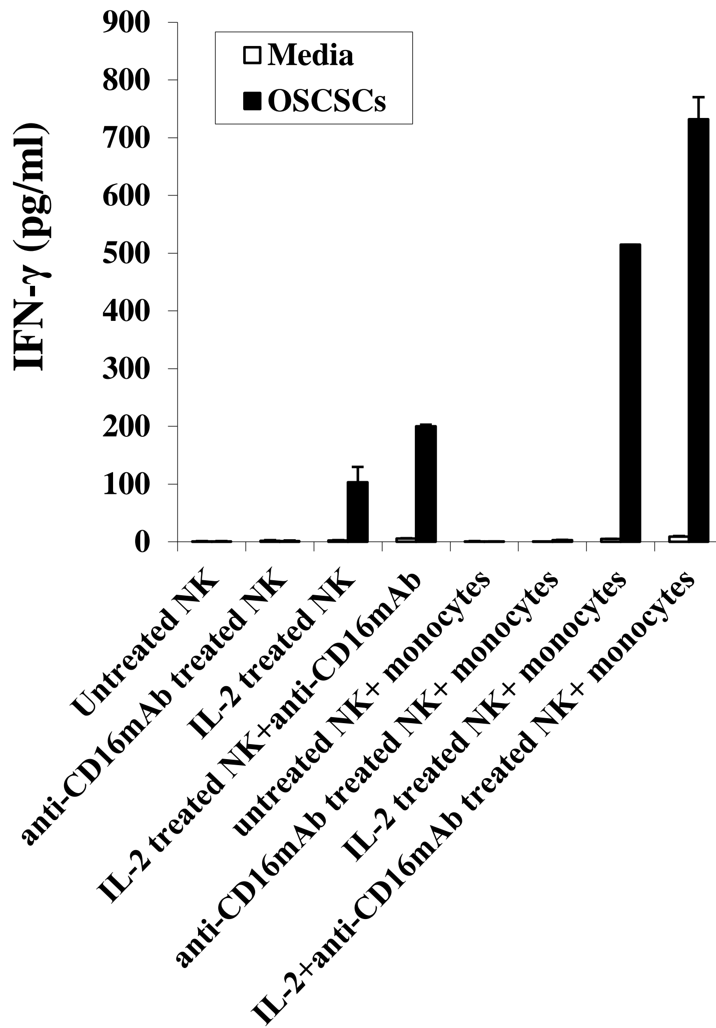


Fig. 2A



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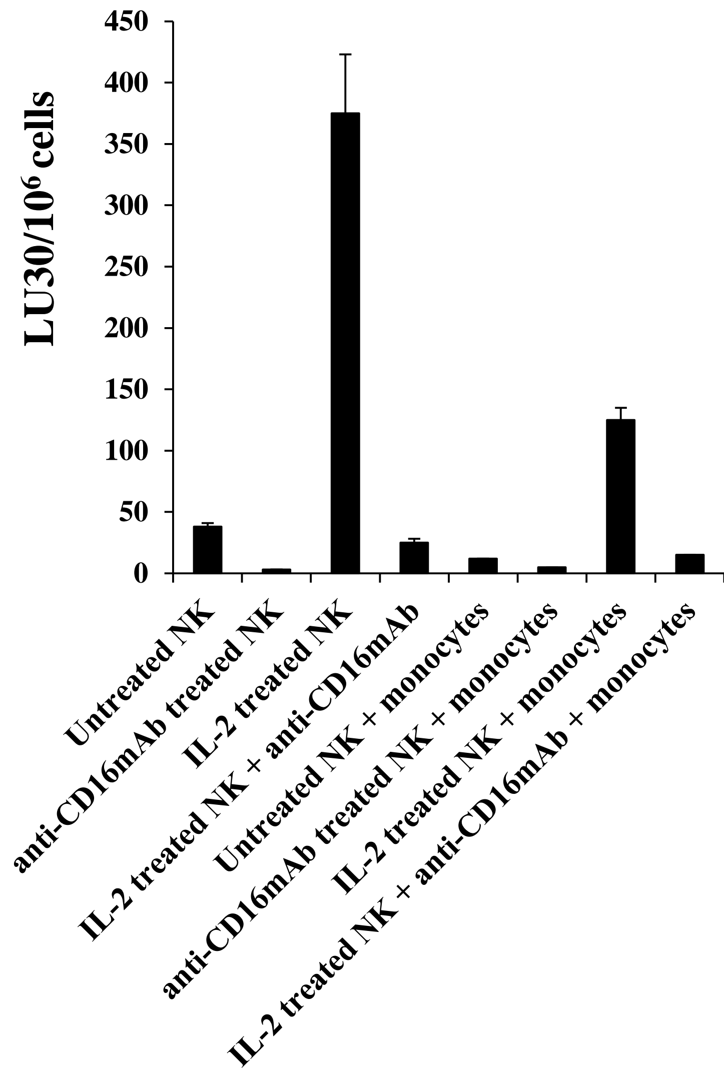

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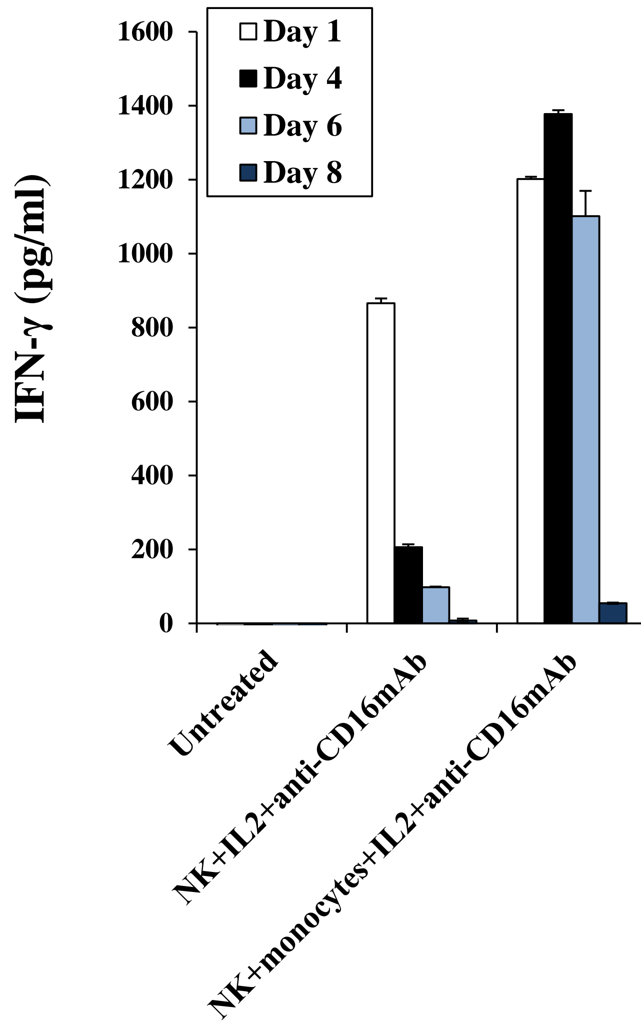
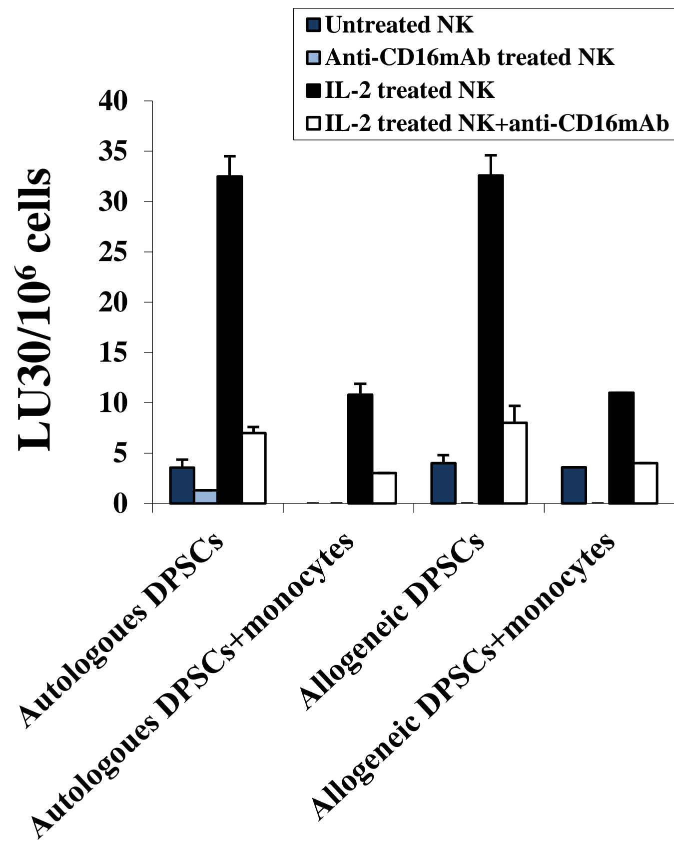


Fig. 2D



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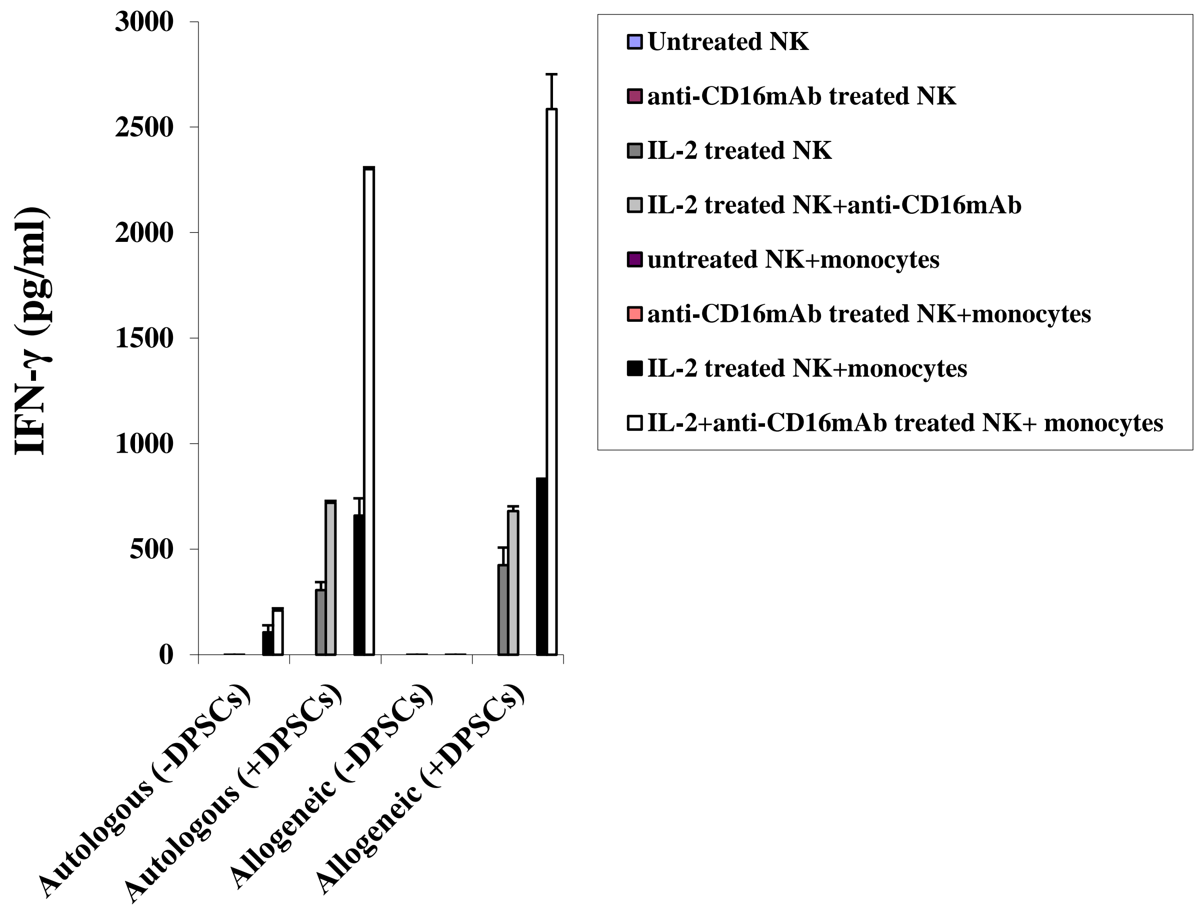


Fig. 3A

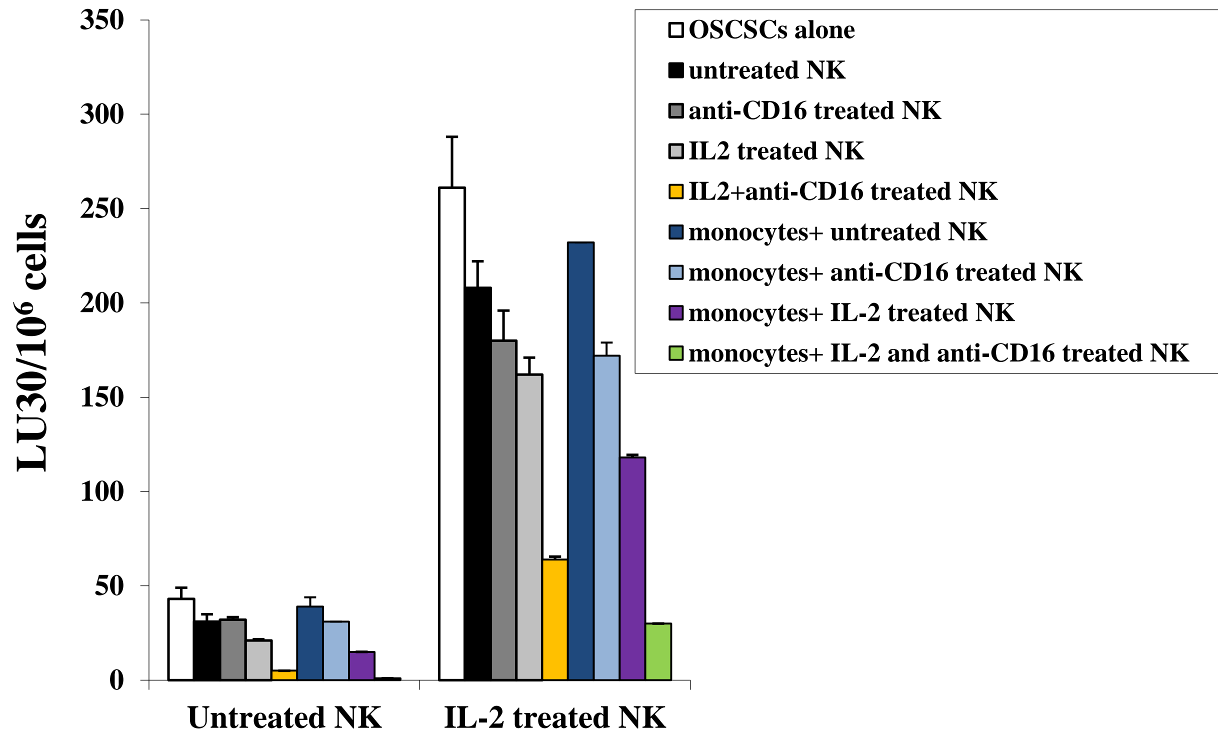


Fig. 3B

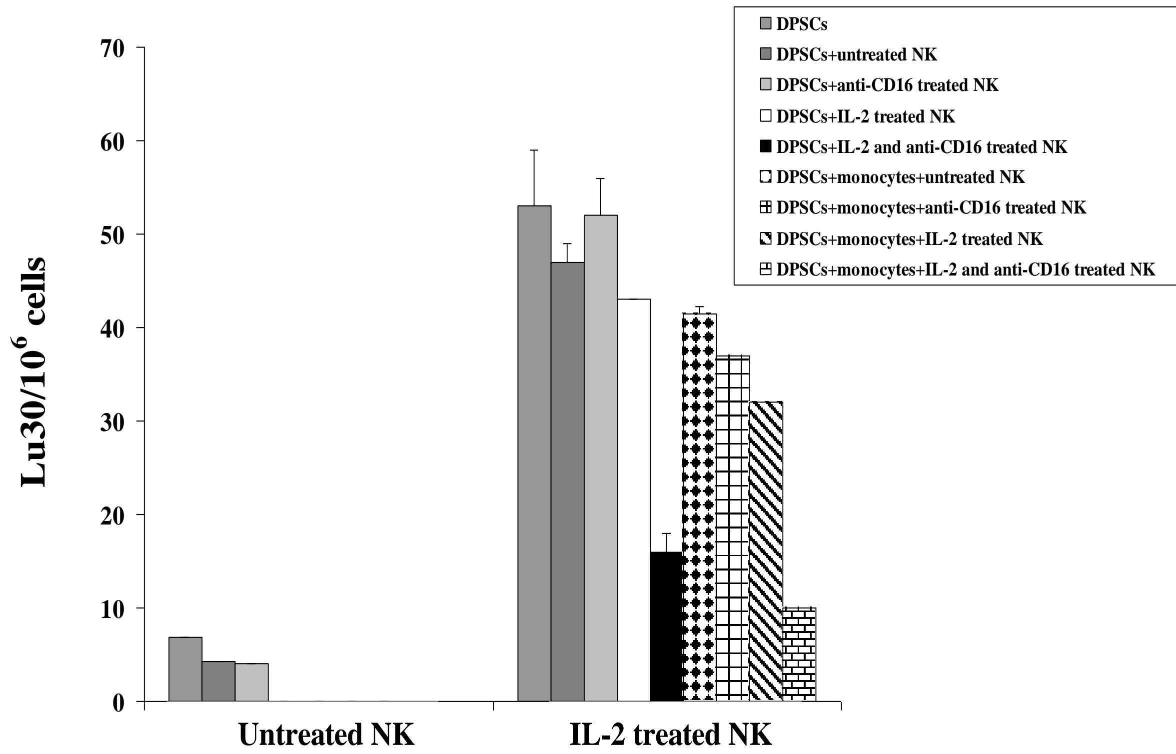
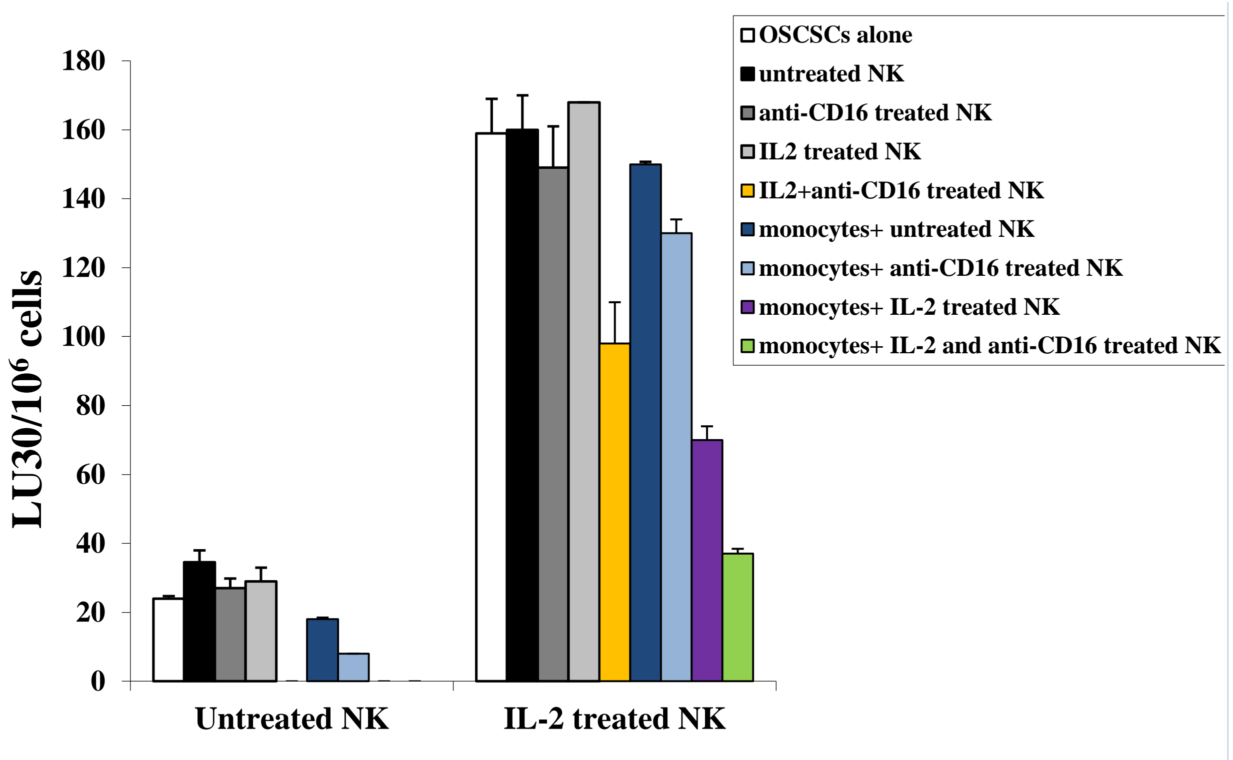


Fig. 3C



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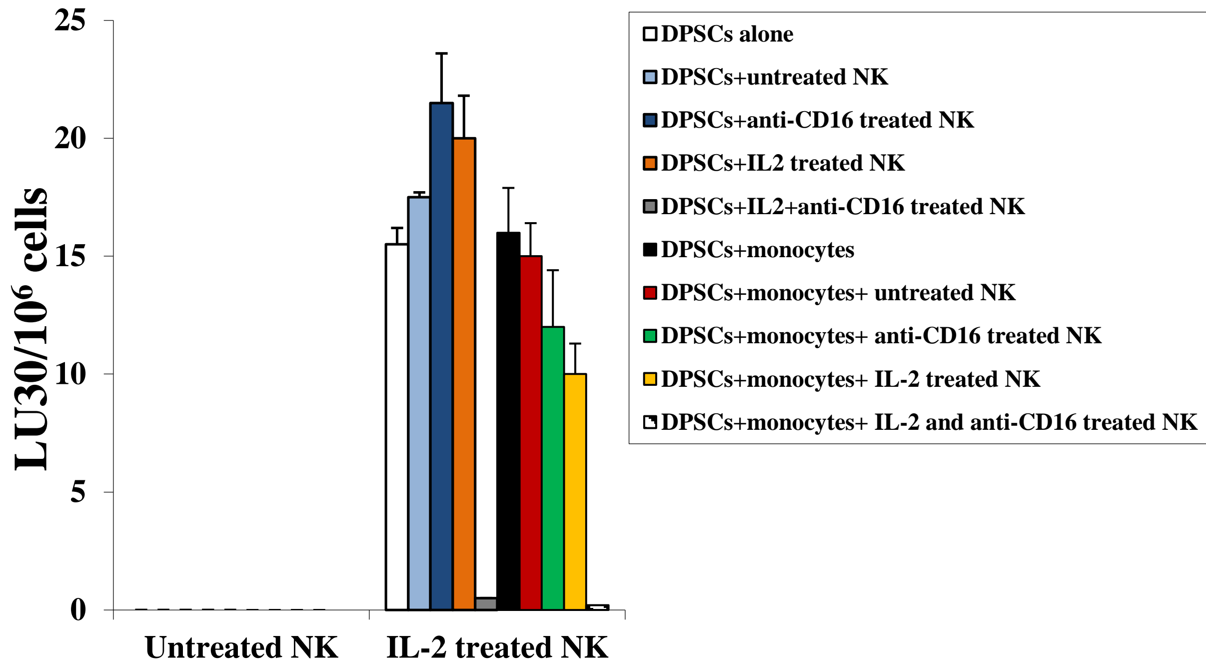

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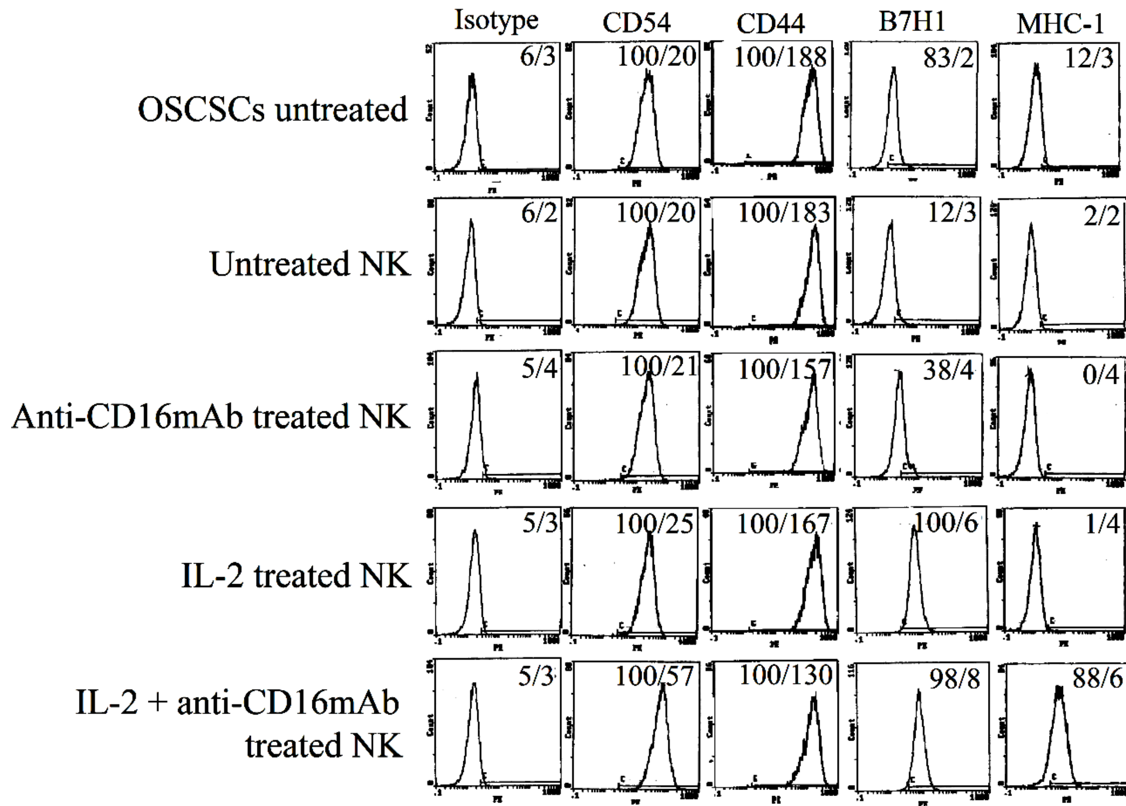

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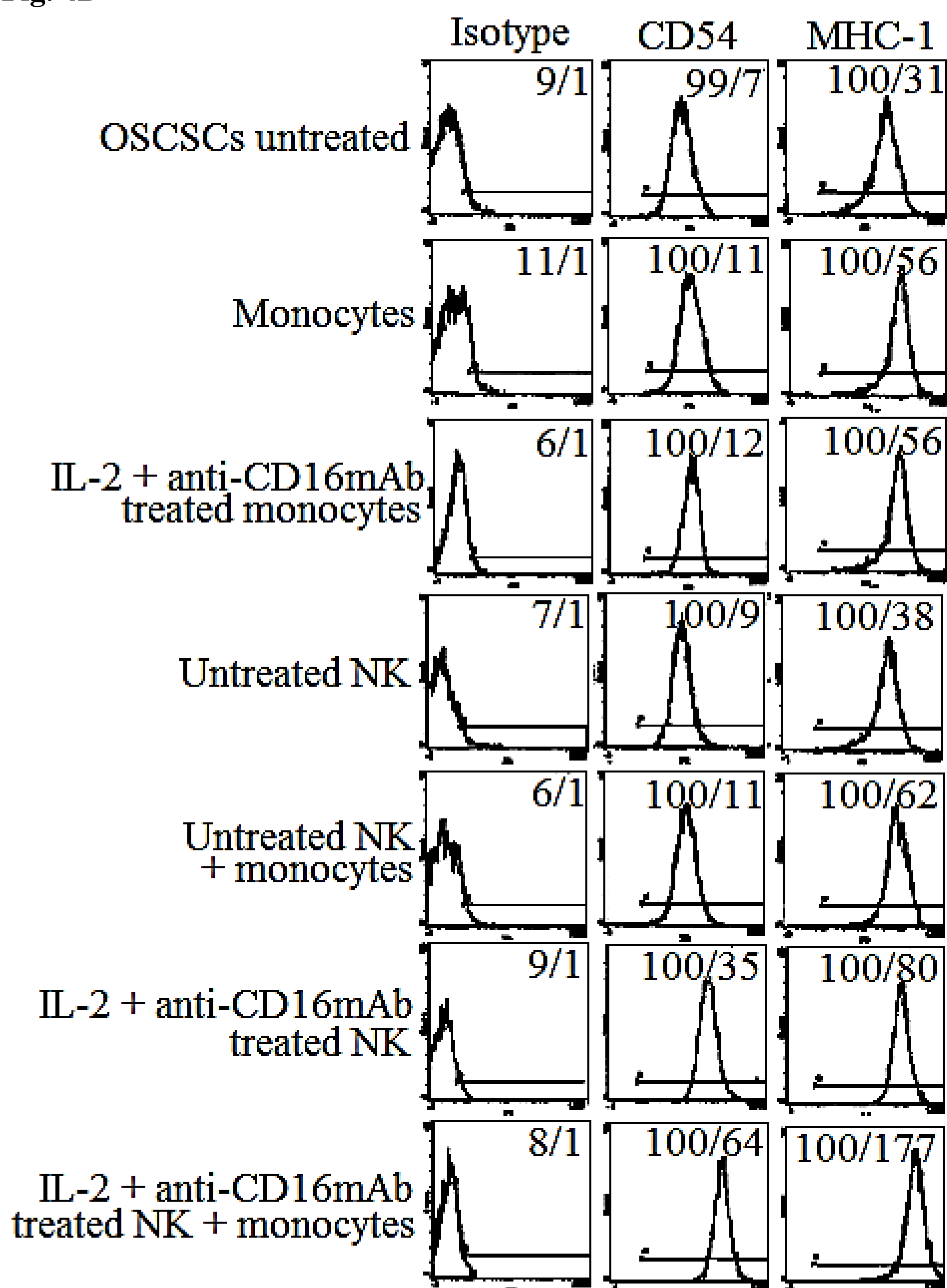


Fig. 4C

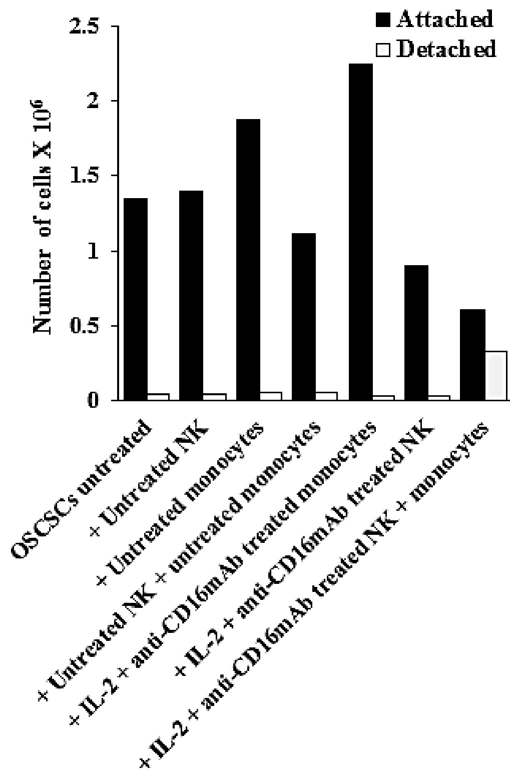


Fig. 4D

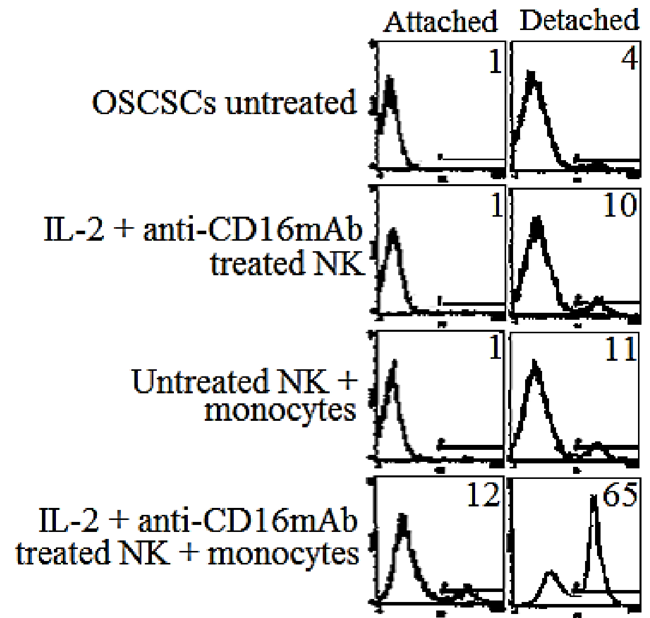
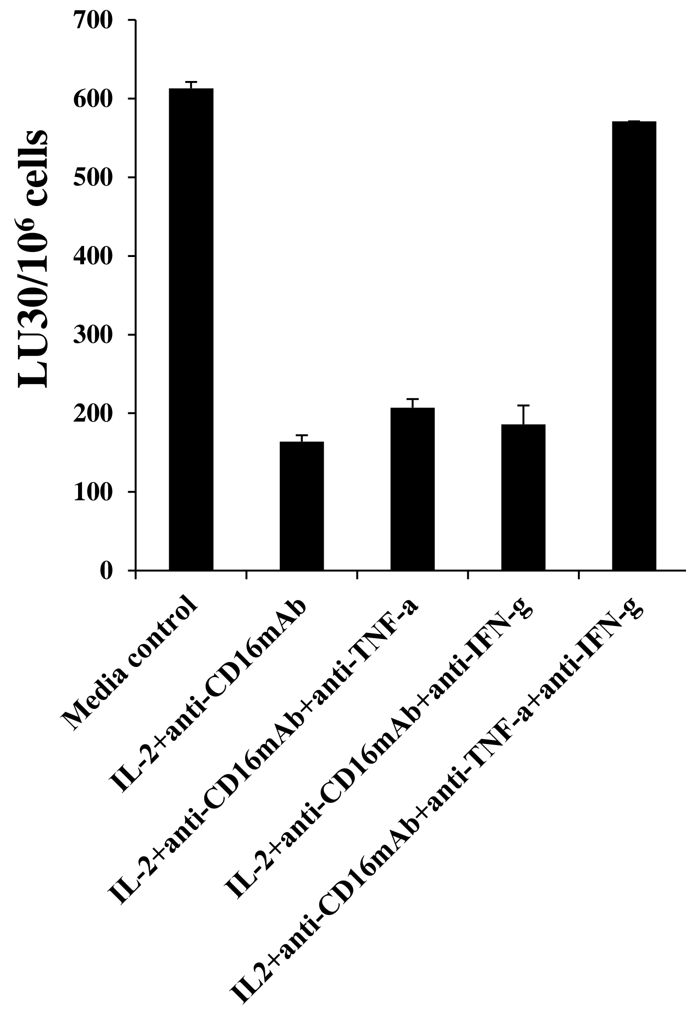


Fig. 5A



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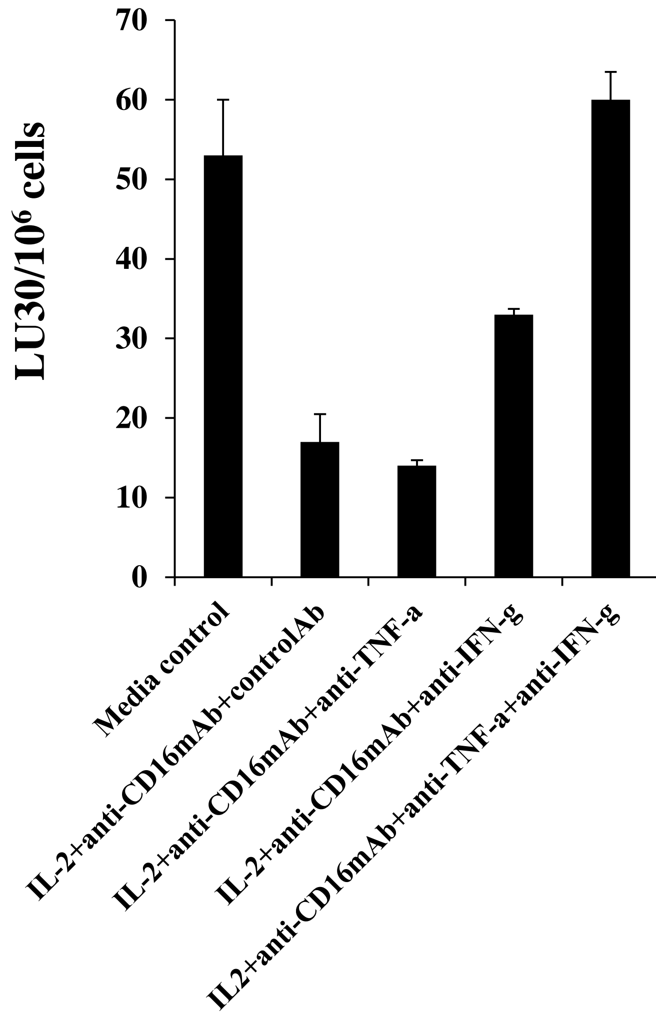

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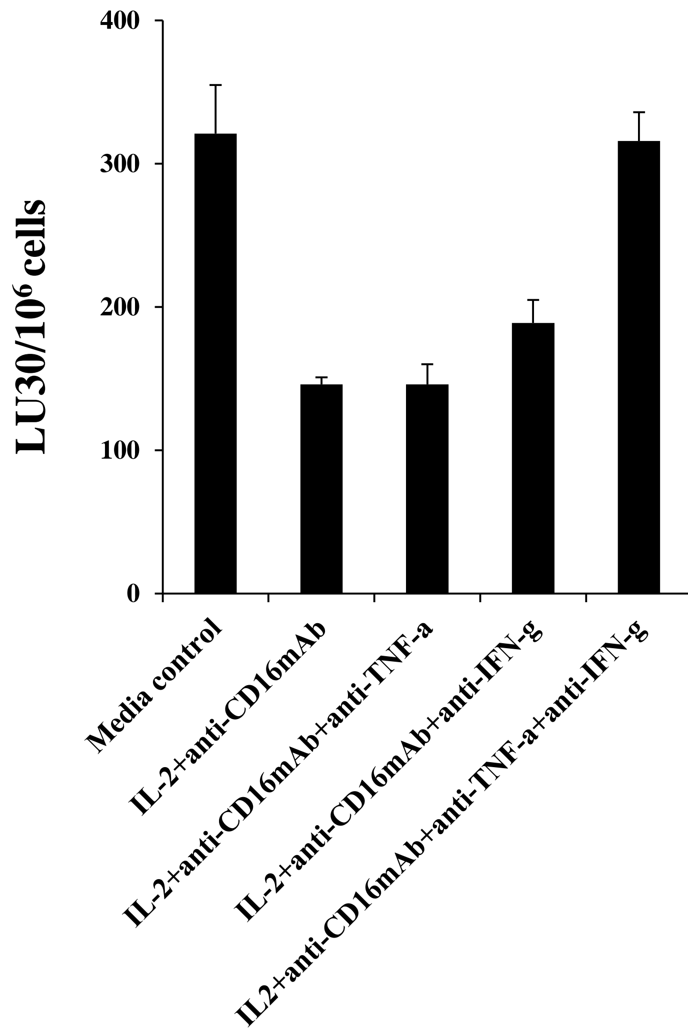
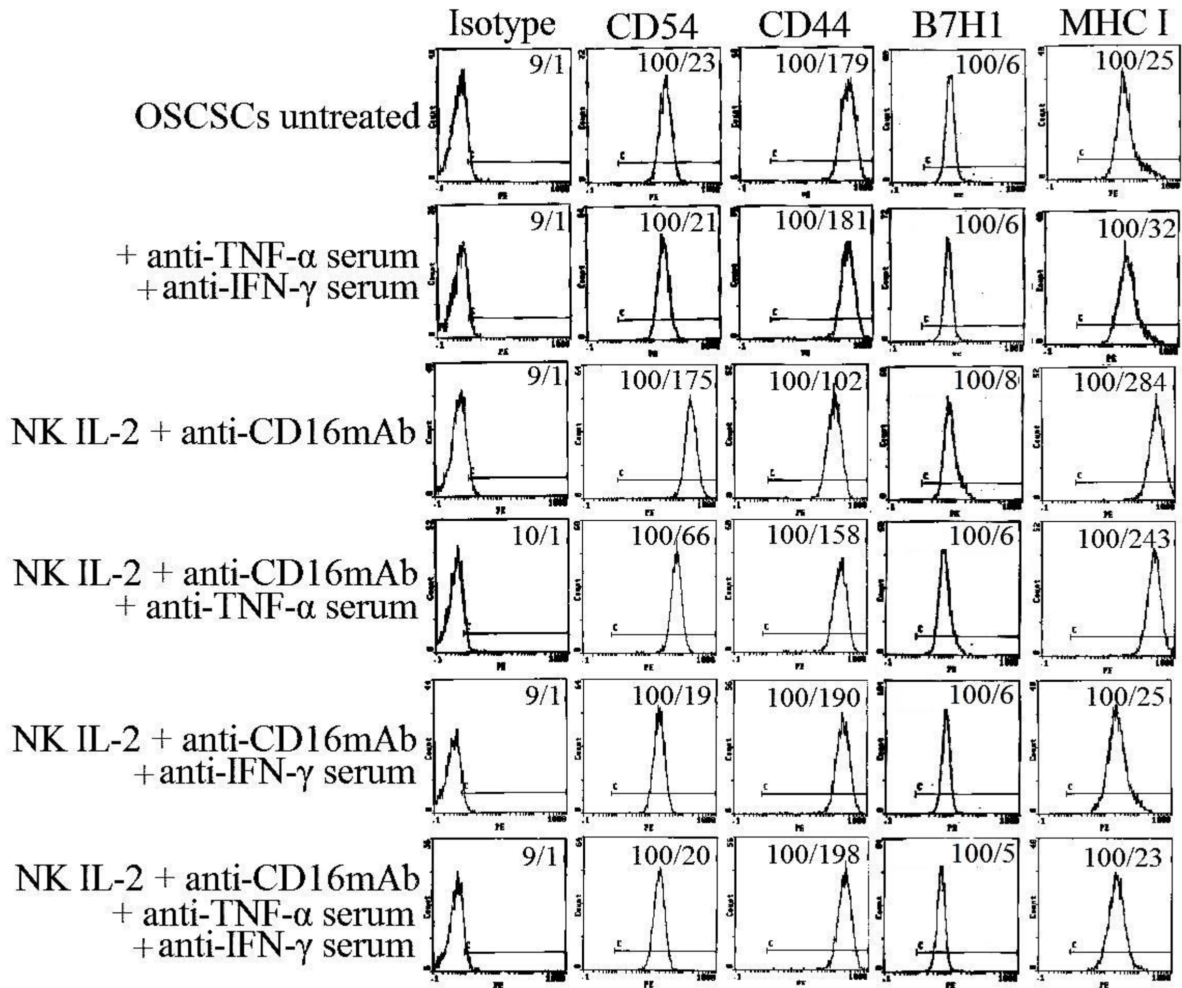


Fig. 5D



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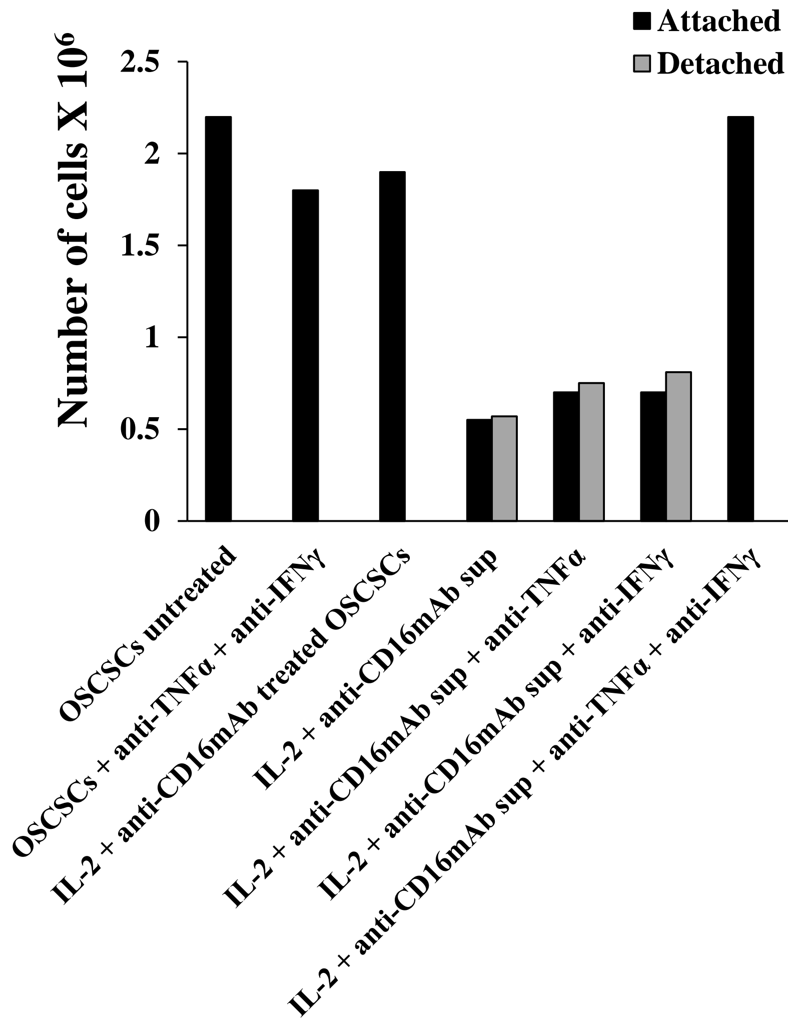

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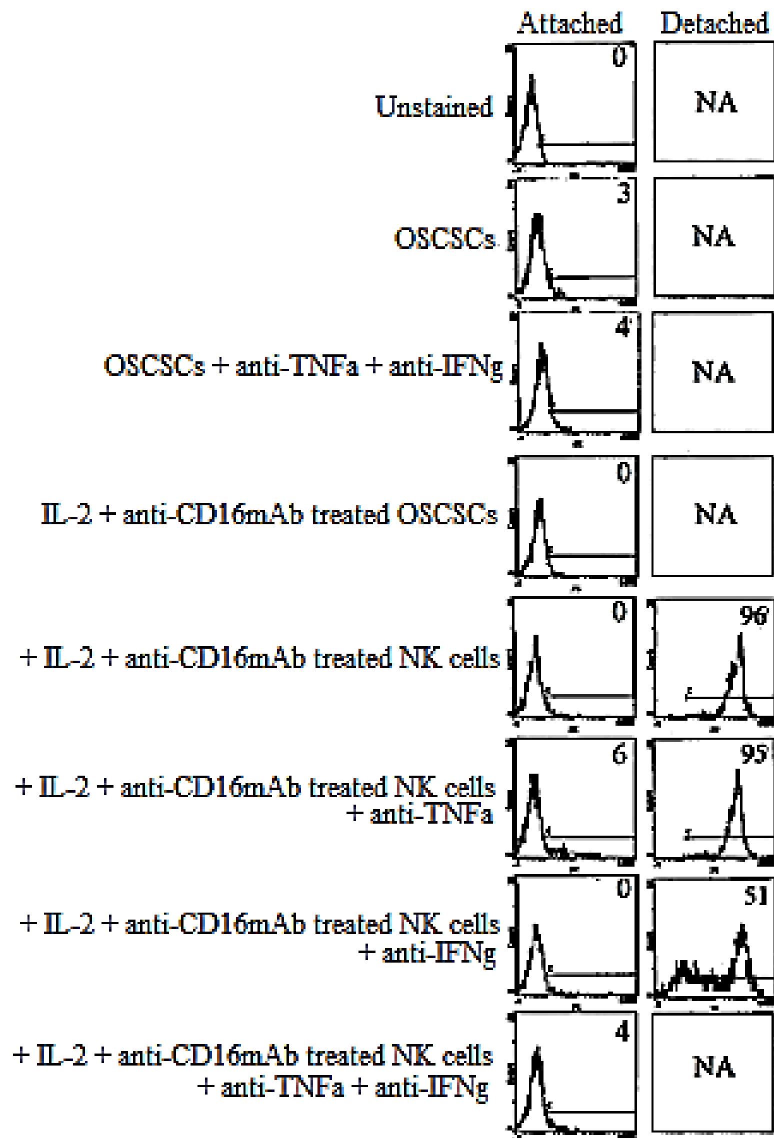
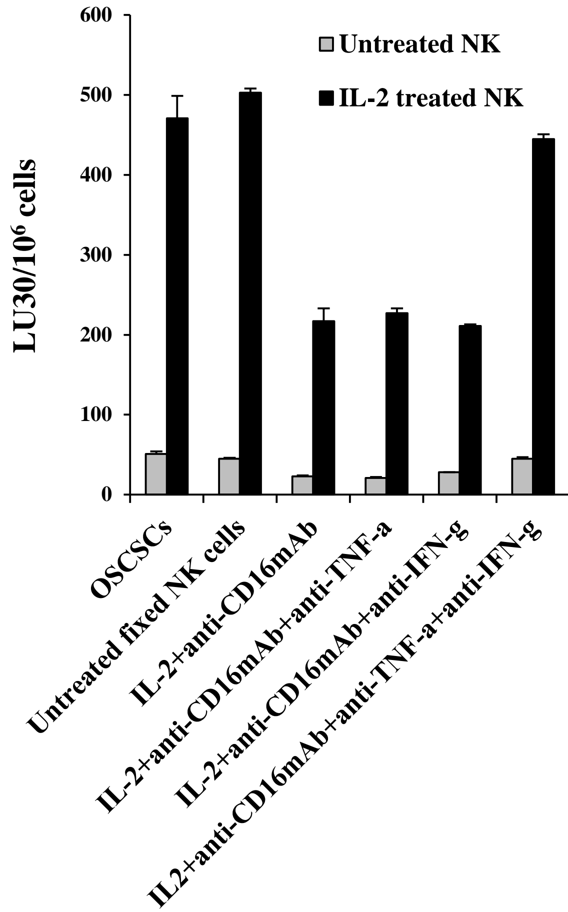


Fig. 6A

Fig. 6B



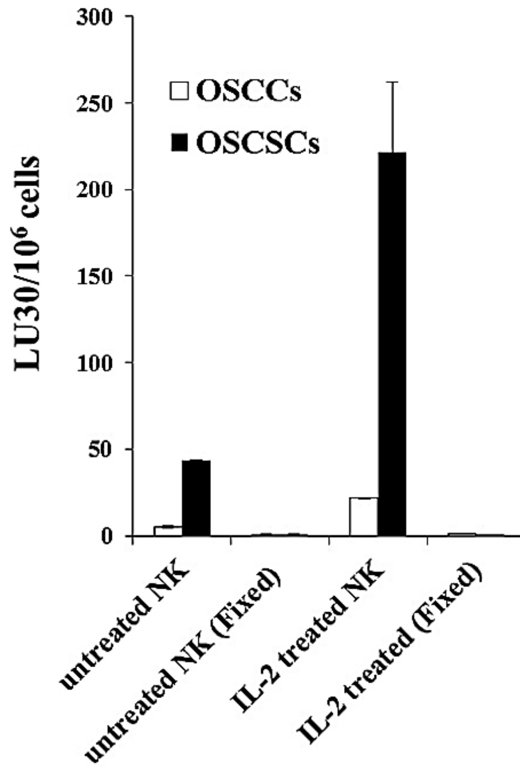


Fig. 6C

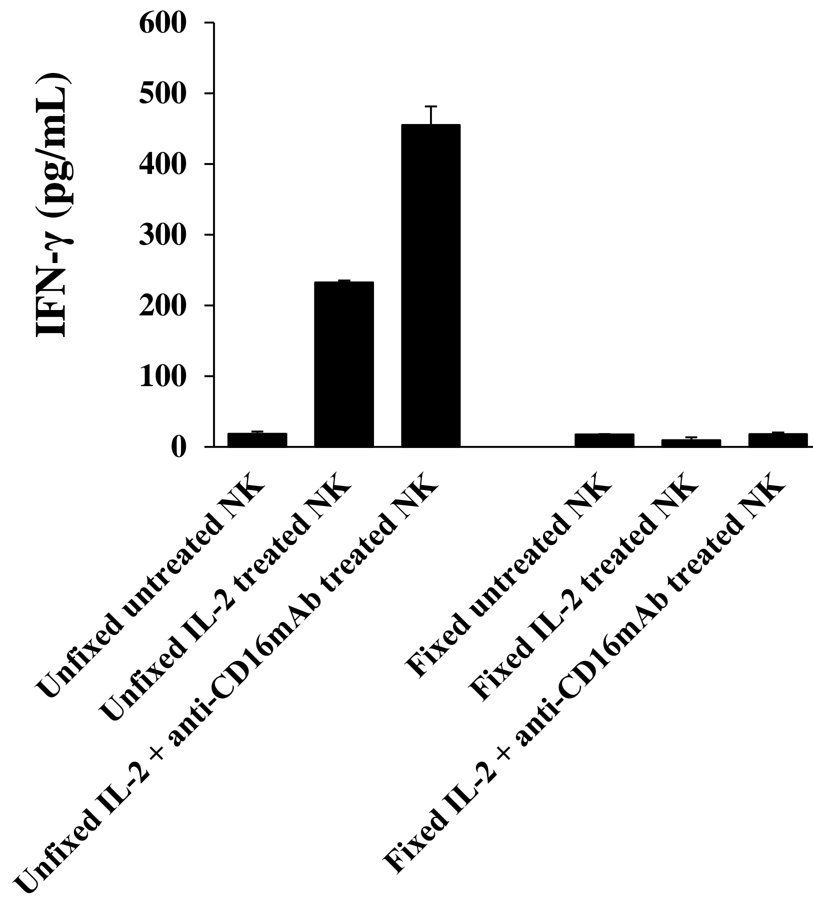


Fig. 6D

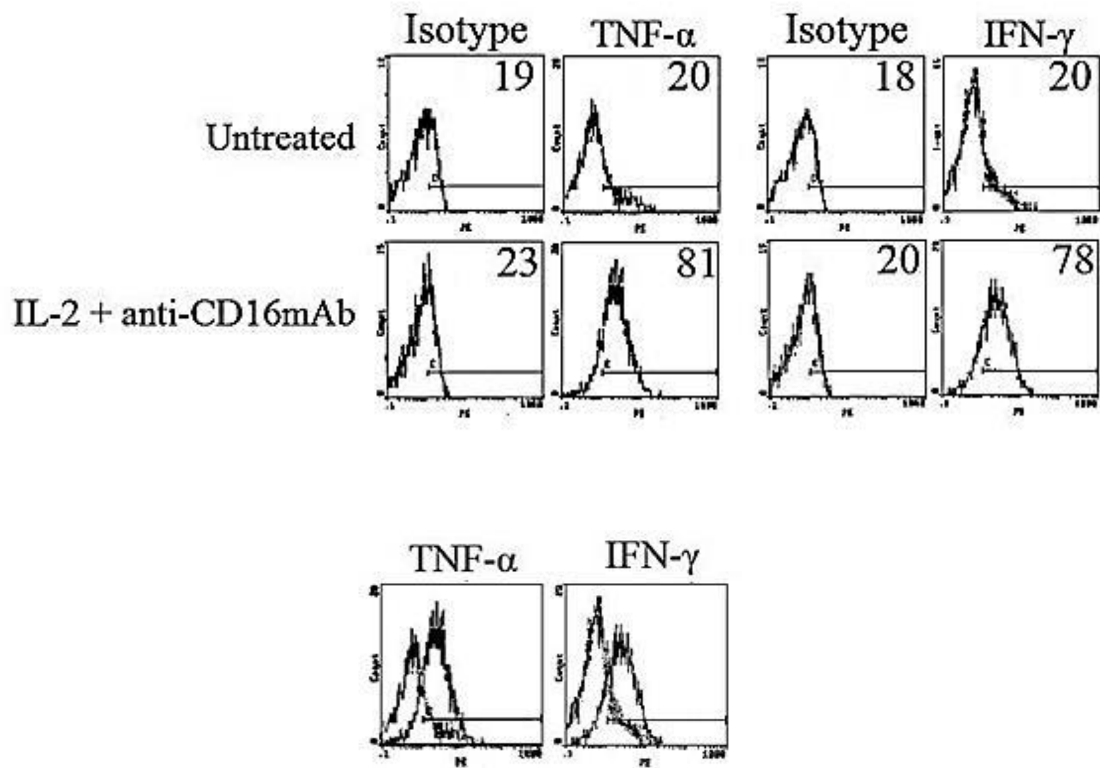


Fig. 6E

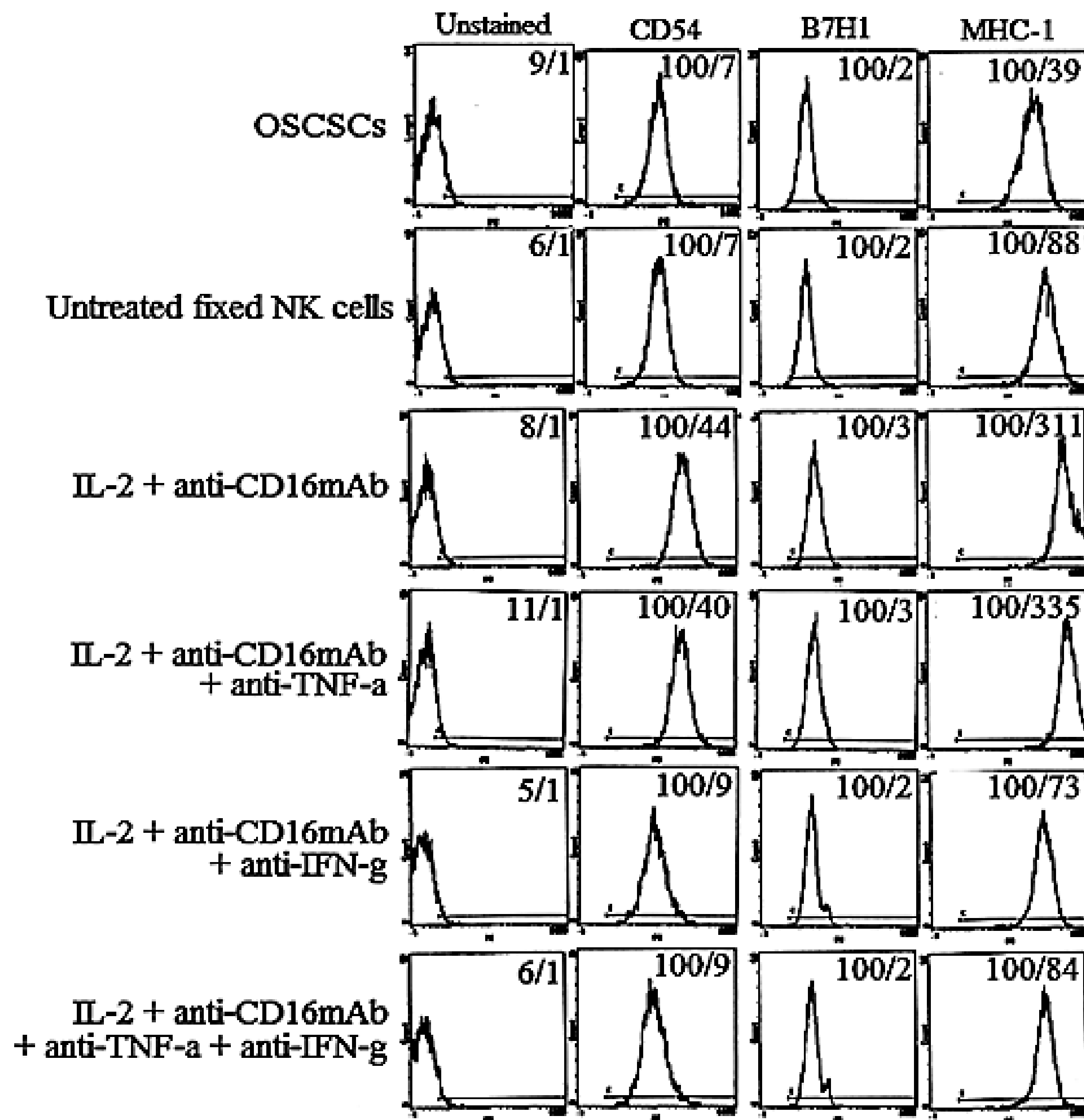


Fig. 6F

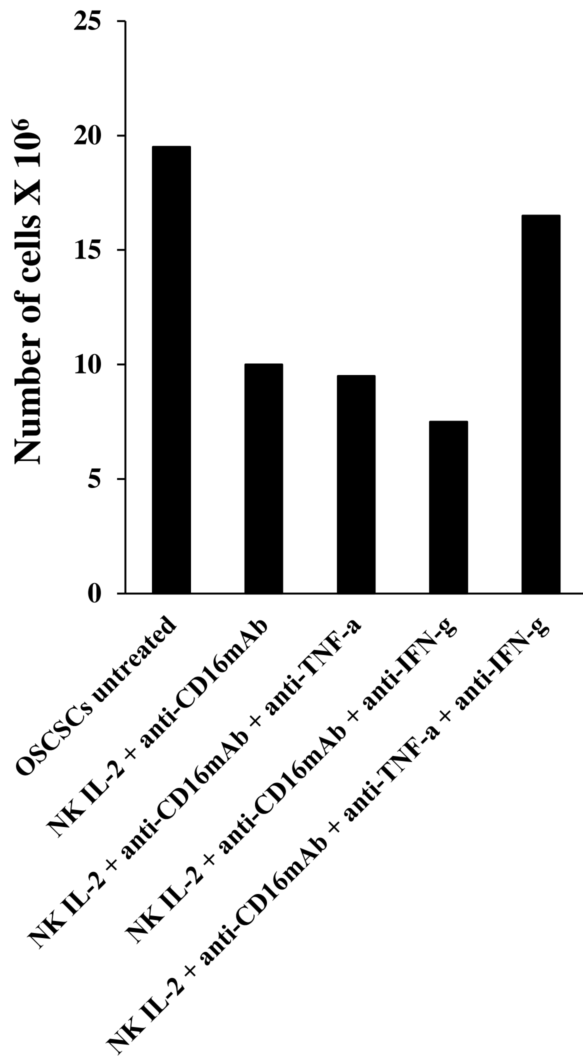


Fig. 6G

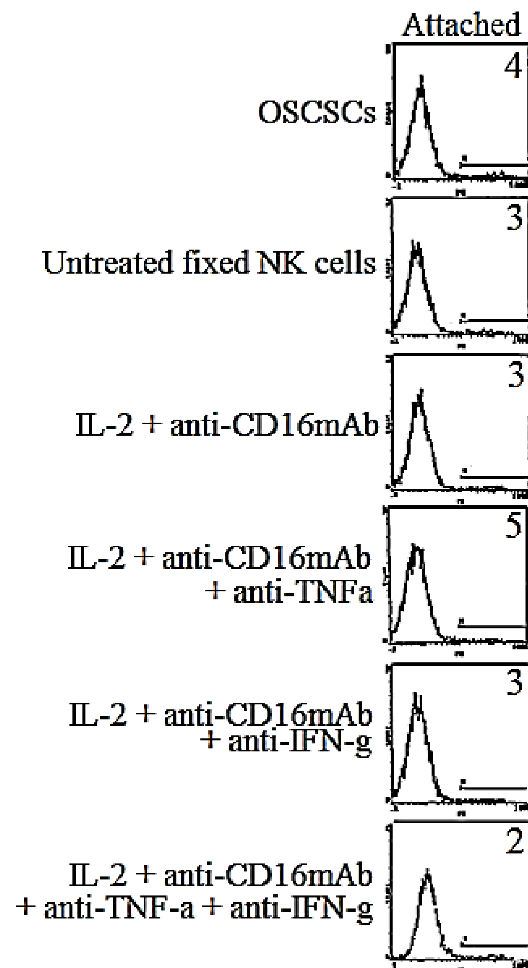


Fig. 6H

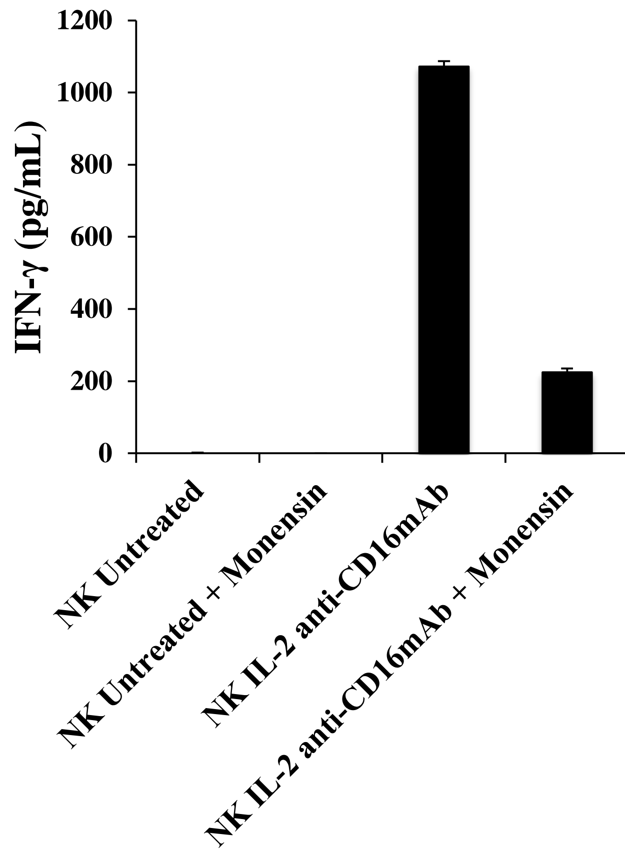
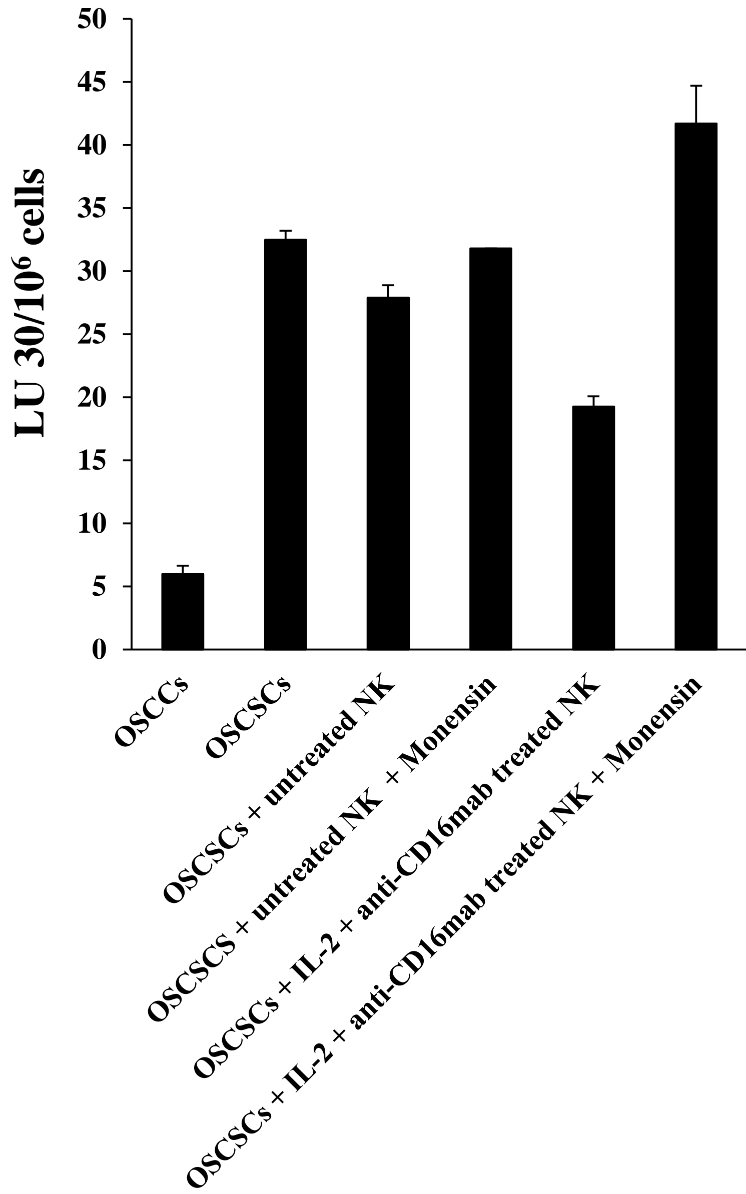


Fig. 6I



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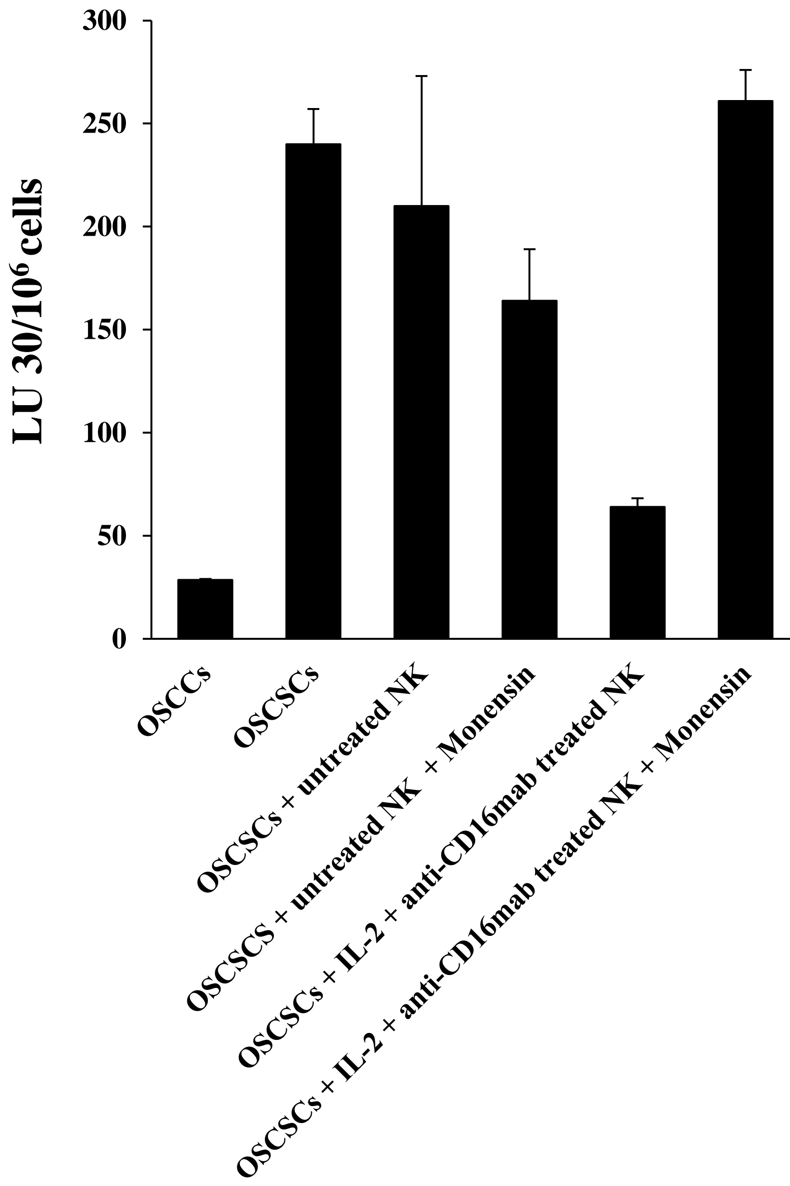

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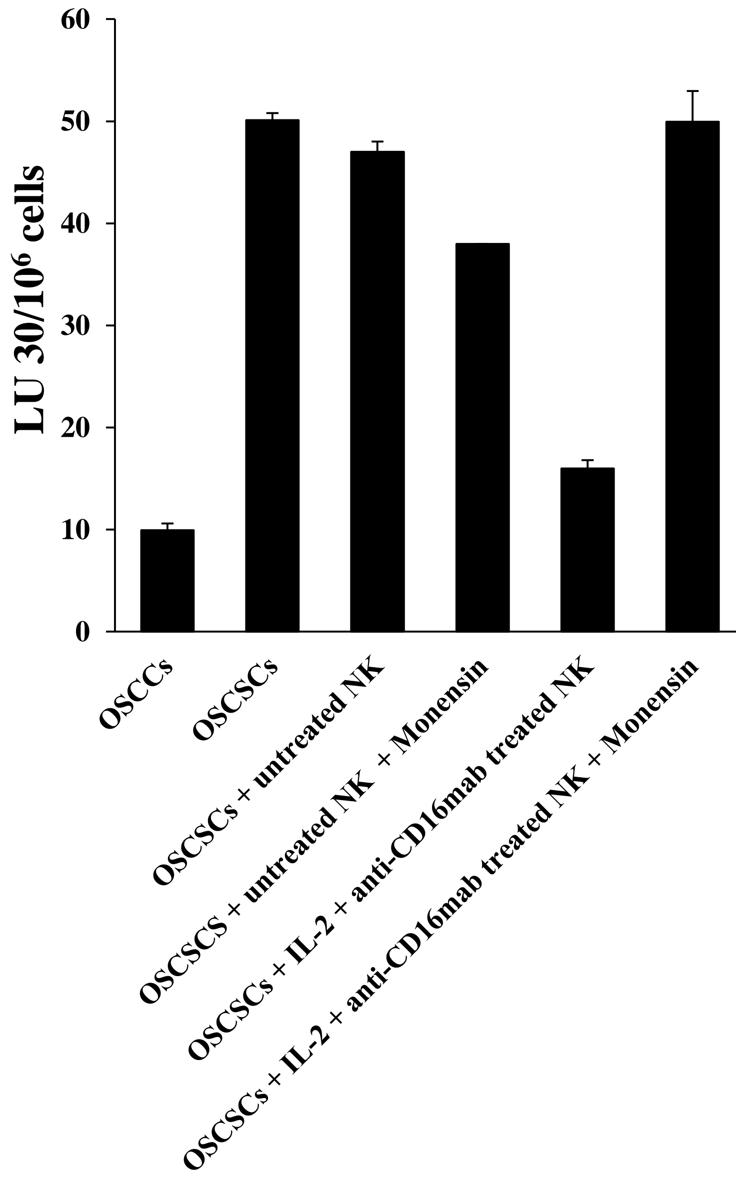


Fig. 6L

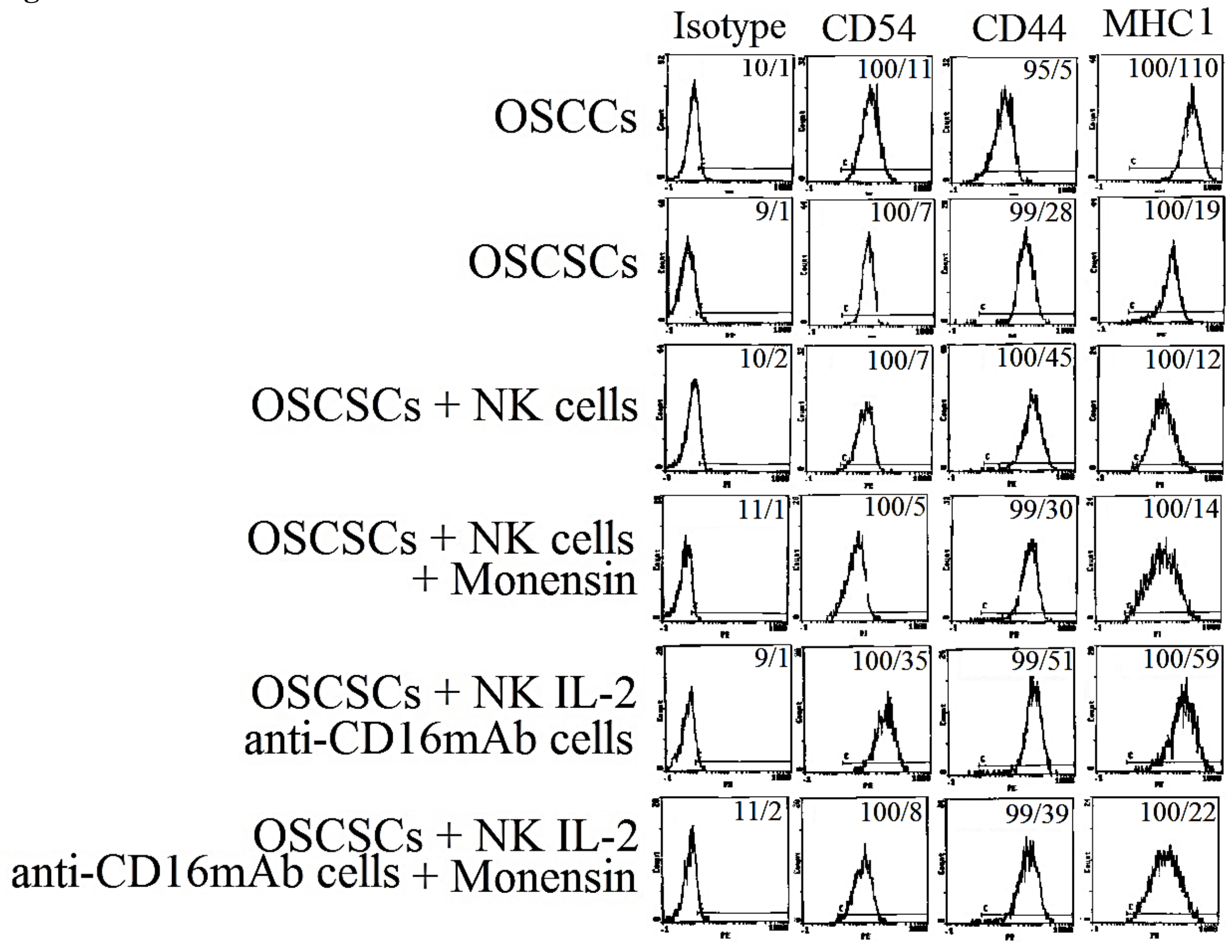


Fig. 6M

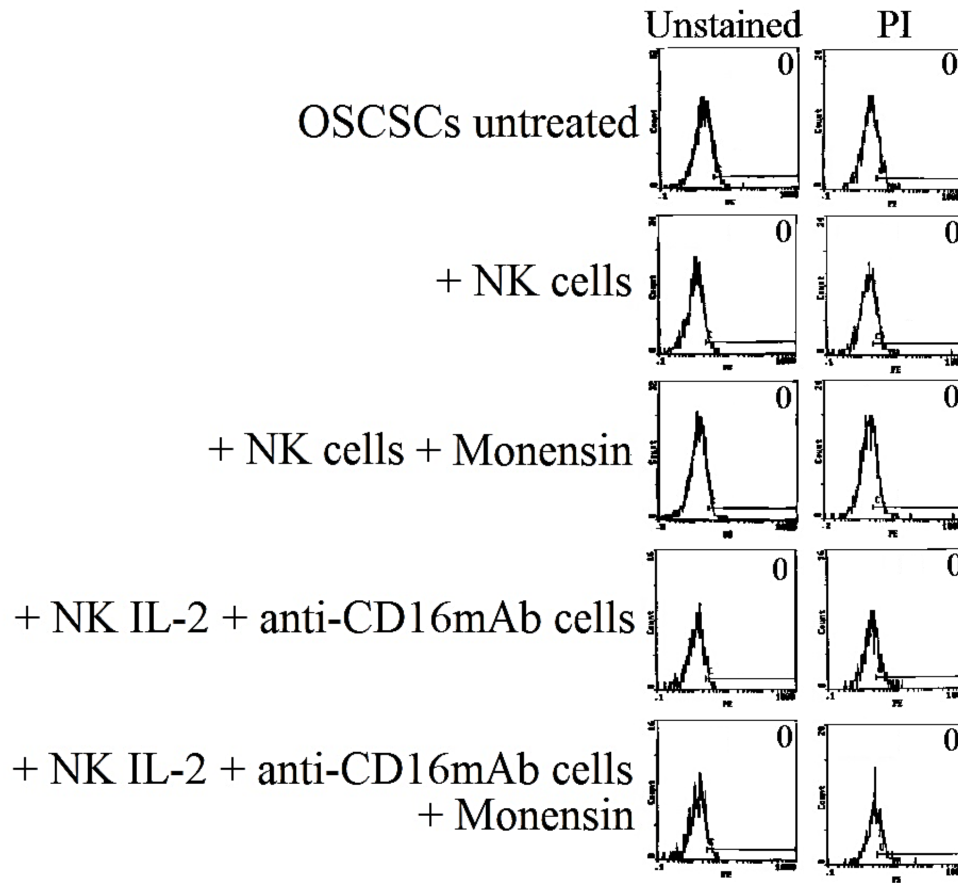
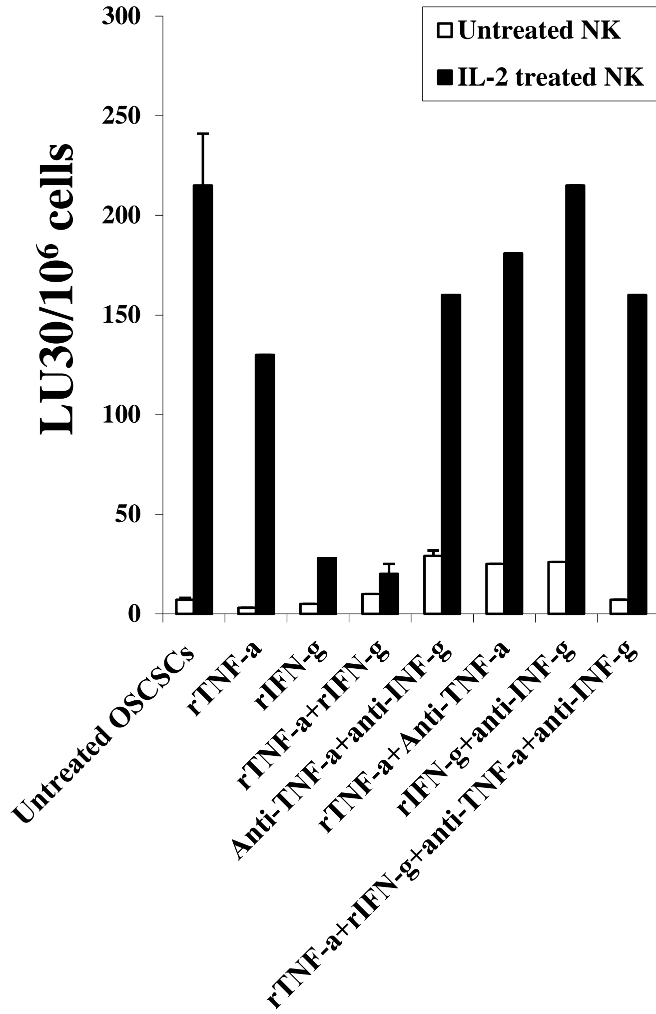


Fig. 7A

Fig. 7B



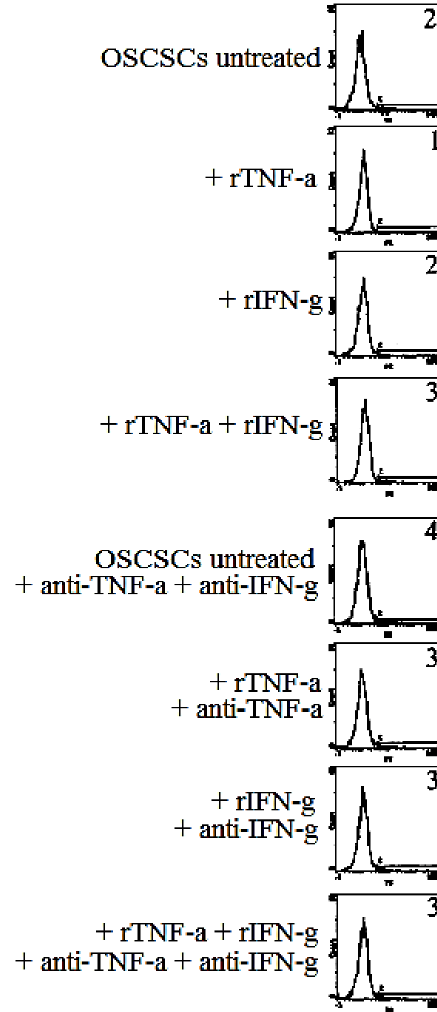


Fig. 7C

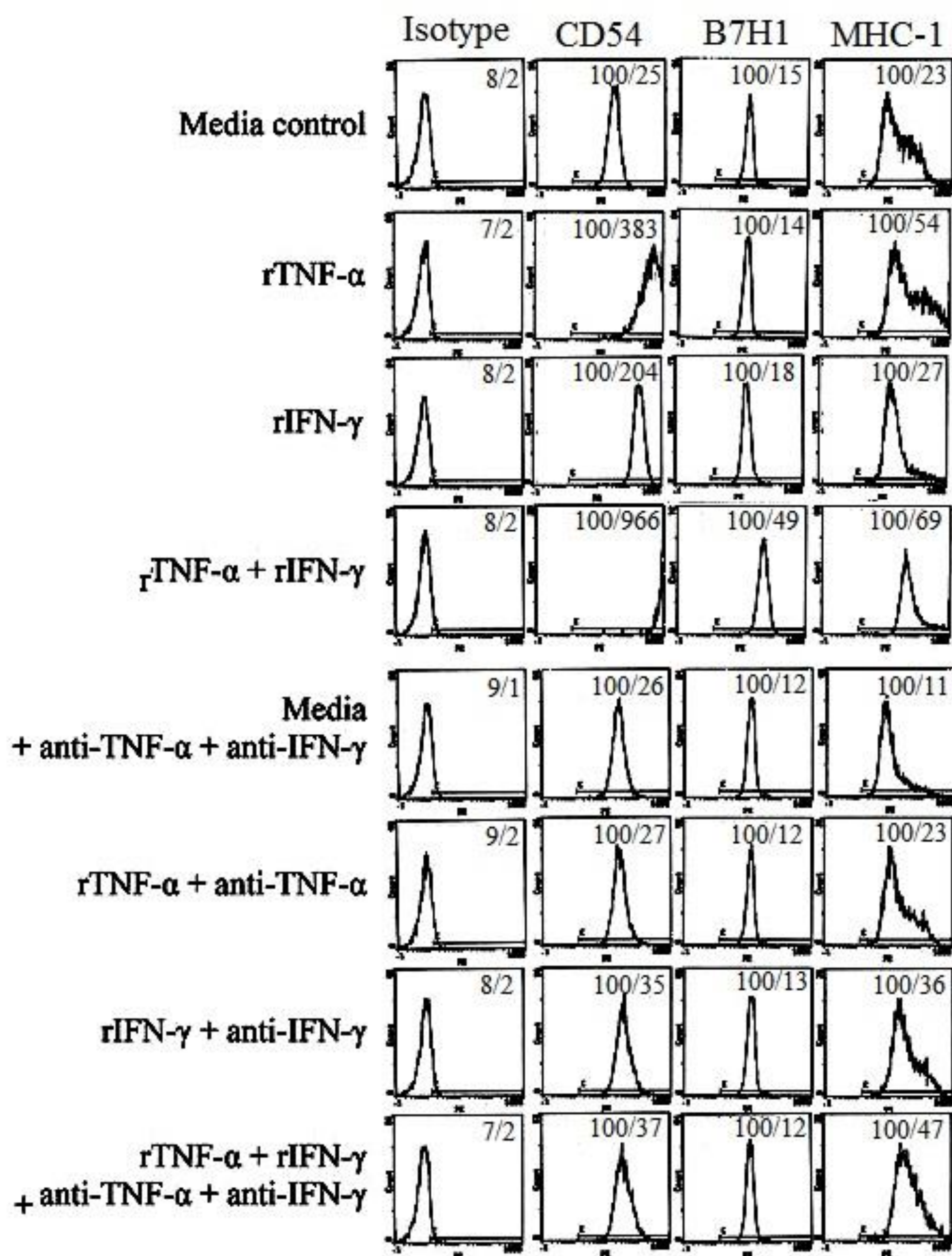


Fig. 8A

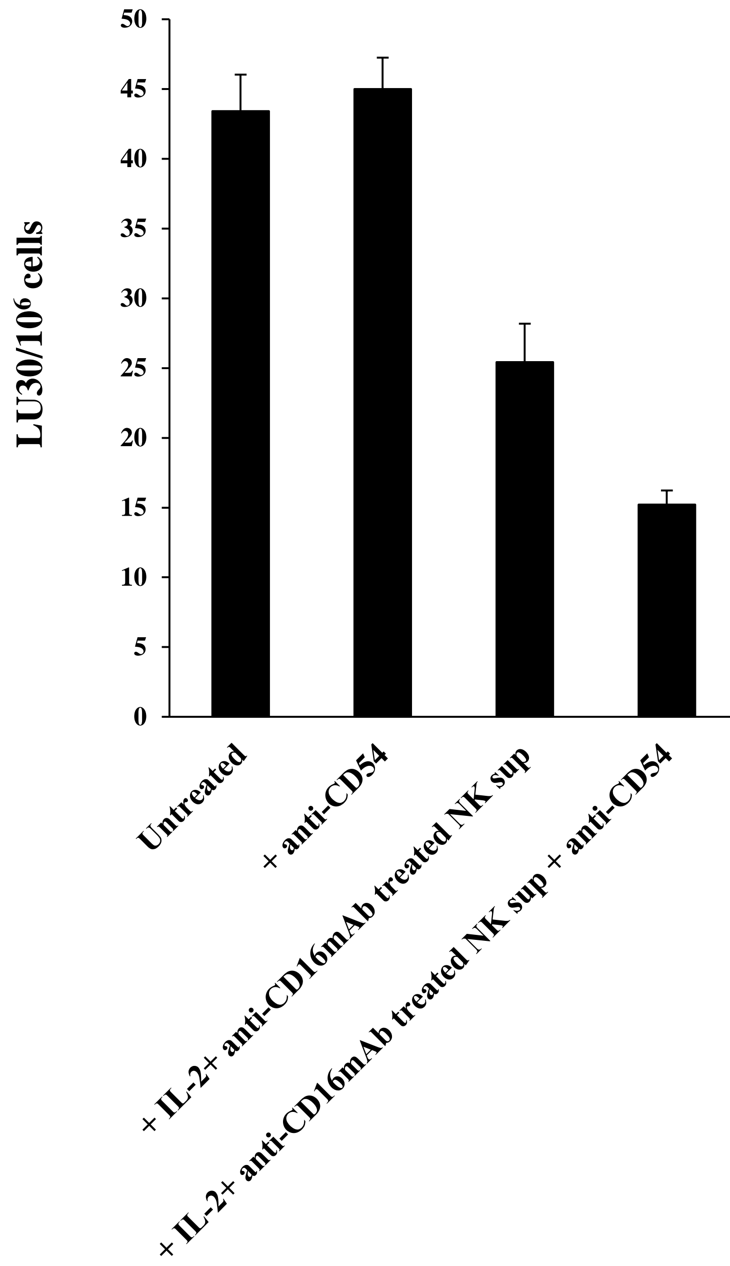


Fig. 8B

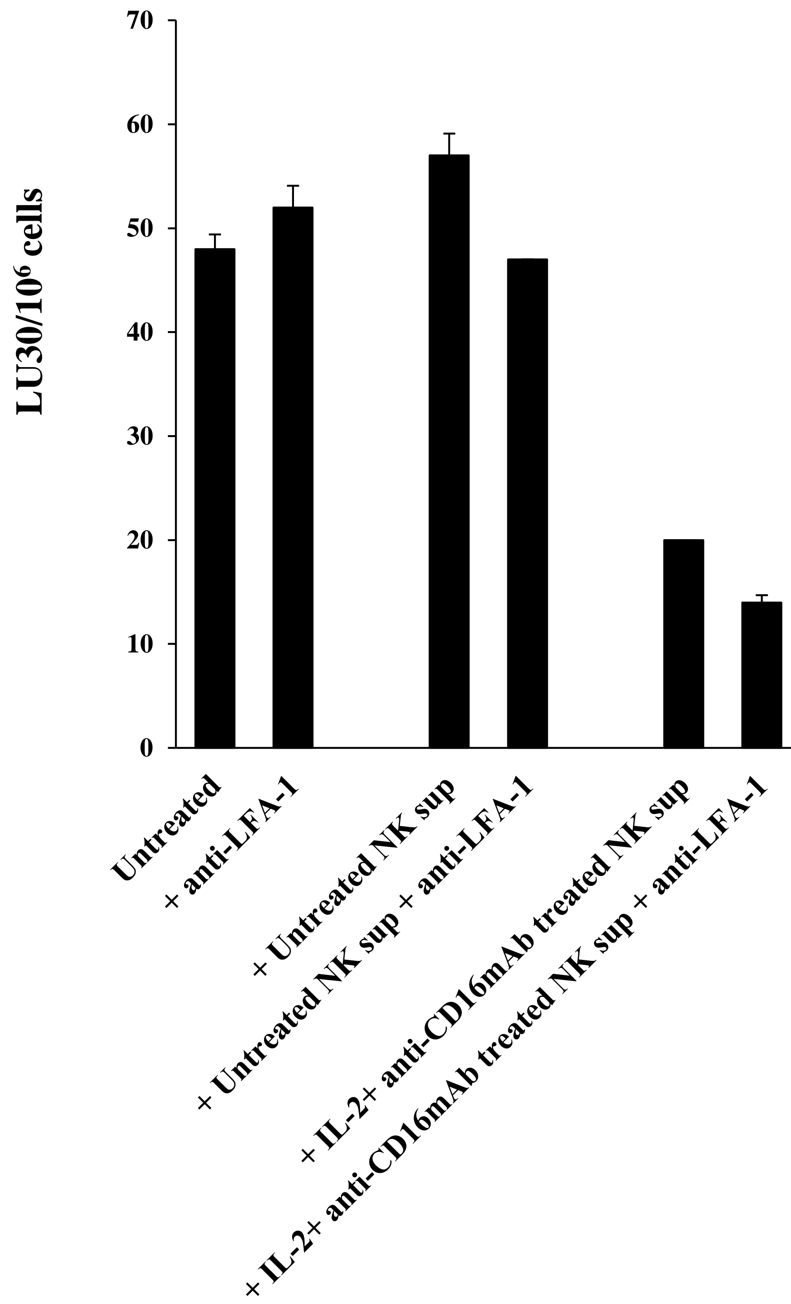


Fig. 9A

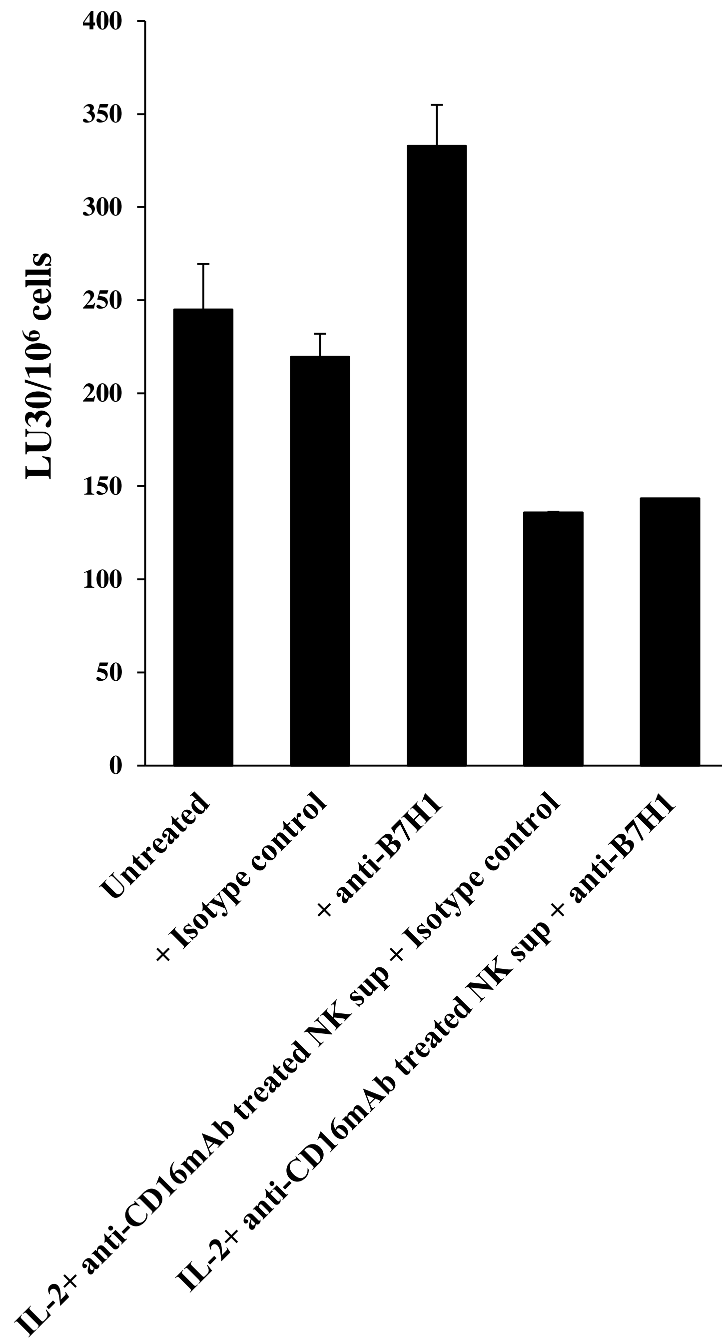


Fig. 9B

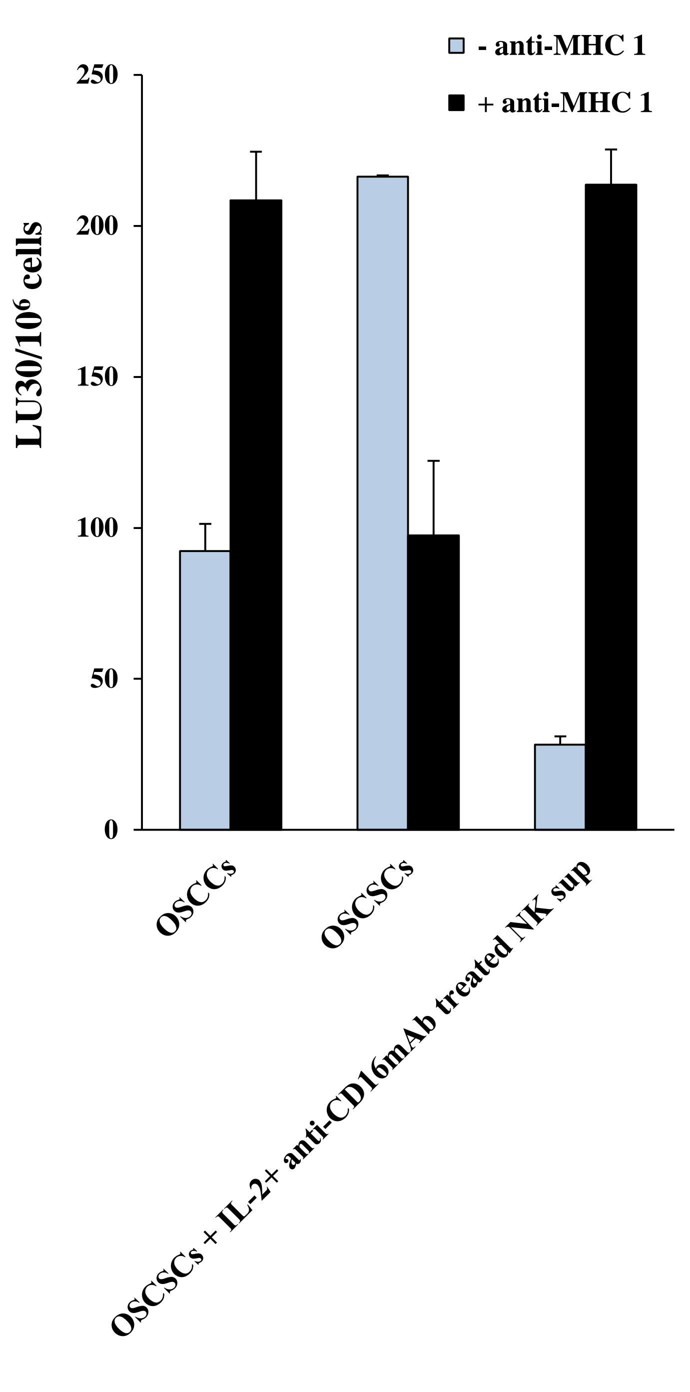
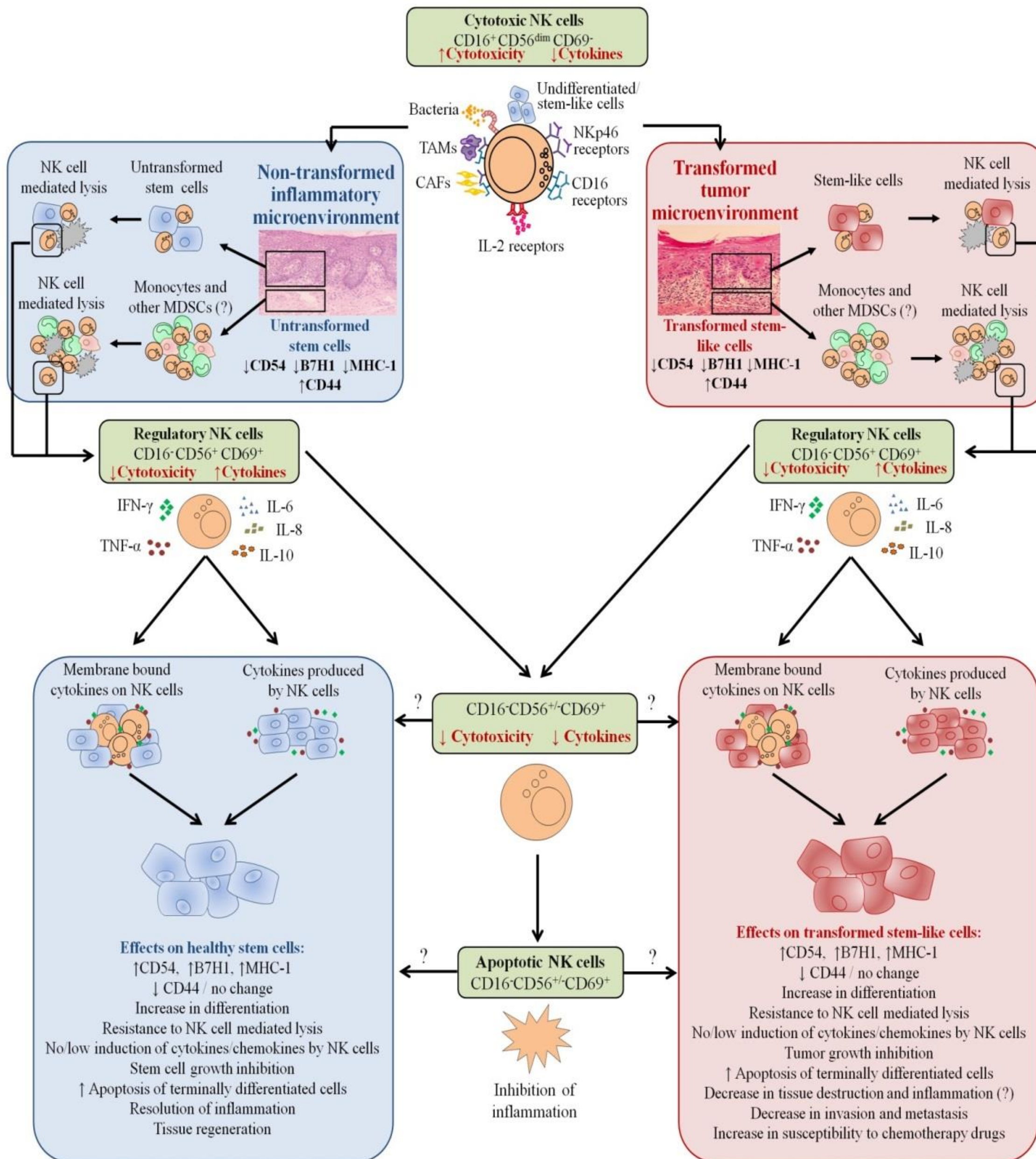


Fig. 10



Supplemental data

Induction of resistance in MP2 and SCAPs to NK cell mediated cytotoxicity by anergized NK cells is mediated by the combination of IFN-γ and TNF-α and not each cytokine alone

Similar results to those obtained with OSCSCs were also observed when undifferentiated or stem-like Mia Paca (MP2) pancreatic tumors or Stem Cell of Apical Papillae (SCAP) were used to treat with the supernatants of NK cells treated with IL-2+anti-CD16mAb in the presence and absence of anti-TNF-α and/or anti-IFN-γ (Fig. 1). Reversal or inhibition of resistance of MP2 (Fig. 1A) and SCAP (Fig. 1B) to NK cell mediated cytotoxicity correlated with the ability of the combination of anti-TNF-α and anti-IFN-γ to block up-regulation of CD54, MHC-class I and B7H1 and down-regulation of CD44 (Fig. 1C and 1D). No or slight modulation of CD54 could be observed on MP2 cells with all the treatments. The effect of anti-IFN-γ mAb in the absence of anti-TNF-α antibody was more dominant for surface receptor expression than cytotoxicity or cell growth since its addition abrogated the increase in surface receptor expression substantially. Similarly, the rate of MP2 (Fig. 1E) and SCAP (Fig. 1G) cell growth was decreased when supernatants obtained from IL-2+anti-CD16 treated NK cells were added, and this decrease was completely inhibited in the presence of the combination of anti-IFN-γ and anti-TNF-α antibodies and not each antibody alone. A slight increase in cell death of MP2 (Fig. 1F) or SCAP (Fig. 1H) could be observed in both attached and detached cells when these cells were treated with the supernatants from IL-2+anti-CD16mAb treated NK cells, however, in contrast to OSCSCs, MP2 and SCAPs had substantially less detached cells, and required higher

amounts of NK supernatants for their differentiation as indicated in the materials and methods section.

Combination of rTNF-α and rIFN-γ induce differentiation and resistance of DPSCs and SCAPs to NK cell mediated cytotoxicity

Similar results to those obtained with OSCSCs were also obtained with SCAPs and DPSCs, although rIFN-γ reduced cytotoxicity by IL-2 activated NK cells, the addition of a combination of rIFN-γ and rTNF-α further reduced IL-2 mediated cytotoxicity (Fig. 2A). Interestingly, the level of CD44 was augmented by the treatment of rTNF-α and rIFN-γ and their combination, and the addition of anti-TNF-α and anti-IFN-γ antibodies blocked the increase substantially on DPSCs (Fig. 2B).

Figure legends

Fig. 1. Induction of resistance of MP2 and SCAPs to NK cell mediated cytotoxicity and inhibition of their growth by IL-2+anti-CD16mAb treated NK cells is mediated by the combination of IFN-γ and TNF-α and not each cytokine alone

Highly purified NK cells were left untreated or treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours, after which the same amounts of supernatants from different NK cell treatments were removed and added to MP2 (A) and SCAP (B) in the presence and absence of anti-TNF-α (1:100) and/or anti-IFN-γ (1:100) for a period of 7 days for MP2 and SCAP. The cytotoxicity against untreated and NK supernatant treated MP2 and SCAP in the presence of antibodies to freshly isolated untreated NK cells or IL-2 treated (1000units/ml) NK cells were assessed using a standard 4 hour ^{51}Cr release assay. Percent cytotoxicity was obtained at different effector to target ratio, and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. The surface expression of CD54, CD44, B7H1 and MHC Class 1 on untreated and NK supernatant treated MP2 (C) and SCAP (D) were assessed after PE conjugated antibody staining using flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities for each histogram. At the end of the incubation of MP2 cells (E and F) or SCAP cells (G and H) with NK cell supernatants, cells which were remained attached to the plate and those which detached during the incubation period were collected separately, and the number of cells (E and G) and their viability (F and H) were assessed using microscopy, and propidium iodide staining followed by flow cytometric analysis respectively.

Fig. 2. Combination of rTNF-α and rIFN-γ induce differentiation and resistance of SCAPs and DPSCs

SCAPs (A) were left untreated or treated with recombinant human TNF-α (20 ng/ml), recombinant human IFN-γ (50 units/ml) or the combination of human TNF-α (20ng/ml) and recombinant human IFN-γ (50 units/ml) in the presence or absence of antibodies against TNF-α (1:100) and/or IFN-γ (1:100) for 24 hours. Afterwards, the cells were detached from the tissue culture plates and labeled with ^{51}Cr and used in a standard 4 hour chromium release assay against untreated and IL-2 (1000units/ml) treated NK cells. Pretreatment of NK cells with IL-2 were carried out for 18-24 hours. Percent cytotoxicity was determined at different effector to target ratio, and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. Surface expressions of CD54, CD44, B7H1 and MHC-1 on DPSCs (B) treated as described in Fig. 2A were determined using staining with PE conjugated antibodies followed by flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentage and the mean channel fluorescence intensities in each histogram. One of minimum three representative experiments is shown in each of Fig. 2A-2B.

Fig. 1A

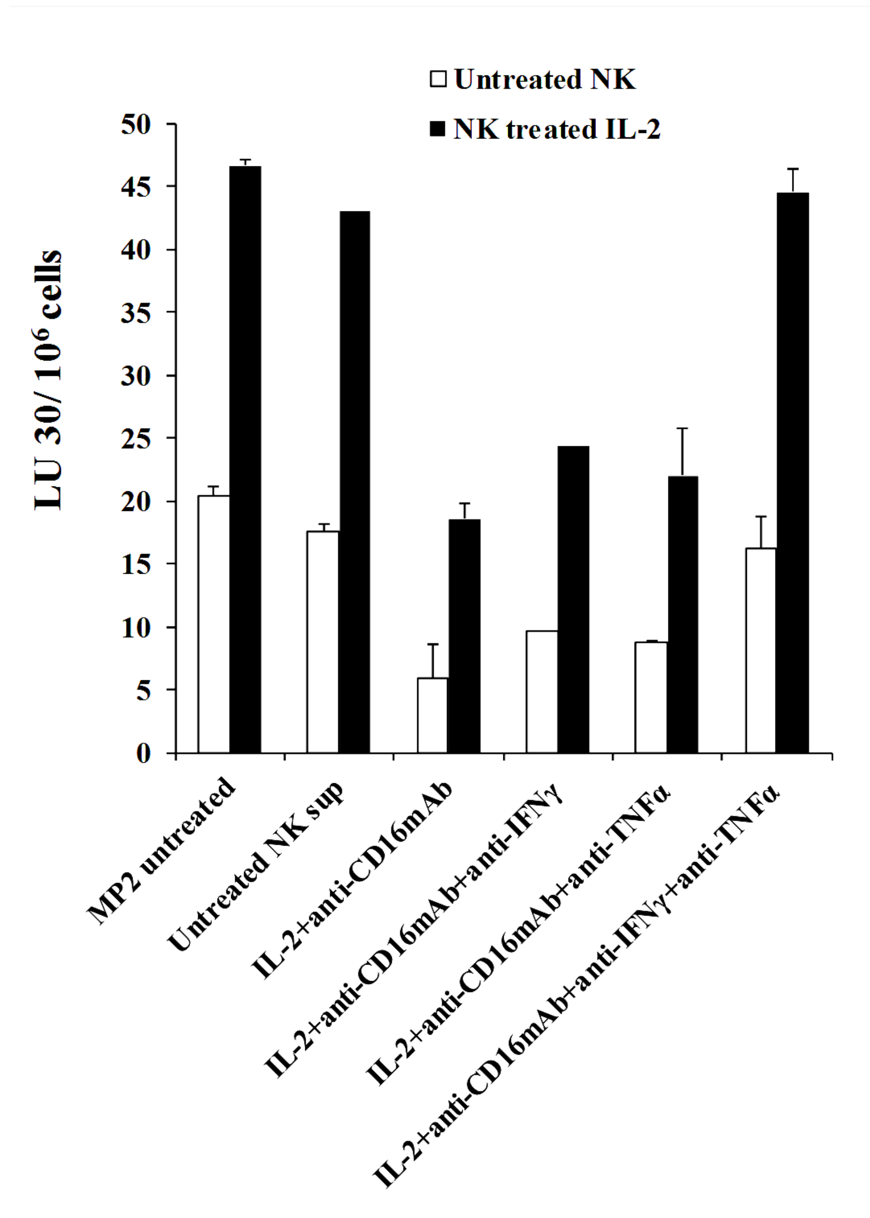


Fig. 1B

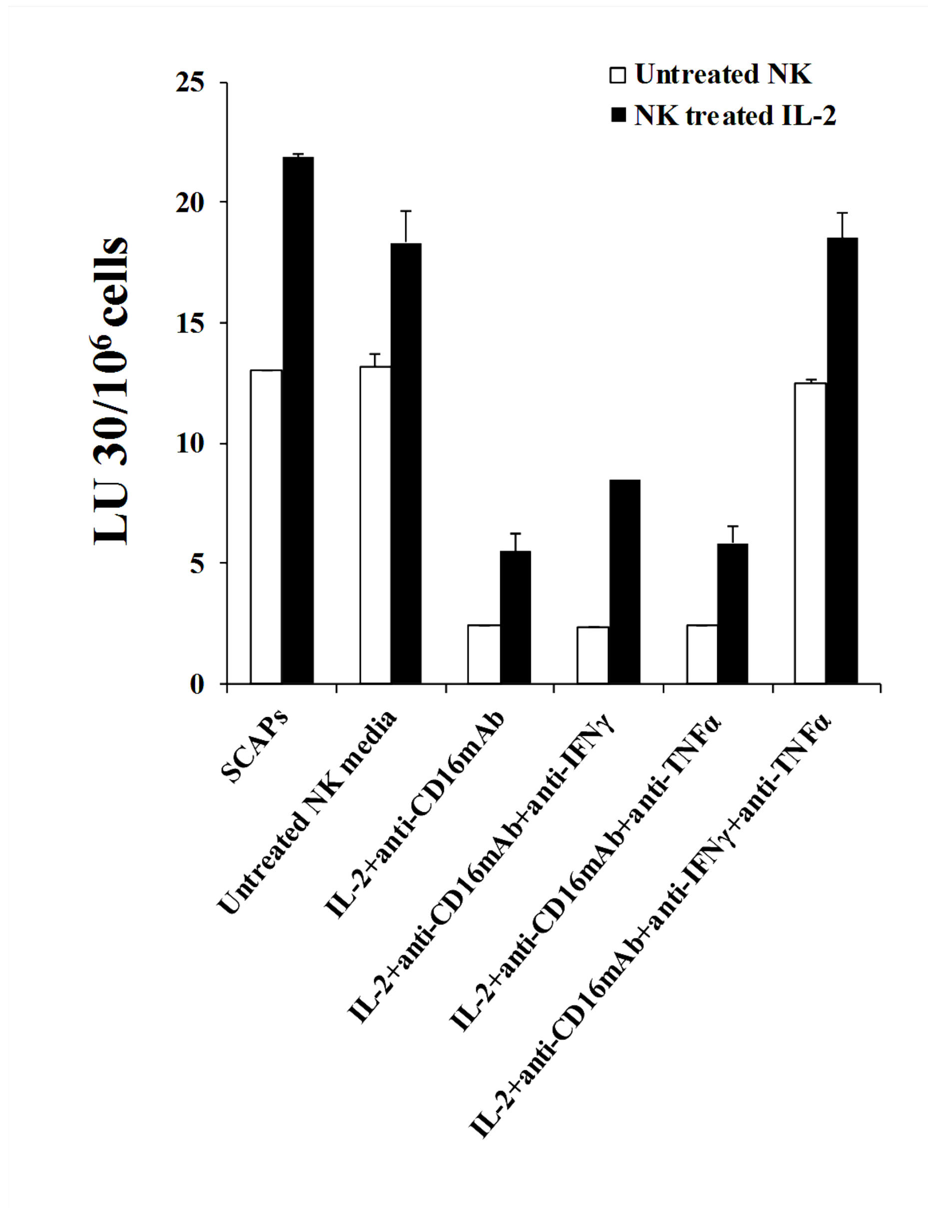


Fig. 1C

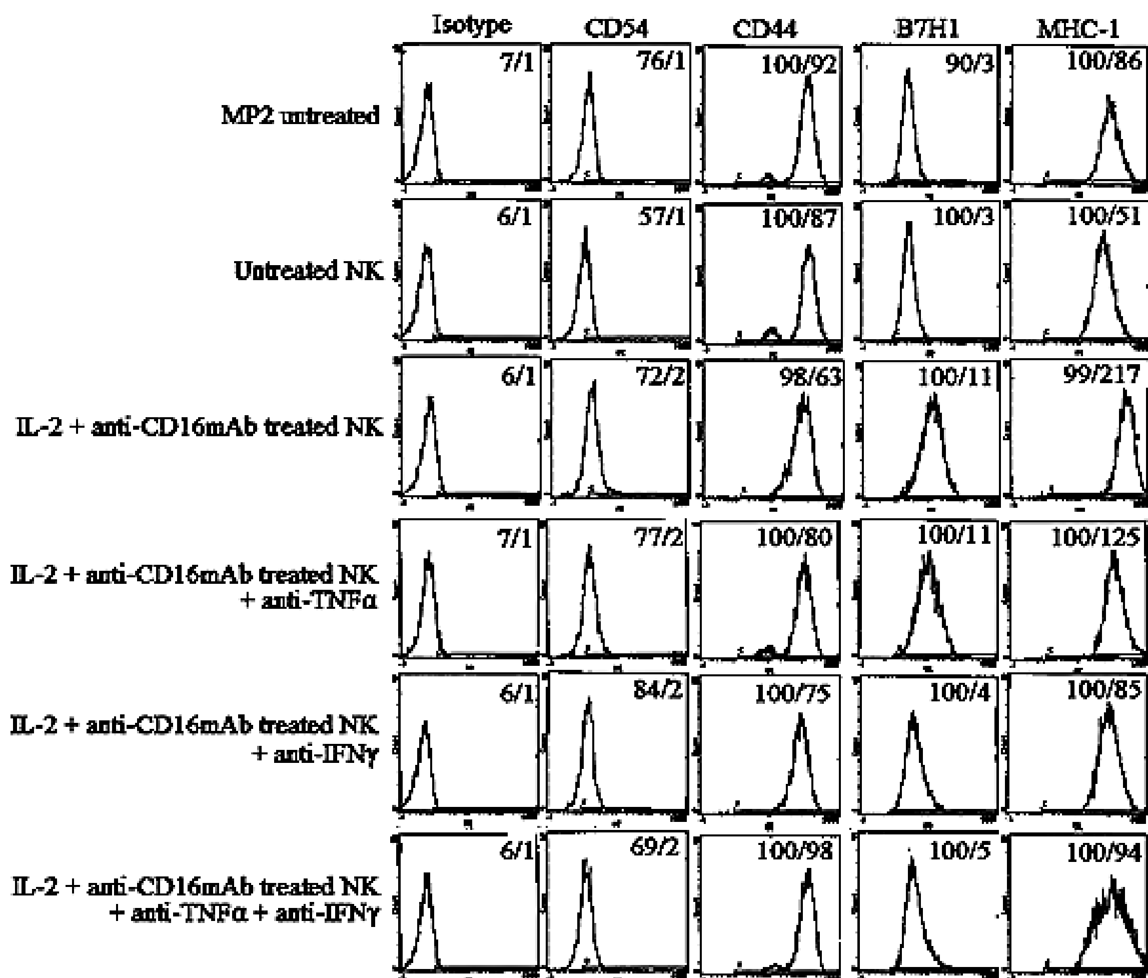


Fig. 1D

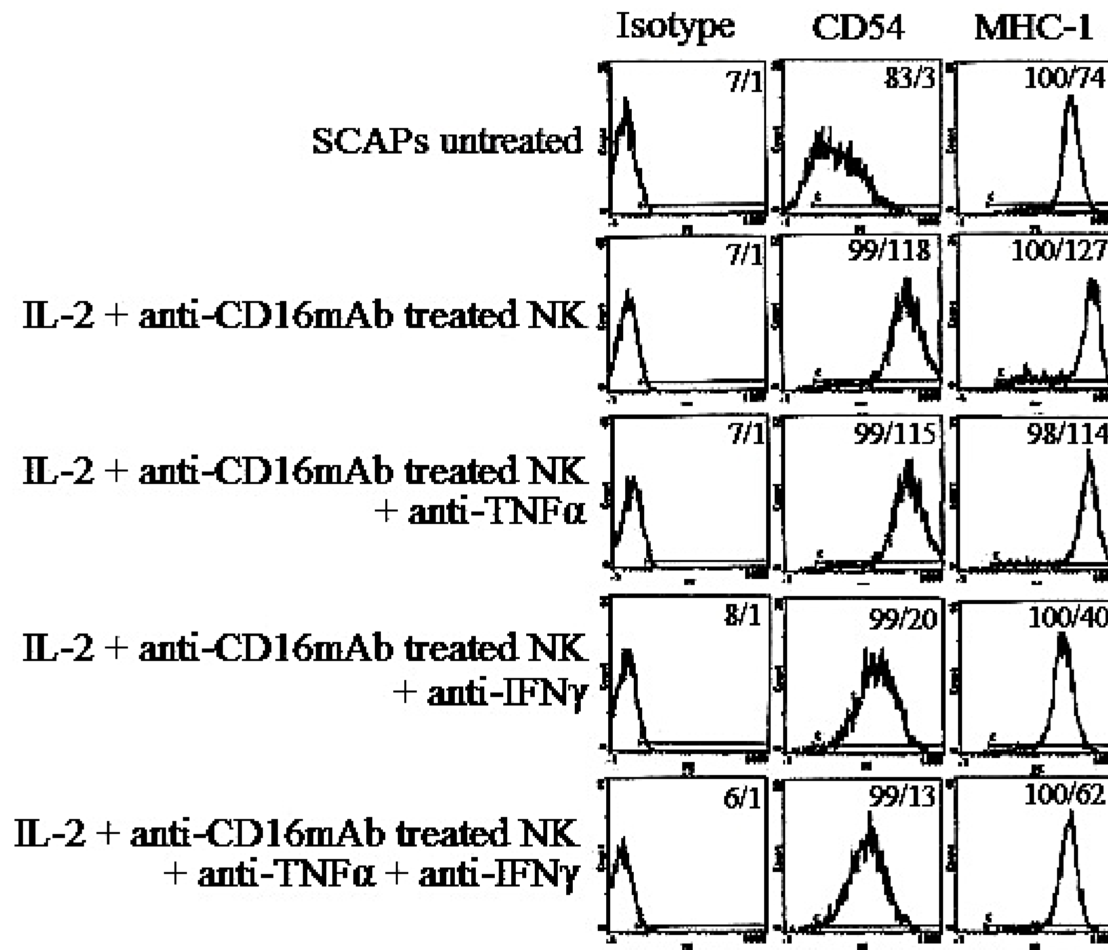


Fig. 1E

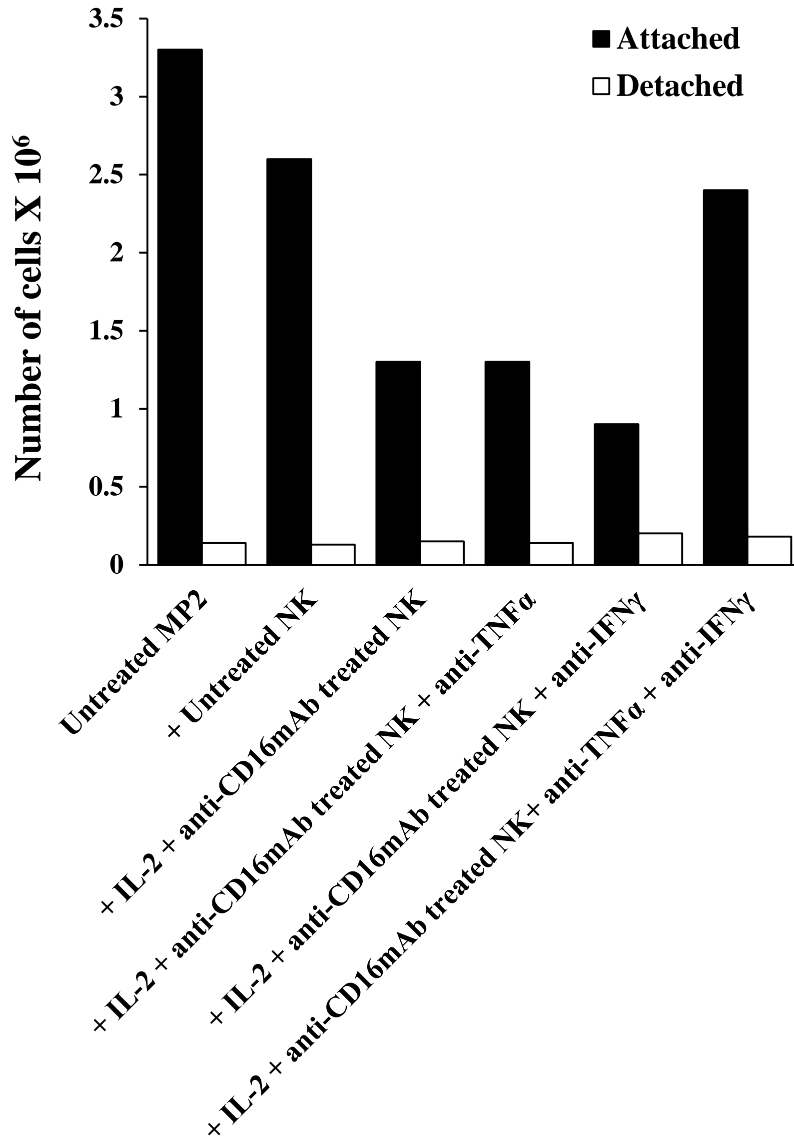


Fig. 1F

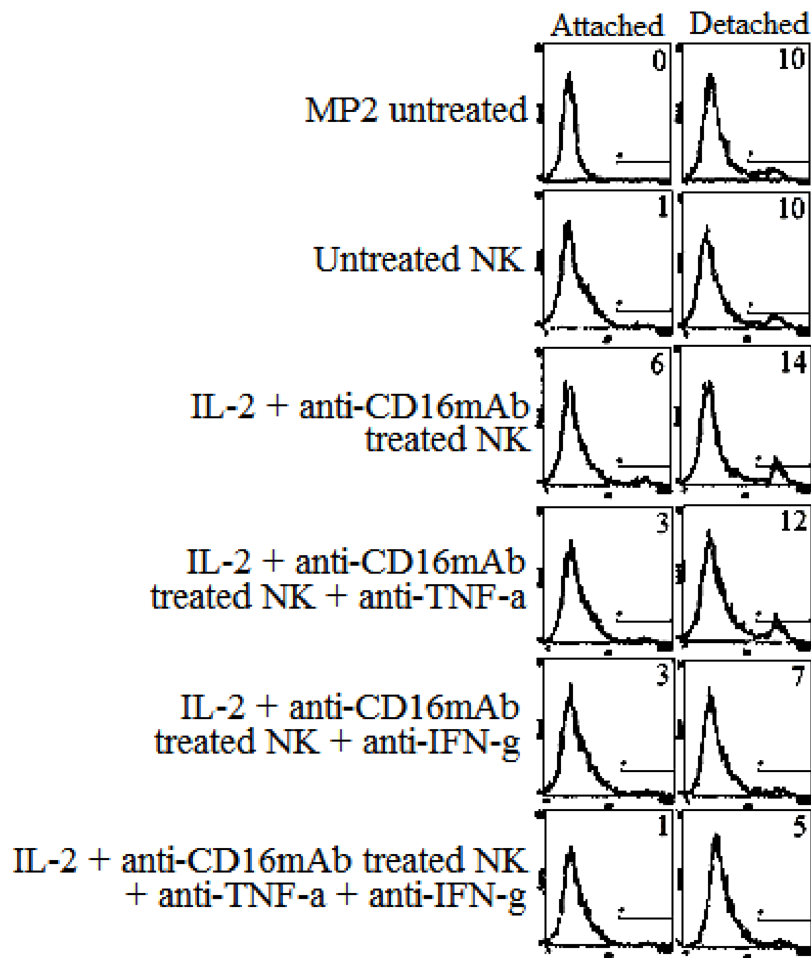


Fig. 1G

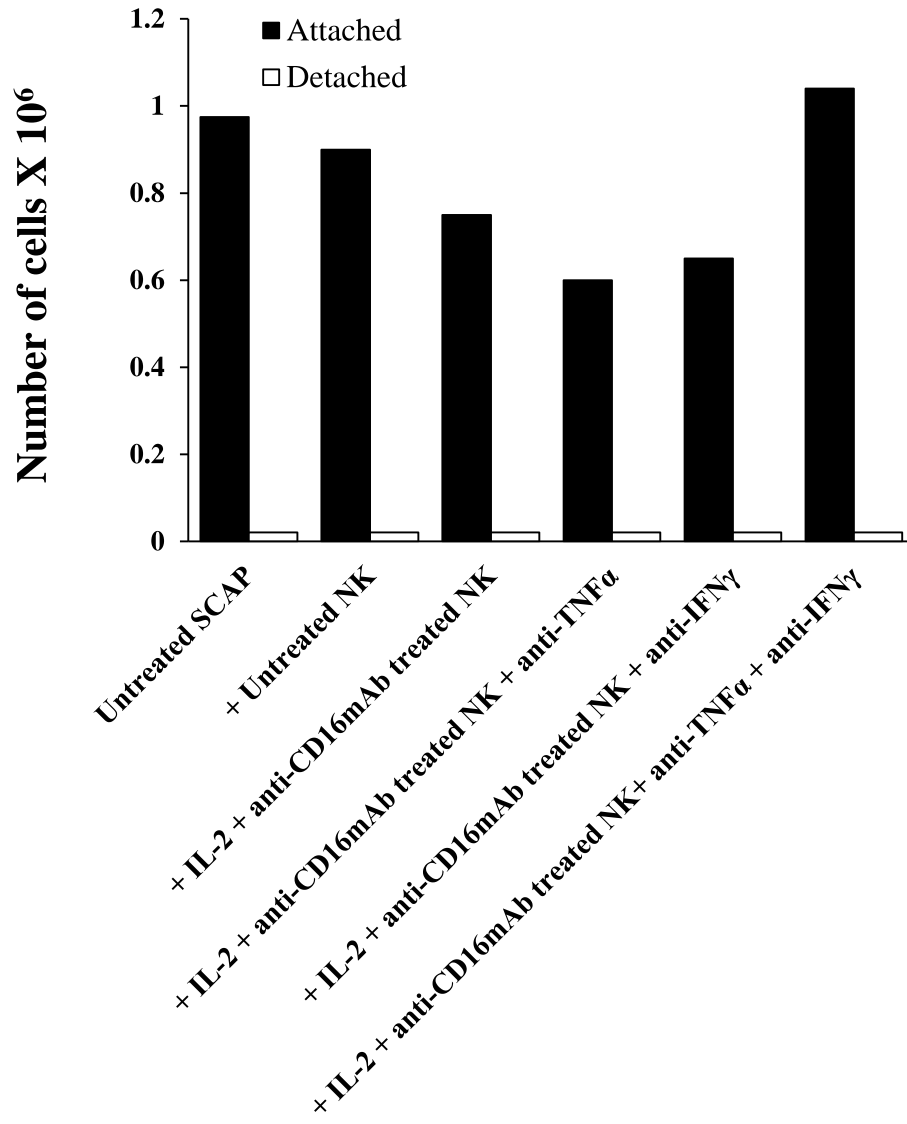


Fig. 1H

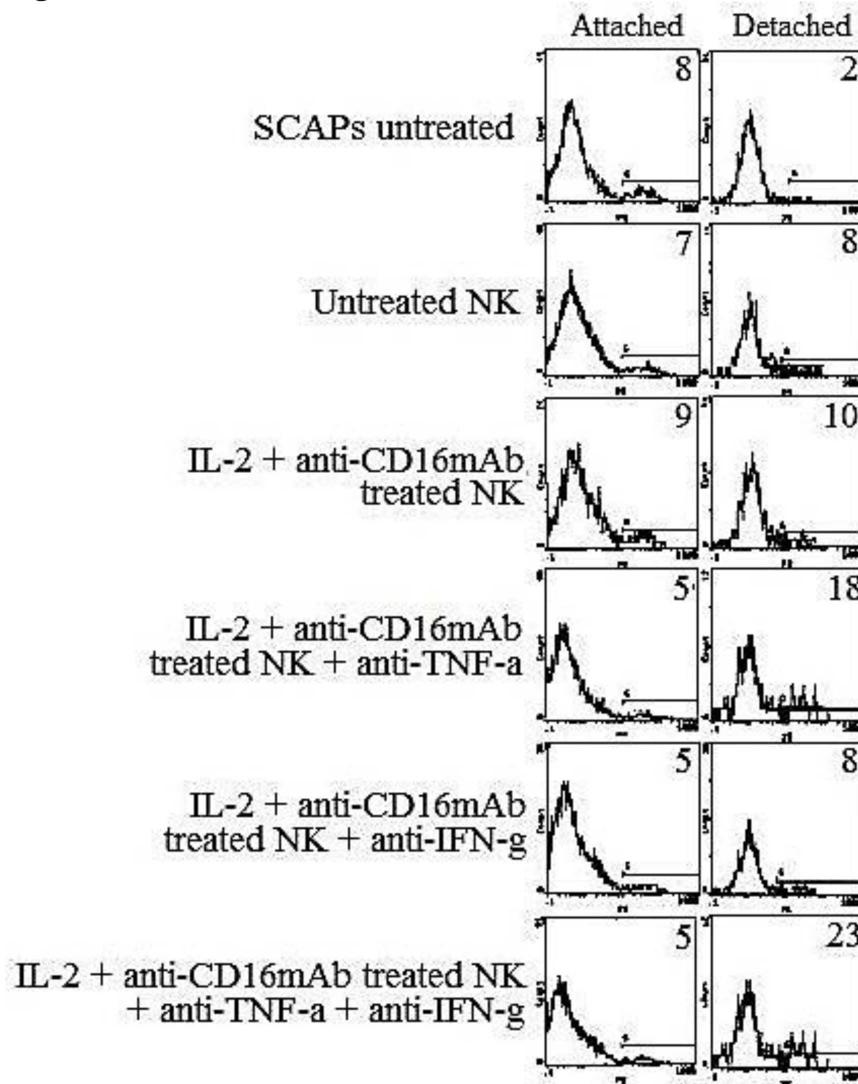


Fig. 2A

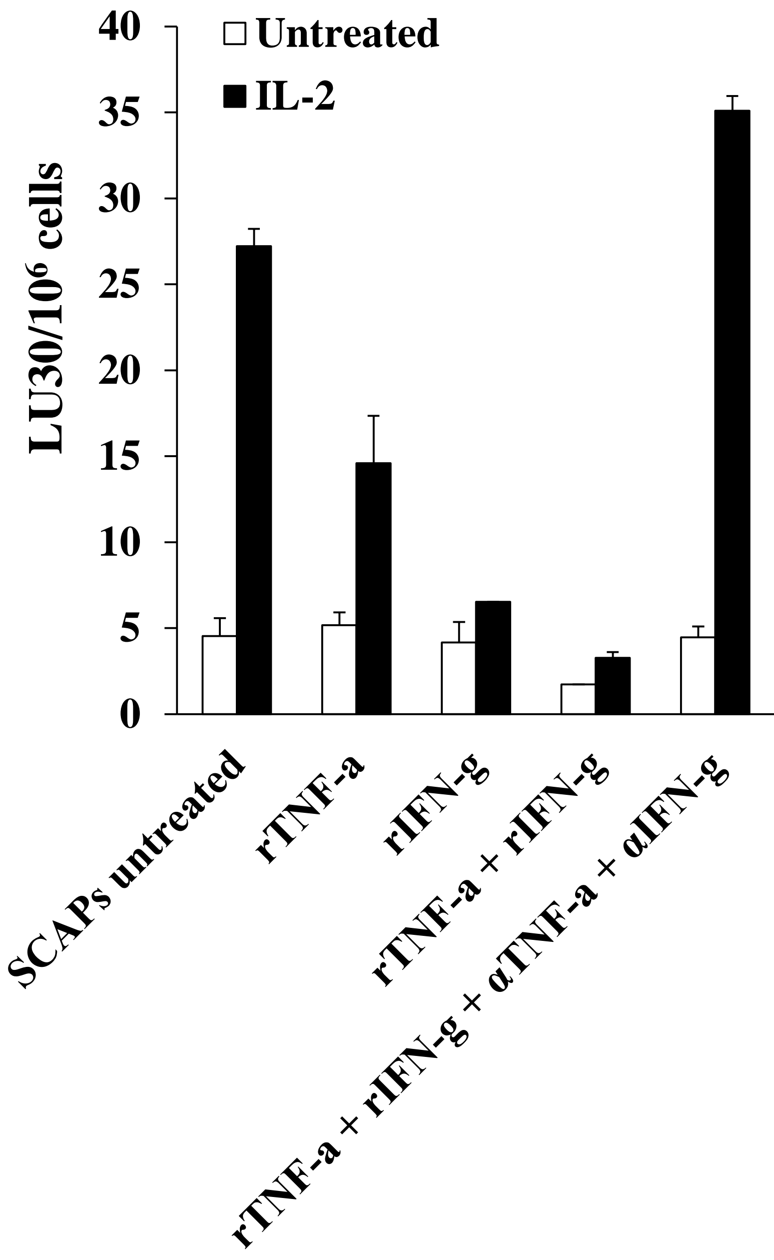
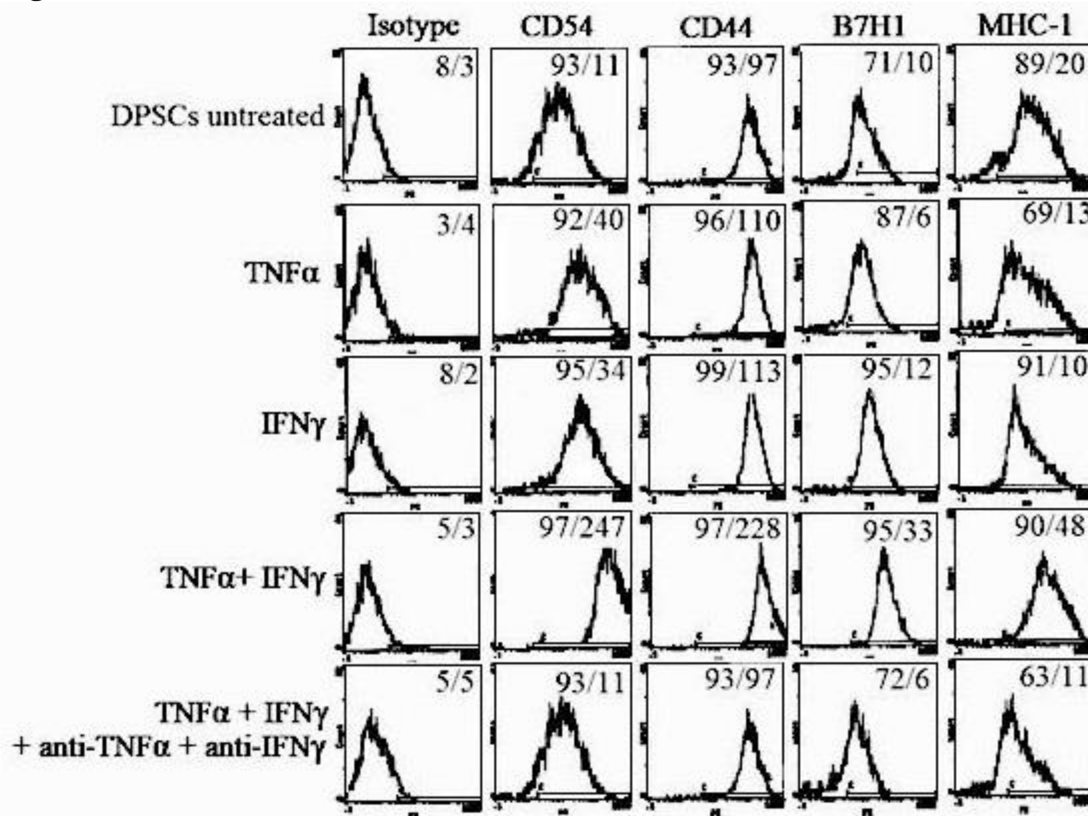


Fig. 2B



CHAPTER 4

Natural Killer cells halt inflammation by inducing stem cell differentiation, resistance to NK cell cytotoxicity and prevention of cytokine and chemokine secretion

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Running Title: Anergized NKs halt inflammation by differentiating stem cells

Keywords: IFN-γ, NK, OSCSCs, OSCCs, cytotoxicity, regulatory NK

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STATEMENT OF TRANSLATIONAL RELEVANCE

This research is directly relevant to human cancer since we used human cancer stem cell model to study the mechanism of suppression of NK cell cytotoxicity. These studies are directly translational since we delineated how NK cells could be used for immunotherapy of tumors. We have also provided mechanisms by which key cytokines, such as IFN-γ and TNF-α, can modulate the differentiation and resistance of cancer stem cells which could provide additional targets of the immunotherapy of the tumors. Unlike many previously published data which tumor resistance is regarded as major block in cancer therapy, our paper provides evidence that tumor resistance may represent a differentiated stage which could be beneficial for patients since they can halt the production of inflammatory mediators which fuel tumor growth and metastasis. Additionally, since NK cells can be used to kill cancer stem cells they are the primary cells for the elimination of cancer stem cells.

Abstract

Purpose: The mechanism of suppression of NK cytotoxicity in cancer patients is not clearly established. In this paper we provide evidence that anergized NK cells induce differentiation of healthy Dental pulp Stem Cells (DPSCs) or transformed Oral Squamous Cancer Stem Cells (OSCSCs) resulting in their resistance to NK cell mediated cytotoxicity and inhibition of the release of inflammatory mediators.

Experimental design: Undifferentiated and NK differentiated OSCSCs and DPSCs were cultured with autologous and allogeneic NK cells and the cytotoxicity, cytokine and chemokine release, surface expression and cell growth were determined in the presence and absence of blocking antibodies to TNF-α and IFN-γ.

Results: Induction of resistance to cytotoxicity in differentiated stem cells correlated with the increased CD54 and MHC class I surface expression, and mediated by the combination of IFN-γ and TNF-α since antibodies to both but not each cytokine alone were able to inhibit resistance. In contrast, inhibition of cytokine and chemokine release was mediated by IFN-γ since the addition of anti-IFN-γ antibody and not anti-TNF-α restored secretion of inflammatory mediators in cultures of NK cells with differentiated DPSCs and OSCSCs. There was a gradual and time dependent decrease in MHC class I and CD54 surface expression which correlated with the restoration of NK cell cytotoxicity, augmentation of cytokine secretion and increased cell growth from days 0-12 post NK supernatant removal.

Conclusions: NK cells may inhibit the progression of cancer by killing and/or differentiation of cancer stem cells which may severely halt inflammation that fuels cancer growth, invasion and metastasis.

Introduction

Immune effectors such as Natural Killer cells and T cells are thought to shape tumor cells and select for cancers with reduced immunogenicity and enhanced capacity to induce immunosuppression [251]. However, the same effector mechanisms are likely responsible for the selection of healthy stem cells with enhanced capacity to induce immunosuppression for the ultimate goal of wound healing, tissue regeneration and cessation of inflammation. Although such mechanisms are thought or speculated to be the underlying cause of immunosuppression in tumors no direct evidence, clear data or even physiological relevance has previously been offered to demonstrate the rationale for tumor mediated immunosuppression. In this paper we provide evidence for the potential role of NK cells in selection, differentiation and cessation of inflammation either during their interaction with healthy stem cells (supplemental data) or when interacting with tumors. This report provides the basis for the understanding of why immunosuppression in tumors may be a key mechanism in controlling tumor growth even though such mechanism can be a double edge sword. In one hand it will control the growth and expansion of the tumors and in other hand it will allow the survival of a selected subpopulation of tumors. Any intervention or strategy to eliminate tumors should consider both mechanisms since they are interrelated.

Much work has been done to identify strategies by which tumor cells evade the immune system. Altered expression of MHC molecules which block recognition and activation of T and NK cells are one of the examples of such mechanism. In addition, tumor cells induce T and NK cell apoptosis, block lymphocyte homing and activation, and decrease macrophage and dendritic cell function by releasing factors such as Fas, VEGF, Interleukin (IL)-6, IL-10, TNF-α,

Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) and IL-1β [175-180]. It has been shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Furthermore, NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [181-184]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [185-187]. In contrast, interaction of NK cells with the resistant tumors does not lead to suppression of NK cell cytotoxicity [137]. Moreover, two key transcription factors, NFκB and STAT3, were identified and shown to increase the production of multiple tumor-derived immunosuppressive molecules [211]. Undoubtedly, the same mechanisms are likely important for normal tissue regeneration and induction of resistance to NK and T cell mediated cytotoxicity.

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity while increasing IFN-γ secretion by the NK cells [136, 137]. On the other hand NK resistant tumors such as RAJI cells do not induce loss of NK cell cytotoxicity nor do they induce IFN-γ secretion [136, 137]. Furthermore, following NK cell cultures with sensitive tumor-target cells, the target binding NK cells undergo phenotypic and functional changes expressing CD16-CD56+/dim/-CD69+ phenotype and a small subpopulation of NK cells undergoes apoptosis [136, 137]. Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in oral and ovarian cancer patients [138, 139]. In addition, down-regulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [140]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [140]. Triggering of CD16 on NK cells by anti-CD16 antibody, which mimics the ligand

binding effect, was also found to result in down-modulation of CD16 receptors causing a great loss of cytotoxicity and gain in cytokine secretion in NK cells which we have previously coined as "split anergy" [136, 137, 141-145]. In addition, a small subpopulation of CD56dimCD16+ NK cells undergoes cell death similar to that seen during the interaction of NK cells with sensitive tumors. Loss of cytotoxicity in NK cells was significantly increased when NK cells were either treated with anti-MHC class I antibody [143] or treated with $F(ab)'_2$ fragment of anti-CD16 mAb [143, 145].

We have previously shown that NK resistant differentiated primary oral epithelial tumors, unlike their cancer initiating or stem cells, demonstrate higher nuclear NFκB activity and secrete significant levels of GM-CSF, IL-1β, IL-6 and IL-8 [18, 190]. Additionally, inhibition of NFκB in tumors leads to a significant increase in NK cell mediated cytotoxicity and augmented secretion of IFN-γ [192, 193]. Moreover, targeted inhibition of NFκB in distinct cells results in the induction of auto-immunity and inflammation [153, 194] .

Therefore, in our previous studies we determined that the stage of maturation and differentiation of the healthy untransformed stem cells, as well as transformed tumorigenic cancer stem cells, is predictive of their sensitivity to NK cell lysis. In this regard we have shown that OSCSCs, which are stem-like oral tumors, are significantly more susceptible to NK cell mediated cytotoxicity; whereas, their differentiated counterpart OSCCs is significantly more resistant [18]. In addition, hESCs, hiPSCs, hMSCs and hDPSCs, were found to be significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts [18]. Based on these results we proposed and recently demonstrated that NK cells play a significant

role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact [20]. In addition, we have shown previously that monocytes, a subset of Myeloid Derived Suppressor Cells (MDSCs), induce significant split anergy in NK cells [19, 236-239]. Such alterations in NK cell effector function is found to ultimately aid in driving differentiation of surviving, healthy, as well as transformed stem cells [19, 236-239]. In cancer patients with advanced disease since the number and function (both the cytotoxic and cytokine secretion) of NK cells may be compromised by the growth and expansion of cancer stem cells, they may not be effective in eliminating and/or differentiating cancer stem cells, thus resulting in the progression of cancer. In this paper we demonstrate that anergized NK cells contribute to the differentiation and resistance to NK cell mediated cytotoxicity of transformed stem cells by secreting key cytokines. More importantly, we also demonstrate that NK differentiated stem cells are not only resist to lysis by the NK cells but they also do not trigger secretion of cytokines or chemokines, potentially contributing to the cessation of inflammation. This will allow the repair of the tissues during normal wound healing whereas during tumorigenesis they may aid in decreasing growth, invasion and metastasis of tumors, while allowing survival of a selected tumor population.

Materials and Methods

Cell Lines, Reagents, and Antibodies

RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA) was used for the cultures of human NK cells and monocytes. OSCCs and stem-like OSCSCs were isolated from the tongue tumors of the patients at UCLA and cultured in RPMI 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1.4% antibiotic antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine, 0.2% gentamicin (Gemini Bio-Products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, PA). The primary tumor cells are tested and authenticated regularly in our laboratory. DPSCs from patients were isolated from the third molars after tooth extraction at UCLA and they were cultured in DMEM complete medium supplemented with 2% FBS and 1% penicillin and streptomycin (Gemini Bio-Products, CA) and used with autologous NK cells.

Recombinant IL-2 was obtained from NIH- BRB. Recombinant TNF-α and IFN-γ were obtained from Biolegend (San Diego, CA). Antibodies to CD16 were purchased from Biolegend (San Diego, CA). Anti-MHC class I were prepared in our laboratory and 1:100 dilution was found to be the optimal concentration to use. PE conjugated anti-CD54 and anti-CD44, were obtained from Biolegend (San Diego, CA). Monoclonal antibodies to TNF-α were prepared in our laboratory from ascites of mice injected with TNF-α hybridomas, after which the antibodies were purified and specificity determined by both ELISA and functional assays against recombinant TNF-α. Polyclonal IFN-γ antibodies were prepared in rabbits, purified and specificity determined with ELISA and functional assays against rIFN-γ. 1:100 dilution of anti-

TNF-α and anti-IFN-γ antibodies was found to be the optimal concentration to block rTNF-α and rIFN-γ function. The human NK purification kits were obtained from Stem Cell Technologies (Vancouver, Canada). Propidium iodide is purchased from Sigma Aldrich (Buffalo, NY).

Purification of NK cells

PBMCs from healthy donors were isolated as described before [137]. Briefly, peripheral blood lymphocytes were obtained after Ficoll-hypaque centrifugation and purified NK cells were negatively selected by using an NK cell isolation kit (Stem Cell Technologies, Vancouver, Canada). The purity of NK cell population was found to be greater than 90% based on flow cytometric analysis of anti-CD16 antibody stained cells. The levels of contaminating CD3+ T cells remained low, at 2.4%±1%, similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures. Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors and all the procedures were approved by the UCLA-IRB.

ELISA and Multiplex assays

Single ELISAs were performed as described previously [137]. Fluorokine MAP cytokine multiplex kits were purchased from R&D Systems (Minneapolis, MN) and the procedures were conducted as suggested by the manufacturer. To analyze and obtain the cytokine and chemokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines provided by the manufacturer. Analysis was performed using the Star Station software.

Surface Staining and cell death assays

Staining was performed by labeling the cells with PE conjugated antibodies or propidium iodide as described previously [137, 201].

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [193]. Briefly, different numbers of purified NK cells were incubated with ^{51}Cr–labeled tumor target cells. After a 4 hour incubation period the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm - spontaneous cpm}}{\text{Total cpm – spontaneous cpm}}$$

LU 30/10^6 is calculated by using the inverse of the number of effector cells needed to lyse 30% of tumor target cells X100.

Stem cell differentiation with NK cell supernatant

Human NK cells were purified from healthy donor's PBMCs as described above. NK cells were left untreated or treated with anti-CD16mAb (3ug/ml), IL-2 (1000 units/ml) or a combination or IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18- 24 hours before the supernatants were removed and used in differentiation experiments. The amounts of IFN-γ produced by the activated NK cells were assessed with IFN-γ ELISA (Biolegend, CA). Differentiation of OSCSCs was conducted with gradual daily addition of increasing amounts of

NK cell supernatant. On average a total of 1000 pg of IFN-γ containing supernatants obtained from IL-2+anti-CD16mAb treated NK cells was added for 5 days to induce differentiation and resistance of OSCSCs to NK cell mediated cytotoxicity. DPSCs required on average a total of 3600pg of IFN-γ containing supernatants obtained from IL-2+anti-CD16mAb treated NK cells during a 5 day treatment to promote differentiation and resistance to NK cell mediated cytotoxicity. Afterwards, target cells were rinsed with 1X PBS, detached and used for experiments.

Statistical analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

Results

Resistance of differentiated but not stem-like tumors to NK cell mediated cytotoxicity

To determine whether NK cells target cancer stem cells and not their differentiated counterparts, NK cells were left untreated or treated with anti-CD16 antibody and/or IL-2 for 18-24 hours before they were used in cytotoxicity assays against OSCSCs and OSCCs. As shown previously and in the supplemental Fig. 1, NK cells mediated much higher lysis of stem like OSCSCs when compared to differentiated OSCCs (P=0.002) (Fig. 1A supplemental) [18]. OSCSCs were found to express a number of stem cell markers and they were $CD133^{+}CD44^{+}CD326^{+}CD26^{+}CD338^{+}CD166^{dim}$ [18, 240-243]. Both untreated and IL-2 treated NK cells mediated higher lysis of OSCSCs when compared to OSCCs in ^{51}Cr release assay (P=0.02) (Fig. 1A supplemental) [18], and IL-2 treated NK cells secreted higher levels of IFN-γ in co-culture with OSCSCs when compared to OSCCs (P=0.008) (Fig. 1B supplemental) [18]. Anti-CD16 mAb treatment inhibited NK cell cytotoxicity against both OSCSCs and OSCCs; however it did not induce any appreciable secretion of IFN-γ (Fig. 1 supplemental) [18]. The addition of the combination of IL-2 and anti-CD16 mAb treatment although inhibited NK cell cytotoxicity significantly against OSCSCs and OSCCs when compared to IL-2 activated NK cells alone (P<0.05) (Fig. 1A supplemental), it induced much higher release of IFN-γ when cultured in the presence and absence of OSCSCs (Fig. 1 supplemental). The levels of IFN-γ secretion remained much less in the co-cultures of IL-2 and/or anti-CD16 mAb treated NK cells with OSCCs when compared to those cultured with OSCSCs (P<0.05) correlating with the decreased cytotoxicity by IL-2 treated NK cells against OSCCs (Fig. 1 supplemental). Therefore, anti-CD16 mAb in combination with IL-2 induced split anergy in NK cells resulting in a great

loss of cytotoxicity but significant gain in secretion of IFN-γ against oral stem-like tumors (Fig. 1 supplemental). Similar results to those obtained with OSCSCs and OSCCs were also obtained with healthy untransformed primary Dental Pulp Stem Cells (DPSCs) and their differentiated counterpart (data not shown) and [18]. Noteworthy, IL-2 treated NK cells mediated much higher lysis of undifferentiated DPSCs when compared to differentiated DPSCs and the addition of the combination of IL-2 and anti-CD16 mAb treatment although inhibited NK cell cytotoxicity against undifferentiated and differentiated DPSCs, it induced higher release of IFN-γ [18].

Supernatants from the combination of IL-2 and anti-CD16 mAb treated NK cells induced resistance of OSCSCs to NK cell mediated cytotoxicity

To determine whether supernatants from split anergized NK cells are capable of inducing differentiation in OSCSCs, NK cells were left untreated or treated with anti-CD16 antibody and IL-2 for 18-24 hours before their supernatants were removed and added to OSCSCs. In addition, we determined the period of time which was required for the NK differentiated tumors to regain sensitivity to NK cell mediated cytotoxicity after the removal of NK supernatants. Treatment of OSCSCs with IL-2+anti-CD16mAb treated NK cell supernatants, but not untreated NK supernatants, decreased NK cell mediated cytotoxicity significantly by freshly isolated untreated or IL-2 treated NK cells (P=0.02) (Fig. 1A). Resistance of OSCSCs to NK cell mediated cytotoxicity could also be observed after their treatment with supernatants from IL-2 treated NK cells, however, the levels of resistance were significantly less when compared to those induced by IL-2+anti-CD16mAb treated NK cell supernatants correlating with the degree of differentiation based on the surface receptor expression [20].

To examine the mechanisms by which OSCSCs become resistant by anergized NK cells, we determined NK cell mediated cytotoxicity when OSCSCs were treated with supernatants of NK cells treated with anti-CD16mAb and IL-2 in the presence and absence of each of IFN-γ and TNF-α antibodies alone or their combination. As shown in Fig. 1A the addition of each of the IFN-γ and TNF-α antibody alone had a slight inhibitory effect on the induction of resistance of OSCSCs, however, the combination of anti-IFN-γ and anti-TNF-α abrogated the resistance of treated OSCSCs completely (Fig. 1A). The inhibition of OSCSCs resistance to NK cell mediated cytotoxicity by the combination of anti-IFN-γ and anti-TNF-α antibodies could be observed when untreated, IL-2 treated or IL-2+anti-CD16mAb treated NK cells (Fig. 1A) were used to assess cytotoxicity. Treatment of OSCSCs with the combination of anti-TNF-α and anti-IFN-γ in the absence of NK supernatants had no effect on NK cell cytotoxicity (Fig. 1A). Similar results to those shown above was also obtained when the supernatants of NK cells were removed from the OSCSCs and they were cultured in media for 2-6 days before they were used in cytotoxicity assay against NK cells. The levels of resistance of OSCSCs to NK cell mediated cytotoxicity were gradually decreased from day 0 to day 2 (Fig. 1B) and to day 6 (data not shown), on day 0 demonstrating the highest resistance, followed by day 2 in which the levels of resistance still remained substantial and by day 6 only 10%-20% resistance could be observed. At day 12 post supernatant removal no differences between IL-2+anti-CD16 mAb supernatant treated OSCSCs and those cultured with supernatants from untreated NK cells (data not shown). Similar results to those seen with OSCSCs were also observed for DPSCs (Fig. 2A supplemental).

Induction of resistance to NK lysis in OSCSCs by supernatants from IL-2 and anti-CD16mAb treated NK cells correlated with the increased expression of CD54 and MHC class I

We then compared NK cell resistance induced by the supernatants from IL-2+anti-CD16mAb treated NK cells in OSCSCs to expression of key cell surface receptors before and after removal of NK supernatants. Among many surface receptors tested, B7H1, CD44, CD54 and MHC class I expression were found to correlate significantly with the differentiation and resistance of OSCSCs to NK cell mediated cytotoxicity [20]. However, in this report we focused on only CD54 and MHC class I. As shown in Fig. 2A the levels of CD54 and MHC class I increased substantially on OSCSCs in the presence of IL-2+anti-CD16mAb treated NK cell supernatants. Supernatants from untreated NK cells did not have significant effect on surface expression of OSCSCs (Fig. 2A). The addition of a combination of anti-TNF-α and anti-IFN-γ antibodies at the initiation of OSCSCs treatment with IL-2+anti-CD16mAb treated NK supernatants prevented the up-regulation of CD54 and MHC class I on OSCSCs (Fig. 2A). The effect of anti-IFN-γ mAb in the absence of anti-TNF-α antibody, however, was more dominant for surface receptor modulation than cytotoxicity or cell growth (please see below) since its addition abrogated the increase in CD54 and MHC class I on OSCSCs (Fig. 2A). Similar results to those shown above were also obtained when the NK supernatants were removed from the OSCSCs on day 0 and they were replaced by media from day 0 to day 6 (Figs. 2A-2C). The levels of MHC class I and CD54 surface receptors gradually decreased from day 0 to day 2 and to day 6 post removal of NK cell supernatants (Figs. 2A-2C). At day 12 post NK supernatant removal only 1.5-2 fold increase in MHC class I and 1-1.2 fold increase in CD54 expression could be observed on OSCSCs cultured with supernatants from IL-2+anti-CD16 mAb treated

NK cells (Figs 2A-2C). Thus, there was a time dependent decrease in the ratios for MHC class I and CD54 surface receptor expression between IL-2+anti-CD16mAb NK supernatant treated OSCSCs versus those cultured with supernatants from untreated NK cells or OSCSCs in the absence of any treatment (Figs 2B and 2C). Similar results to those seen with OSCSCs were also observed for DPSCs [20].

Treatment of OSCSCs by supernatants from IL-2 and anti-CD16mAb treated NK cells severely inhibited secretion of cytokines and chemokines by the NK cells

We next determined whether decrease in NK cytotoxicity correlates with a decrease in cytokine and chemokine secretion in cultures of OSCSCs treated with supernatants from split anergized NK cells. In addition, we determined the period of time which was required for the NK differentiated tumors to regain sensitivity to NK cells and increase cytokine and chemokine secretion after the removal of NK supernatants. Treatment of OSCSCs with IL-2+anti-CD16mAb treated NK cell supernatants, but not unstimulated NK supernatants, significantly decreased secretion of chemokines IL-8 ($p=0.001$), MCP-1 and MIP1β (Fig. 3A) and cytokines IL-6 ($p=0.01$) and IFN-γ ($p=0.0009$) (Fig. 3B) by freshly isolated untreated and IL-2 treated NK cells. To examine the mechanisms by which NK supernatant treated OSCSCs decrease cytokine secretion by NK cells, we determined secretion in the presence and absence of each of IFN-γ and TNF-α antibodies alone or their combination. As shown in Fig. 3 the addition of TNF-α antibody in the absence of anti-IFN-γ to OSCSCs treated with IL-2+anti-CD16mAb NK supernatants had no or low effect on the increase in secretion of cytokines and chemokine. In contrast, the addition of anti-IFN-γ in the absence of anti-TNF-α increased the levels of IL-8, MCP-1, MIP-1β, IL-6 and IFN-γ in the co-cultures of NK cells with IL-2+ anti-CD16mAb treated OSCSCs to the

levels when OSCSCs treated with the unstimulated NK supernatants were cultured with NK cells (Fig. 3). The increase in the secretion of cytokines and chemokines in the co-cultures of NK cells with IL-2+anti-CD16mAb treated OSCSCs were also observed when they were cultured in the presence of both anti-TNF-α and anti-IFN-γ antibodies (Fig. 3). Similar results to those shown above were also obtained when the supernatants of IL-2+anti-CD16mAb treated NK cells were removed from the OSCSCs and were replaced by media from day 0-6 (data not shown). The levels of cytokines gradually rose from day 0-6 and by day 12 no differences could be seen between OSCSCs treated with the supernatants from IL-2+anti-CD16mAb stimulated NK cells and those cultured with untreated OSCSCs (Fig. 3C). As the levels of CD54 and MHC class I gradually decreased from day 0-12 (Fig. 2), the levels of IFN-γ secretion in the co-cultures of NK cells with IL-2+anti-CD16mAb NK supernatant differentiated OSCSCs gradually rose to the same levels obtained in the co-cultures of NK cells with OSCSCs cultured with supernatants from untreated NK cells (Fig. 3C). Thus, there was a time dependent decrease in the expression of CD54 and MHC class I which correlated with restoration of cytokine secretion in co-cultures of NK cells with IL-2+anti-CD16mAb NK supernatant differentiated OSCSCs when NK supernatants were removed and replaced with media from day 0-12 (Figs. 2-3). Similar results to those seen with OSCSCs were also observed for DPSCs (Fig. 2B supplemental).

Treatment of OSCSCs by supernatants from IL-2 and anti-CD16mAb treated NK cells inhibited proliferation of OSCSCs

To determine growth dynamics of OSCSCs after treatment with the NK supernatants, the numbers of OSCSCs were counted after treatment with the NK cell supernatants by microscopic evaluation, and the levels of cell death were determined by staining with propidium iodide

followed by flow cytometric analysis. As shown in Fig. 4A there was a decrease in the numbers of OSCSCs after their treatment with IL-2 and anti-CD16mAb treated NK cell supernatants when compared to untreated OSCSCs or those cultured with untreated NK cell supernatants (Fig. 4A). In addition, the decrease in the rate of cell growth was completely inhibited in the presence of the combination of anti-IFN-γ and anti-TNF-α antibodies and not each antibody alone [20]. Interestingly, at day 12 of post supernatant removal the rate of growth in OSCSCs treated with supernatants from IL-2+anti-CD16mAb treated NK cells increased 2 fold when compared to OSCSCs cultured either with untreated NK cell supernatants or untreated OSCSCs (Fig. 4A). Moreover, when the viability of OSCSCs were determined after the addition of supernatants from the IL-2+anti-CD16 mAb treated NK cells, no significant cell death in the OSCSCs were seen at day 0 (Fig. 4B) or at days 2-12 after supernatant removal (data not shown).

Discussion

In this paper we provide evidence that conditioned or anergized NK cells have the ability to induce differentiation of cancer stem cells and limit inflammation through the release of TNF-α and IFN-γ. Similar results to those observed with supernatants were also obtained when fixed IL-2+anti-CD16mAb treated NK cells were used to differentiate OSCSCs [20]. In addition, monensin treated and fixed NK cells lost the ability to induce resistance and differentiation of OSCSCs [20]. Although supernatants or fixed NK cells treated with IL-2 in the absence of anti-CD16mAb had some effect on resistance of stem cells, the magnitude of resistance was significantly less when compared to those induced by IL2 with anti-CD16mAb treated NK cells [20].

One of the most important observations reported in this paper is that the differentiation of OSCSCs by anergized NK cells inhibited greatly the secretion of cytokines and chemokines in the cultures of NK cells with differentiated tumors. This observation is of great significance since it indicates that cellular differentiation is an important step in inhibition and prevention of inflammation. Indeed, the levels of cytokines and chemokines secreted in the co-cultures of NK cells with anergized NK supernatant differentiated OSCSCs was in general similar or slightly higher than those secreted by the NK cells in the absence of tumors. Another intriguing observation in our previous studies is inhibition of bFGF in stem cells by anergized NK cells [18]. Since bFGF is important for the maintenance of stemness in a variety of cell types, inhibition of bFGF by anergized NK cells may be one of the mechanisms by which NK cells prevent growth and proliferation of stem cells and promote their differentiation [252, 253].

We also determined whether differentiation of OSCSCs with supernatants from anergized NK cells were long or short lived. There was a gradual and time dependent decrease in the expression of both MHC class I and CD54 which correlated with the increased cell growth and restoration of NK cell cytotoxicity and cytokine secretion in cultures of NK cells with differentiated OSCSCs from days 0-12 post NK supernatant removal. These experiments indicated that for the OSCSCs to remain differentiated, a continuous exposure to cytokines are necessary since after their removal, the cells revert to their undifferentiated phenotype and become sensitive to NK cell mediated cytotoxicity, and trigger the release of cytokines and chemokines. It is also possible that a few undifferentiated OSCSCs were able to escape differentiation when exposed to the anergized NK cell supernatant, and thus they were able to expand after the removal of NK cell cytokines. However, this possibility is less likely since all of the cells at the peak of differentiation were MHC class I and CD54 positive indicating that the cells had gone through differentiation and upon removal of the NK cytokines they reverted to an undifferentiated phenotype. These results may explain the mechanisms underlying chronic auto-immune inflammation in which the patients suffer from the bouts of exacerbation and remission. Such plasticity in differentiated tumors may explain the need for continuous presence of immune cells in the tumor microenvironment for the inhibition of tumor invasion and metastasis. Indeed, patients which have tumors with infiltrating immune cells have a better prognosis than those which lack infiltration of immune effectors.

Lack of NK cytotoxic function against differentiated tumors may be due to the release of immunosuppressive cytokines such as TGF-β and IL-10 which regulate cytotoxicity as well as TNF-α and IFN-γ release. Our recent studies indicated that IL-10 is an important regulator which

limits NK cell mediated tumor differentiation through inhibition of IFN-γ secretion during monocyte mediated induction of NK anergy (manuscript in prep). The potential role of TGF-β on NK cell mediated differentiation should await future studies.

It is interesting to note that anergized NK differentiated OSCSCs express higher levels of CD54; however, they are not/less susceptible to NK cell mediated cytotoxicity even though the increase in CD54 expression on tumors is shown to increase NK cell mediated cytotoxicity. It is clear from these experiments that CD54 binding and function in cytotoxicity may be limited depending on the differentiation status of the tumors.

OSCSCs do not secrete IL-6, however, the levels of IL-6 secretion are significantly elevated in the cultures of untreated NK but not IL-2 treated NK cells with OSCSCs. This increase could be due to the elevated IL-6 release by the untreated NK cells, or OSCSCs or both. Untreated or IL-2 treated NK cells fail to trigger IL-6 secretion by NK differentiated OSCSCs.

Induction of split anergy in NK cells is an important NK cell conditioning step responsible for the differentiation of cells during pathological processes. In tumors, since the generation and maintenance of cancer stem cells is chronically high, the majority of NK cells including those of the circulating NK cells, may become conditioned to support differentiation of the cells and as such the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble that of the anergic NK cells [236-239, 254]. Therefore, our results suggest two very important functions for the NK cells. One potential function of NK cells is to limit the number of stem cells and second to support differentiation of the stem cells. In

respect to the oral squamous cell carcinomas since the majority of immune effectors are found at the connective tissue area (Fig. 5) [236-239, 254], it is likely that NK cells may first encounter and interact with either the other immune effectors or the effectors of connective tissue such as fibroblasts. However, there is also the possibility that NK cells may first encounter the basal epithelial stem cells in which case by eliminating the stem cells, they too can become anergized (Fig. 5). By eliminating a subset of stem cells or other immune inflammatory cells or effectors of connective tissue NK cells could then be in a position to support differentiation of remaining cells since they will be conditioned to lose cytotoxicity and induce cytokine and growth factor secretion. It is interesting to note that all of the immune effectors isolated from oral gingival tissues of healthy as well as diseased gingiva have CD69+ phenotype, with the exception that the numbers of immune effectors are much less in the healthy oral gingival tissues when compared to diseased tissues [236-239, 254] (manuscript in prep). In addition, lack of significant infiltration of NK cells in the tumor nest and the localization of NK cells in the immune rich compartment which surrounds the tumor in other tumor types, also provides the means for the induction of split anergy in NK cells primarily by other immune effectors in the tumor microenvironment [254]. Such mechanisms of NK cell conditioning by Myeloid Derived Suppressor Cells (MDSCs) may explain why the cytotoxic function of NK cells are greatly reduced in the tumor microenvironment as well as in circulating NK cells.

There should be two distinct strategies to eliminate tumors, one which targets stem cells and the other which targets differentiated cells. Since cancer stem cells are resistant to chemotherapeutic drugs but sensitive to NK cell mediated killing while differentiated oral tumors are more resistant to NK cell mediated killing but more susceptible to chemotherapeutic drugs,

combination therapy should be effective for the elimination of tumors [20]. In addition, since a great majority of patients' NK cells have modified their phenotype to support differentiation of the cells, they may not be effective in eliminating cancer stem cells. Therefore, these patients may benefit from repeated allogeneic NK cell transplantation for elimination of cancer stem cells. In this regard depletion of NK anergizing effectors such as MDSCs in the tumor microenvironment before allogeneic NK cell transplantation should in theory provide such strategy. However, this strategy may also halt or decrease the ability of NK cells to drive optimal differentiation of the tumors and tilt the balance towards a more inflammatory tumor microenvironment which could run the risk of fueling the growth and expansion of more cancer stem cells. Alternatively, a strong tumor differentiating microenvironment may be induced by the MDSCs anergized NK cells, in hope that most if not all of the newly arising cancer stem cells are induced to differentiate (Fig. 5). The benefit of such approach will be the ability of chemotherapeutic drugs to target the differentiated tumors in addition to the lack of differentiated tumors to metastasize. Indeed, our recent in vivo data indicated that pancreatic cancer stem cells have the ability to grow faster and metastasize, whereas their differentiated tumors grew slower and remained localized for a long period of time without metastasizing (manuscript in prep).

It is possible that the successful cancer therapy may lie between a balance in the two abovementioned approaches. The most devastating outcome of the cancer is its ability to deplete NK cells and other immune inflammatory cells (Fig. 5). In this case, not only cancer stem cells will be surviving but they will also remain poorly differentiated. NK cell immunotherapy in these patients should be highly beneficial.

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Figure legends

Fig. 1. Induction of resistance to NK cell mediated lysis of OSCSCs treated with IL-2+anti-CD16mAb NK cells supernatant is mediated by the combination of IFN-γ and TNF-α and not each cytokine alone

Highly purified NK cells were left untreated or treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours, after which the supernatants were removed and used for the treatment of OSCSCs. Untreated OSCSCs and those treated with anti-TNF-α (1:100) and anti-IFN-γ (1:100) in the absence of NK supernatants were used as controls. Same amounts of supernatants from untreated NK cells and those cultured with IL-2+anti-CD16mAb treated NK cells in the presence and absence of anti-TNF-α (1:100) and/or anti-IFN-γ (1:100) were used to treat OSCSCs for a period of 4 days to induce differentiation. Differences between untreated OSCSCs and those stimulated with IL-2+ anti-CD16mAb treated NK supernatants with or without the addition of either anti-TNF-α or anti-IFN-γ alone were significant at a p value of <0.05 (*) (A). OSCSCs were treated as described in Fig. 1A for a period of 4 days before they were washed extensively and cultured in medium in the absence of NK supernatants for 2 days. Differences between untreated OSCSCs and those stimulated with IL-2+ anti-CD16mAb treated NK supernatants with or without the addition of anti- TNF-α alone were significant at a p value of <0.05 (*) (B). The cytotoxicity against untreated OSCSCs and those treated with anti-TNF-α and anti-IFN-γ in the absence of NK supernatants, and OSCSCs cultured with either untreated NK supernatants or those cultured with the supernatants from IL-2 + anti-CD16mAb treated NK cells in the presence and absence of antibodies to TNF-α and IFN-γ were assessed using untreated, IL-2 treated and the combination of IL-2 and anti-CD16mAb treated freshly isolated NK cells using a standard 4 hour ^{51}Cr release assay. Percent cytotoxicity

was obtained at different effector to target ratio and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100.

Fig. 2. Increased expression of CD54 and MHC class I on OSCSCs differentiated with supernatants from IL-2 and anti-CD16mAb treated NK cells

OSCSCs were treated with NK supernatants in the presence and absence of anti-TNF-α and anti-IFN-γ antibodies as described in figure 1A and the surface expression of CD54 and MHC Class 1 on untreated and NK supernatant treated OSCSCs were assessed after 4 days of differentiation. After differentiation, OSCSCs were washed and cultured in normal culture medium without the addition of NK supernatants for a period of 2 days, 6 days and 12 days. Surface expression of CD54 and MHC Class I at each time point was assessed after PE conjugated antibody staining followed by flow cytometric analysis. Isotype antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities in each histogram (A). The ratios of MHC class I (B) or CD54 (C) at days 0, 2, 6 and 12 were determined by using the mean channel fluorescence of the IL-2+anti-CD16mAb treated NK supernatant differentiated OSCSCs to the mean channel fluorescence of the untreated OSCSCs.

Fig. 3. OSCSCs cultured with supernatants from IL-2 and anti-CD16mAb treated NK cells significantly inhibited the production of IL-6 and IFN-γ cytokines and IL-8, MCP-1 and MIP-1β chemokines by NK cells.

Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/ml) and/or anti-CD16mAb (3 μg/ml) for 18 hours. Afterwards, NK cells were added to OSCSCs treated

with NK cell supernatants as described in Fig. 1A at an effector to target ratio of 0.5 to 1. After an overnight incubation, the supernatants were removed from the co-cultures and the levels of IL-8, MCP-1, MIP-1β chemokines (A) and IL-6 and IFN-γ cytokines (B) secretions were determined using specific ELISAs as well as in a multiplexed format using Luminex technology. Identical results for cytokine and chemokine secretions were obtained using either single or the multiplexed format. Differences between untreated OSCSCs and those stimulated with IL-2+ anti-CD16mAb treated OSCSCs with or without the addition of anti-TNF-α were significant at a p value of <0.05 (*). The ratios of IFN-γ production by IL-2 treated NK cells at day 0, 2, 6 and 12 were determined by comparing the amounts of IFN-γ secreted in the co-cultures of NK cells with differentiated OSCSCs with the supernatants from IL-2+anti-CD16 mAb treated NK cells with those secreted in the co-cultures of NK cells with untreated OSCSCs (C).

Fig. 4. Decreased proliferation of OSCSCs after treatment with supernatants from IL-2 and anti-CD16mAb treated NK cells.

OSCSCs were treated with supernatants from IL-2 and anti-CD16mAb treated NK cells for 4 days after which treated OSCSCs were washed and cultured in media for an additional 2, 6 and 12 days. The ratios of tumor growth were determined by comparing the number of IL-2+anti-CD16mAb treated NK supernatant differentiated OSCSCs with untreated OSCSCs at each time point (A). The viability of cells was assessed using propidium iodide staining followed by flow cytometric analysis (B).

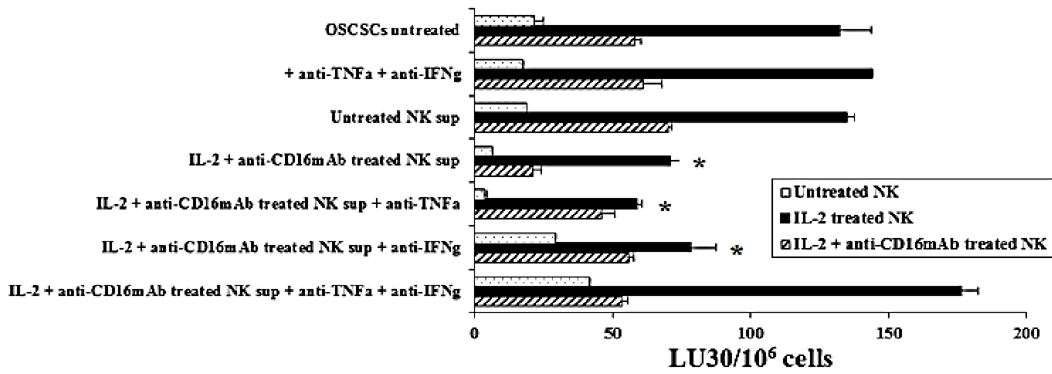
Fig. 5. Hypothetical model of induction of anergized/regulatory NK cells by immune inflammatory cells and by the effectors of connective tissue to support differentiation of

non-transformed stem cells and cancer stem cells resulting in their resistance to NK cell mediated cytotoxicity.

Induction of NK cell anergy in microenvironment is shown. Significant infiltration of immune effectors are likely to anergize NK cells to lose cytotoxicity and gain the ability to secrete cytokines, a term which we previously coined ‘split anergy’ in NK cells, and to support differentiation of stem cells. NK cells are likely to encounter and interact with other immune effectors such as monocytes/macrophages, other myeloid-derived suppressor cells (MDSCs) or with cancer-associated fibroblasts, in order to be conditioned to form anergized/regulatory NK (NKreg) cells. NK cells may also directly interact with stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they can become conditioned to support differentiation of other stem cells. NK cell-differentiated epithelial cells will no longer be killed or induce cytokine secretion by the NK cells, resulting in the resolution of inflammation.

Fig. 1

A (day 0)



B (day 2)

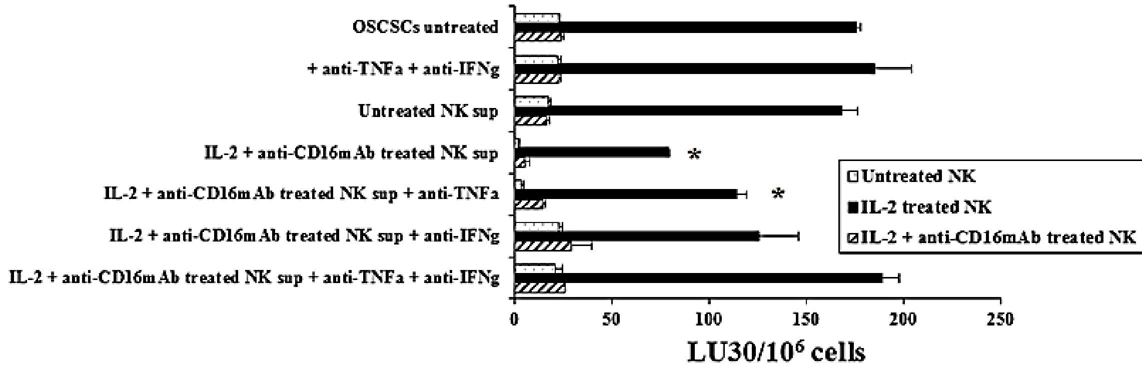
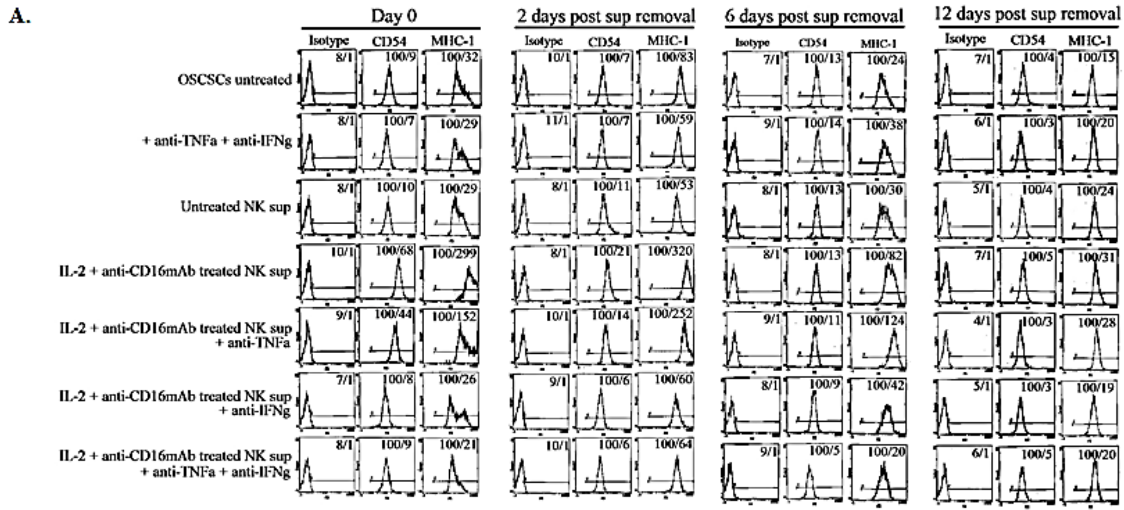
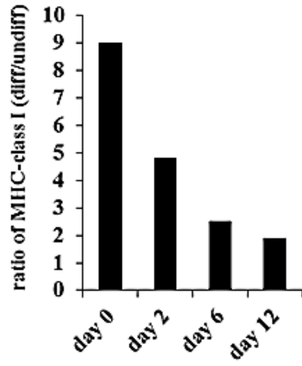


Fig. 2





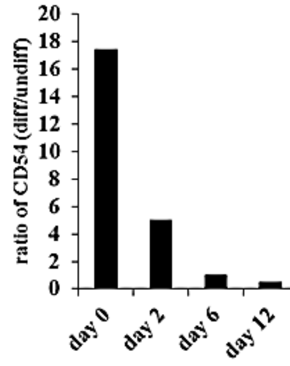
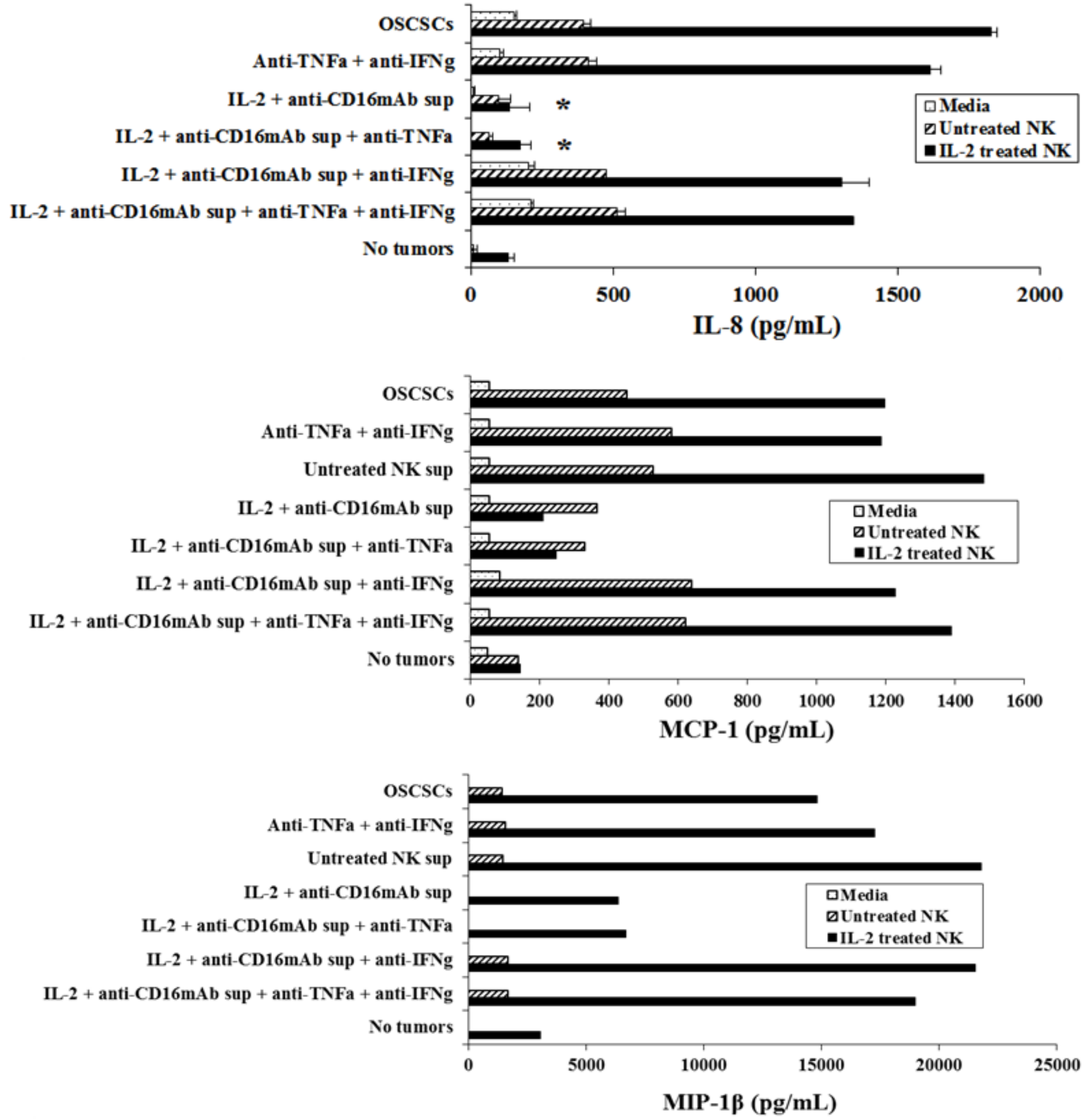


Fig. 3



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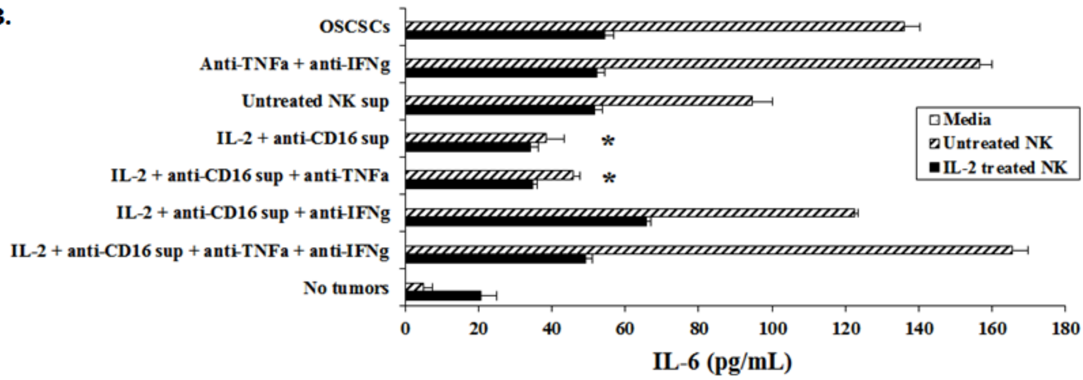
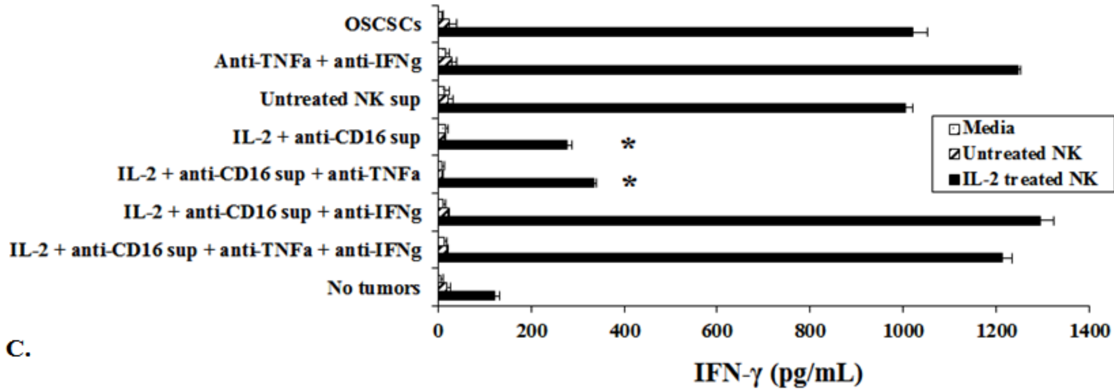




C.

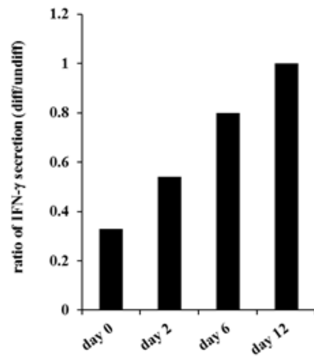
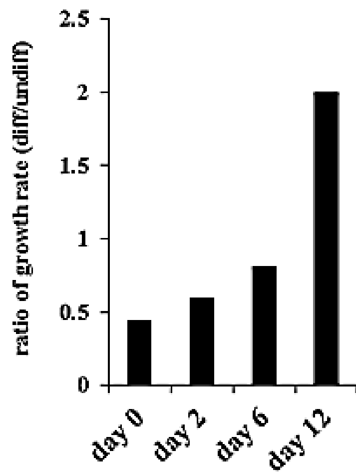


Fig. 4

A.



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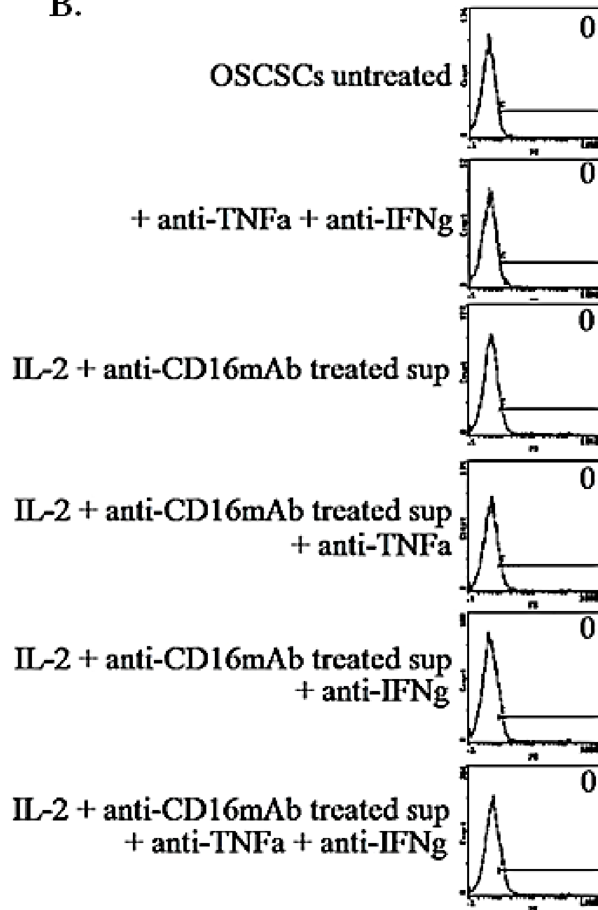
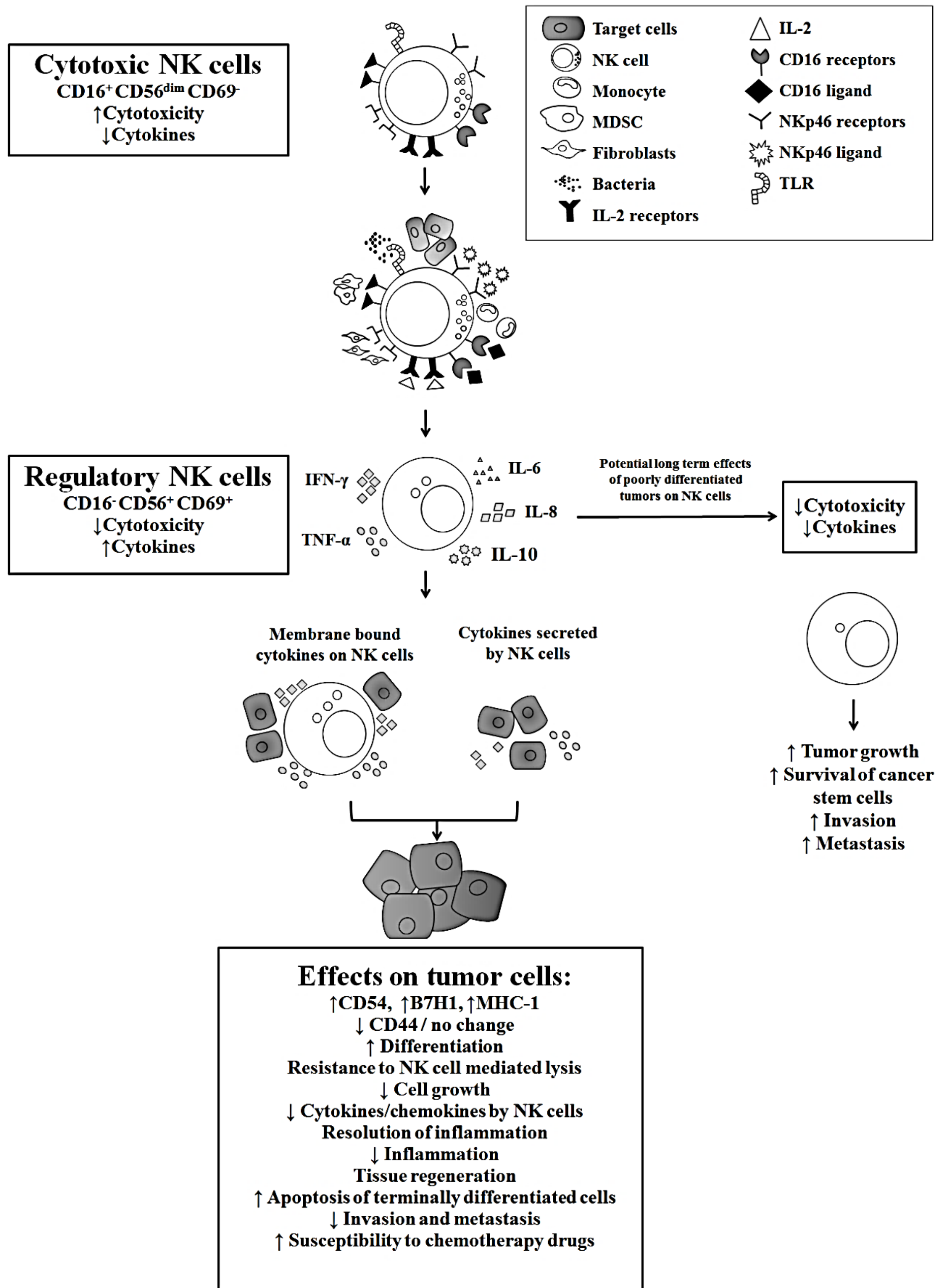


Fig. 5



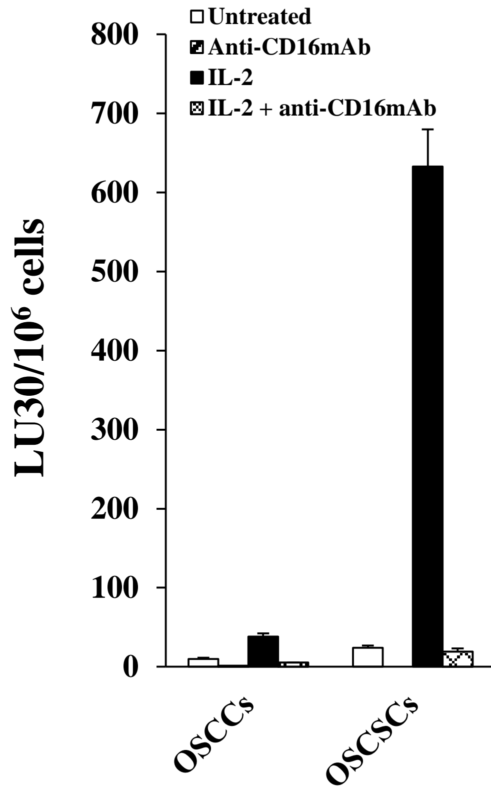
Supplemental Data

Fig. 1 supplemental

Fig. 1 Caption: NK cells are activated by OSCSCs but not by their differentiated OSCCs counterpart

NK cells were left untreated or treated with IL-2 (1000 units/ml), anti-CD16mAb (3 μg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 μg/ml) for 18 hours before they were added to ^{51}Cr labeled OSCSCs and OSCCs. NK cell mediated cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100 (A). NK cells were treated as described in Fig. 1A and each NK sample was cultured in the absence or presence of OSCSCs and OSCCs at an NK cell to target cell ratio of 0.5:1. After an overnight incubation, the supernatants were removed from the co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs (B). One of three representative experiments is shown in each figure.

A.



B.

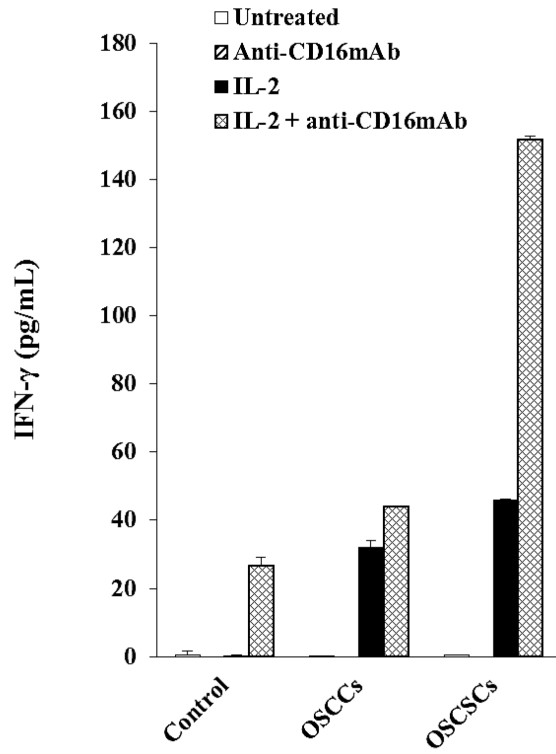
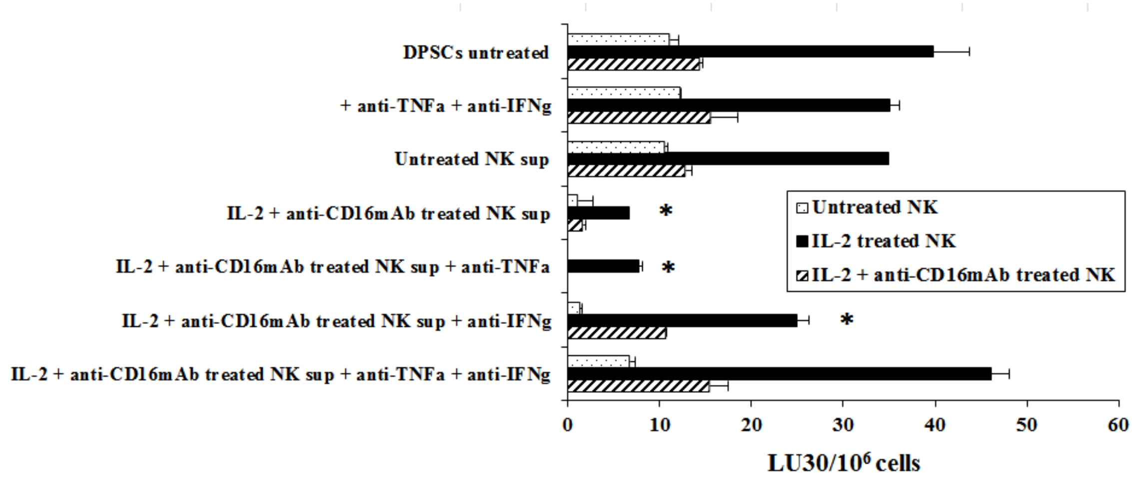


Fig. 2 supplemental

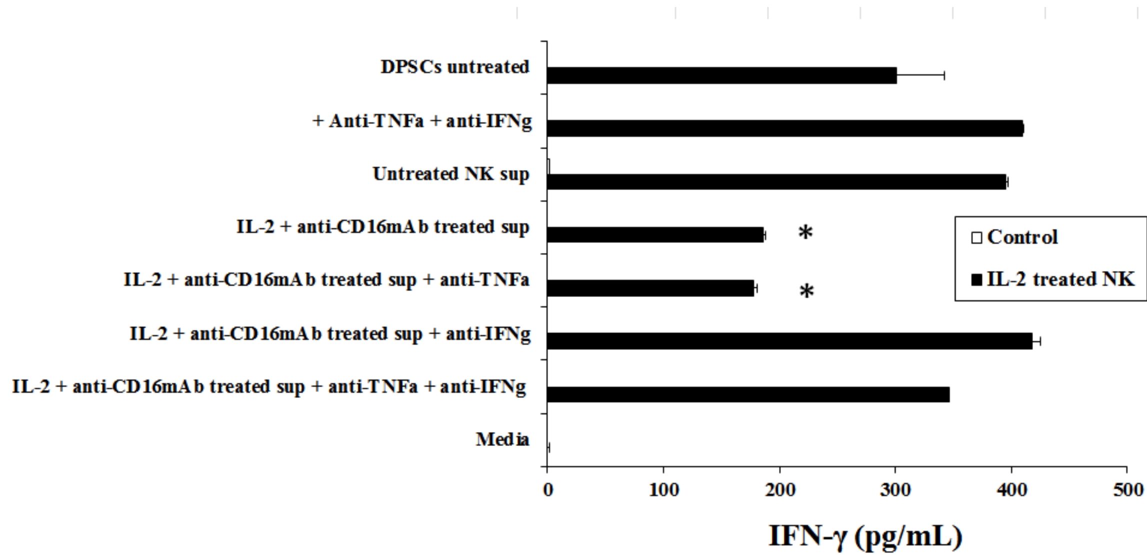
Fig. 2 Caption: Similar to OSCSCs, NK cells trigger autologous DPSCs differentiation resulting in their resistance to NK cell cytotoxicity, and prevention of IFN-γ secretion

Highly purified NK cells were left untreated or treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 18 hours, after which supernatants were removed and added to autologous DPSCs in the presence and absence of anti-TNF-α (1:100) and/or anti-IFN-γ (1:100) for a period of 4 days. The cytotoxicity of untreated, IL-2 treated and IL-2 + anti-CD16mAb treated NK cells against autologous NK supernatant treated DPSCs were assessed using a standard 4 hour ^{51}Cr release assay. Percent cytotoxicity was obtained at different effector to target ratio, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. Differences between untreated DPSCs and those stimulated with IL-2+ anti-CD16mAb treated NK supernatants with or without the addition of either anti-TNF-α or anti-IFN-γ alone were significant at a p value of <0.05 (*) (A). Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/ml) for 18 hours. Afterwards, NK cells were added to DPSCs treated as indicated in the figure 2A at an effector to target ratio of 0.5 to 1. After an overnight incubation, the supernatants were removed from the co-cultures and the levels of IFN-γ secretions were determined using specific ELISAs Differences between untreated DPSCs and those stimulated with IL-2+ anti-CD16mAb treated DPSCs with or without the addition of anti-TNF-α were significant at a p value of <0.05 (*) (B).

A.



B.



CHAPTER 5

Persistent inflammation with loss of NK cell cytotoxicity after differentiation of brain cancer stem cells by NK cells

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& These authors contributed equally to the paper.

Running Title: NK cells kill brain stem cells

Keywords: IFN-γ, NK, GBMs, brain stem cells

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Abstract

We have recently shown that Natural Killer (NK) cells control survival and differentiation of Cancer Stem Cells (CSCs) through two distinct phenotypes of effector (cytotoxic) and regulatory (anergized) NK cells respectively. In this report, brain CSCs and their differentiated counterparts were studied and compared to Oral Squamous Cancer Stem Cells (OSCSCs). Serum and NK cell differentiated brain CSCs were significantly less susceptible to NK and CTL direct cytotoxicity as well as NK cell mediated Antibody Dependent Cellular Cytotoxicity (ADCC) whereas their CSCs were highly susceptible. Induction of differentiation and resistance to cytotoxicity in brain CSCs correlated with the increased expression of CD54, B7H1 and MHC class I and were mediated by the combination of IFN-γ and TNF-α secreted by the NK cells. Unlike OSCSCs, brain CSCs expressed lower surface expression of TNF-αRs and IFN-γRs and remained inflammatory due to their inability to cease secretion of cytokines and chemokines after differentiation. Addition of anti-MHC class I antibody inhibited NK and CTL mediated cytotoxicity against CSCs, whereas it increased cytotoxicity against differentiated tumors. Loss of NK cell cytotoxicity in the presence of persistent inflammation is a potential mechanism for survival and metastasis of GBM CSCs.

Introduction

Immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, however, neither underlying physiological relevance nor the exact mechanisms by which immunosuppression occurs are well understood. It is shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [181-184]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [185-187]. In contrast, interaction of NK cells with the resistant tumors did not result in suppression of NK cell cytotoxicity [137, 255].

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed against OSCSCs when compared to differentiated Oral Squamous Carcinoma Cells (OSCCs) [18]. More importantly, OSCSCs expressed $CD44^{high}$ $CD326^{high}$ $CD26^{high}$ $CD166^{low}$ $CD338^{+}$ oral stem cell markers [18, 256]. We have also demonstrated that NK cells were able to lyse hMSCs, hDPSCs, hESCs and hiPSCs significantly more than their differentiated counterparts [18]. In addition, de-differentiation by blocking NFκB in oral tumors, was able to increase NK cell mediated cytotoxicity [18, 19, 144] and elevated CD44 surface receptor expression (data not shown).

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity while increasing IFN-γ secretion by the NK cells, a term which we coined as split anergy [136, 137, 141-145]. On the other hand NK resistant tumors such as RAJI cells do not induce split anergy in NK cells [136, 137]. Significant down-modulation of CD16 receptor and

decreased NK cell cytotoxic function were also seen in patients with cancer including those of the oral and ovarian cancer patients [138, 139].

Glioblastomas (GBMs) are among the tumors with poor prognosis when diagnosed. The current chemo- and radio-therapeutic strategies against GBMs have had limited success in either limiting the disease or establishing long lasting regression of these tumors in the majority of the patients, therefore, there is a clear need for better therapies to control these tumors. In this regard, T cell based immune-therapies have gained some popularity in recent years and was shown to have some success in certain patients, however, because of a lower MHC class I expression on GBMs, the effectiveness of CTL based therapies may be limited. NK cells, on the other hand, should be able to target such tumors since they are known to kill tumors with no or decreased levels of MHC class I expression. Indeed, it is shown that NK cells are recruited to the brain and CNS during pathological diseases such as Multiple Sclerosis and during non-pathological human brain and have a significant role in immune regulation [257]. In GBMs, NK cells are found in the tumor microenvironment and are shown to be suppressed by the tumor and the effectors of the immune system [257]. In addition, targeting GBMs with the NK cells increases the survival of the animals [258]. Therefore, it is clear from the previous studies that NK based immunotherapies may have beneficial effect in patients.

In this paper we studied the function of NK cells against a number of GBM CSCs. Overall, our data indicates that NK cells are potent effectors which can be used to eliminate cancer stem-like cells. However, inactivation of NK cell cytotoxic function by the effectors of

the tumor microenvironment and by the CSCs and persistent inflammation after differentiation of CSCs may significantly limit their effectiveness in lysis of CSCs.

Materials and Methods

Cell Lines, Reagents, and Antibodies

RPMI 1640 supplemented with 10% FBS was used for the cultures of human NK cells. Stem-like GBMs were isolated from freshly resected human tumor tissues as described previously [148], and they were seeded at $1X10^5$ cells/mL into culture flasks pre-coated with laminin (1mg/mL; BD Biosciences, CA) and Poly-L-Ornithine (15ug/mL; Sigma Aldrich, MO). The cells were cultured in medium containing DMEM/F12 (Gemini Bio-Products, CA) supplemented with penicillin G, streptomycin sulfate, B-27 (1:50; Gemini Bio-Products, CA), recombinant human Fibroblast Growth Factor (hFGF-2, 20ng/mL, R&D Systems, MN), recombinant human Epidermal Growth Factor (hEGF, 20ng/mL, R&D Systems, MN) and Leukemia Inhibitory Factor (LIF, 1000U/mL). The medium was refreshed every 3 days. Recombinant IL-2 was obtained from NIH- BRB. The human NK purification kits were obtained from Stem Cell Technologies (Vancouver, Canada). The anti- CD44 were obtained from biolegend (San Diego, CA). Antibodies for CD16 were purchased from biolegend (San Diego, CA). EGFR antibody (Erbitux) was purchased from UCLA pharmacy.

Transplantation of CSCs into the immunodeficient mice

Tumor formation was performed as described previously [259]. 45-47 days later, all mice injected with two Glioblastoma CSCs X01GB, X02GB and one anaplastic oligoastrocytoma X03AOA developed malignant brain tumors. Intracranial tumors generated by all three tumor lines demonstrated high infiltration into the surrounding cerebral cortex as evidenced by an unclear demarcation between the tumors and the normal tissues.

Purification of NK cells and monocytes

NK cells and monocytes were purified from healthy donors using negative isolation kits from Stem Cell Technologies, Vancouver, Canada as described previously [137]. Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors and all the procedures were approved by the UCLA-IRB.

Purification of T cells and the generation of CTLs

X02GB cells were irradiated at 7,000 rads from a ^{60}Co source. PBMCs were cultured with X02GB cells for 14 days at a responder to stimulator ratio of 15:1 in RPMI-1640 containing 10% FBS and 60 IU IL-2/mL. Daily lactic acid concentrations were used to determine if replenishment with fresh culture medium was necessary. CD8+ T cells were purified negatively using T cell isolation kit (Stem Cell Technologies, Vancouver, Canada).

XO2GB CSC differentiation with NK cell supernatant

To differentiate with serum the brain CSCs were cultured in DMEM medium supplemented with 10% FBS for a period of 4 weeks (XO1D, XO2D and XO3D). To differentiate with NK cell supernatants, the NK cells were left untreated or treated with the combination of anti-CD16mAb (3ug/ml), IL-2 (1000 units/ml) in the presence or absence of autologous monocytes (1:1 NK: monocyte) for 18- 24 hours before the supernatants were removed and used in differentiation experiments. The amounts of IFN-γ produced by activated NK cells were assessed with IFN-γ ELISA (Biolegend, CA). XO2Ds were further selected for differentiation with the NK cell supernatants. Differentiation of OSCSCs and XODs was

conducted with gradual daily addition of increasing amounts of NK cell supernatants. On average a total of 0. 001pg of IFN-γ containing supernatants obtained from IL-2+anti-CD16mAb treated NK cells was added per tumor cell for 5 days to induce differentiation and resistance of OSCSCs to NK cell mediated cytotoxicity. XO2D cells required on average a total of 0.035pg of IFN-γ containing supernatants from IL-2+anti-CD16mAb treated NK cells per tumor cell during a 7 day treatment, whereas XO2GB CSCs required 0.037 - 0.070pg of IFN-γ containing supernatants from IL-2+anti-CD16mAb treated NK cells per tumor cell for 7-10 days to promote differentiation and resistance to NK cell mediated cytotoxicity. Initially $1X10^6$ tumor cells were cultured and treated with NK supernatants for differentiation. Afterwards, target cells were rinsed with 1X PBS, detached and used for experiments.

ELISA

ELISAs for IFN-γ, IL-6 and IL-8 (Biolegend, CA) measurement were performed as described previously [137].

Surface Staining

Staining was performed by labeling the cells with antibodies as described previously [137, 142, 201].

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [193] and the LU $30/10^6$ was calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X100.

Statistical analysis

An unpaired, two-tailed student t-test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

Results

Characterization of brain tumor stem cells and their differentiated counterparts

X01GB and X02GB and X03AOA CSCs [148-150] were used to determine surface expression of key receptors before and after serum differentiation. The brain CSCs exhibited a rounded morphology, which upon differentiation with serum demonstrated flat fibroblast-like spindle shape cells (supplementary Fig. S1). CSCs formed tumors in the brain of immunodeficient mice with high proliferative capacity (Fig. 1A). The levels of CD44 were the highest in X01GB and X02GB and lower in X03AOA CSCs (Fig. 1B). Differentiation caused significant decreases of CD44 surface expression in all 3 CSCs; however, X03AOA exhibited the least decrease in CD44 expression after differentiation (Fig. 1B). EGF Receptor expressions were significantly higher in all 3 brain CSCs when compared to their differentiated counterparts (Fig. 1B). X02GB had the highest EGF receptor expression, followed by X03AOA and the least expression was observed on X01GB. In contrast, no consistent differences could be observed for the expression of MHC class I, and the levels were not changed significantly after differentiation of CSCs (Fig. 1B). The profiles of U87 GBM cell line were similar to those of the differentiated primary GBM tumors (Fig. 1B).

X02GBs were lysed significantly more than X01GB and X03AOA CSCs

Both untreated and IL-2 treated NK cells mediated the highest lysis against X02GB, followed by X01GBs and the least NK cell mediated lysis was observed against X03AOA (Fig. 2A). Treatment of NK cells with anti-CD16 mAb with and without IL-2 significantly diminished

NK cell cytotoxicity against all three brain tumor cells. Lysis of brain CSCs by NK cells was significantly more when compared to U87 GBM cell line (Fig. 2A).

Differentiation of brain CSCs with serum decreases susceptibility to NK cell mediated cytotoxicity

Differentiation of all three brain CSCs resulted in a significant decrease in NK cell mediated cytotoxicity (Fig. 2B). Decrease in NK cell mediated cytotoxicity was observed in untreated as well as IL-2 and IL-2+anti-CD16 mAb treated NK cells when added to differentiated brain tumor cells (Fig. 2B). The highest decrease in NK cell mediated cytotoxicity could be observed in the most sensitive stem cells X02GB after differentiation. XO3AOA had the lowest decrease in IL-2 mediated cytotoxicity when differentiated (Fig. 2B).

Differentiation of stem cells with serum decreased expression of EGFR and NK cell ADCC against brain tumors

The levels of ADCC were found to be different from direct cytotoxicity and it correlated with the levels of expression of EGFR on the surface of the brain stem cells (Figs. 1B and 2C). X02GB had the highest expression of EGFR (Fig. 1B) and therefore, NK cells mediated the highest levels of ADCC against these cells (Fig. 2C). X03AOA expressed the next highest EGFR expression therefore, unlike the direct cytotoxicity where NK cells mediated the least cytotoxicity; they had the second highest ADCC when compared to the other 2 lines (Fig. 2C). The lowest ADCC was observed against X01GB which expressed the lowest surface expression of EGFR (Fig. 2C).

Differentiation of brain CSCs decreased direct cytotoxicity as well as NK cell mediated ADCC (Fig. 2C). Differentiation of the CSCs decreased the levels of EGFR expression on brain tumor cells and resulted in a decreased NK cell mediated ADCC against X02GB and X03AOA tumors (Figs. 1B and 2C). Similar results were obtained when differentiated XO1GB were used with NK cells in the presence of Cetuximab (data not shown).

Induction of split anergy in NK cells after treatment with IL-2 and anti-CD16 mAb

Anti-CD16 mAb treated NK cells lost significant cytotoxicity and were unable to elevate IFN-γ secretion; however, those treated with a combination of IL-2 and anti-CD16 mAb were able to secrete IFN-γ while demonstrating loss of NK cell cytotoxicity against tumors, a term which we have coined as split anergy (Fig. 3). IL-2 activated NK cells were able to lyse X02GB brain stem cells significantly (Fig. 3A), and they were also able to induce significant secretion of IFN-γ (Fig. 3B). However, the levels of IFN-γ secretion was less when compared to the combination of IL-2 with anti-CD16 mAb triggered secretion of IFN-γ by the NK cells co-cultured with X02GB brain stem cells. Untreated and IL-2 treated NK cells lysed U87 GBMs significantly less than X02GB, which correlated with decreased secretion of IFN-γ by the IL-2 treated NK cells co-cultured with U87 GBMs. Increase in the induction of IFN-γ secretion in the presence of decreased NK cell cytotoxicity could also be seen in IL-2 and anti-CD16mAb treated NK cells cultured with U87 GBM tumors. IL-2 treated NK cells in the absence of brain tumors also secreted IFN-γ, however, the levels were less than those secreted by IL-2 treated NK cells cultured with either U87 or X02GB (Fig. 3). Combination of IL-2 with anti-CD16mAb treated NK cells secreted significantly higher levels of IFN-γ in the absence of tumors and the levels increased when cultured with either U87 or X02GB brain CSCs (Fig. 3).

T cells mediated lysis of brain tumor stem cells but not their differentiated counterparts

Cytotoxic T cells were generated against XO2GB and used in killing assay against both the stem cells and their differentiated counterparts. As shown in Fig. 4A $CD8^{+}$ T cells purified from PBMCs pre-stimulated with irradiated XO2GB to generate CTLs killed XO2GB CSCs, but they were unable to lyse their differentiated counterparts. Addition of anti-MHC-Class I antibody significantly blocked CTL mediated lysis of XO2GB stem cells. CTLs were unable to lyse either NK cell sensitive K562 or NK cell resistant Raji or NK cell sensitive OSCSCs (Fig. 4). The levels of CTL mediated lysis of XO2GB stem cells were significantly less than that mediated by the NK cells obtained from the same donor (Fig. S2A). Addition of anti-MHC class I antibody did not change the resistance of XO2D cells against CTLs. In contrast to CTLs, NK cells purified from the same donor lysed OSCSCs and K562s but not Raji cells significantly (Fig. S2A and S2B) and the addition of anti-MHC class I antibody inhibited NK cell mediated lysis of OSCSCs similar to that seen when anti-MHC class I antibody was added to the cultures of CTLs with XO2GB stem cells (Fig. S2A).

Supernatants from the combination of IL-2 and anti-CD16 mAb treated NK cells induced resistance of XO2D and less in XO2GB to NK cell mediated cytotoxicity

We next determined differences between XO2GB, serum differentiated XO2Ds, and XO2GB and XO2Ds differentiated by the supernatants from the IL-2+anti-CD16mAb treated NK cells in regards to resistance to NK cell mediated cytotoxicity. Treatment of XO2Ds with IL-2+anti-CD16mAb treated NK cell supernatants decreased NK cell mediated cytotoxicity significantly by freshly isolated untreated, IL-2 treated or IL-2+anti-CD16 mAb treated NK cells

($P<0.05$) (Fig. 5A). Resistance of XO2Ds to NK cell mediated cytotoxicity could also be observed after their treatment with supernatants from IL-2 treated NK cells, however, the levels of resistance were significantly less when compared to those induced by IL-2+anti-CD16mAb treated NK cell supernatants (data not shown). The following profile emerged from these studies. XO2GB in the absence of serum or supernatants from anergized NK cells were the most sensitive to NK cell mediated cytotoxicity, followed by serum differentiated XO2Ds and the most resistance could be observed when supernatants from IL-2+anti-CD16mAb treated NK cells were used to differentiate XO2Ds (Fig. 5A).

To examine the mechanisms by which XO2Ds became resistant by anergized NK cells, we determined NK cell mediated cytotoxicity when XO2Ds were treated with supernatants of NK cells treated with IL-2 + anti-CD16mAb in the presence and absence of each of IFN-γ and TNF-α antibodies alone or their combination. As shown in Fig. 5A the addition of TNF-α antibody alone had a slight inhibitory effect whereas antibody to IFN-γ had significant inhibitory effect on the induction of resistance of XO2Ds by the supernatants of NK cells and the combination of anti-IFN-γ and anti-TNF-α abrogated the resistance of treated XO2Ds completely (Fig. 5A). The inhibition of XO2Ds resistance to NK cell mediated cytotoxicity by either anti-IFN-γ antibody alone or the combination of anti-IFN-γ and anti-TNF-α antibodies could be observed when untreated, IL-2 treated or IL-2+anti-CD16mAb treated NK cells (Fig. 5A) were used to assess cytotoxicity. Treatment of XO2Ds with the combination of anti-TNF-α and anti-IFN-γ in the absence of NK supernatants had no effect on NK cell cytotoxicity (Fig. 5A).

When XO2GB, and not XO2Ds, were differentiated with supernatants from IL-2+anti-CD16 mAb treated NK cells 68% inhibition in the cytotoxicity could be observed when untreated NK cells were used to assess cytotoxicity. However, only 36% decrease could be seen with IL-2 treated NK cells, whereas with XO2Ds 75% and 74% inhibition with untreated and IL-2 treated NK cells could be seen respectively (Fig. 5B). These experiments indicated that supernatants from IL-2+anti-CD16mAb treated NK cells were able to block cytotoxicity mediated by serum differentiated XO2Ds more efficiently than XO2GBs after induction of differentiation.

Induction of NK resistance in XO2Ds and XO2GB by supernatants from IL-2 + anti-CD16mAb treated NK cells correlated with the increased expression of CD54 and MHC class I

We then compared NK cell resistance induced by the supernatants from IL-2+anti-CD16mAb treated NK cells in XO2GB and XO2Ds for expression of key cell surface receptors. Among many surface receptors tested, CD44, CD54 and MHC class I expression was found to correlate significantly with the differentiation and resistance of NK sup-differentiated XO2GB and XO2Ds (Fig. 5C). As shown in Fig. 5C, the levels of CD54 and MHC class I increased substantially on XO2Ds in the presence of IL-2+anti-CD16mAb treated NK cell supernatants. Supernatants from untreated NK cells did not have significant effect on surface expression of XO2Ds (Fig. 5C). The addition of a combination of anti-TNF-α and anti-IFN-γ antibodies at the initiation of XO2D treatment with IL-2+anti-CD16mAb treated NK supernatants prevented the up-regulation of CD54 and MHC class I on XO2Ds (Fig. 5C). Both anti-TNF-α and anti-IFN-γ were able to decrease CD54 and MHC class I and the addition of the combination of antibodies

abrogated the increase in CD54 and MHC class I, although anti-IFN-γ appears to be more dominant in blocking the increase in CD54 and MHC class I surface receptors on XO2Ds (Fig. 5C). Similar results to those seen for XO2D was also seen when supernatants from IL-2+anti-CD16mAb treated NK cells were used to differentiate XO2GB (Fig. 5C and 5D).

We have previously shown that monocytes induce significant split anergy in NK cells, therefore, we determined the expression of CD54, B7H1, and MHC class I on XO2Ds which were treated with supernatants from the combination of IL-2+anti-CD16mAb treated NK cells with irradiated monocytes (Fig. 5E). As shown in Fig. 5E, the addition of irradiated monocytes in the absence of NK cells did not change the CD54, B7H1 and MHC class I surface expression significantly. However, when added to NK cells which were treated with IL-2+anti-CD16mAb they synergistically increased the expression of all three receptor expression (Fig. 5E). Accordingly, the levels of resistance to NK cell mediated cytotoxicity in XO2Ds rose significantly higher when compared to XO2Ds treated with supernatants treated with IL-2+anti-CD16mAb treated NK cells in the absence of monocytes (data not shown).

To determine growth dynamics of XO2Ds after treatment with the NK supernatants the numbers of attached XO2Ds were counted after treatment with the supernatants by microscopic evaluation and the levels of cell death were determined by staining with propidium iodide followed by flow cytometric analysis. As shown in Fig. 5F there was a decrease in the numbers of XO2Ds after their treatment with IL-2 + anti-CD16mAb treated NK cell supernatants when compared to untreated XO2Ds or those treated with untreated NK cell supernatants (Fig. 5F). However, the highest decrease was observed when XO2Ds were cultured with the supernatants

obtained from the combination of IL-2+anti-CD16mAb treated NK cells with monocytes (data not shown). Anti-TNF-α antibody restored the numbers of IL-2+anti-CD16mAb differentiated XO2Ds, and anti-IFN-γ or the combination of anti-TNF-α and anti-IFN-γ antibodies restored the levels of cell growth to the levels observed with untreated XO2Ds (Fig. 5F). No detached XO2Ds could be observed in any of the treatments tested (Data not shown). In addition, no significant cell death could be observed in XO2Ds treated with the supernatants from the IL-2+anti-CD16 mAb treated NK cells in the absence and presence of irradiated monocytes (Fig. 5F).

Treatment of XO2D with supernatants from IL-2 + anti-CD16mAb treated NK cells was unable to inhibit secretion of cytokines and chemokines by the NK cells

Unlike OSCSCs in which treatment with IL-2+anti-CD16mAb inhibited NK cell cytotoxicity, and increased CD54 and MHC class I and blocked secretion of cytokines and chemokines significantly (Figs. S3A, S3B, S3C, S3D and S3E), treatment of XO2Ds did not decrease or inhibit secretion of cytokine IFN-γ and chemokine IL-8 by freshly isolated untreated and IL-2 treated NK cells when compared to either untreated XO2D, or that treated with anti-IFN-γ and anti-TNF-α in the absence of NK supernatants or treated with supernatants from untreated NK cells (Fig. 5G). The addition of anti-TNF-α antibody and anti-IFN-γ to XO2D treated with the supernatants of NK cells activated with IL-2+anti-CD16mAb did not change the secretion of cytokines and chemokine. IL-2 + anti-CD16mAb treated X02Ds secreted significantly higher levels of IL-6 and IL-8 as compared to untreated or untreated NK supernatant treated X02Ds (Fig. 5G). The addition of anti-TNF-α and anti-IFN-γ to IL-2 + anti-CD16mAb differentiated X02Ds inhibited the increase in IL-6 and IL-8 (Fig. 5G). We then

assessed the levels and function of TNFR I and II and IFN-γ Rα and IFN- γ Rβ on XO2Ds and compared the levels to those expressed on OSCSCs to determine whether the magnitude of functional differences observed in differentiation could relate to the lower expression of these receptors on XO2Ds. As shown in Fig. 5H both the levels and function of these receptors were less on XO2Ds than on OSCSCs (Fig S4A, S4B). Much higher expression of TNFRI and IFN-γ Rα and IFN-γ Rβ could be seen on OSCSCs and differentiation with IL-2+anti-CD16mAb treated NK supernatants decreased the levels of TNFRI, II and IFN-γ Rβ, whereas it did not change the levels of IFN-γ Rα (Figs. S4A and S4B). Similarly, the addition of the combination of rTNF-α and rIFN-γ to OSCSCs decreased the levels of TNFRI, II and IFN-γ Rβ, but not IFN-γ Rα (Figs. S4A and S4B).

Inverse cytotoxicity of NK cells against CSCs and differentiated XO2GBs in the presence of MHC class I antibody

As shown in Fig. 6, the addition of anti-MHC class I mAb to the cultures of NK cells with stem like XO2GB (Fig 6A) or XO2Ds (Fig. 6B) decreased NK cell cytotoxicity moderately, whereas it augmented NK cell cytotoxicity against XO2Ds differentiated with the supernatants from IL-2+anti-CD16mAb treated NK cells (Fig. 6C). Increase in NK cell cytotoxicity in the presence of MHC class I antibody in IL-2+anti-CD16 mAb supernatant differentiated XO2Ds correlated with the increased expression of MHC class I on XO2Ds (Fig. 5C). Both XO2GB and XO2D in the absence of supernatants from IL-2+anti-CD16mAb treated NK cells exhibited no or lower expression of MHC class I (Fig. 5D and 5E). Similarly, anti-MHC class I mAb decreased NK cell mediated cytotoxicity when added to the cultures of NK cells with OSCSCs, whereas it increased when added to IL-2+anti-CD16mAb treated NK supernatant differentiated OSCSCs

(Fig. S2A and [256]. The addition of antibodies to MHC class I increased NK cell cytotoxicity significantly when added to well- differentiated OSCCs isolated from oral cancer patient which exhibited higher surface expression of MHC class I [256].

Discussion

In this report we present evidence that NK cells target brain CSCs and not their differentiated counterparts directly and through ADCC. Both NK and CTLs were lysed brain CSCs significantly more than their differentiated counterparts, albeit the levels of NK cell cytotoxicity were much higher than those obtained by CTLs. Indeed, peptide specific targeting of GBM stem-like tumors but not their differentiated tumors by specific CTLs was shown previously [260].

Inhibition of NK cell cytotoxicity was seen when antibody to MHC class I was added to the cultures of NK cells with either GBM or OSCSC stem-like cells whereas increased cytotoxicity was seen when the cells were differentiated (Tseng et al, "in press"). Stem-like tumors which were NK supernatant differentiated or were isolated from well differentiated tumors exhibited increased NK cell mediated cytotoxicity in the presence of anti-MHC class I antibody correlating with the increased expression of surface MHC class I [256]. Indeed, we have previously demonstrated that anti-MHC class I antibody when added in the absence of CD16 triggering on NK cells had moderate inhibitory activity on cytotoxic function of NK cells, however, it mediated significant inhibition when added in the presence of CD16 triggering [143]. Based on these observations we speculated that since XO2GB and OSCSCs mediate significant receptor mediated activation of NK cells, the addition of anti-MHC class I antibody may serve to inhibit NK cell mediated cytotoxicity as seen in Fig. 6. However, when stem cells are differentiated, since NK cells are not activated, they are not inhibited by the addition of anti-MHC class I antibody, therefore, they can mediate lysis of the high MHC expressing tumors in

the presence of anti-MHC class I mAb. Lysis of differentiated tumors in the presence of anti-MHC antibodies could be due to either blocking of inhibitory MHC class I signals which activates NK cells or it could be due to the cross-linking of MHC class I on the tumor cells which delivers a death signal to the tumors or both. These possibilities are under investigation in our laboratory and are the subject of a follow up study. The observations with the anti-MHC class I antibody have significant implications regarding the function of NK and CTLs. It indicates that these cells may have a common mode of action when they encounter stem cells, however, their mode of action may differ when interacting with differentiated cells.

We have previously demonstrated that the stage of maturation and differentiation of tumors is predictive of their sensitivity to NK cell lysis [18, 19, 236-239, 250]. Based on the accumulated results from our laboratory, we proposed that NK cells may play a significant role in differentiation and subsequent resistance of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact [256]. In addition, in order to drive differentiation, NK cells will have to be conditioned by either immune effectors such as monocyte-macrophages or the effectors of connective tissue such as fibroblasts in the tumor stroma in order to lose cytotoxicity and gain in cytokine producing phenotype [236-239, 250].

To induce resistance to the NK cell mediated cytotoxicity, decrease in tumor proliferation and increase CD54, B7H1 and MHC class I expression, significantly higher amounts of NK supernatant containing IFN-γ was required to differentiate XO2GB in comparison to OSCSCs. Interestingly, significantly lower amounts of NK supernatants was not only able to induce resistance to NK cell mediated cytotoxicity and decreased proliferation of

tumors, but also it inhibited cytokine and chemokine secretion in the cultures of NK cells with OSCSCs. In contrast, although treatment of XO2Ds with higher amounts of NK supernatants inhibited NK cell cytotoxicity significantly, it only decreased IFN-γ secretion moderately with enhancing effects on IL-6 and IL-8 secretion in the cultures of XO2Ds with NK cells. In addition, unlike OSCSCs in which NK supernatants could promote cell death in a subpopulation of OSCSCs after differentiation [256], much higher amounts of supernatants containing IFN-γ was unable to induce cell death in XO2Ds. These experiments revealed crucial differences between GBMs and other tumor types in their responses to NK cell mediated differentiation, which is likely one of the reasons for GBMs aggressive behavior and poor prognosis in patients. GBMs may not only survive because of a lack of tumor death by direct NK cell mediated cytotoxicity but also they may survive because of a lack of death by death inducing cytokines such as TNF-α and Fas Ligand and remain inflammatory since they are unable to decrease the release of cytokines and chemokines after differentiation. Lack of cell death and decreased ability to control the release of inflammatory cytokines and chemokines by NK cells relates to the decreased surface expression and function of TNF-α and IFN-γ receptors on XO2Ds. OSCSCs express much higher levels of TNFRI and IFN-γ Rα and IFN-γ Rβ when compared to XO2Ds. Accordingly, upon differentiation with IL-2+anti-CD16mAb treated NK supernatants OSCSCs down-modulate both TNFRI and II and IFN-γ Rβ, suggesting an increased receptor signaling and function, whereas no changes could be seen for XO2Ds. Therefore, strategies to augment both TNF-α and IFN-γ receptors on GBMs should be beneficial for the patients.

Although large amounts of NK supernatants containing IFN-γ could cause decreased tumor proliferation and resistance to NK cell mediated cytotoxicity in XO2Ds, the same amount

of supernatants induced less resistance of XO2GB to NK cell cytotoxicity. This was especially evident when assessed by IL-2 treated NK cells, and did not decrease tumor proliferation (data not shown) even though it was able to increase CD54 and MHC class I expression on the cells by two fold.

There should be two distinct strategies to eliminate GBM tumors, one which targets stem cells and the other which targets differentiated cells. Since CSCs are found to be more resistant to chemotherapeutic drugs but sensitive to NK cell mediated cytotoxicity while differentiated tumors are more resistant to NK cell mediated killing but relatively more sensitive to chemotherapeutic drugs [261], combination chemotherapy and immunotherapy should be effective for the elimination of both undifferentiated and differentiated tumors [256]. In addition, since a great majority of patient NK cells have modified their phenotype to support differentiation of the cells, these patients may benefit from repeated allogeneic NK cell transplantation for elimination of CSCs. In this regard depletion of NK anergizing effectors such as monocytes via radiation or chemotherapeutic drugs before allogeneic NK cell transplantation should in theory provide such strategy for targeting CSCs by the NK cells. However, this strategy may also halt or decrease the ability of NK cells to drive differentiation of the tumors due to the removal of synergizing effect of monocytes with NK cells for secretion of TNF-α and IFN-γ. Alternatively, a strong tumor differentiating microenvironment may be induced by the increased release and membrane expression of IFN-γ and TNF-α by the NK cells anergized by monocytes, in the presence of increased expression of IFN-γ and TNF-α surface receptors on glioblastoma tumor cells in order to drive differentiation of CSCs. The benefit of such approach is the ability of chemotherapeutic drugs to target differentiated tumors in addition to the

prevention of tumor metastasis. Indeed, our recent in vivo data indicated that CSCs have the ability to grow faster and metastasize, whereas their differentiated tumors grew slower and remained localized for a long period of time without metastasizing (manuscript in prep). The most dangerous and devastating outcome of the cancer is its ability to deplete NK cells and other immune effectors. In this case, not only CSCs will be surviving but they will also remain poorly differentiated, which are the likely reasons for cancer invasion and metastasis to distant organs. NK cell immunotherapy in these patients should be highly beneficial in containing tumor progression and metastasis.

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Figure legends

Fig. 1. X01GB, X02GB and X03AOA CSCs formed tumors in the brain of immunodeficient mice and expressed significantly higher surface expression of CD44 and EGFR when compared to their differentiated counterparts

X01GB, X02GB and X03AOA cells ($2x10^5$ cells) were injected orthotopically into the brain of immunodeficient nude mice as described in the Materials and Methods section and histological analysis performed by H&E staining (A). Right panel in Fig.1A demonstrated that these tumors have high proliferative capacity *in vivo*. X01GB, X02GB and X03AOA CSCs and their differentiated counterparts were stained with either isotype control antibody or PE conjugated antibodies to CD44 mAb (B, left panel), EGF-R mAb or MHC class I mAb (B, right panel) as described in the Materials and Methods section. The levels of CD44, EGF receptor and MHC class I expression on each cell type were determined by flow cytometric analysis. The number on the upper right hand corner of each histogram is the mean channel fluorescent. One of three representative experiments is shown in this figure.

Fig. 2. X02GB were lysed significantly more than X01GB and X03AOA CSCs and differentiation of stem cells decreased susceptibility of NK cell mediated cytotoxicity and increased resistance against NK cell mediated ADCC

Highly purified NK cells ($1X10^6$ cells/ml) were either left untreated or treated with IL-2 (1000u/ml), anti-CD16 mAb (3µg/ml) or a combination of IL-2 (1000u/ml) and anti-CD16 mAb (3µg/ml) for 12-24 hours and used in cytotoxicity assay against X01GB, X02GB, X03AOA CSCs and U87 GBMs (A) and their differentiated counterparts (B). Purified NK cells were left

untreated or treated with IL-2 (1000 units/ml) for 12-24 hours and then added to ^{51}Cr labeled X01GB, X02GB and X03AOA CSCs and their differentiated counterparts treated with and without Cetuximab (10μg/ml) (C). NK cell cytotoxicity was determined using a standard ^{51}Cr release assay and the lytic units 30/10^6 were determined using inverse number of effectors required to lyse 30% of the tumor cells X 100. Differences between untreated, anti-CD16mAb treated or IL-2 and/or anti-CD16mAb treated NK cell killing against all 4 cell lines were significant at a p value of <0.05. One of four representative experiments is shown in this figure.

Fig. 3. Induction of split anergy in NK cells after treatment with IL-2 + anti-CD16 mAb significantly decreases NK cell cytotoxicity against U87 GBMs and X02GB CSCs while increasing secretion of IFN-γ

NK cells were treated as described in Fig. 2A and then added to ^{51}Cr labeled U87 GBMs and XO2GB stem cells. NK cell cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay and the lytic units 30/10^6 were determined using inverse number of NK cells required to lyse 30% of the U87 GBMs or XO2GB stem cells X 100. One of three representative experiments is shown in this figure (A). NK cells were treated as described in Fig. 2A and each NK sample (1X10^5/ml) were either cultured in the absence or presence of U87 GBMs and XO2GB stem cells at an NK to U87 GBMs or XO2GB stem cells ratio of 0.5:1. After an overnight culture, supernatants were removed from the cultures and the levels of IFN-γ secretion were determined using specific ELISAs (B). One of two representative experiments is shown in this figure.

Fig. 4. CTLs mediate cytotoxicity against stem-like GBMs but not their differentiated counterparts

X02GB cells were irradiated at 7,000 rads from a ^{60}Co source. PBMCs were cultured with X02GB cells for 14 days at a responder to stimulator ratio of 15:1 in RPMI-1640 containing 10% FBS and 60 IU IL-2/mL. Daily lactic acid concentrations were used to determine if replenishment with fresh culture medium was necessary. CD3^{+} T cells were purified negatively using T cell isolation kit and cultured with stem-like ^{51}Cr labeled XO2GB and differentiated XO2D at different E:T ratios and cytotoxicity were determined in a 4 hour ^{51}Cr release assay. Anti-MHC class I antibody was added at a 1:100 dilution. NK sensitive OSCSCs and K562 and NK resistant Raji cells were used as controls. Supernatants were then harvested and radioactivity counted using a gamma counter. Lytic units 30/10^{6} cells were determined using inverse number of effector cells required to lyse 30% of the target cells X 100.

Fig. 5. Increased resistance to NK cell mediated cytotoxicity and increased expression of CD54 and MHC class I on X02GB and X02D differentiated with supernatants from IL-2 + anti-CD16mAb treated NK cells

Highly purified NK cells were left untreated or treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours and after which the supernatants were removed and used for the treatment of X02D cells for 7 days. Untreated X02GB and X02D and those treated with anti-TNF-α (1:100) and anti-IFN-γ (1:100) in the absence of NK supernatants were also used as controls. Similar amounts of supernatants from untreated NK cells and those cultured with IL-2+anti-CD16mAb treated NK cells in the presence and absence of anti-TNF-α (1:100) and/or anti-IFN-γ (1:100) were used to treat X02D for a period of 7 days

to induce differentiation. Afterwards, the cells were rinsed with 1X PBS, detached from the tissue culture plate and used in a standard ^{51}Cr release assay against freshly isolated untreated NK, IL-2 (1000 units/ml) and the combination of IL-2 (1000 units/ml) and anti-CD16mAb (3µg/ml) treated NK cells (A). Differentiation of X02GB and X02D was performed as described in figure 5A. Percent inhibition against untreated and IL-2 (1000 units/ml) treated NK cell mediated lysis was calculated against NK supernatant differentiated X02GB and X02D cells (B). X02D (C) and X02GB (D) were differentiated with NK supernatants in the presence and absence of anti-TNF-α and anti-IFN-γ antibodies as described in figure 5A and the surface markers on untreated and NK supernatant treated cells were assessed after 7 days of differentiation. Surface expression of CD54, CD44 and MHC Class I were assessed after PE conjugated antibody staining followed by flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities in each histogram. Highly purified NK cells were treated with IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) with or without autologous monocytes for 18-24 hours. IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) were added to monocytes as control. Afterwards, the supernatant was removed and added to X02D for 7 days. CD54, B7H1 and MHC Class I surface expression on untreated and NK supernatant differentiated X02D was assessed after PE conjugated antibody staining followed by flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities in each histogram (E). X02D cells were differentiated as described in figure 5A and the number of attached cells at the end of the experiment was determined by microscopic evaluation and the levels of cell death were determined by staining with Propidium iodide followed by flow cytometric analysis (F). Freshly isolated NK cells were

left untreated or treated with IL-2 (1000 units/ml) and for 18 hours. Afterwards, NK cells were added to XO2D treated with NK cell supernatants as described in Fig. 5A at an effector to target ratio of 0.5 to 1. After an overnight incubation, the supernatants were removed from the co-cultures and the levels of IFN-γ, IL-8 and IL-6 secretions were determined using specific ELISAs (G). Highly purified NK cells were treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours, after which the supernatants were removed and used for the treatment of XO2D. IL-2+anti-CD16mAb treated NK supernatants were used to treat XO2Ds (35000 pg of IFN-γ) for a period of 7 days to induce differentiation, and the levels of TNF-α RI, TNF-α RII, IFN-γ Rα and IFNγ Rβ on X02D were determined using PE conjugated antibodies followed by flow-cytometric analysis. The histogram on the left is the isotype control staining and on the right the receptor staining. The numbers on the right hand corner are the percent positive cells and the mean channel fluorescence for each receptor expression (H).

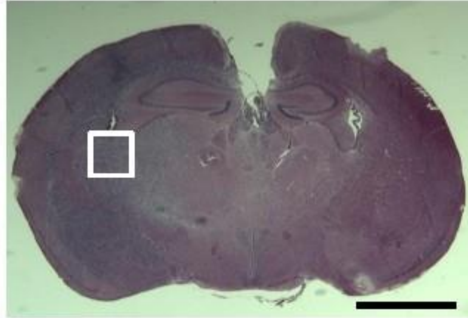
Fig. 6. Antibody to MHC-class I inhibits NK cell mediated cytotoxicity against stem-like XO2GB and XO2D whereas it increases substantially against IL-2+anti-CD16mAb NK supernatant differentiated XO2D

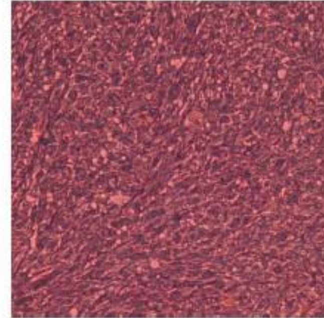
NK cells ($1X10^6$/ml) were treated with IL-2 (1000 units/ml) 12-24 hours before they were cultured with ^{51}Cr labeled XO2GB, XO2D or NK supernatant differentiated X02D cells at different E:T ratios in the presence and absence of anti-MHC-Class I mAb (1:100 dilution). X02D differentiation by NK supernatant was performed as described in figured 5A. NK cell mediated cytotoxicity against XO2GB (A), XO2D (B) and IL-2+anti-CD16mAb NK supernatant treated XO2D (C) were determined in the absence and presence of anti-MHC class I antibody (1:100). NK cells were left untreated or treated with IL-2 (1000 units/ml) for 12-24 hours before

they were added to ^{51}Cr labeled tumors. Percent cytotoxicity was obtained at different effector to target ratio, and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100.

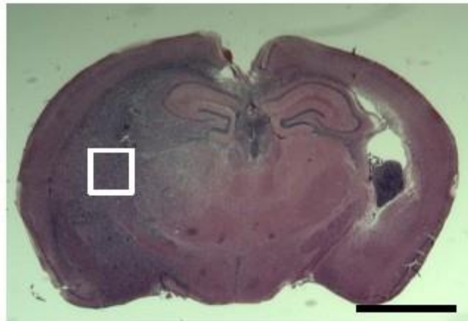
Fig. 1A

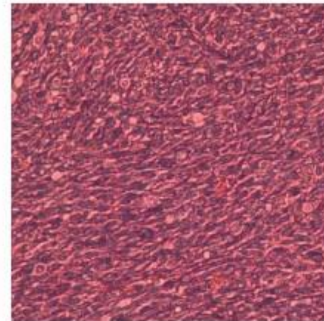
X01GB
47 Days



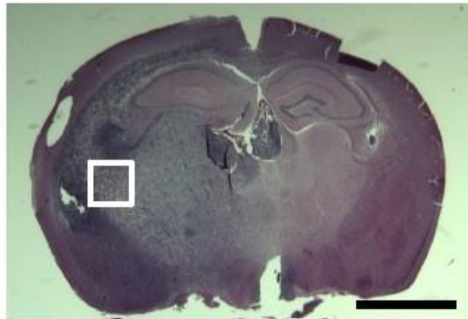


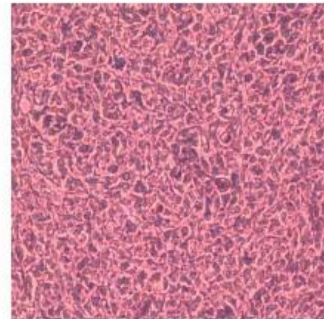
X02GB
46 Days





X03AOA
45 Days





Scale bar: 2mm

Fig. 1B

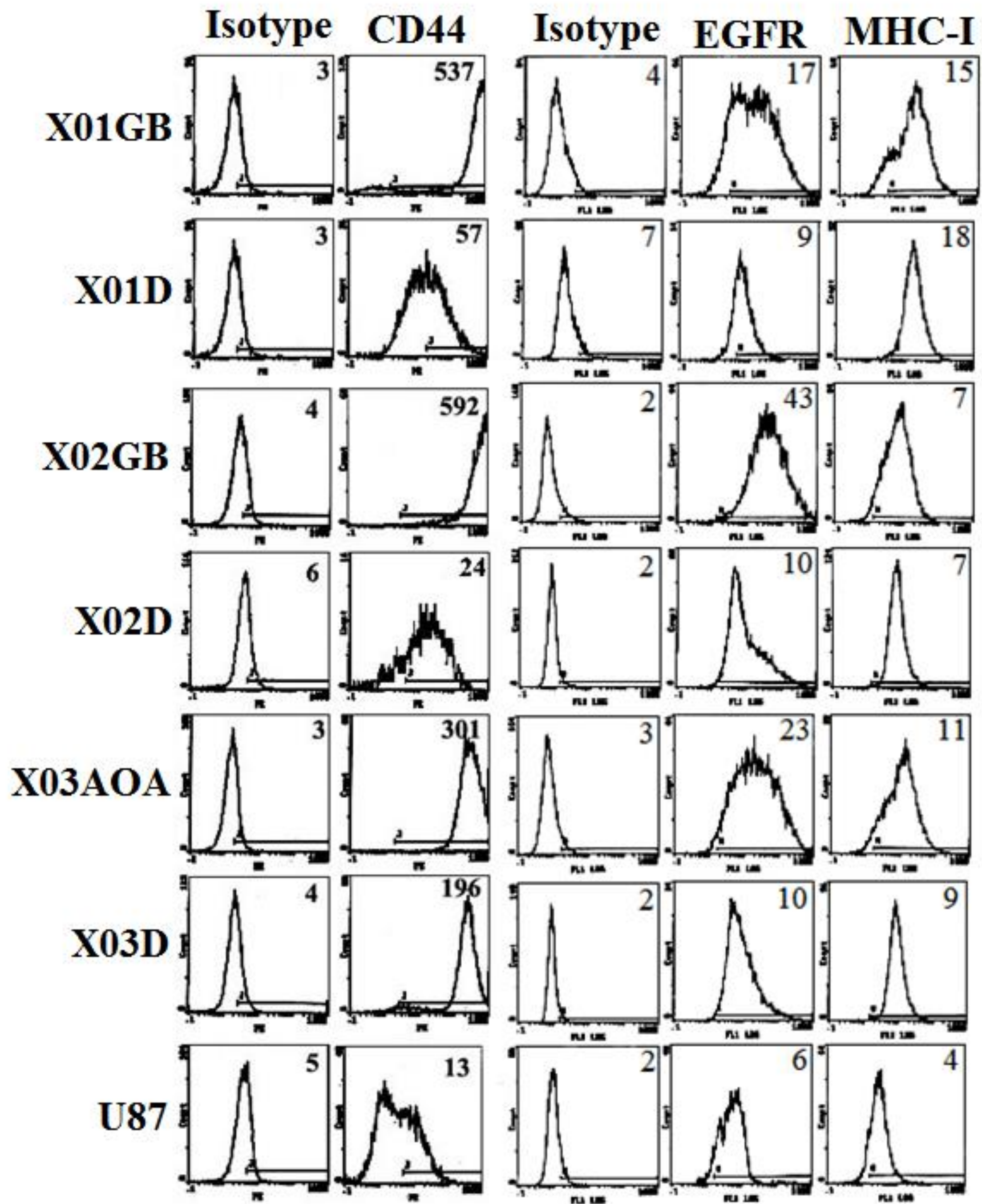


Fig. 2A

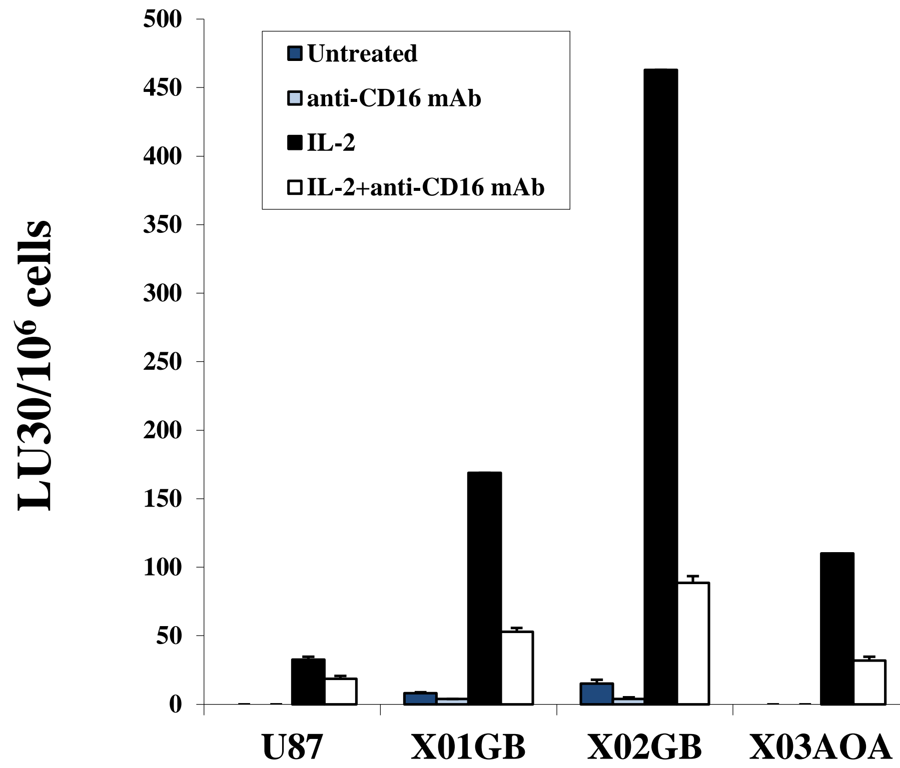


Fig. 2B

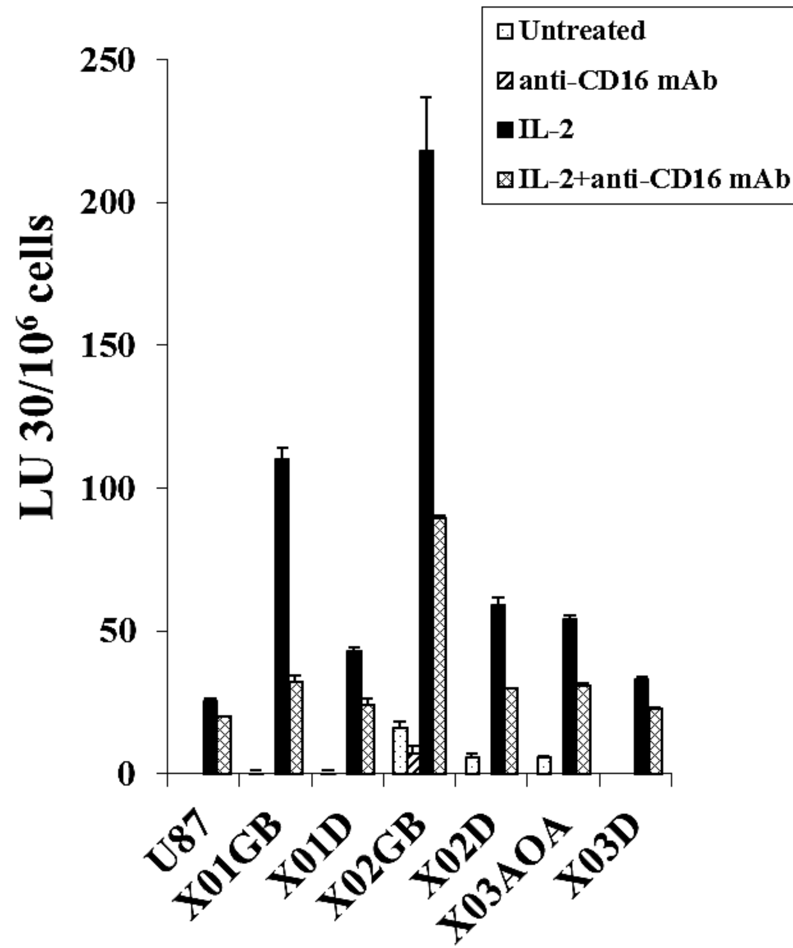
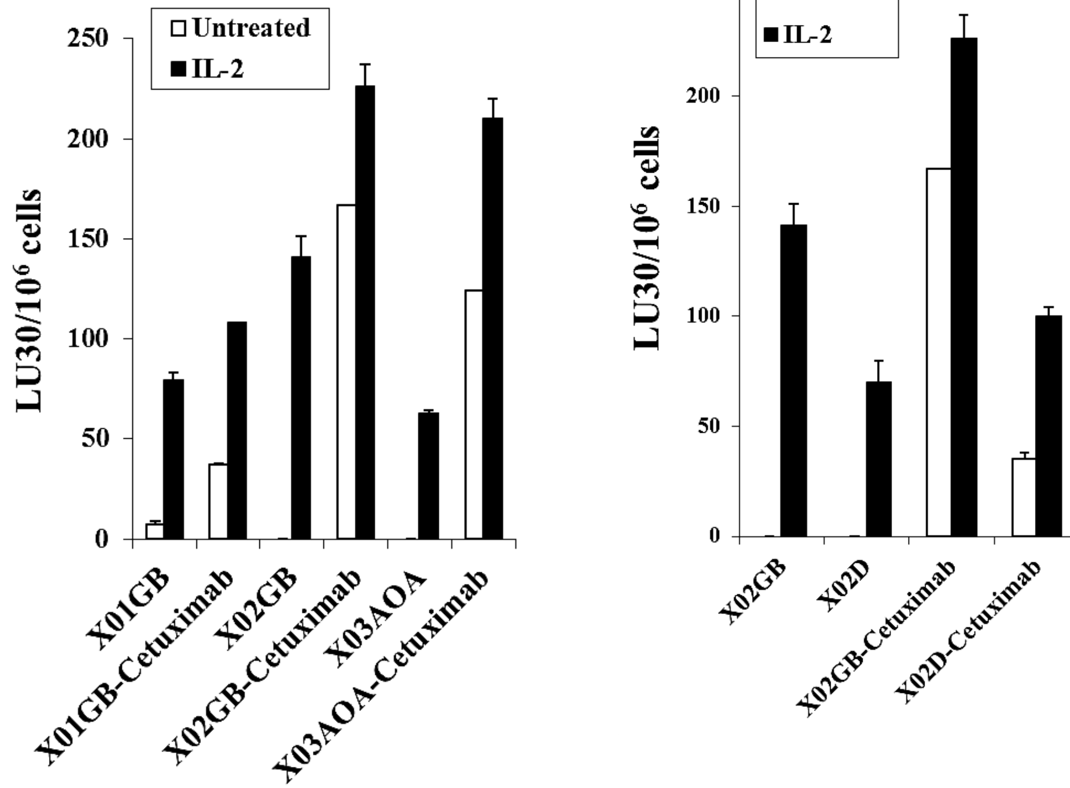


Fig. 2C



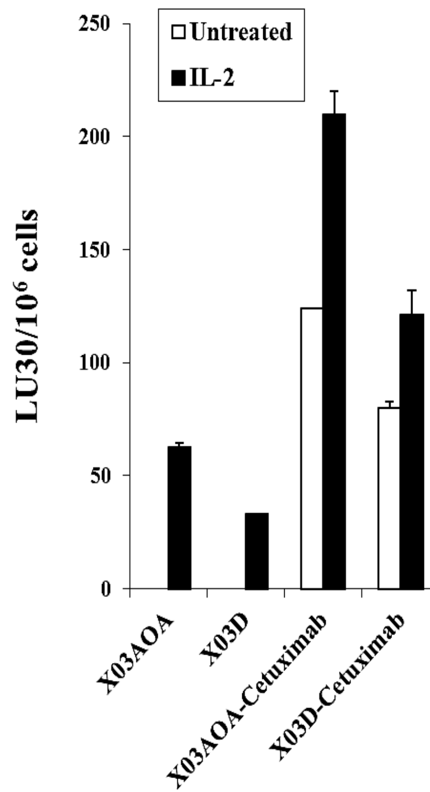


Fig. 3

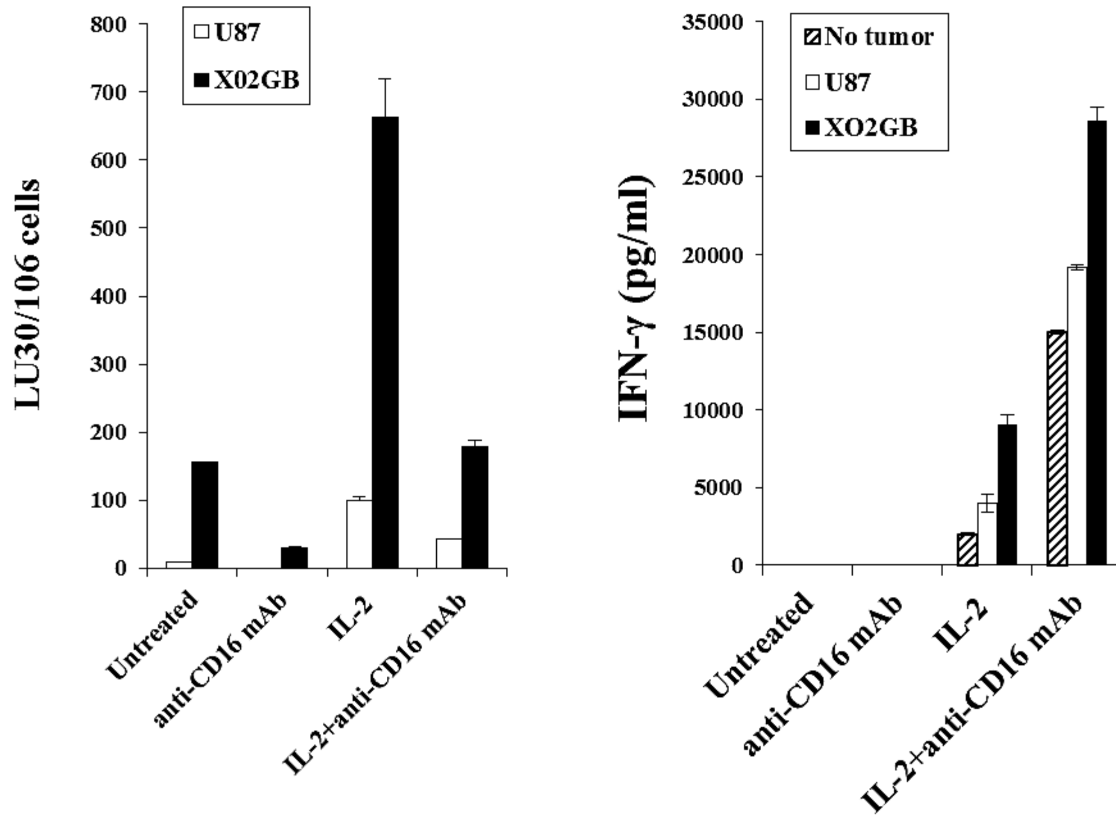


Fig. 4

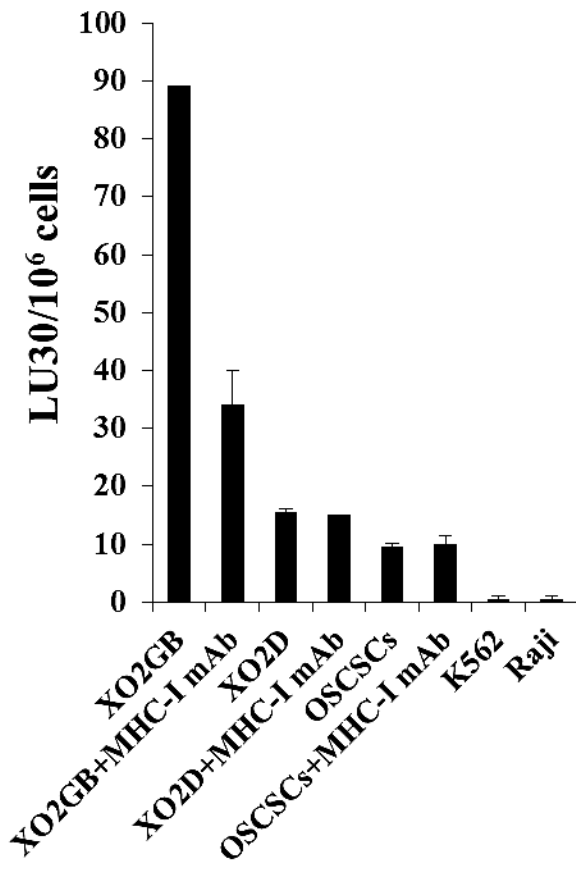


Fig. 5A

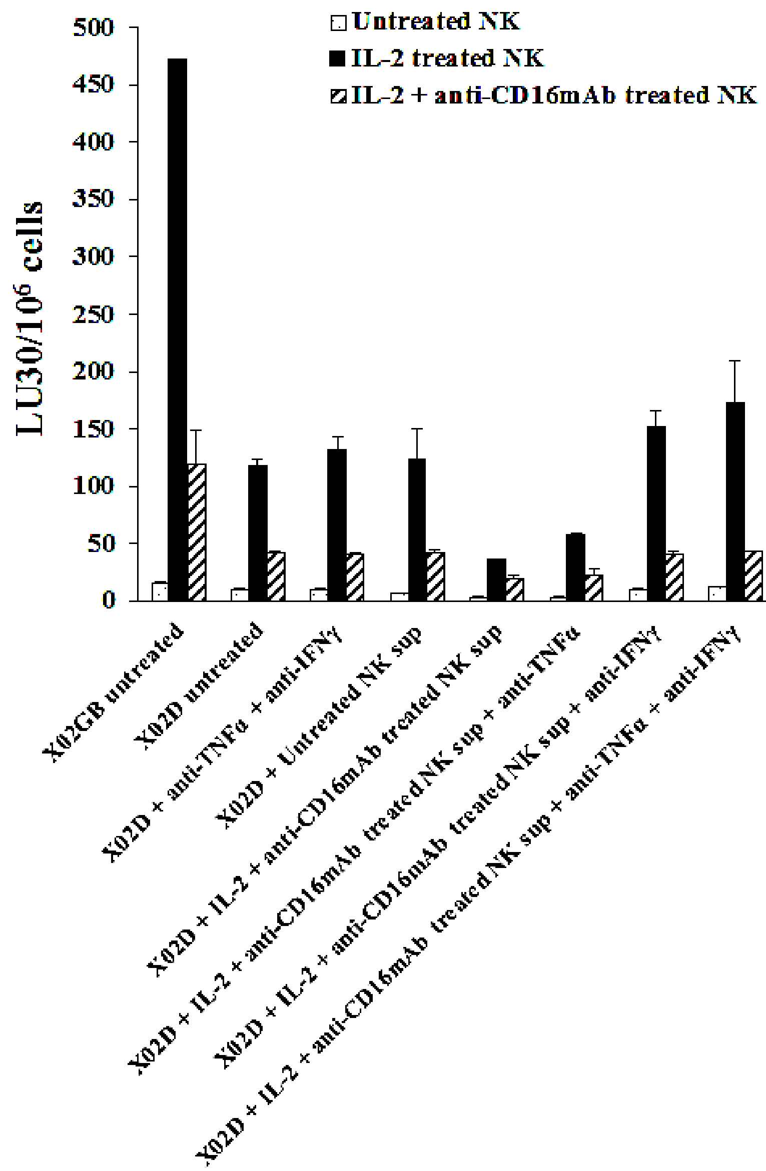


Fig. 5B

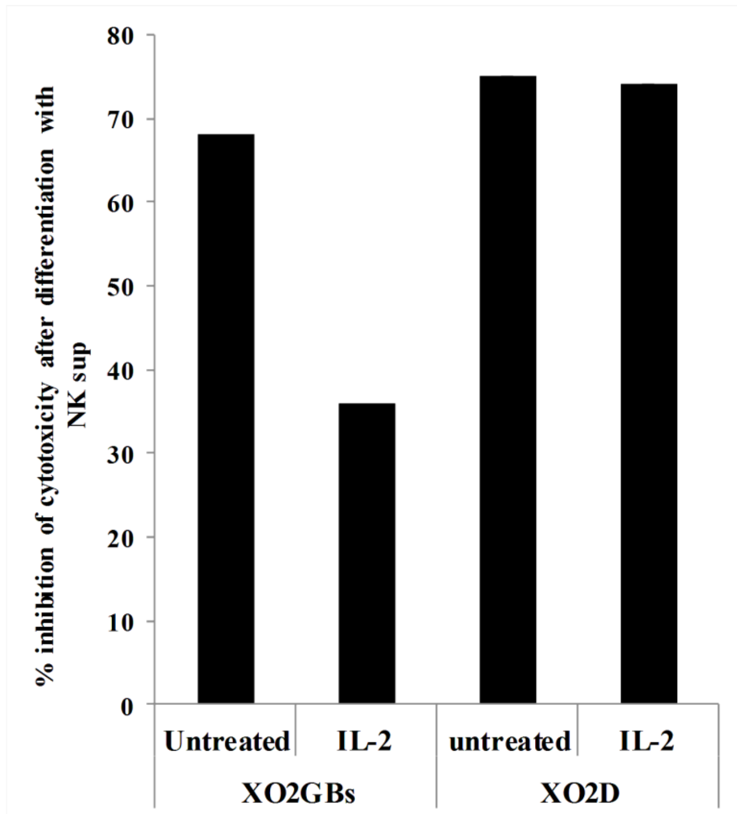


Fig. 5C

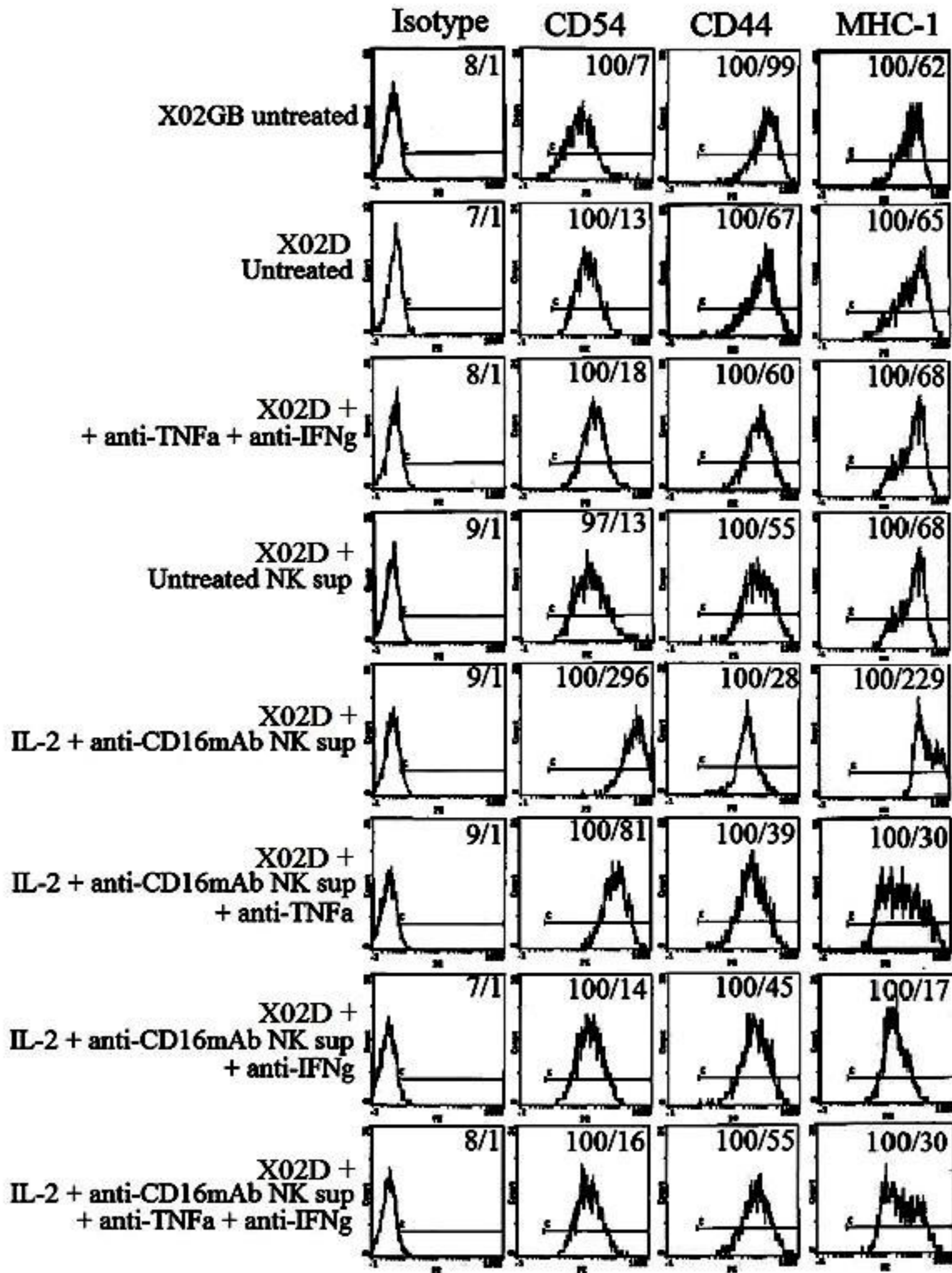


Fig. 5D

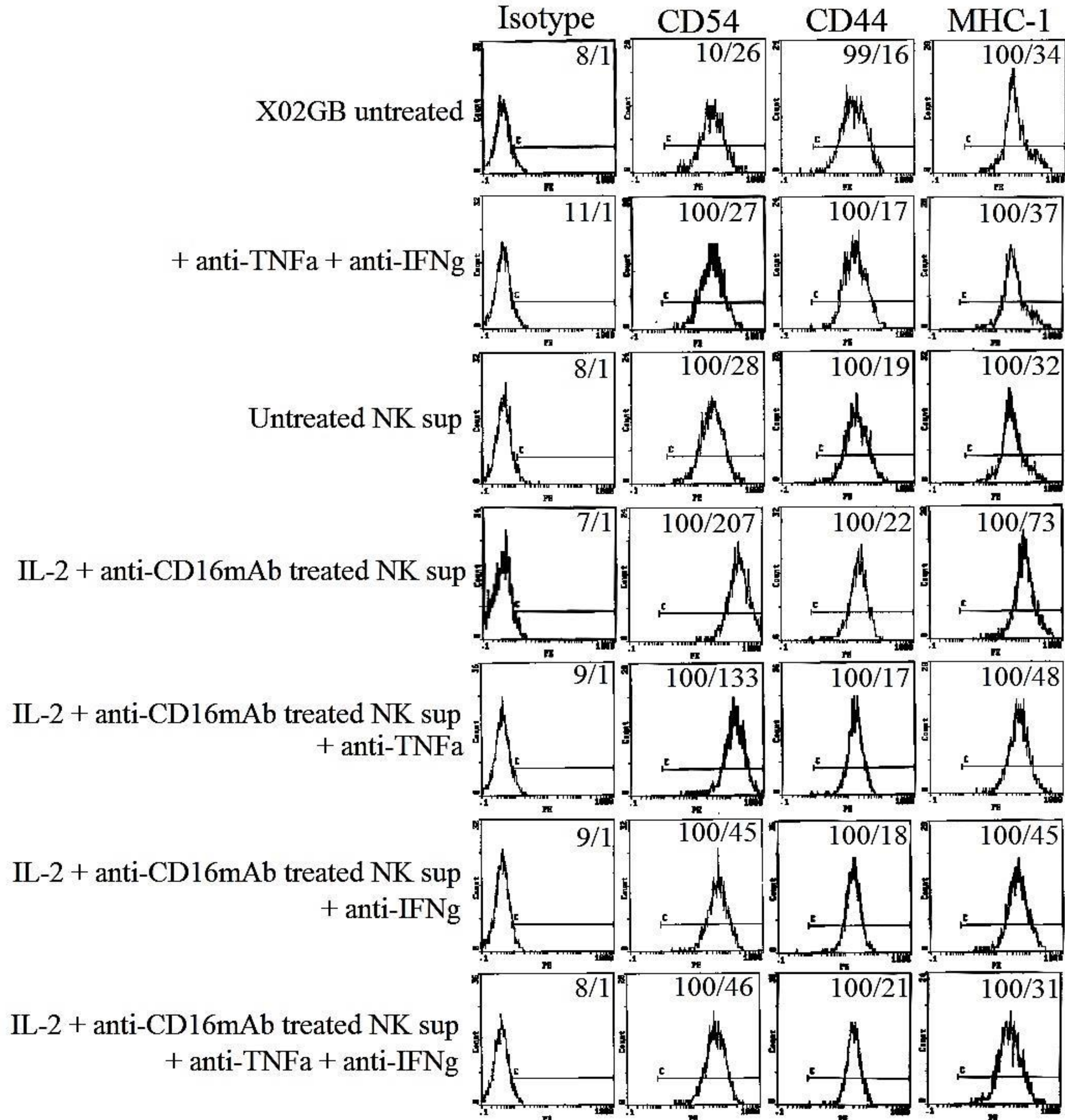


Fig. 5E

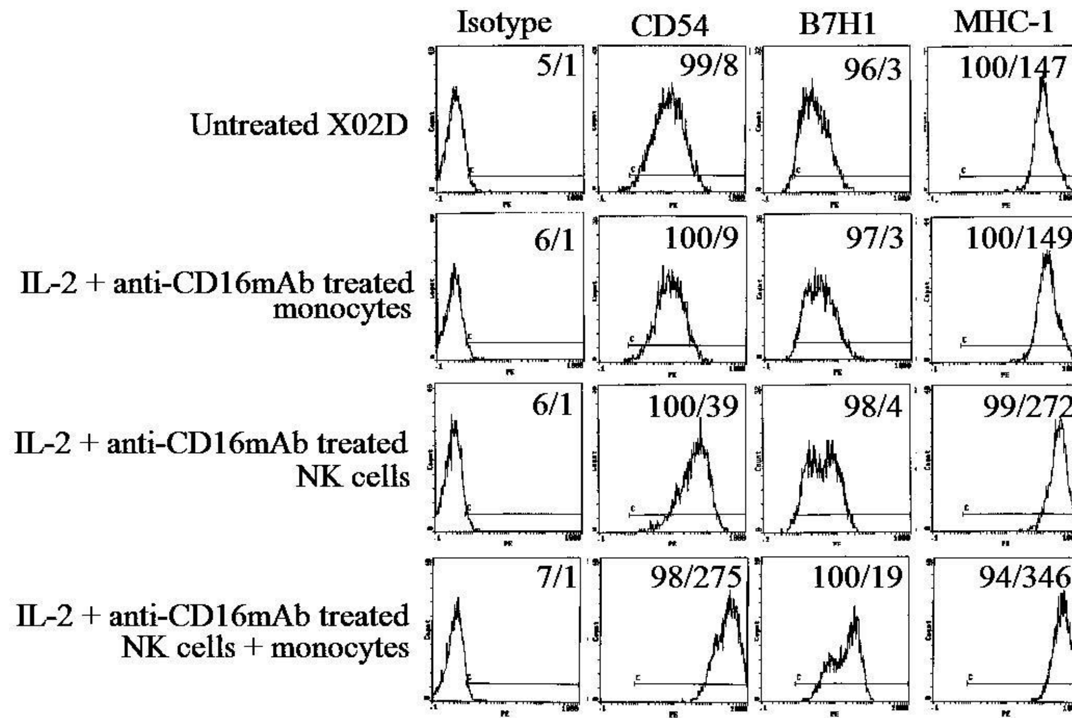


Fig. 5F

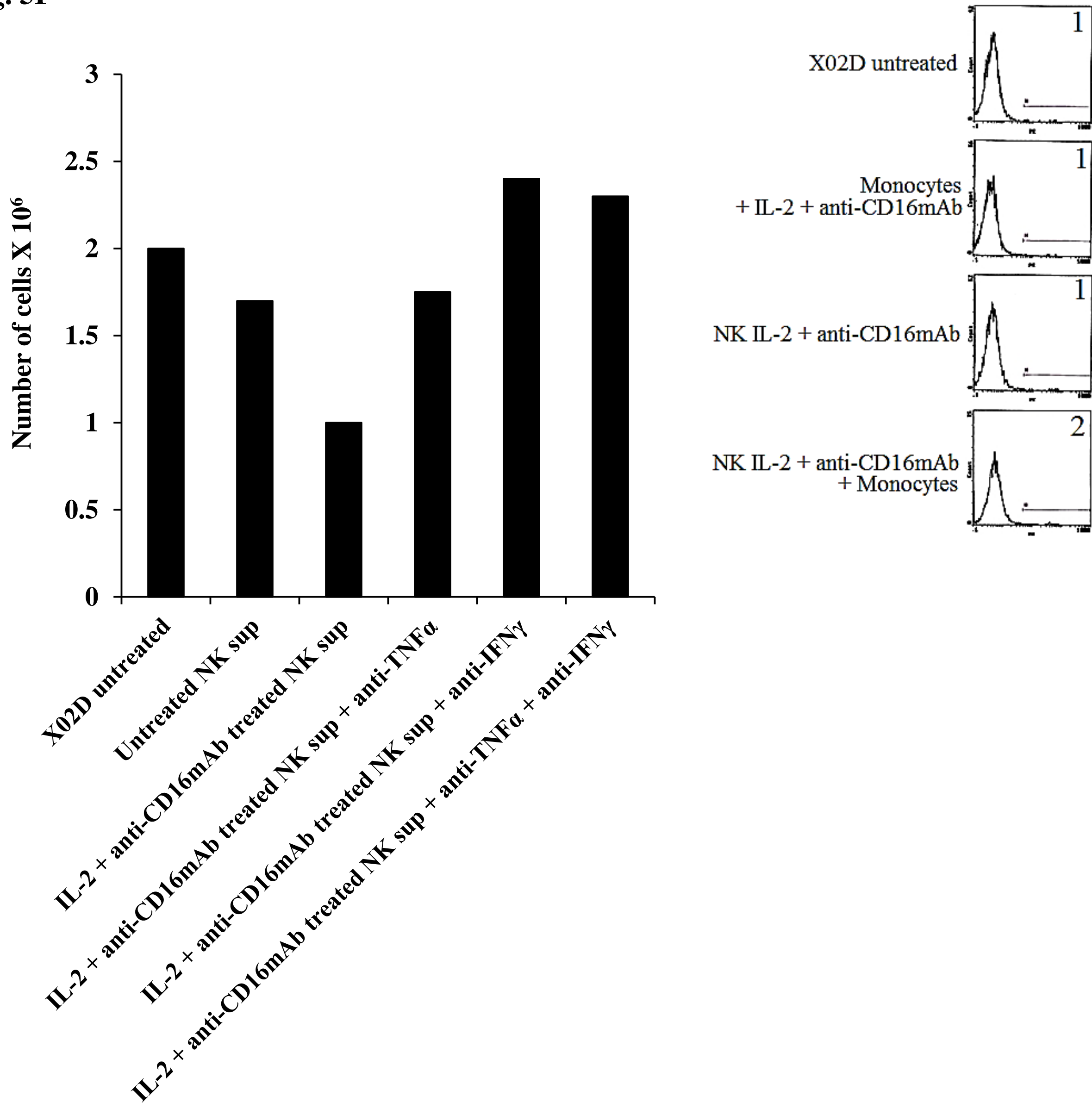


Fig. 5G

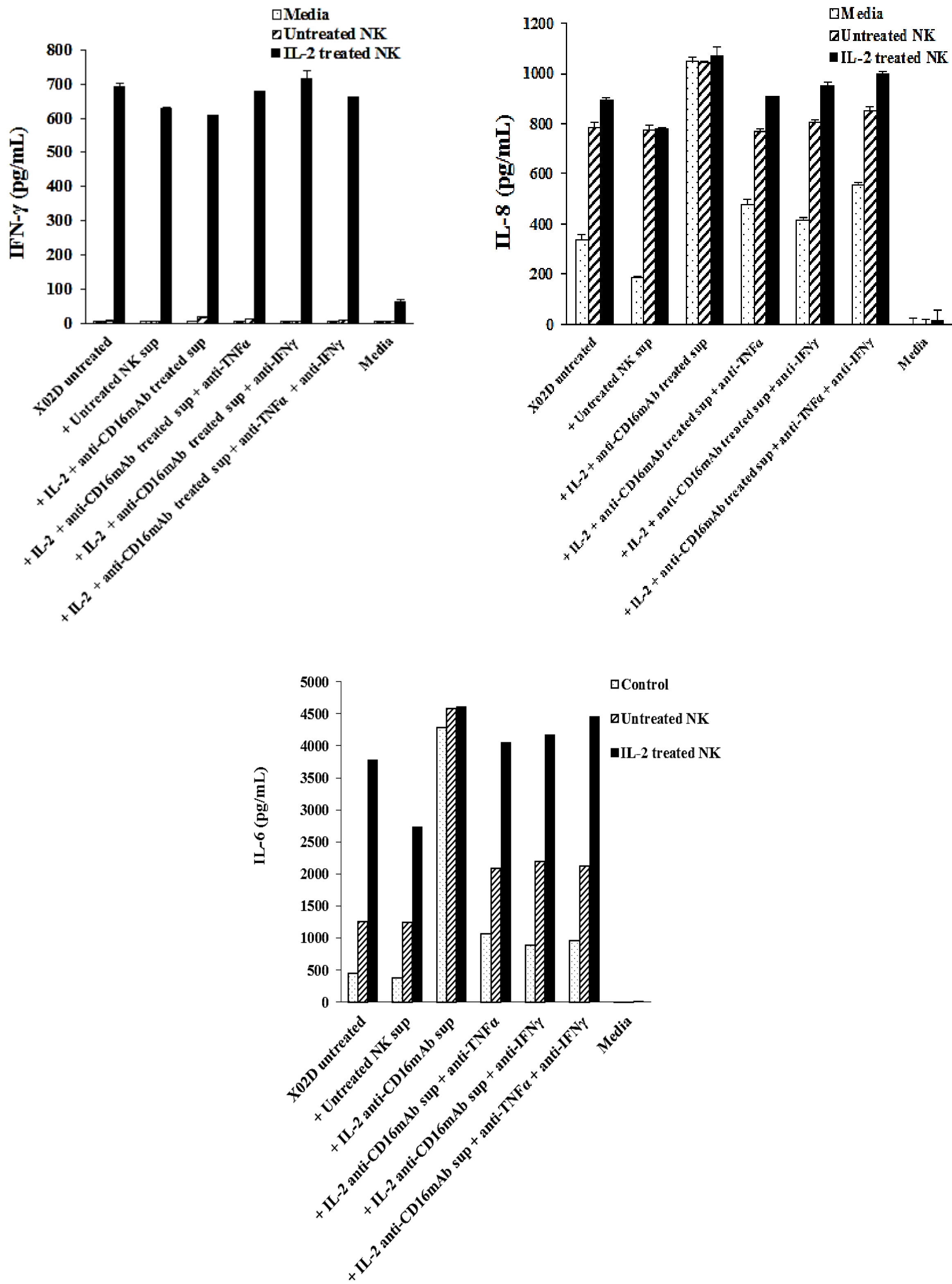


Fig. 5H

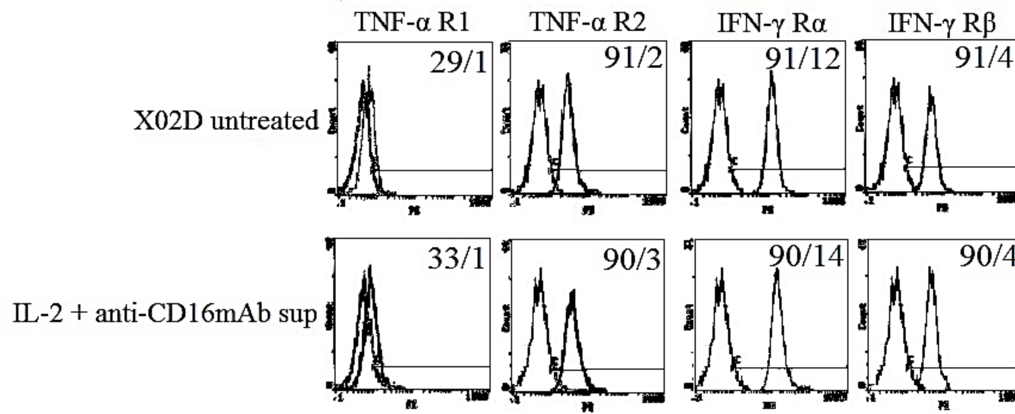
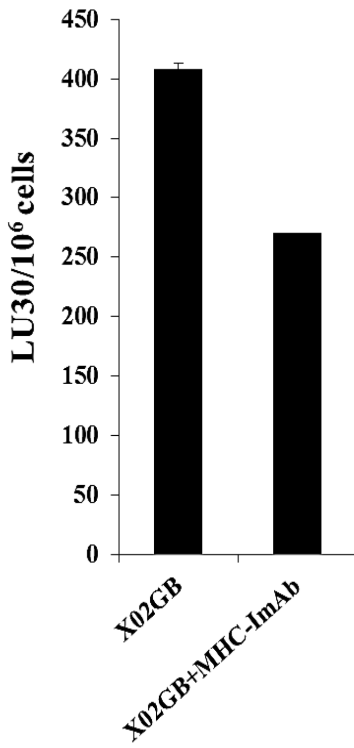
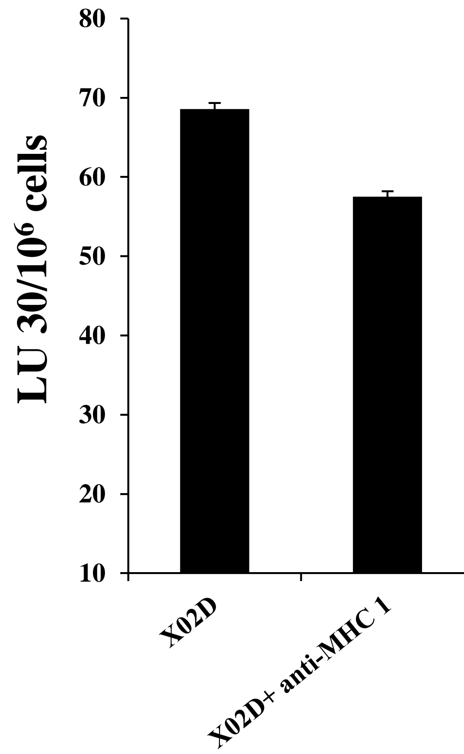


Fig. 6

A.



B.



C.

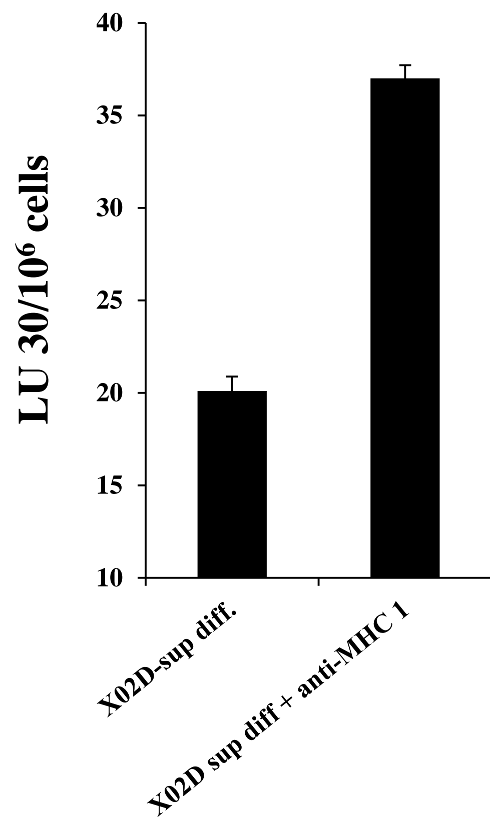


Figure Legends

Fig. S1 (supplementary).

X01GB, X02GB and X03AOA stem cells exhibited spindle shape morphology upon differentiation. X01GB, X02GB and X03AOA cells were cultured and either left undifferentiated or differentiated in the presence of serum culture as described in the materials and methods section. As shown in the figure the brain stem cells exhibited more of a rounded morphology, which upon differentiation with serum demonstrated flat fibroblast-like spindle shape cells.

Fig. S1

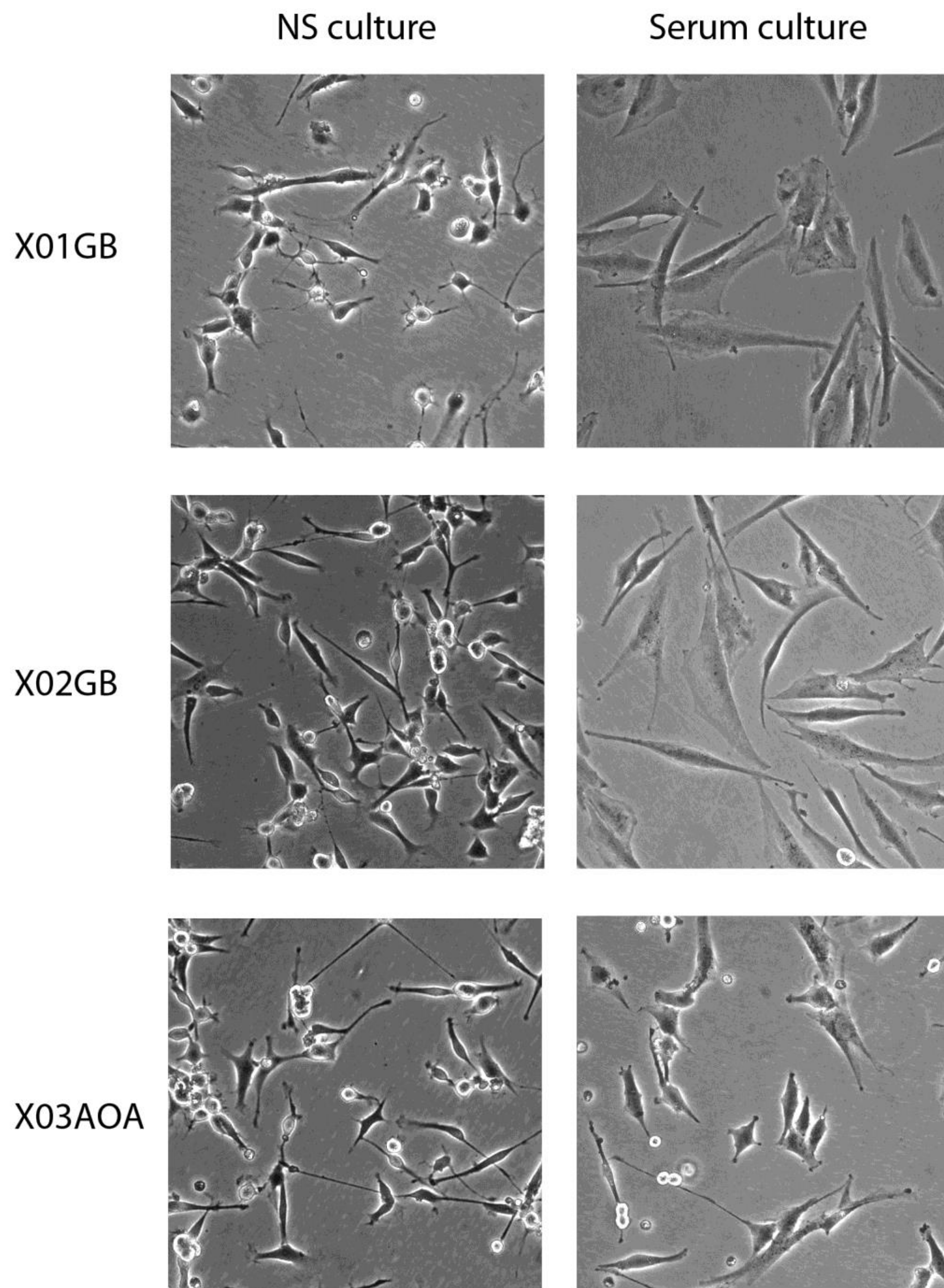


Fig. S2. Inhibition of NK cell mediate cytotoxicity against OSCSCs by anti-MHC class I antibody

Highly purified NK cells ($1X10^6$ cells/ml) were either left untreated or treated with IL-2 (1000u/ml), anti-CD16 mAb (3µg/ml) or a combination of IL-2 (1000u/ml) and anti-CD16 mAb (3µg/ml) for 12-24 hours and used in cytotoxicity assay against ^{51}Cr labeled OSCSCs at different E:T ratios in the presence and absence of anti-MHC-Class I mAb (1:100 dilution) (A) and K562 and Raji cells as control (B) in a standard 4 hour ^{51}Cr release assay. Supernatants were then harvested and radioactivity counted using a gamma counter. Lytic units $30/10^6$ cells were determined using inverse number of effector cells required to lyse 30% of the target cells X 100.

Fig. S2A

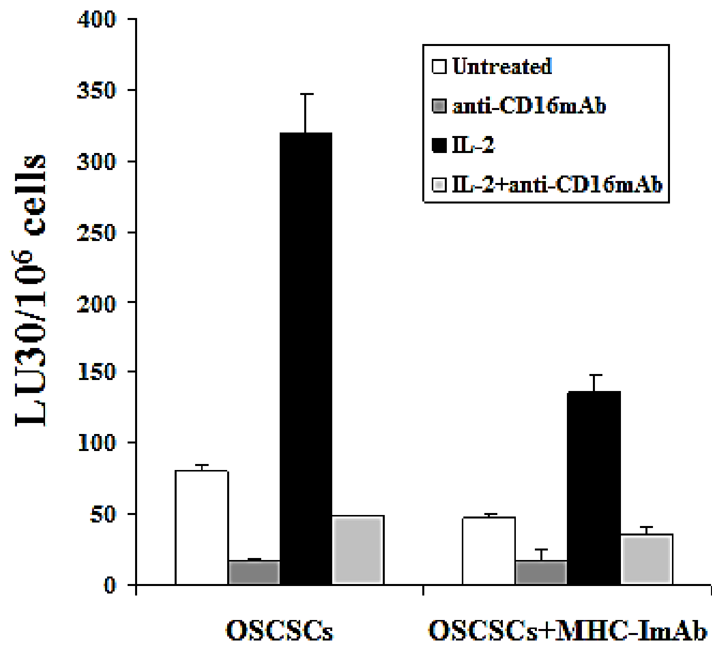


Fig. S2B

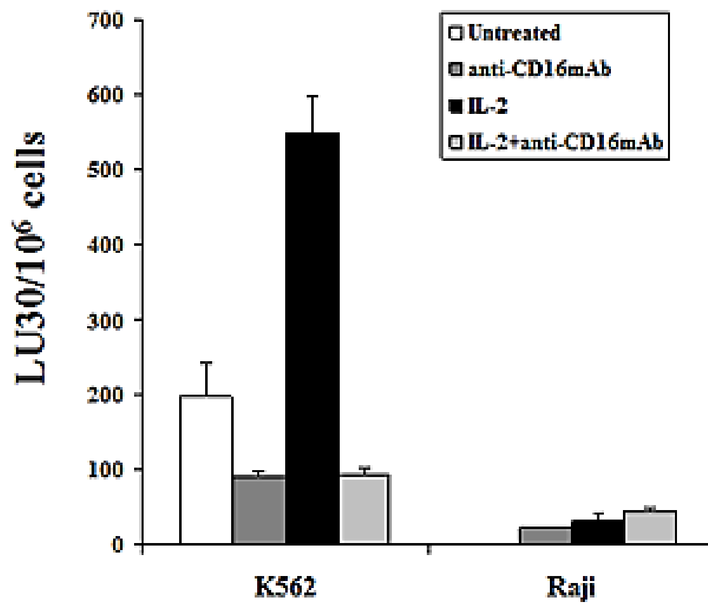


Fig. S3. Induction of NK resistance in OSCSCs by supernatants from IL-2 + anti-CD16mAb treated NK cells correlated with the increased expression of CD54 and MHC class I and significantly inhibited secretion of cytokines and chemokines by the NK cells

Highly purified NK cells were treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours and after which the supernatants were removed and used for the treatment of OSCSCs. Untreated OSCSCs and those treated with anti-TNF-α (1:100) and anti-IFN-γ (1:100) in the absence of NK supernatants were also used as controls. Same amounts of supernatants from IL-2+anti-CD16mAb treated NK cells in the presence and absence of anti-TNF-α (1:100) and/or anti-IFN-γ (1:100) were used to treat OSCSCs for a period of 5 days to induce differentiation. The cytotoxicity against untreated and treated OSCSCs was assessed using freshly isolated untreated (A) and IL-2 treated (B) NK cells using a standard 4 hour ^{51}Cr release assay. Percent cytotoxicity was obtained at different effector to target ratio and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100. OSCSCS were differentiated as described in figure supplemental 3A and the surface expression of CD54 and MHC Class 1 on untreated and NK supernatant treated OSCSCs cells were assessed after PE conjugated antibody staining followed by flow cytometric analysis (C). Surface expression of MHC Class I and CD44 on OSCCs, OSCSCs and NK supernatant differentiated OSCSCs, as described in supplemental figure 3A, were assessed after PE conjugated antibody staining followed by flow cytometric analysis (D, left). Highly purified NK cells were left untreated or treated with IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) with or without autologous monocytes for 18-24 hours. IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) were added to monocytes as control. Afterwards, the supernatant was removed and added to OSCSCs for 5 days. CD54 and MHC Class I surface expression on untreated and NK

supernatant differentiated OSCSCs was assessed after PE conjugated antibody staining followed by flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities in each histogram (D, right). Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/ml) and/or anti-CD16mAb (3 μg/ml) for 18 hours. Afterwards, NK cells were added to OSCSCs treated with NK cell supernatants as described in supplemental figure 3A at an effector to target ratio of 0.5 to 1. After an overnight incubation, the supernatants were removed from the co-cultures and the levels of IFN-γ and IL-8 secretions were determined using specific ELISAs (E).

Fig. 3A

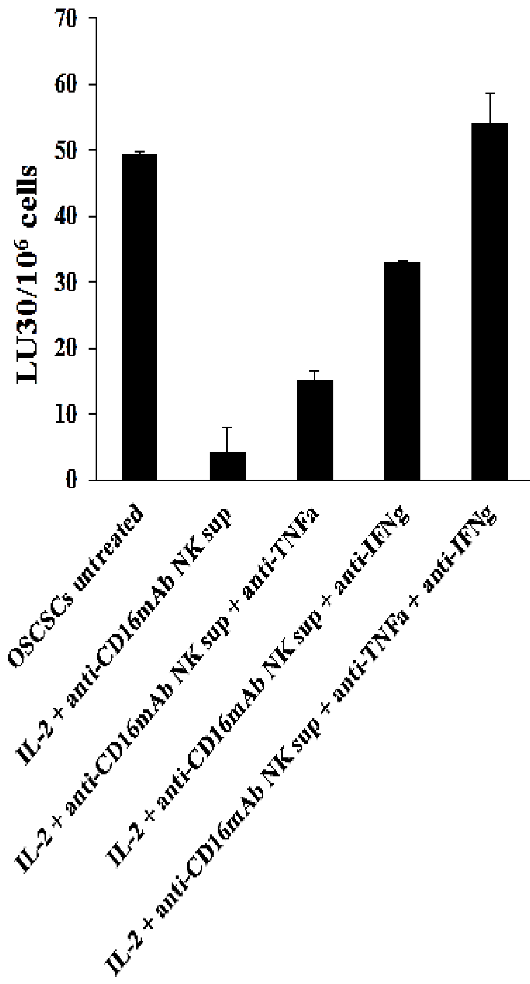


Fig. 3B

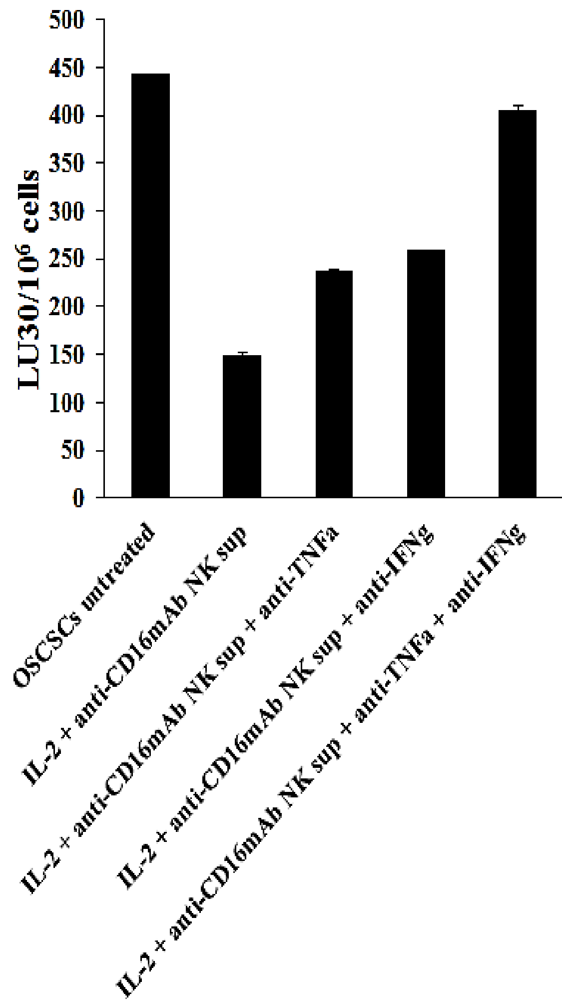


Fig. 3C

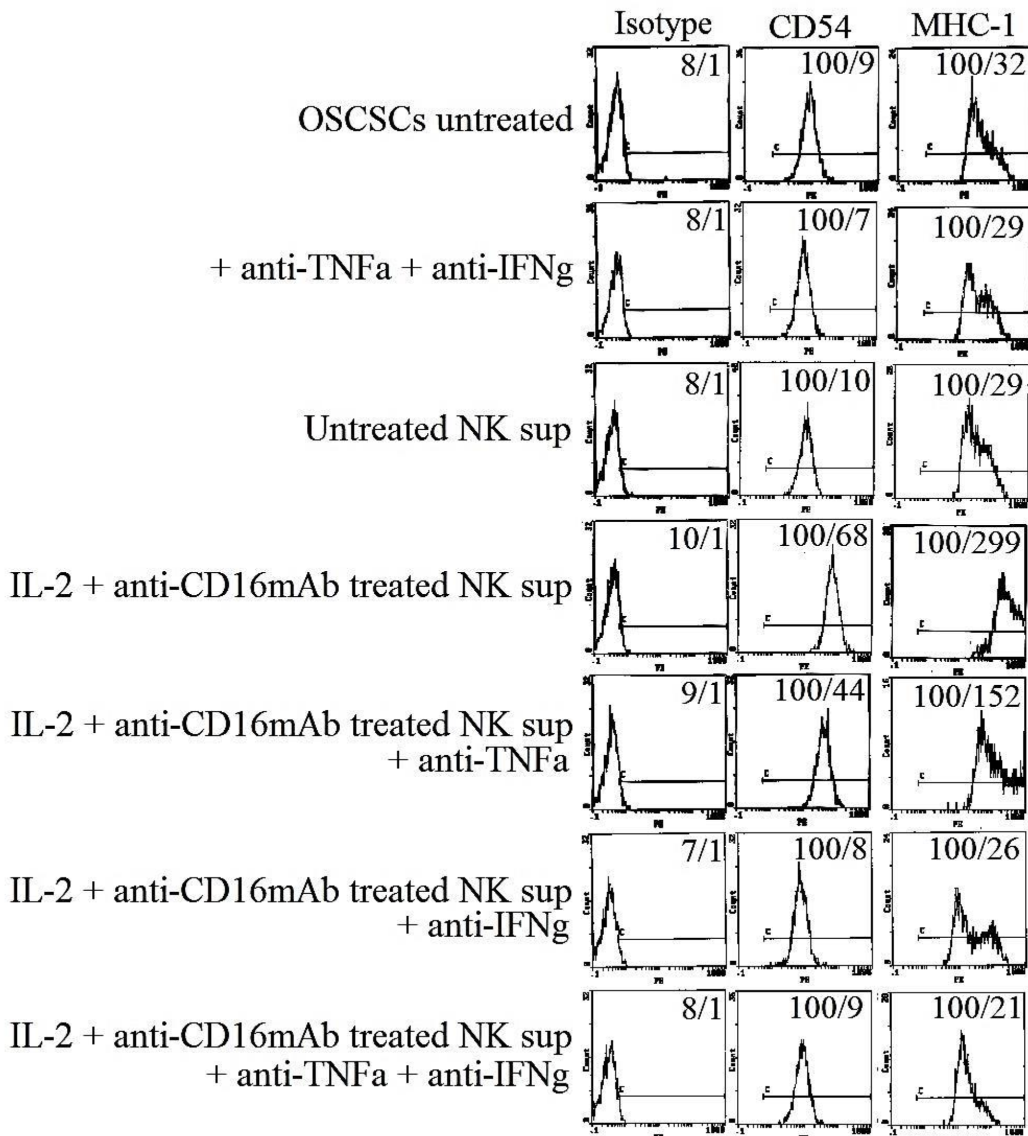


Fig. 3D

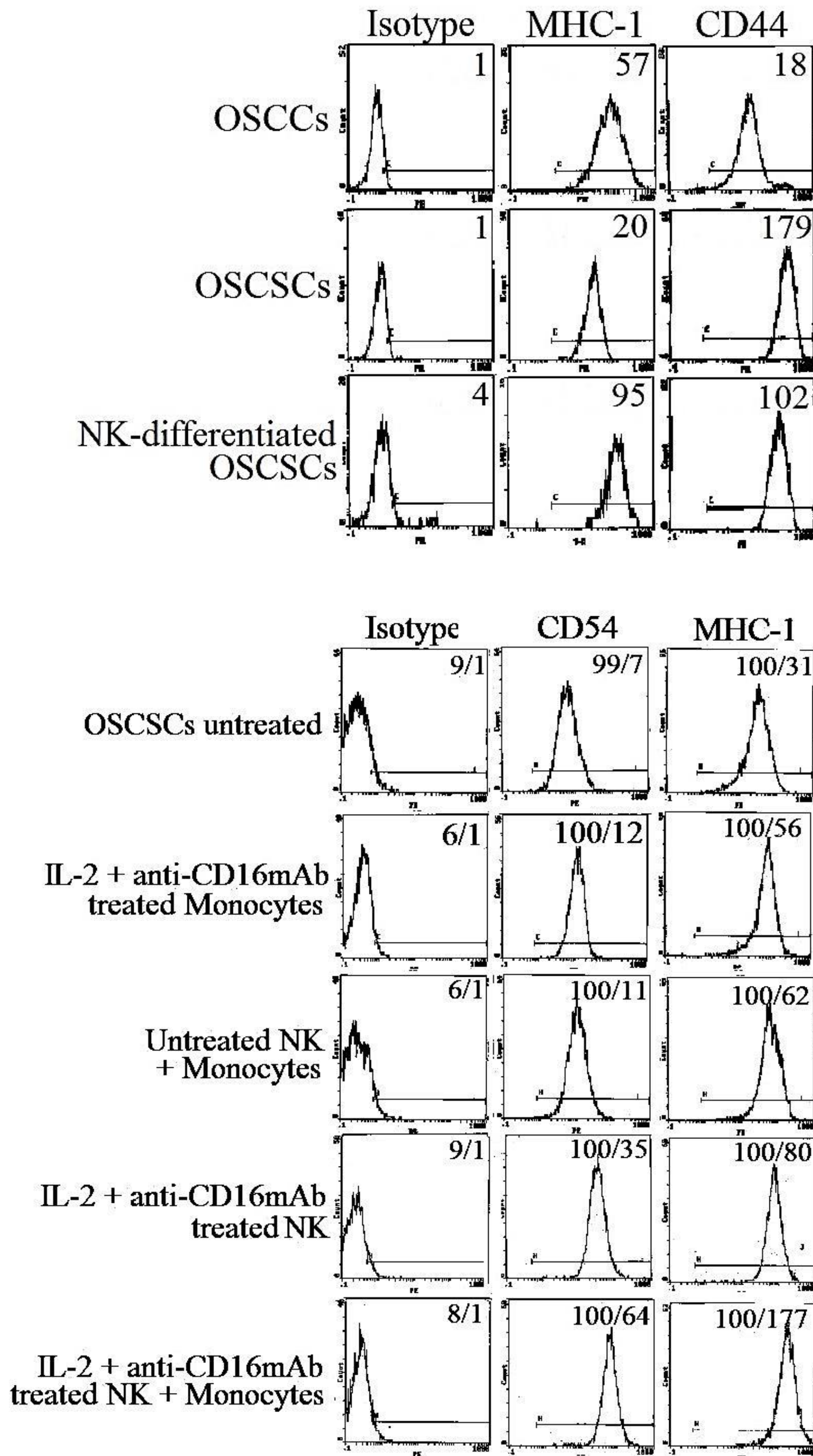
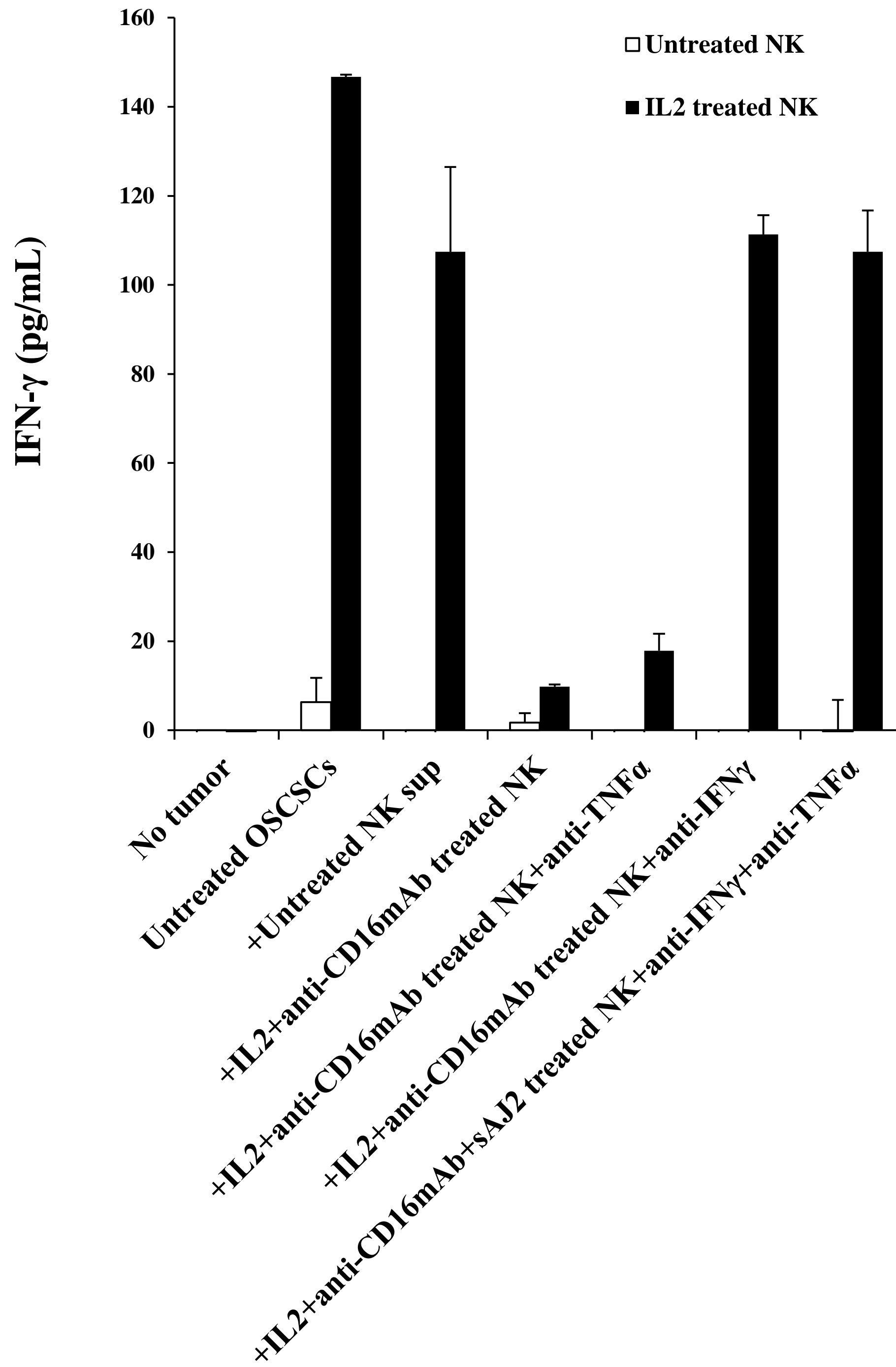
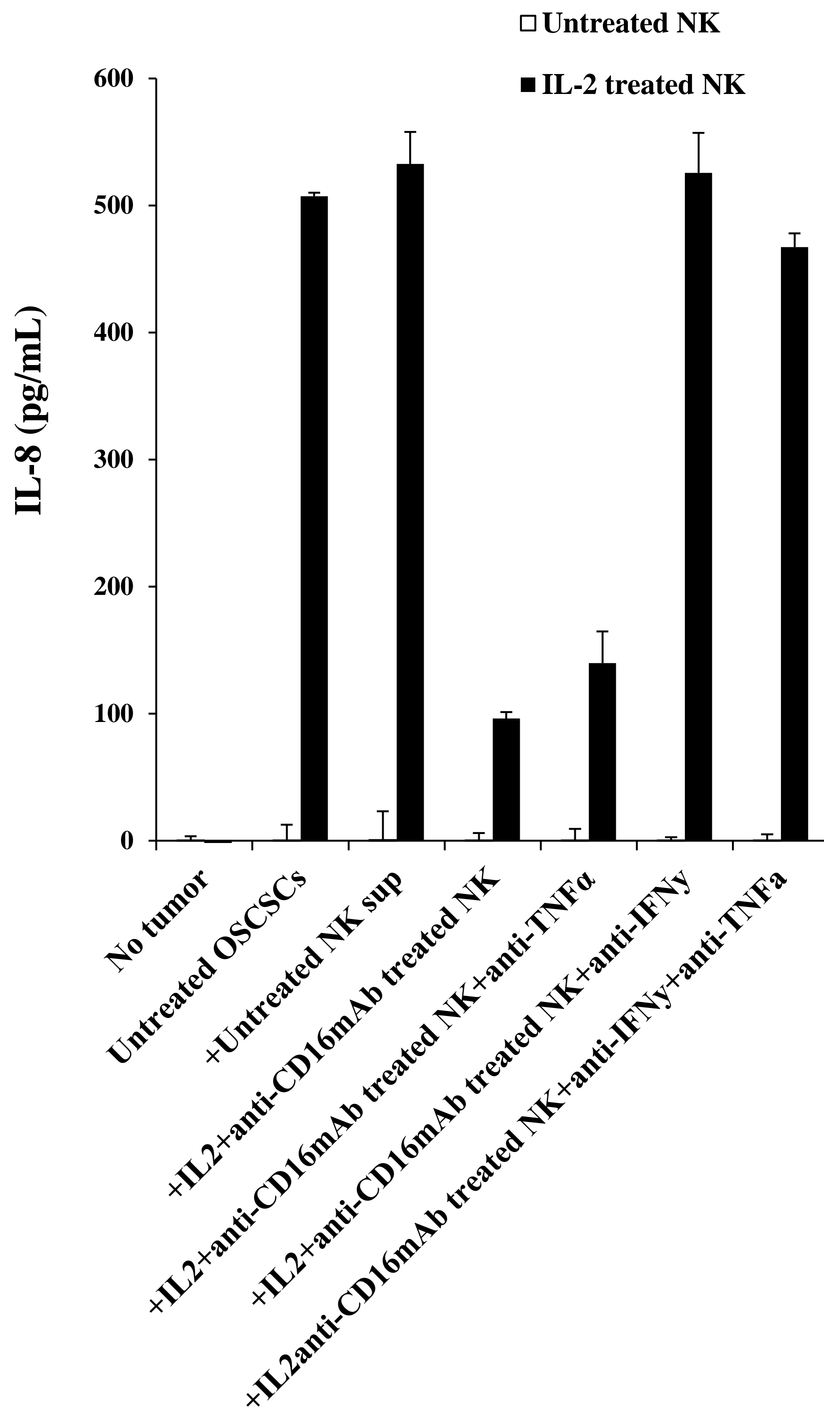


Fig. 3E





Untreated NK
IL-2 treated NK
IL-8 (pg/mL)
600
500
400
300
200
100
0
No tumor
Untreated OSCSCs
+Untreated NK sup
+IL2+anti-CD16mAb treated NK
+IL2+anti-CD16mAb treated NK+anti-TNFα
+IL2+anti-CD16mAb treated NK+anti-IFNγ
+IL2anti-CD16mAb treated NK+anti-IFNγ+anti-TNFa

Fig. S4. Increased levels and function of TNF-αRs and IFNγRs on OSCSCs

Highly purified NK cells were treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 24 hours, after which the supernatants were removed and used for the treatment of OSCSCs. IL-2+anti-CD16mAb treated NK supernatants (1000pg of IFN-γ) were used to treat OSCSCs for a period of 5 days to induce differentiation, and the levels of TNFRI, TNFRII, IFN-γRα and IFNγRβ on OSCSCs (A) were determined using PE conjugated antibodies followed by flow-cytometric analysis. OSCSCs were treated with rTNF-α (20ng/ml) and/or rIFN-γ (2.5ng/ml) for 18 hours and the levels of TNFRI, TNFRII, IFN-γRα and IFNγRβ were determined using PE conjugated antibodies followed by flow-cytometric analysis (B). The histogram on the left is the isotype control staining and on the right the receptor staining. The numbers on the right hand corner are the % positive cells and the mean channel fluorescence for each receptor expression.

Fig. S4A

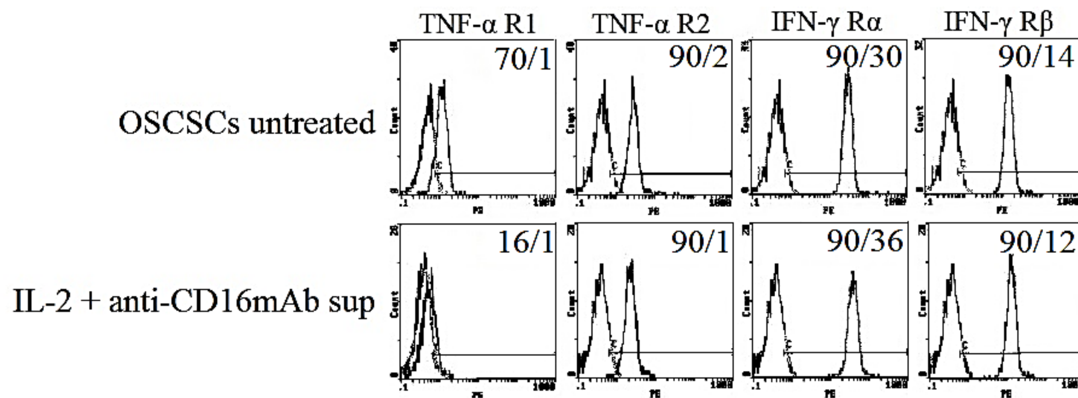
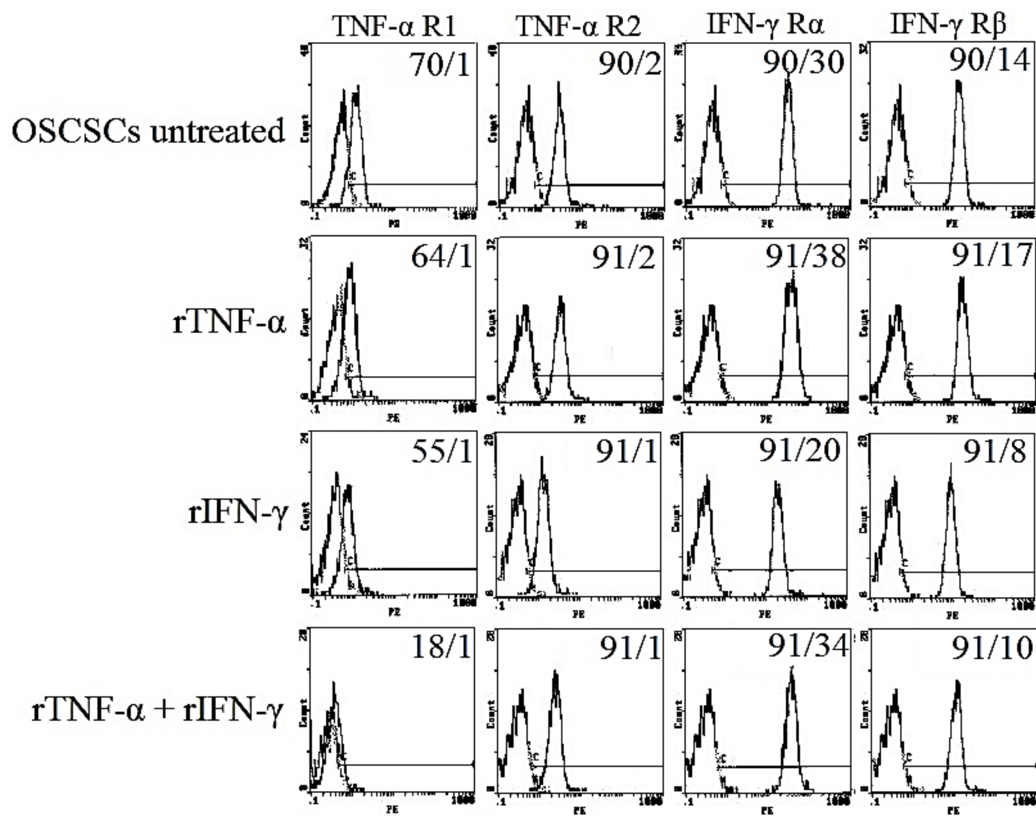


Fig. S4B



CHAPTER 6

NK differentiated stem-like pancreatic tumors while are resistant to NK mediated cytotoxicity become susceptible to chemo-drug induced cell death and do not metastasize

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Running Title: NK differentiated pancreatic tumors do not metastasize

Keywords: IFN-γ, NK, pancreatic cancers, cytotoxicity, regulatory NK

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Abstract

In this paper we provide evidence that anergized NK cells are capable of inducing differentiation of Mia-Paca-2 (MP2), poorly differentiated stem-like pancreatic tumors, resulting in their resistance to NK cell mediated cytotoxicity. Increase in NK cell resistance and differentiation in pancreatic cancer cells directly correlated with the increased expression of CD54, B7H1 and MHC class I and a decrease in the proliferative rate of MP2. The induction of differentiation is mediated by the combination of both TNF-α and IFN-γ secreted by NK cells since antibodies to both cytokines, and not each alone, were able to inhibit the differentiation process and restore the sensitivity of MP2 to NK cells. The effect of anti-IFN-γ in the absence of anti-TNF-α was more dominant for surface receptor modulation than cytotoxicity since its addition significantly abrogated the increase in surface receptor expression. MP2 differentiated by anergized NK cells do not trigger IFN-γ and IL-8 secretion by freshly isolated NK cells as compared to untreated MP2. Differentiation by anergized NK cells rendered MP2 to become highly susceptible to CDDP and it apoptotic effect can be inhibited by the addition of N-Acetyl Cysteine (NAC). Similarly, Paclitaxel also mediated high levels of cell death in differentiated MP2 and a synergistic effect was observed with the addition of NAC. Orthotopic injection of differentiated MP2 into NOD/SCID mice resulted in an inhibition of tumor growth and metastasis to the liver and lung and additionally it prolonged the survival rate of those animals. Thus, anergized NK cells are important in inhibiting the progression of pancreatic cancer by differentiation which severely halted cancer growth, invasion and metastasis.

Introduction

Pancreatic ductal adenocarcinomas are among the tumors with poor prognosis when diagnosed, with the overall 5-year survival rate among patient to be less than 5% [262]. The causes of pancreatic cancer remain unknown; however, several factors have been implicated in the developmental and the progression of the disease, such as tobacco use, excess alcohol and coffee consumption [263-265]. Recent data suggest that the development of pancreatic cancer is attributed to an accumulation of genetic mutation, causing intraepithelial neoplasia [266]. The progression from pancreatic intraepithelial neoplasia grades 1A and 1B to a more aggressive phenotype (grades 2 and 3) is parallel to the activation of the *KRAS2* oncogene, inactivation of *CDKN2A* and *TP53* and deletion of SMAD family member 4 gene [267-270]. Although surgery is an option, multiple large randomized clinical trials revealed disappointing outcome for patients diagnosed with early pancreatic cancer. Adjuvant chemotherapy with gemcitabine provided prolong survival rate after complete resection of the pancreatic tumor, however recurrence occurs [271-273]. Therefore, there is a clear need for better therapies to control the growth of these tumors.

Previous studies conducted in our laboratory demonstrated that the stage of maturation and differentiation of both healthy untransformed stem cells and transformed tumorigenic cancer stem cells is predictive of their sensitivity to NK cell mediated lysis [18]. We have shown that Oral Squamous Carcinoma Stem Cells (OSCSCs) express a number of stem cell markers and they were $CD133^+$ $CD44^+$ $CD326^+$ $CD26^+$ $CD338^+$ $CD166^{dim}$ [18, 240-243] and are significant more susceptible to NK cell lysis as compared to their differentiated counterpart Oral Squamous Carcinoma Cells (OSCCs) [18]. In addition, Human embryonic stem cells, Human induced

pluripotent stem cells, Human mesenchymal stem cells and Human dental pulp stem cells were all found to be significantly more sensitive to NK cells compared to their differentiated counterparts or parental lines in which the cells were derived from [18]. CD16 receptor triggering and interaction with stem cells trigger NK cells to secrete high levels of IFN-γ and TNF-α. Based on our data, we proposed that NK cells may play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact.

In addition, we have shown previously that $CD14^{+}HLADR^{-}$ monocytes can condition NK cells to lose cytotoxicity and secrete inflammatory cytokines [19, 236-239]. The signals received from the stem cells or monocytes alter the phenotype of NK cells and cause NK cells to lose cytotoxicity and change into cytokine producing cells. These alterations in NK cell effector function are thought to ultimately aid in driving differentiation of a minor population of surviving, healthy, as well as transformed stem cells. We recently provided the proof of concept and demonstrated that anergized NK cells, conditioned NK cells that have lost the ability to mediate cytotoxicity but secrete high levels of cytokines, were able to differentiate OSCSCs and glioblastoma stem cells [261] (manuscript submitted). More importantly, we also demonstrate that NK differentiated stem cells are not only resist to lysis by the NK cells but they also do not trigger secretion of cytokines or chemokines, potentially contributing to the cessation of inflammation. This will allow the repair of the tissues during normal wound healing whereas during tumorigenesis they may aid in decreasing growth, invasion and metastasis of tumors, while allowing survival of a selected tumor population.

Currently available chemotherapy treatments for patients suffering pancreatic cancer include cisplatin or paclitaxcel in conjunction with gemcitabine. Cisplatin is a potent antitumor agent and it acts by interacting with DNA to form DNA adducts which ultimate culminate in the activation of apoptosis. The combination of gemcitabine and cisplatin has been used in the treatment of metastatic pancreatic cancer and was reported to be tolerable with minor side effects [274]. Paclitaxcel on the other hand mediate cytotoxicity by arresting cells in the G2/M phase of the cell cycle and prevent normal cell division [275]. The median overall survival was 8.5 months for patients who received paclitaxcel with gemcitabine but adverse effects, such as neutropenia, fatigue and neuropathy were observed [276].

This report provides the basis for the understanding of why immunosuppression in pancreatic tumors may be an important mechanism in controlling tumor growth even though it may result in the expansion of a selected minor population of tumors. The combination of NK cell therapy and chemotherapy drugs may be beneficial in the elimination, if not retards the progression of pancreatic cancer.

Materials and Methods

Cell Lines, Reagents, and Antibodies

RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA) was used for the cultures of human NK cells. Human pancreatic cancer cell lines Panc-1, MIA PaCa-2 (MP2), BXPC3, HPAF, Capan were generously provided by Dr. Guido Eibl (UCLA David Geffen School of Medicine) and PL12 was provide by Dr. Nicholas Cacalano (UCLA Jonsson Comprehensive Cancer Center). Panc-1, MP2 and BXPC3 were cultured DMEM in supplement with 10% FBS and 2% Penicillin-Streptomycin (Gemini Bio-Products, CA). HPAF, Capan and PL12 were cultured in RMPI 1640 medium supplemented with 10% FBS and 2% Penicillin-Streptomycin. Recombinant IL-2 was obtained from NIH- BRB. Recombinant TNF-α and IFN-γ were obtained from Biolegend (San Diego, CA). Antibodies to CD16 were purchased from Biolegend (San Diego, CA). Anti-MHC class I were prepared in our laboratory and 1:100 dilution was found to be the optimal concentration to use. PE conjugated anti-CD54, anti-CD44 anti-CD166, and anti-B7H1, were obtained from Biolegend (San Diego, CA). Monoclonal antibodies to TNF-α were prepared in our laboratory from ascites of mice injected with TNF-α hybridomas, after which the antibodies were purified and specificity determined by both ELISA and functional assays against recombinant TNF-α. Polyclonal IFN-γ antibodies were prepared in rabbits, purified and specificity determined with ELISA and functional assays against rIFN-γ. 1:100 dilution of anti-TNF-α and anti-IFN-γ antibodies was found to be the optimal concentration to block rTNF-α and rIFN-γ function. The human NK purification kits were obtained from Stem Cell Technologies (Vancouver, Canada). Propidium iodide and N-Acetyl Cysteine (NAC) were purchased from Sigma Aldrich (St. Louis, MO).

Cisplatin and Paclitaxel were purchased from Ronald Reagan UCLA Medical Center Pharmacy (Los Angeles, CA).

Purification of NK cells

PBMCs from healthy donors were isolated as described before [137]. Briefly, peripheral blood lymphocytes were obtained after Ficoll-hypaque centrifugation and purified NK cells were negatively selected by using an NK cell isolation kit (Stem Cell Technologies, Vancouver, Canada). The purity of NK cell population was found to be greater than 90% based on flow cytometric analysis of anti-CD16 antibody stained cells. The levels of contaminating CD3+ T cells remained low, at 2.4%±1%, similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures. Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors and all the procedures were approved by the UCLA-IRB.

ELISA

Single ELISAs were performed as described previously [137]. To analyze and obtain the cytokine and chemokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines provided by the manufacturer. Analysis was performed using the Star Station software.

Surface Staining and cell death assays

Staining was performed by labeling the cells with antibodies or propidium iodide as described previously [137, 142, 201].

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [193]. Briefly, different numbers of purified NK cells were incubated with ^{51}Cr–labeled tumor target cells. After a 4 hour incubation period the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm - spontaneous cpm}}{\text{Total cpm – spontaneous cpm}}$$

LU 30/10^6 is calculated by using the inverse of the number of effector cells needed to lyse 30% of tumor target cells X100.

Stem cell differentiation with NK cell supernatant

Human NK cells were purified from healthy donor's PBMCs as described above. NK cells were left untreated or treated with anti-CD16mAb (3ug/ml), IL-2 (1000 units/ml) or a combination or IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18- 24 hours before the supernatants were removed and used in differentiation experiments. The amounts of IFN-γ produced by the activated NK cells were assessed with IFN-γ ELISA (Biolegend, CA). Differentiation of MP2 was conducted with gradual daily addition of increasing amounts of NK cell supernatant. On average a total of 5000pg of IFN-γ containing supernatants obtained from IL-2+anti-CD16mAb treated NK cells was added for 5 days to induce differentiation and resistance of MP2 to NK cell mediated cytotoxicity.

Analysis of human pancreatic cancer cell growth in immunodeficient mice

In vivo growth and metastatic behavior of human pancreatic cancer cell lines were determined by orthotopic cell implantation into 8-10 week-old severe combined immunodeficient (SCID) mice (NOD/SCID; IL-2Rγ-/-, lacking T, B, and natural killer cells), as previously described [277]. To establish orthotopic tumors, mice were first anesthetized with ketamine (100 mg/kg) and xylzine (10 mg/kg), and then the pancreas was exposed through an abdominal incision (laparotomy). Tumor cells were then transferred by direct injection of a single cell suspension ($5X10^5$ cells) into the pancreas or transplantation of a subcutaneous tumor fragment ($2mm^3$) onto the pancreas, secured by a 7-0 Prolene suture. After tumor implantation, all mice were monitored at least twice weekly for disease progression by abdominal palpation and for overall signs of morbidity such as ruffled fur, hunched posture, and immobility. Moribund mice were euthanized by isofluorane or CO2 inhalation. For survival studies, mice were followed until death or euthanized when signs of morbidity were evident. Tumor size was measured with a caliper. Pancreatic tumors, livers, and lungs were harvested from mice at the end of the experiment following orthotopic tumor implantation or when tumor size reached 2cm diameter.

Statistical analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

Results

The stage of differentiation in pancreatic tumors correlates with susceptibiltiy to NK cell meidated cytotoxicity; Loss of NK cell cytotoxicity and gain in secretion of IFN-γ after NK cell receptor triggering

Six pancreatic tumor cells were used to determine surface expression, susceptibilty to NK cell mediated cytotoxicity and secretion of cytokiens when cultured with NK cells. Poorly differentiated MP-2 and Panc-1 expressed higher amounts of CD44 and moderate or low levels of MHC class I and CD54. Moderately differentiated BXPC3 and HPAF expressed moderate to high levels of CD44 and CD54 and higher levels of MHC class I. Well differentiated Capan and PL12 had much lower levels of CD44 and higher levels of CD54 and MHC class I (Fig. 1A). A direct correlation between the stage of differentiation and susceptibility to NK cell mediated cytotoxicity was observed in pancreatic tumor cells. Undifferentiated MP2 and Panc-1 exhibited the highest whereas PL-12 and Capan well differentiated tumors demonstrated the lowest sensitivity to NK mediated lysis (Fig. 1B). Moderately differentiated BXPC3 and HPAF demonstrated intermediate sensitivity to NK cell lysis (Fig. 1B). IL-2 treated NK cells had the highest increase in cytotoxicity and the addition of anti-CD16mAb to both untreated and IL-2 treated NK cells decreased NK cell mediated cytotoxicity significantly against all the tumors (Fig. 1B). Significant secretion of IFN-γ in the co-cultures of IL-2 treated NK cells with MP2 was observed, and the levels were significantly higher than that seen when NK cells were cultured with PL12 (Fig. 1D). The combination of IL-2 and anti-CD16mAb treatment of NK cells although inhibited NK cell cytotoxicity, it induced higher release of IFN- γ when cultured with and without MP2 and PL12 tumors (Fig. 1D). The levels of IFN- γ secretion remained less in the co-cultures of NK cells with PL12 when compared to those cultured with MP2.

The induction of resistance of MP2 cells to NK cell mediated cytotoxicity by IL-2+anti-CD16mAb treated NK cells is mediated by the combination of IFN-γ and TNF-α and not each cytokine alone

Treatment of MP2 with IL-2+anti-CD16mAb treated NK cell supernatants decreased NK cell mediated cytotoxicity significantly by both untreated and IL-2 treated NK cells ($P<0.05$) (Fig. 2A). A lower decrease in susceptibility of MP2 cells to NK cell mediated cytotoxicity could also be observed when MP2 cells were treated with supernatants obtained from untreated NK cells (Fig. 2A). To examine the mechanism by which MP2 tumors become resistant, we determined NK cell mediated cytotoxicity after the addition of each of IFN-γ and TNF-α antibodies alone or in combination with IL-2+anti-CD16mAb treated NK supernatants to MP2 tumors. As shown in Fig. 2A, the addition of each of the IFN- γ and TNF-α antibodies alone had a slight inhibitory effect on the induction of resistance of MP2 cells by the supernatants of NK cells treated with IL-2+anti-CD16mAb; however, the combination of anti-IFN-γ and anti-TNF-α antibodies abrogated the resistance of treated MP2 cells completely (Fig. 2A). The restoration of MP2 susceptibility to NK cell mediated lysis by the combination of anti-IFN-γ and anti-TNF-α antibodies could be observed when either untreated or IL-2 treated NK cells were used to assess cytotoxicity (Fig. 2A). Resistance of MP2 to NK cell mediated cytotoxicity induced by supernatants from IL-2+anti-CD16mAb treated NK cells correlated with the increased expression of CD54, B7H1 and MHC-1 as shown in figure 3B, and the addition of the combination of anti-IFN-γ and anti-TNF-α antibodies abrogated the increase completely (Fig. 2B). The effect of anti-IFN-γ mAb in the absence of anti-TNF-α antibody, however, was more dominant for surface receptor modulation than cytotoxicity, since its addition abrogated the

increase in surface receptor expressions substantially (Fig. 2B). In addition, the rate of MP2 cell proliferation was decreased when supernatants obtained from IL-2+anti-CD16mAb treated NK cells were added, and this decrease was significantly inhibited in the presence of the combination of anti- IFN-γ and anti-TNF-α antibody, and not each antibody alone (Fig. 2C). No cell death could be observed in MP2 cells treated with any of the abovementioned NK supernatants (Fig. 2C).

The levels of MHC class I gradually decreased from day 0-12 after NK supernatant removal from the differentiated MP2 tumors (data not shown). Indeed there was a two-fold decrease in MHC class 1 expression from day 0 of the removal of NK cell supernatants to day 2 of the cultures (Fig. S1) and by day 12 the levels were similar to that obtained with untreated MP2 cells (data not shown). Thus, there was a time dependent decrease in the expression of MHC class I which correlated with restoration of sensitivity to NK cell mediated cytotoxicity and increased cytokine secretion in co-cultures of NK cells with IL-2+anti-CD16mAb NK supernatant differentiated MP2 tumors when NK supernatants were removed and replaced with media from day 0-12 (Fig. S1).

Combination of rTNF-α and rIFN-γ induce differentiation and resistance of MP2 cells to NK cell mediated cytotoxicity

MP2 and Capan were treated with rTNF-α and rIFN-γ and their susceptibility to NK cell mediated lysis was assessed in a standard 4 hour ^{51}Cr release assay. As shown in figure S2A, the addition of rTNF-α to MP2 was able to induce moderate resistance against NK cell mediated cytotoxicity whereas IFN-g induced significant resistance. Combination of rTNF-α and rIFN-γ were able to upregulate CD54, MHC-1 and B7H1 and down modulate CD44 in MP-2 tumors.

Both TNF-a and IFN-g were able to increase surface expression of CD54 and MHC class I, however, only IFN-g was able to upregulate B7H1 (Fig. S2B). As indicated above Capan expressed higher surface expression of CD54, MHC class I and B7H1 and the levels further increased by TNF-a and IFN-g and the their combination (Fig. S2B).

Treatment of MP2 with supernatants from IL-2 and anti-CD16mAb treated NK cells significantly inhibited the secretion of IFN-γ and IL-8 by NK cells

We next determined whether decrease in NK cytotoxicity correlated with a decrease in cytokine and chemokine secretion in co-cultures of NK cells with MP2 treated with supernatants from anergized NK cells. Treatment of MP2 with IL-2+anti-CD16mAb treated NK cell supernatant significantly decreased secretion of IFN-γ (Fig. 3A) and IL-8 (Fig. 3B) by freshly isolated untreated, IL-2 treated and IL-2 + anti-CD16mAb treated NK cells. The addition of anti-TNF-α antibody or anti-IFN-g antibody to co-cultures of NK cells with MP2 tumors treated with supernatants from NK cells stimulated with IL-2 + anti-CD16mAb increased the levels of both IFN-γ (Fig. 3A) and IL-8 (Fig. 3B). However, the increase in IFN-g and IL-8 with anti-IFN-g antibody was more substantial than that mediated by anti-TNF-a. The increase in the secretion of IFN-γ and IL-8 was also observed when they were cultured in the presence of the combination of anti-TNF-α and anti-IFN-γ (Fig. 3).

Increased induction of cell death by NAC in well differentiated PL12, Capan and OSCCs and not in poorly differentiated stem-like MP2 and OSCSCs; effect on CDDP mediated cell death.

The effect of N-Acetyl Cystein (NAC), previously characterized as a differentiation agent from our laboratory, was determined on MP2 and OSCSCs and differentiated PL12, Capan and OSCCs. As shown in Fig. 4A addition of NAC to either MP2 or OSCSCs had no significant effect on cell viability, however, when added to PL-12, Capan or OSCCs induced signficant cell death. Addition of CDDP to MP2 and OSCSCs had no or moderate effect in inducing cell death, whereas when added to PL-12, Capan and OSCCs it induced significant cell death (Figs. 4B and 4C). Addition of NAC not only induced higher death in untreated PL12, Capan and OSCCs but also it inhibited CDDP mediated cell death in all cells tested (Figs. 4B and 4C). In contrast to CDDP, NAC synergistically increased Paclitaxel mediated cell death in PL-12, Capan and OSCCs, whereas had slight to moderate effect on Paclitaxel mediated cell death in MP2 and OSCSCs (Fig. 4D and 4E). Paclitaxel had dose dependent effect on induction of death in PL12, Capan (Fig. 4D). Both MP2 and OSCSCs were resistant to Paclitaxel mediated cell death, although at very high concentration of Paclitaxel (1000nM) significant cell death could be observed against OSCSCs but not MP2s (Figs. 4D and 4E).

NAC, CDDP and Paclitaxel induce significant cell death in MP2s differentiated with IL-2+anti-CD16mAb treated NK supernatant

Differentiation of MP2s with supernatants from IL-2+anti-CD16mAb treated NK cells resulted in a significant susceptibility to CDDP which was significantly blocked by the addition of NAC (Fig, 4F). Similar to those seen with PL-12, Capan and OSCCs addition of NAC to MP2s differentiated with supernatants from IL-2+anti-CD16mAb treated NK cells mediated increased cell death (Fig. 4F). Blocking of IL-2+anti-CD16mAb treated NK supernatant differentiated MP2 cells with anti-IFN-g and anti-TNF-a substantially decreased the levels of cell

death induced by either NAC or CDDP to the levels that were seen with untreated or those treated with the untreated NK cell supernatant treated MP2s (Fig. 4F). Similarly, paclitaxel mediated higher cell death of IL-2+anti-CD16 treated NK supernatant differentiated MP2s and NAC significantly increased paclitaxel mediated cell death (Fig. 4G). Blocking of IL-2+anti-CD16mAb treated NK supernatant differentiated MP2 cells with anti-IFN-g and anti-TNF-a substantially decreased the levels of cell death induced by either Paclitaxel and/or NAC to the levels that were seen with untreated MP2s (Fig. 4G).

Lack of tumor growth and metastasis and long term survival of mice after orthotopic injection of IL-2+anti-CD16mAb stimulated NK cell supernatant differentiated MP2 tumors in pancreas

To determine whether IL-2+anti-CD16 mAb stimulated NK cell supernatant differentiated MP2 loses the ability to grow significantly and establish in the pancreas of NOD/SCID; IL-2Rγ-/- mice we first injected mice (n=9) with MP2 stem-like tumors and compared the growth rate and metastatic ability to PL12 well differentiated tumors (n=9). As shown in Fig. 5A MP2 stem-like tumors grew within 4 weeks and metastasized to both liver and lungs and killed the animals whereas mice injected with PL12 generated very small tumors within 12 weeks and did not metastasize nor kill the animals (Figs. 5A and 5B). Injection of IL-2+anti-CD16 mAb stimulated NK cell supernatant differentiated MP2s to pancreas (n=6) did not exhibit growth nor metastasized to liver and lung and all mice survived at 12 weeks when the experiment was terminated (Fig. 5C). Since all of the IL-2+anti-CD16 mAb stimulated NK cell supernatant differentiated MP2s reverted to their stem-like phenotype after 12 days of incubation when NK supernatants were removed from the cells and replaced by control media, as evidenced

by gradual decrease in expression of MHC class I (Fig. 2S) and CD54 and B7H1 and gain in susceptibility to NK cell mediated cytotoxicity (data not shown), we injected the reverted MP2 cells (Diff-MP2-R) in the pancreas (n=3) and observed their growth and metastatic potential. Although Diff-MP2-R grew to a smaller size than the untreated MP2s (Fig. 5D), it retained its metastatic potential to liver (Fig. 5C).

Discussion

In this paper we provide evidence that anergized NK cells are capable of secreting pro-inflammatory cytokines to induce differentiation of pancreatic cancer stem cells and limit tumor growth and metastasis. The data is in agreement with our previous finding in which anergized NK cells can differentiate OSCSCs and limit inflammation through the release of TNF-α and IFN-γ in both the secreted form as well as membrane bound [20]. We screen a panel of pancreatic tumor cell lines and based on their surface expression of CD54, CD44, MHC-1 and CD166 we categorized MP2 and Panc-1 to be stem-like cells, followed by BXPC3 and HPAF which are moderately differentiated and lastly Capan and PL12 which are most differentiated cells. We also observed high expression of CD166 on PL12 and low express on MP2 cells. We previously have demonstrated that the level of differentiation in target cells is predictive to their susceptibility to NK cell mediated lysis [18]. Similarly, we report that pancreatic cancer stem cells, MP2 and Panc-1, were most sensitive to both untreated, IL-2 treated and IL-2+anti-CD16mAb treated NK cells, whereas moderately differentiated cells were sensitive too but not to the same extent as MP2 or Panc-1. Capan and PL12, which are the most differentiated pancreatic cell lines, were resistant against NK cell mediated lysis. MP2 cultured with NK cells also triggered the highest secretion of IFN-γ as compared to NK cells cultured with PL12 or without any tumor cells.

One of the most important observations reported in this paper is that differentiation of MP2 by anergized NK cells significantly inhibited their sensitivity to NK cell mediated lysis. We also demonstrate that the induction of differentiation is dependent upon the combination of both

IFN-γ and TNF-α since antibody to each cytokine alone was not able to restore the sensitivity of differentiated MP2 to its control level. The increase in resistance to NK cells after MP2 differentiation correlated with a modulation in the surface expression of key receptors and reduction in growth rate. Differentiated MP2 cells by anergized NK cells expressed higher levels of B7H1, MHC-1 and lower levels of CD44 and the change in surface expression was abrogated by the addition of anti-IFN-γ and anti-TNF-α.

In addition, the differentiation of MP2 by anergized NK cells inhibited greatly the secretion of IFN-γ and IL-8 in the cultures of NK cells with differentiated tumors. This observation is of great significance since it indicates that cellular differentiation is an important step in inhibition and prevention of inflammation. Indeed, the levels of cytokines and chemokines secreted in the co-cultures of NK cells with anergized NK supernatant differentiated MP2 was in general similar or slightly higher than those secreted by the NK cells in the absence of tumors. The amount of anergized NK cells necessary to cause differentiation in pancreatic cancer stem cells is significantly more than OSCSCs and glioblastoma cancer stem cells and the amount required may not be within the physiologically possible. Therefore, further investigation on strategies to expand and/or enhance the function of anergized NK cells is needed.

To investigate the role of anergized NK cells in an *in vivo* system, we orthotopically injected MP2 and PL12 into the pancreas of NOD SCID animals and monitored tumor growth for 12 weeks. Animals injected with MP2 resulted in large primary tumor growth in the pancreas while the growth is minor in animals injected with PL12. Extensive metastasis in the liver and lungs were observed in animals with MP2 while none was detected in animals with PL12. By

differentiating MP2 with anergized NK cells, we observed an increase in survival rate and the reduction in primary tumor growth and metastasis in the lungs and liver. We recently demonstrated that there is a gradual and time dependent decrease in the expression of both MHC class I and CD54 which correlated with the increased cell growth and restoration of NK cell cytotoxicity and cytokine secretion in cultures of NK cells with differentiated OSCSCs from days 0-12 post NK supernatant removal. These experiments indicated that for the OSCSCs to remain differentiated, a continuous exposure to cytokines are necessary since after their removal, the cells revert to their undifferentiated phenotype and become sensitive to NK cell mediated cytotoxicity, and trigger the release of cytokines and chemokines [261]. As shown in this study, without continuous presence of anergized NK cells, differentiated MP2 cells can revert back to its stem-like state which correlated with extensive primary tumor growth in the pancreas and metastasis in the liver and lungs. The rate of reversion is fast since two weeks without anergized NK cells was able to revert differentiated MP2 cells back to a phenotype similar to untreated MP2 cancer stem cells.

CDDP and Paclitaxel are common chemotherapy drugs used to treat many types of cancer. Differentiated cells, such as PL12, Capan and OSCCs are more sensitive to the cytotoxicity of both CDDP and paclitaxel, while cancer stem cells are more resistant. The addition of NAC to differentiated cells treated with CDDP offered a protective effect and inhibited the apoptotic effect of CDDP. The inhibition of cell death conferred by NAC was more prominent in conditions of differentiated cells while cells without treatment of CDDP had higher levels of cell death. PL12, Capan or OSCCs treated with increasing concentration of paclitaxel had an increasing amount of apoptosis and the effect was enhanced with the addition of NAC.

While untreated MP2 cells were relatively unaffected by CDDP and Paclitaxel, the differentiation of MP2 cells by anergized NK cells caused an increase in sensitivity to both chemotherapy drugs. By inhibiting both IFN-γ and TNF-α with specific antibodies the effects of differentiation was abrogated and thus restoring resistance to both CDDP and Paclitaxel.

Collectively, our results suggest two very important functions for NK cells. The first role of NK cells is to limit the number of cancer stem cells and followed by the induction of differentiation of cancer stem cells. NK cells have been shown to be present in the surrounding area next to tumor nest in an immune rich compartment which provides a microenvironment to induce split anergy in NK cells. Such mechanisms of NK cell conditioning by Myeloid Derived Suppressor Cells (MDSCs) may explain why the cytotoxic function of NK cells are greatly reduced in the tumor microenvironment as well as in circulating NK cells. There should be two distinct strategies to treat patients with pancreatic cancer, one which targets cancer stem cells and the other to target differentiated cells. Allogeneic transplantation of cytotoxic NK cells should be used to target cancer stem cells, such as MP2 and OSCSCs, since these cells are resistant to Cisplatin and Paclitaxel but are highly sensitive to cytotoxic NK cells. Whereas, chemotherapeutic drugs should be used to target differentiated cells, such as PL12, OSCCs and differentiated MP2 cells by anergized NK cells. Since a great majority of patients' NK cells have modified their phenotype to support differentiation of the cells, they may not be effective in eliminating cancer stem cells. Therefore, these patients may benefit from repeated allogeneic NK cell transplantation for elimination of cancer stem cells. In this regard depletion of NK anergizing effectors such as MDSCs in the tumor microenvironment before allogeneic NK cell transplantation should in theory provide such strategy. However, this strategy may also halt or

decrease the ability of NK cells to drive optimal differentiation of the tumors and tilt the balance towards a more inflammatory tumor microenvironment which could run the risk of fueling the growth and expansion of more cancer stem cells. Alternatively, a strong tumor differentiating microenvironment may be induced by the MDSCs anergized NK cells, in hope that most if not all of the newly arising cancer stem cells are induced to differentiate. The benefit of such approach will be the ability of chemotherapeutic drugs to target the differentiated tumors in addition to the lack of differentiated tumors to metastasize.

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Figure legends

Fig. 1. The stage of differentiation in six pancreatic tumor cell lines correlates with susceptibiltiy to NK cell mediated cytotoxicity and IFN-γ secretion by NK cells

The surface expression of CD44, CD54, MHC-1 on six pancreatic cell lines were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies (A). The surface expression of CD166 on MP2 and PL12 was assessed by flow cytometric analysis after staining with PE conjugated CD166 antibody (B). Isotype control antibodies were used as control. The numers on the right hand corner are the percentages and the mean channel fluoresence intensities for each histogram. Freshly isolated NK cells were left untreated or treated with anti-CD16mAb (3ug/ml), IL-2 (1000 units/ml) or the combination of anti-CD16mAb (3ug/ml) and IL-2 (1000 units/ml) for 18 hours before they were added to ^{51}Cr labeled MP2, Panc-1, BXPC3, HPAF, Capan and PL12. NK cell mediated cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay and the lytic units 30/10^{6} cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100 (C). NK cells were treated as described in figure 1C and each NK sample was either cultured in the presence or absence of MP2 and PL12 at an NK cell to target ratio of 0.1:1. After an overnight incubation, the supernatant was removed from the co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs. One of minimum three representative experiments is shown (D).

Fig. 2 Induction of differentiation, inhibition of cell growth and resistance to NK cell mediated cytotoxicity in MP2 differentiated by IL-2+anti-CD16 treated NK cells are mediated by the combination of IFN-γ and TNF-α and not each cytokine alone

Highly purified NK cells were treated with the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 ug/ml) for 24 hours and after which the same amounts of supernatants from different treatments were removed and added to MP2 cells in the presence and absence of anti-TNF-α (1:100) and/or anti-IFN-γ (1:100) for a period of 5 days. The cytotoxicity against untreated and NK supernatant differentiated MP2 cells in the presence of antibodies to freshly isolated untreated NK cells or IL-2 (1000 units/ml) treated NK cells were assessed using a standard 4 hour ^{51}Cr release assay. Percent cytotoxicity was obtained at different effector to target ratio and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100 (A). The surface expression of CD54, B7H1, MHC Class 1 and CD44 on untreated and NK supernatant treated MP2 cells as described above were assessed after PE conjugated antibody staining using flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities in each histogram (B). At the end of the incubation of MP2 cells with NK cell supernatants, MP2 cells which were remained attached to the plate and those which detached during the incubation period were collected separately and the number of cells were assessed using microscopy (C).

Fig. 3 Treatment of MP2 cells with supernatants from IL-2 and anti-CD16mAb treated NK cells significantly inhibited the secretion of IFN-γ and IL-8 by NK cells

Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours. Afterwards, NK cells were added to untreated MP2 and MP2 differentiated with NK cell supernatants as described in Fig. 2A at an effector to target ratio of 0.1 to 1. After an overnight incubation, the supernatants were removed from the co-cultures and the levels of IFN-γ cytokine (A) and IL-8 chemokine (B) secretions were determined using specific ELISAs. Differences between untreated MP2 cells and those stimulated with IL-2+anti-CD16mAb treated OSCSCs with or without the addition of anti-TNF-α were significant at a p value of <0.05 (*).

Fig. 4 The effect of cell death induced by NAC, CDDP and Paclitaxel in well differentiated PL12, Capan and OSCCs but not in stem-like MP2 and OSCSCs

MP2, PL12 and Capan (A) and OSCCs and OSCSCs (B) ($1X10^5$ cells/well) were seeded in 24-well plate. After an overnight incubation the cells were treated with CDDP (100ug/ml) with or without NAC (20nM) for 24 hours. Percent cell death was measured by propidium iodide stain followed by flow cytometric analysis. MP2, PL12 and Capan (C) and OSCCs and OSCSCs (D) were seeded as describe in figure 4A and were left untreated or treated with Paclitaxel (10, 20, 600 and 1000nM) in the presence or absence of NAC (20nM) for 24 hours. After an overnight treatment period, the viability of the cells was determined by propidium iodide and analyzed with flow cytometry. NK cells supernatant were prepared as described in figure 2A and added to MP2 for a period of 5 days. Afterwards, untreated MP2 and those treated with either untreated NK cell supernatant or IL-2+anti-CD16mAb treated NK cell supernatant were incubated with or without NAC (20nM) in presence of CDDP (100ug/ml) (E) or Paclitaxel (10,

200, 600nM) (F) for 18-24 hours. Afterwards, the viability of untreated and treated MP2 cells were then determined using propidium iodide and analyzed with flow cytometry.

Fig. 5. Orthotopic injection of MP2 cells differentiated with IL-2+anti-CD16mAb treated NK cell supernatant resulted in a lack of tumor growth and metastasis and long term survival.

Successful orthotopic cell implantation of MP2 and PL12 (5 X 10^5 cells) into 8-10 week old NOD/SCID mice is shown. Method of implantation is described as in Materials and Methods. After 4 weeks (MP2) and 12 weeks (PL12) post implantation, the animals were euthanized by isoflorane or CO_2 inhalation and the pancreas, liver and lungs were analyzed for primary tumor growth and metastases (A). Highly purified NK cells were treated with IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 24 hours before the supernatant was collected and added to MP2 cells for 5 days (Diff-MP2). The rate of survival between animals injected with PL12, MP2 and Diff-MP2 (5 X 10^5 cells) were tracked for 12 weeks (B). MP2 cells were differentiated as described in figure 5B (Diff-MP2). A second set of Diff-MP2 cells were replenished with fresh medium after the differentiation period without supernatant from IL-2+anti-CD16mAb treated NK cells for 2 weeks (Diff-MP2-R). Afterwards, MP2, Diff-MP2 and Diff-MP2 tumor cells were orthotopically implanted into NOD/SCID mice (5 X 10^5 cells). Mice with injected Diff-MP2 tumor cells were sacrificed at 10 weeks and mice with Diff-MP2-R tumor cells were sacrificed at 8 weeks. After the animals were euthanized, images of the pancreas, liver and lungs were taken (C) and the tumor weight was measured (D).

Fig. 1A

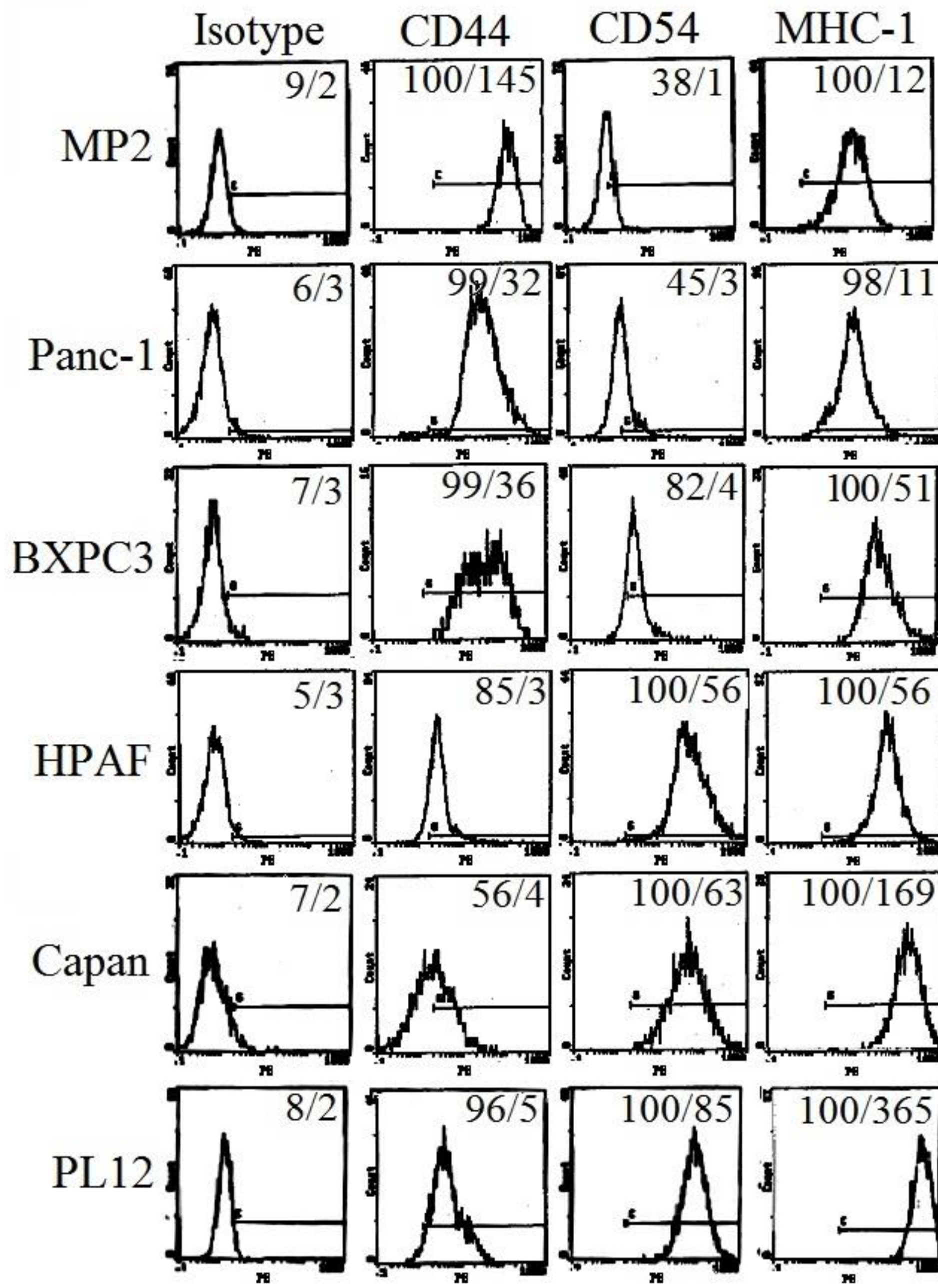


Fig. 1 B

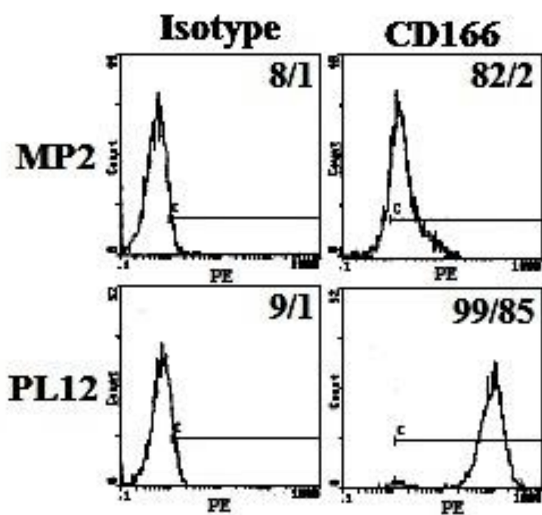


Fig. 1C

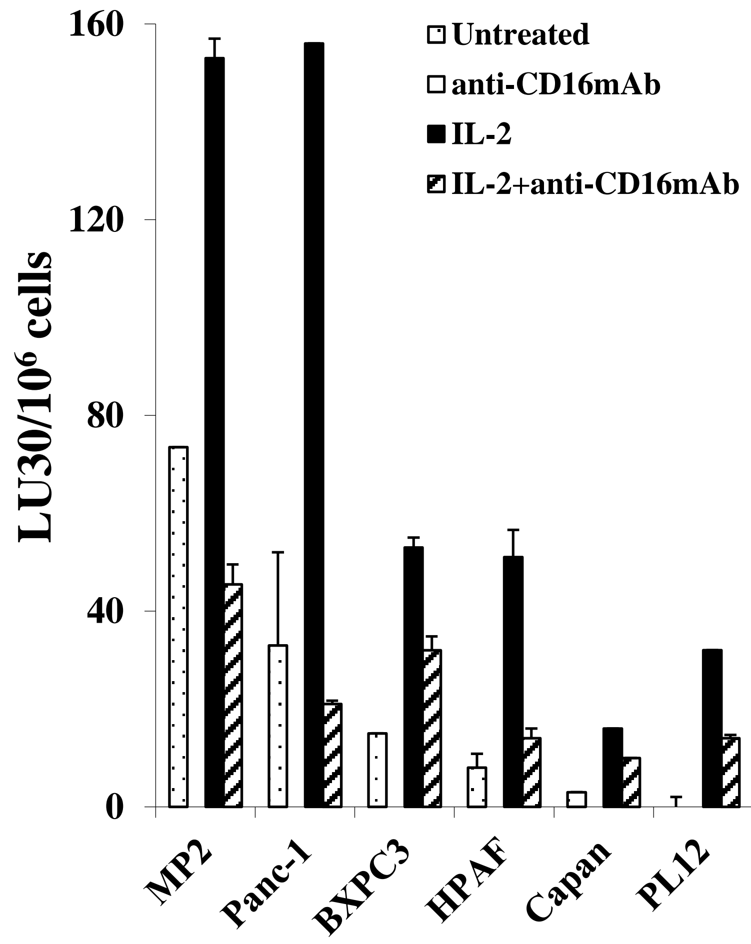


Fig. 1D

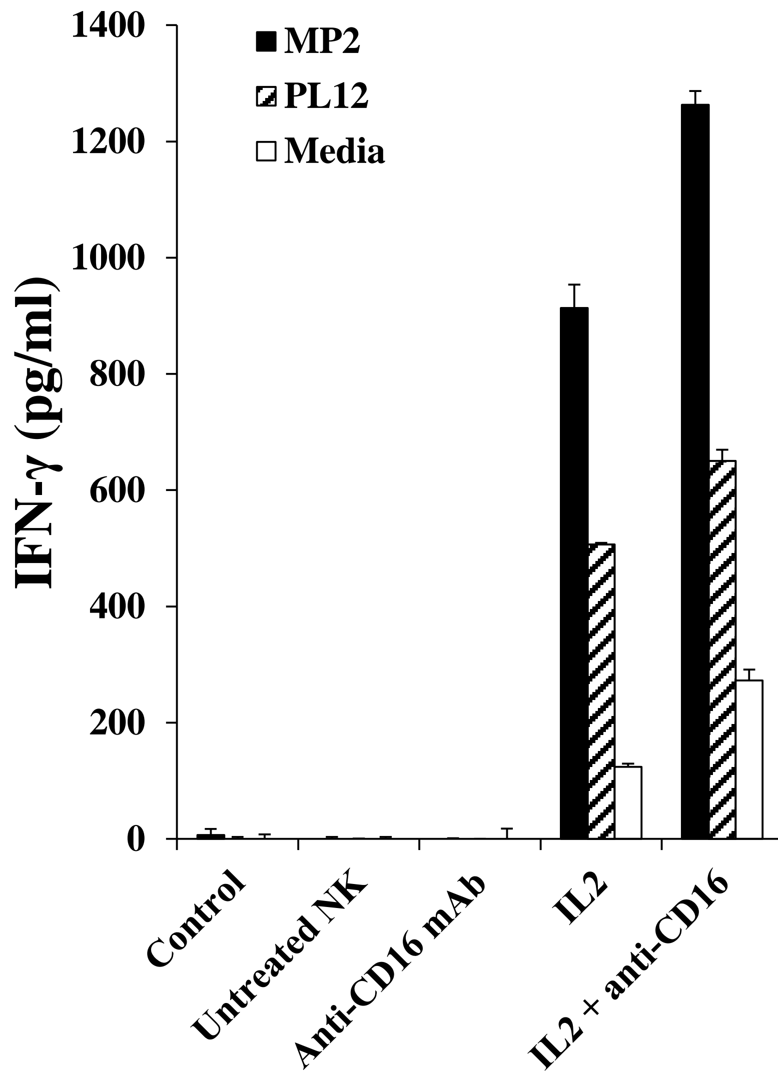


Fig. 2A

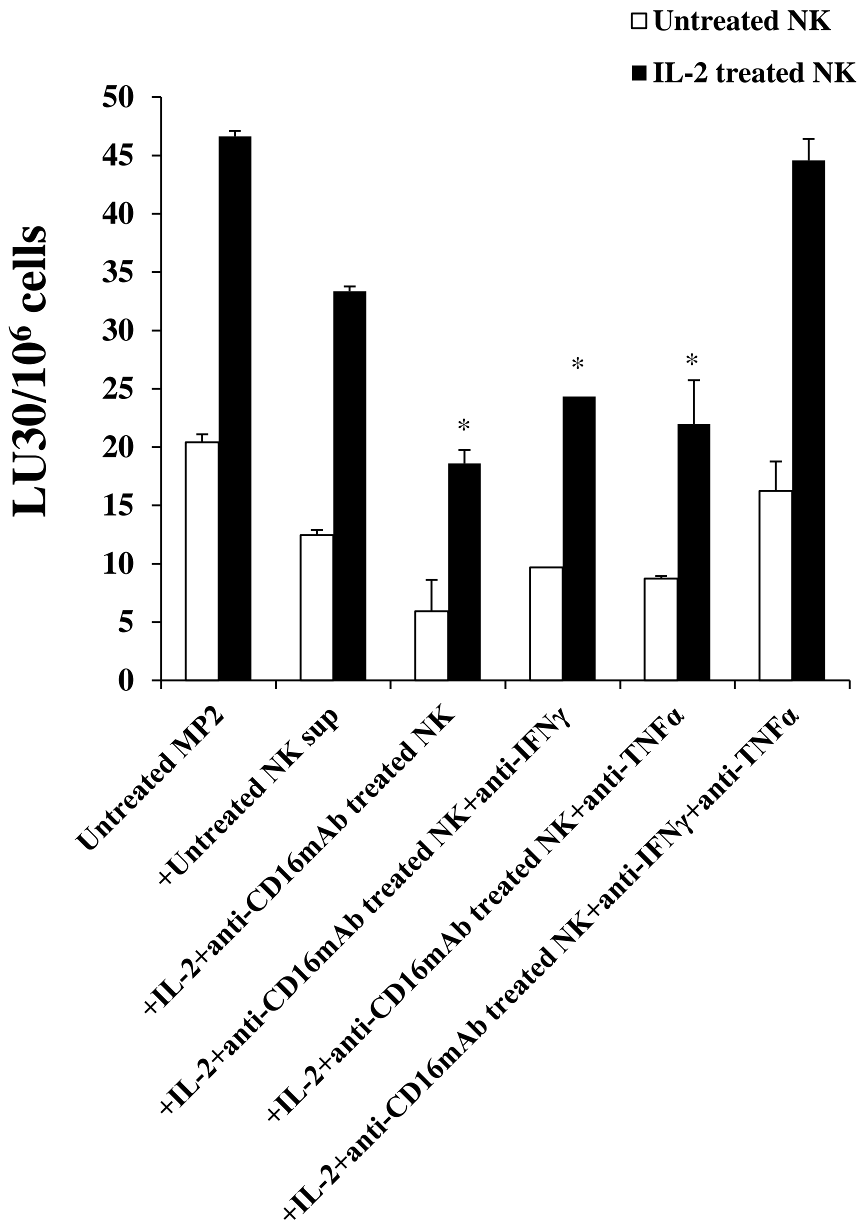


Fig. 2B

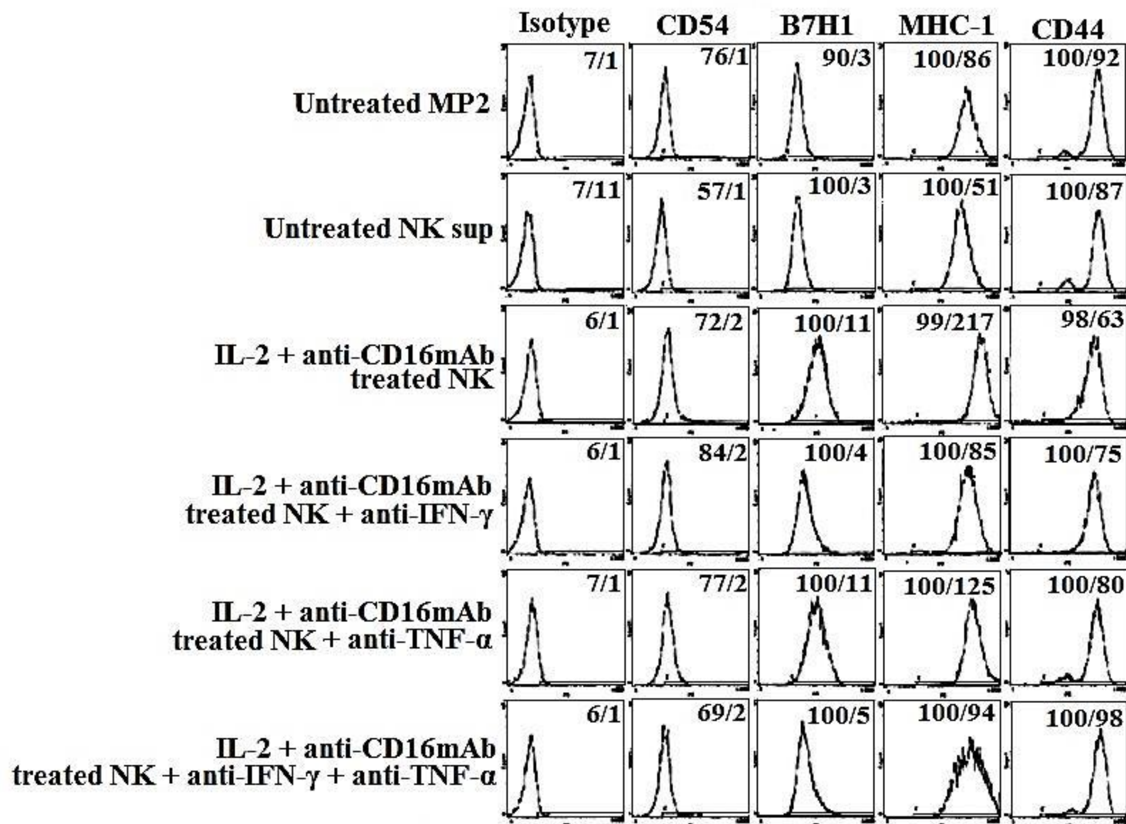
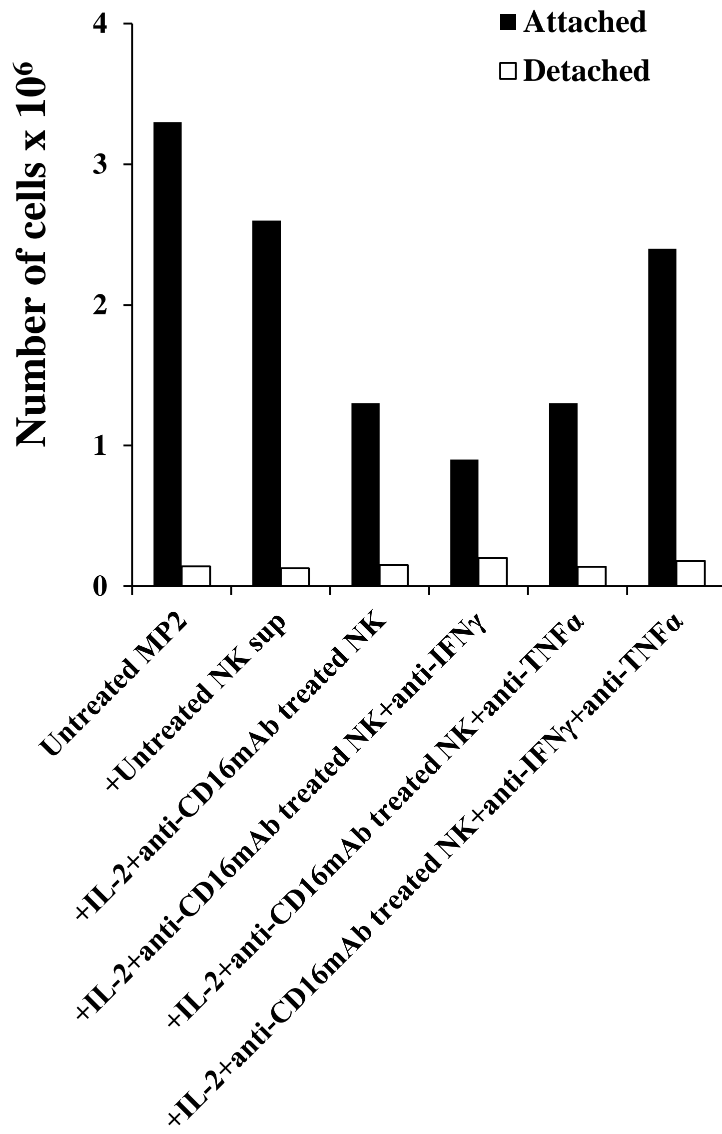


Fig. 2C



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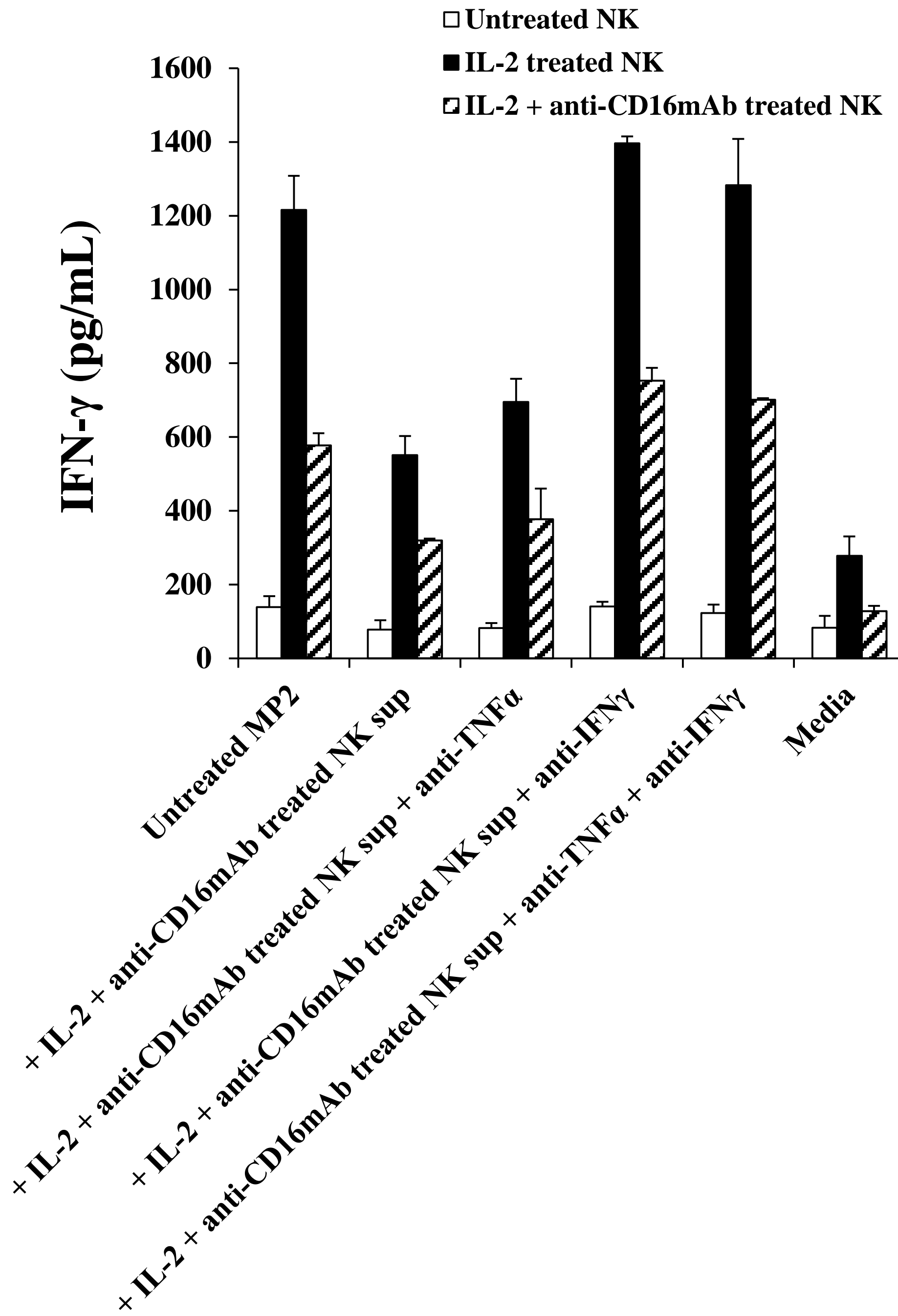

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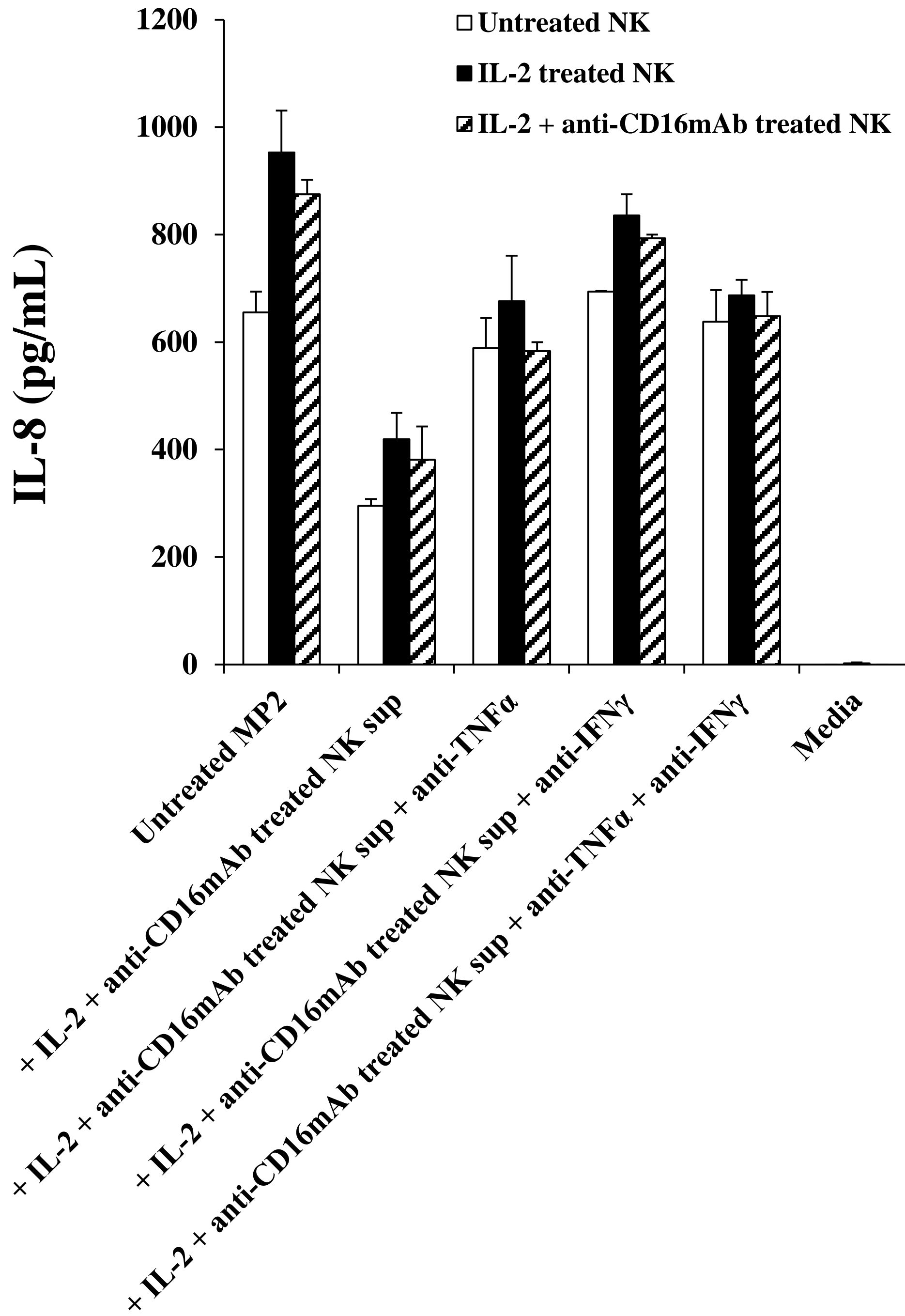

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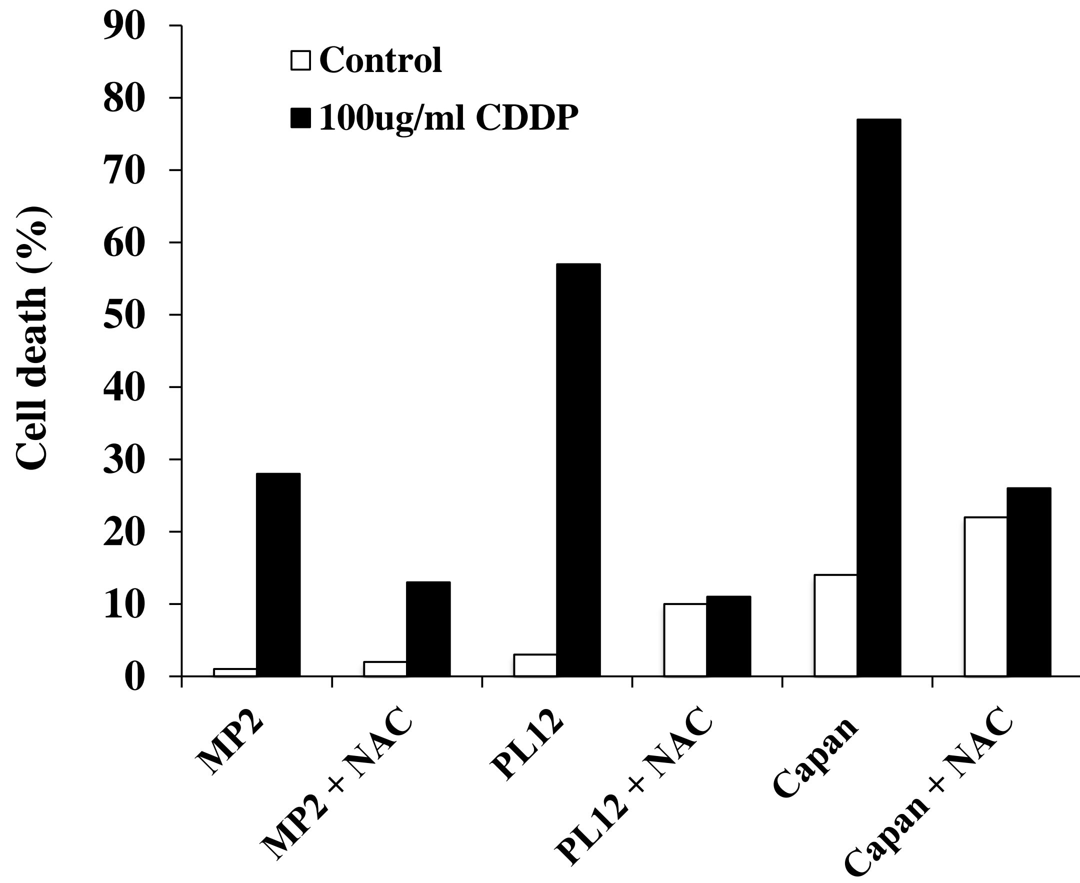
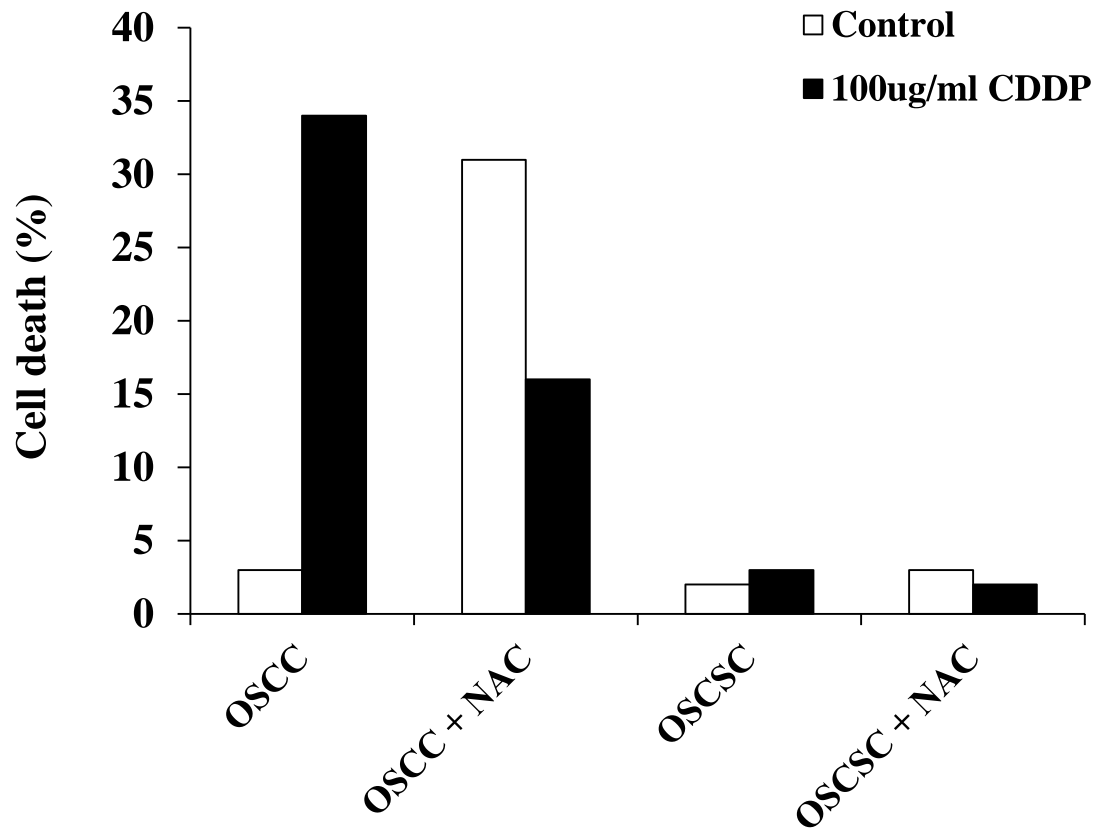


Fig. 4B



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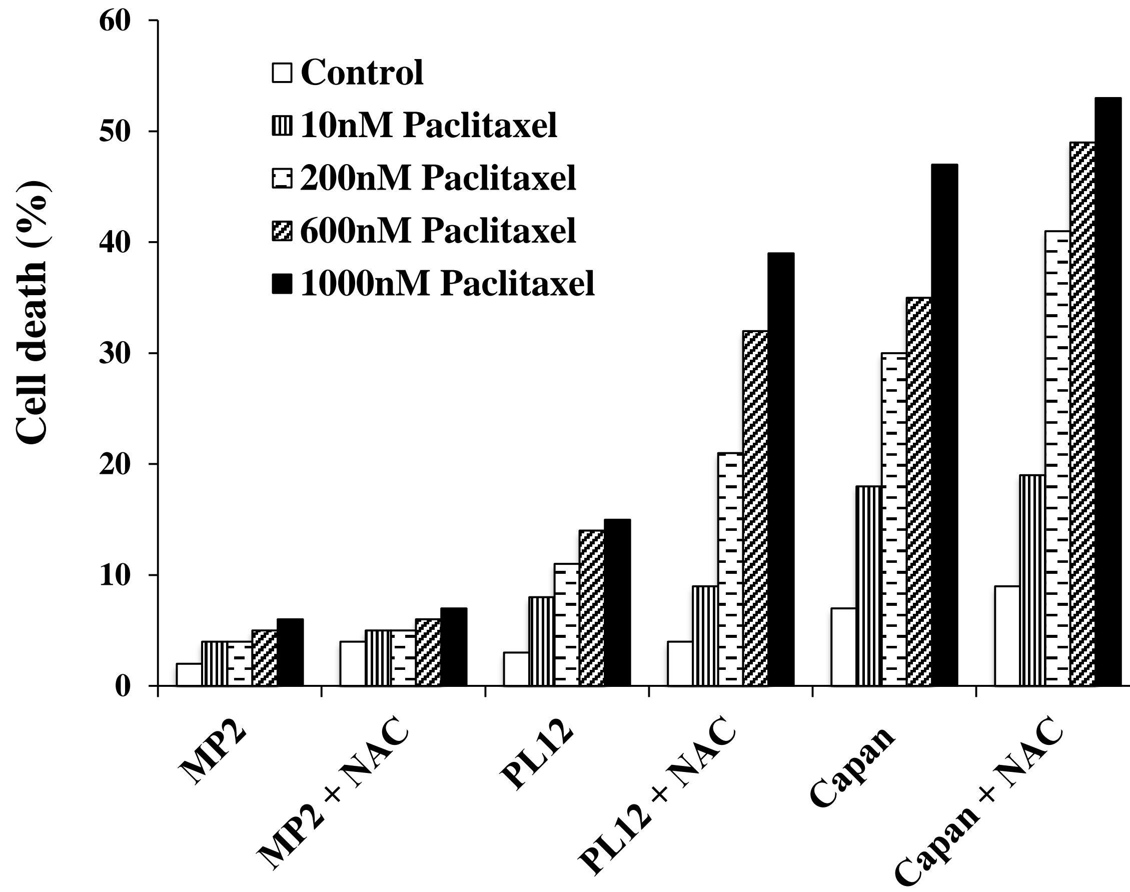
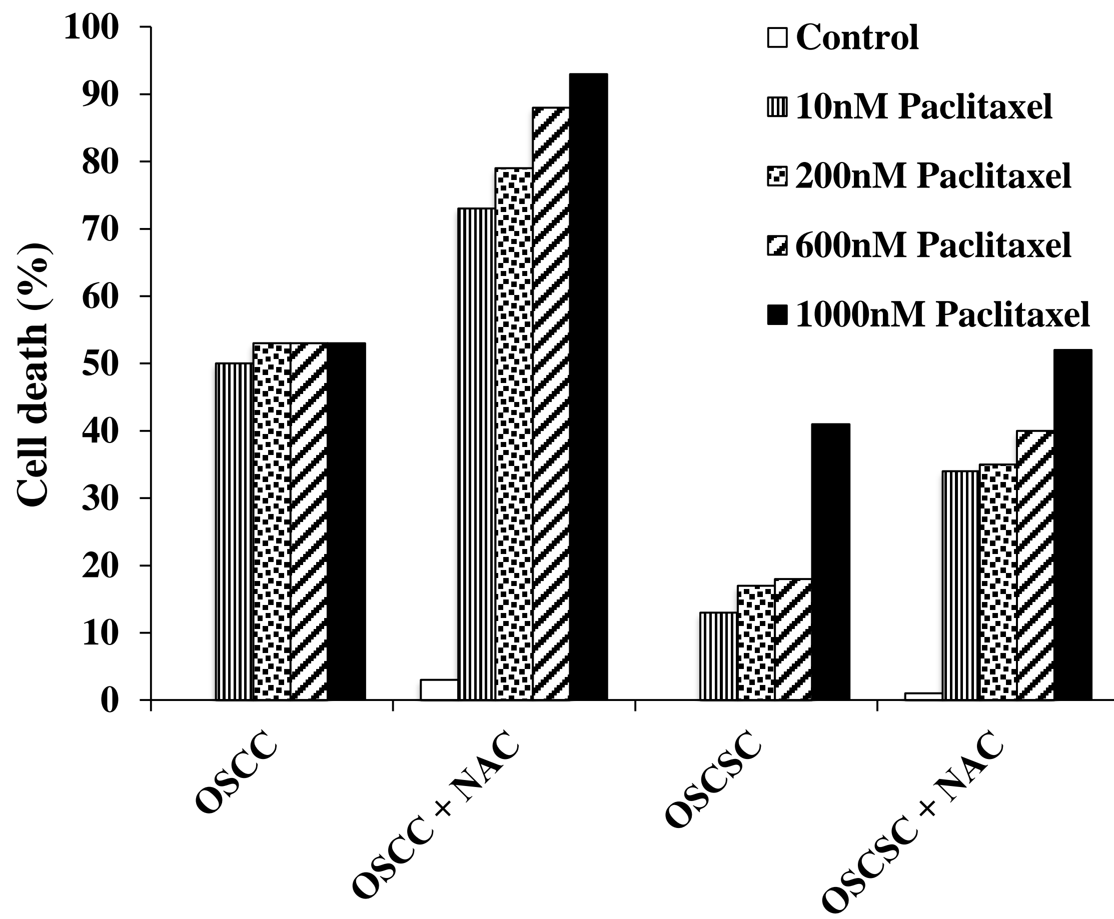


Fig. 4D



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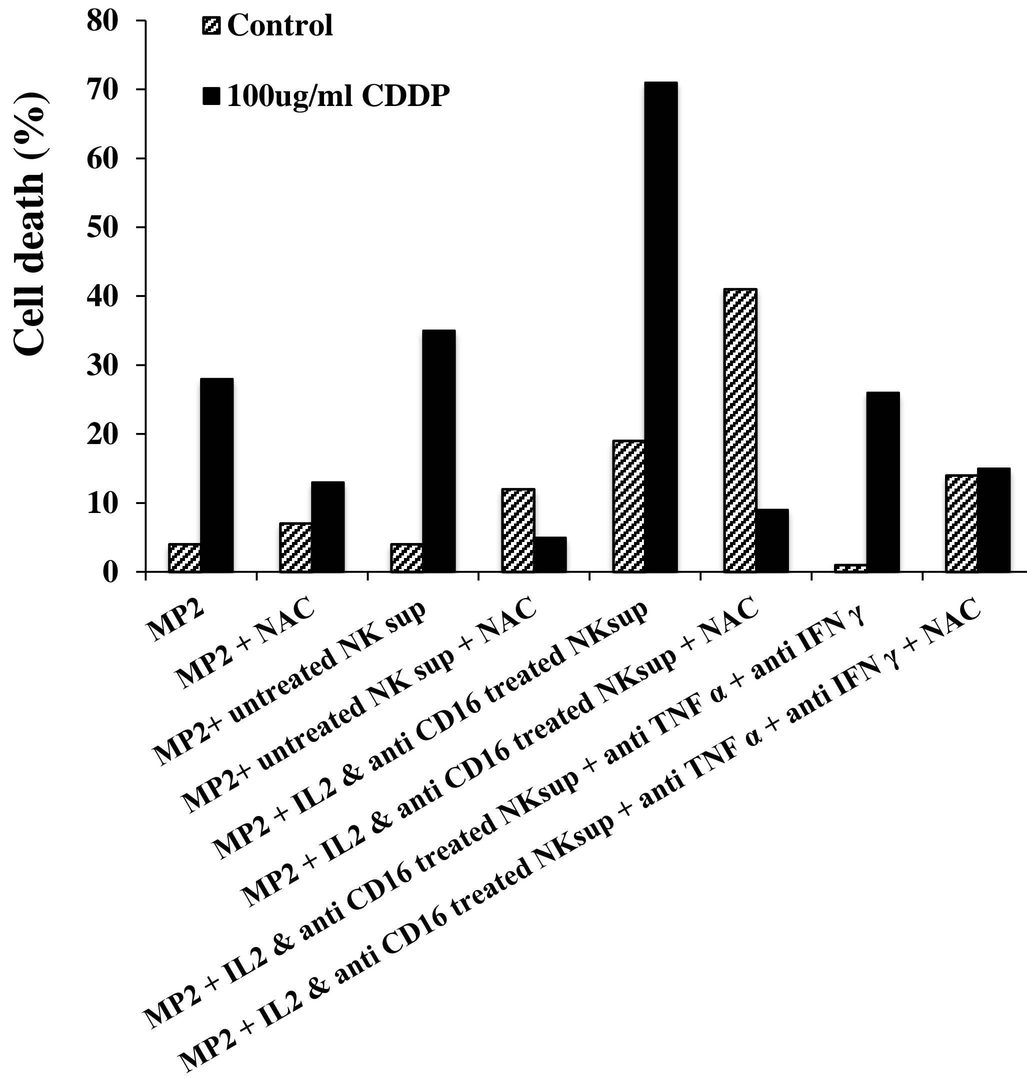


Fig. 4F

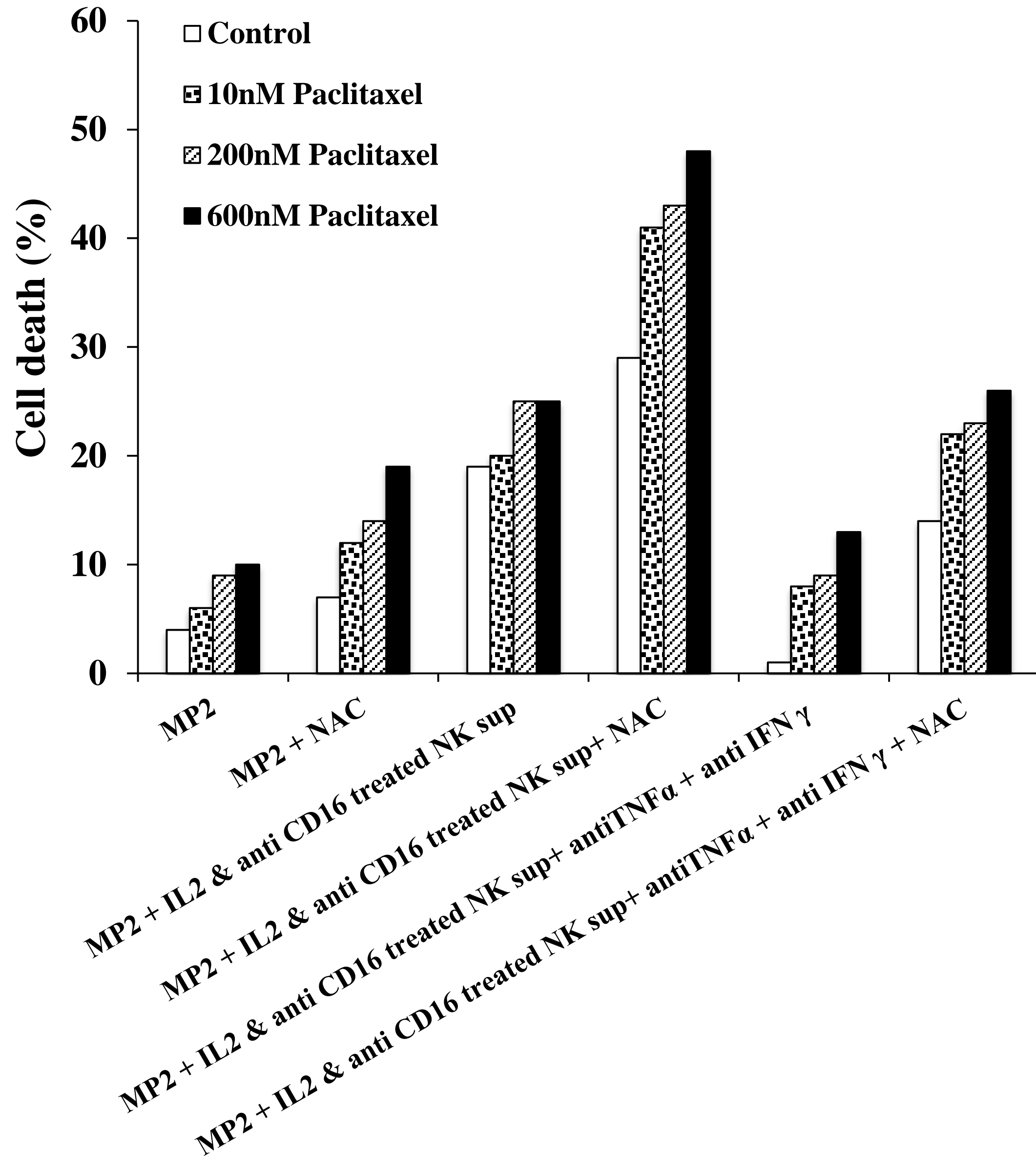


Fig. 5A

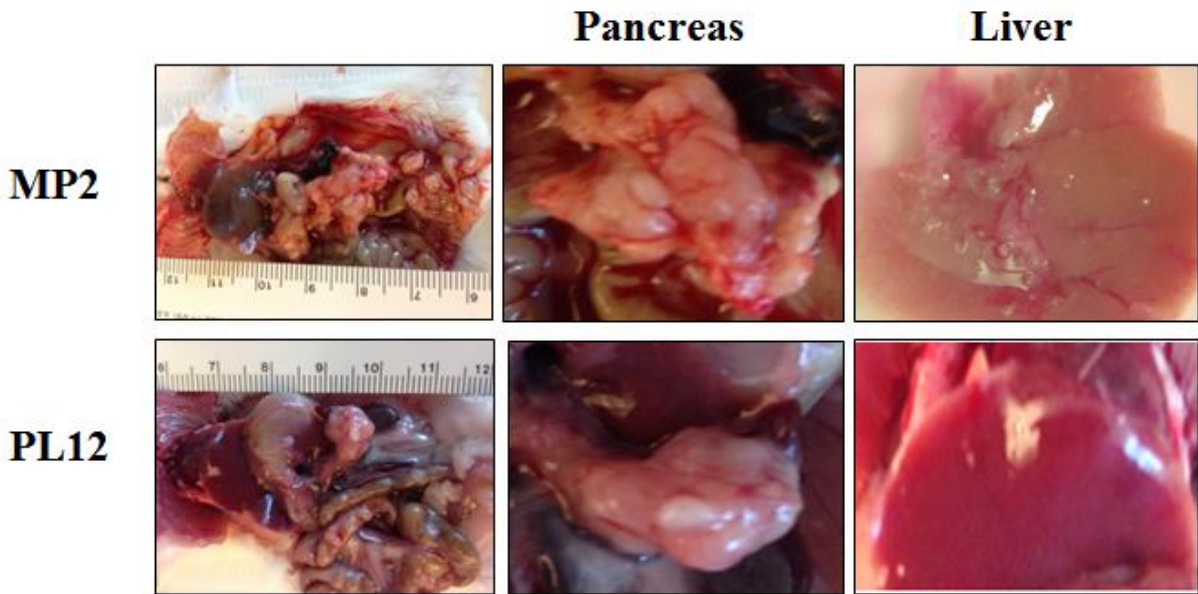
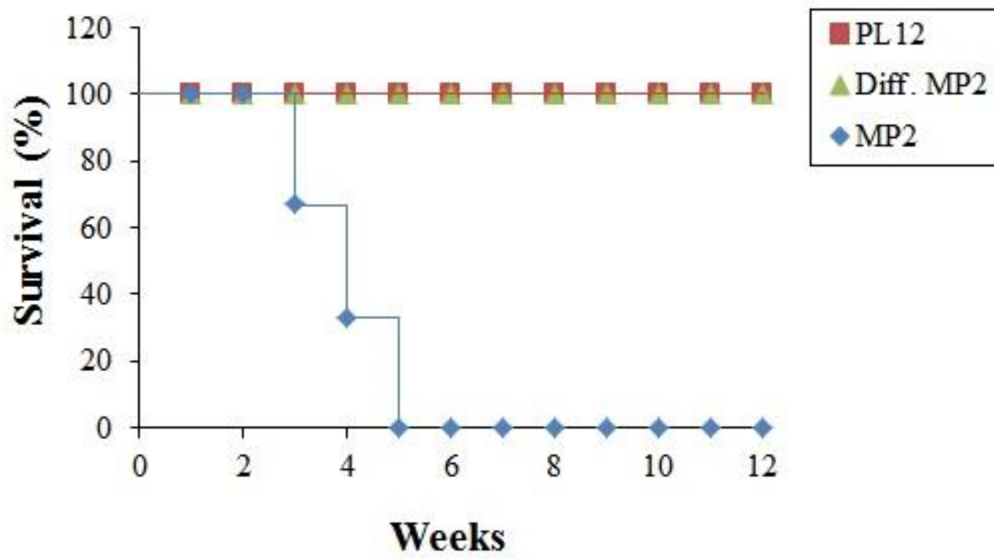


Fig. 5B



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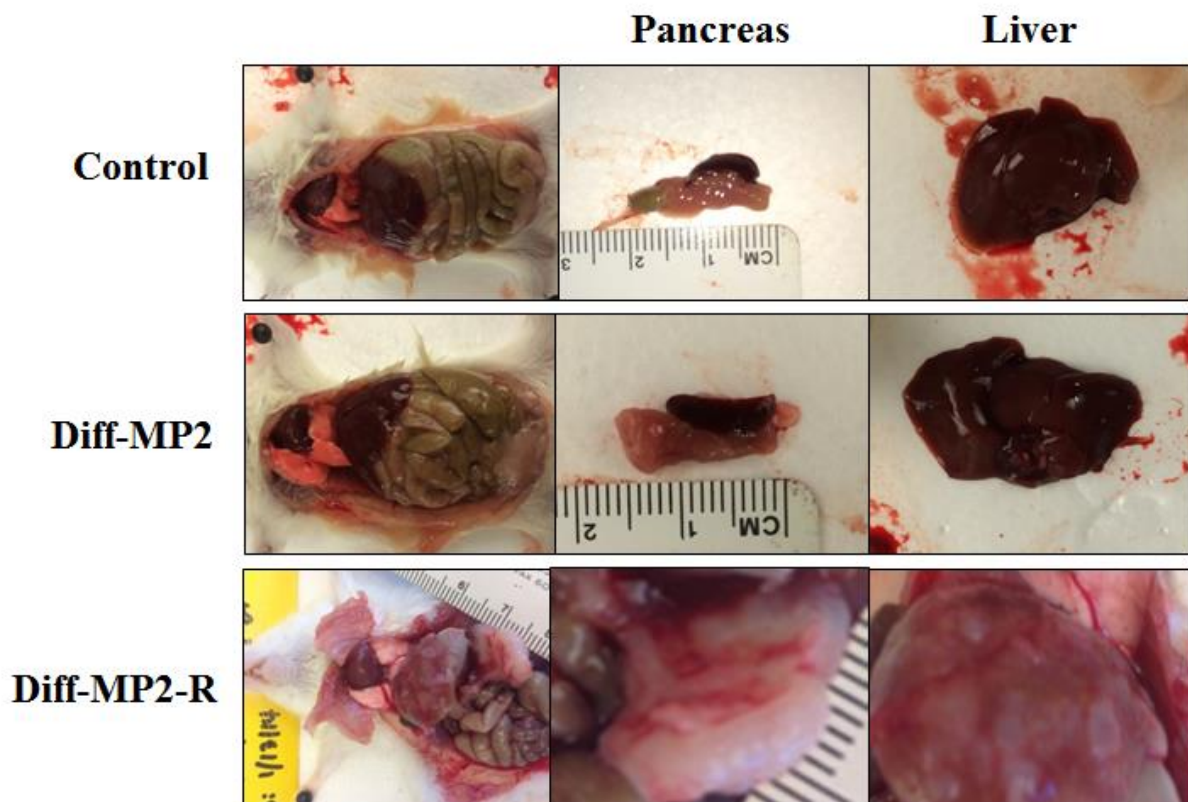
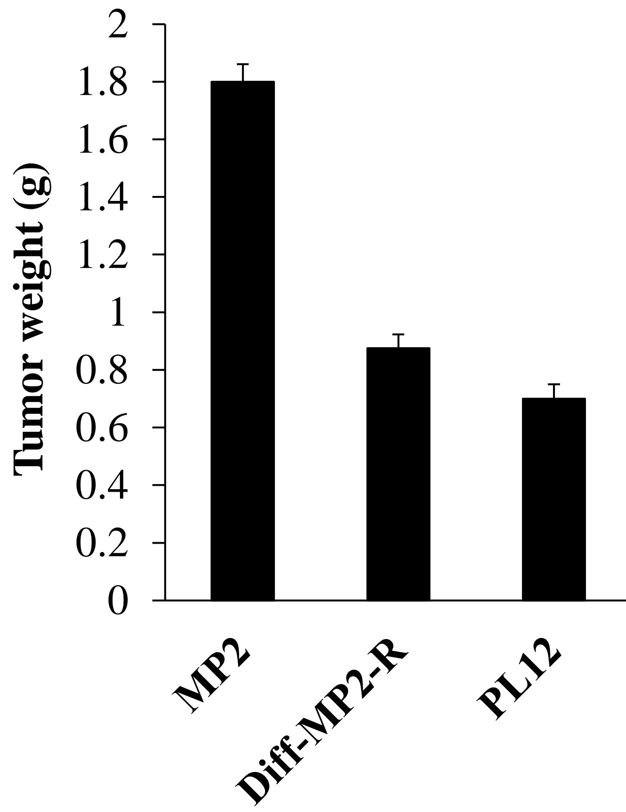


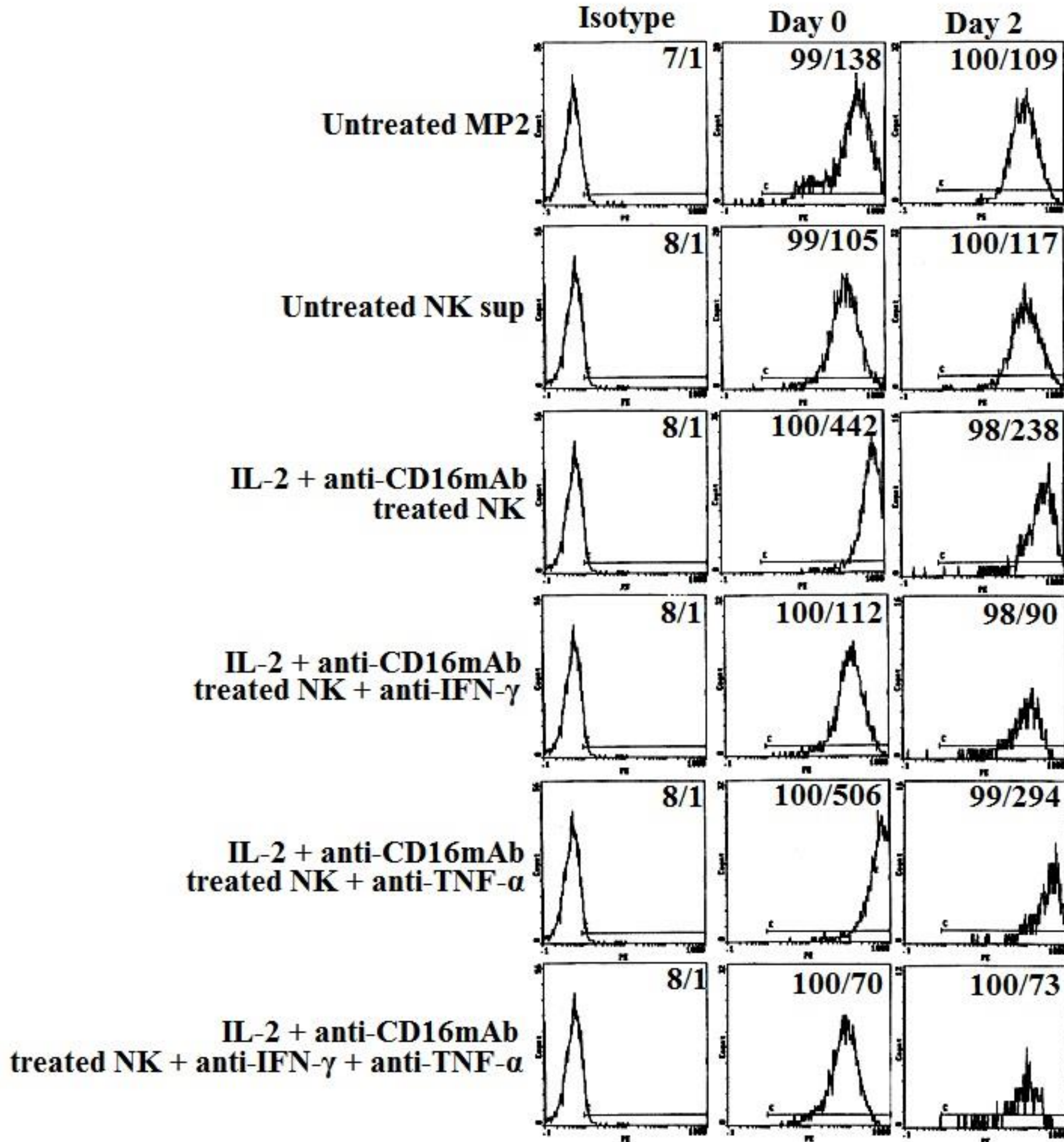
Fig. 5D



Supplemental Figure 1.

Fig. S1 Gradual decrease in MHC-I expression after the removal of IL-2+anti-CD16mAb treated NK cell supernatant from differentiated MP2 tumors

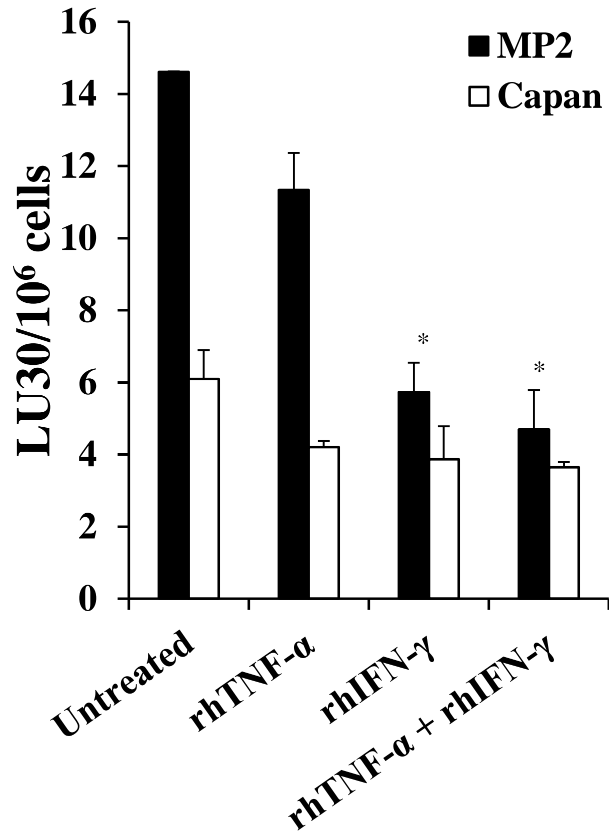
NK cells were purified from healthy donors and left untreated or treated with the combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 24 hours, after which supernatant was removed and added to MP2 tumor cells in the presence and absence of anti-TNF-α (1:100) and/or IFN-γ (1:100) for a period of 5 days. The surface expression of MHC class I on untreated MP2 cells and those treated with either untreated NK supernatant or those treated with IL-2+anti-CD16mAb treated NK supernatant in the presence of antibody blocking were assessed after PE-conjugation antibody staining using flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities in each histogram.


MHC-1
Isotype
Day 0
Day 2
Untreated MP2
7/1
99/138
100/109
Untreated NK sup
8/1
99/105
100/117
IL-2 + anti-CD16mAb treated NK
8/1
100/442
98/238
IL-2 + anti-CD16mAb treated NK + anti-IFN-γ
8/1
100/112
98/90
IL-2 + anti-CD16mAb treated NK + anti-TNF-α
8/1
100/506
99/294
IL-2 + anti-CD16mAb treated NK + anti-IFN-γ + anti-TNF-α
8/1
100/70
100/73

Supplemental Figure 2. Combination of rTNF-α and rIFN-γ induce differentiation and resistance of MP2 cells to NK cell mediated cytotoxicity

MP2 and PL12 cells were left untreated or treated with recombinant human TNF-α (10ng/ml), recombinant human IFN-γ (50U/ml) or the combination of human TNF-α (10ng/ml) and recombinant human IFN-γ (50U/ml) for 24 hours. Afterwards, the cells were detached from the tissue culture plates and labeled with 51Cr and used in a standard 4 hour chromium release assay against IL-2 (1000 units/ml) treated NK cells. Pre-treatment of NK cells with IL-2 were carried out for 18-24 hours. Percent cytotoxicity was obtained at different effector to target ratio and the lytic units 30/106 cells were determined using the inverse number of NK cells required to lyse 30% of the tumor cells X 100 (A). Surface expression of CD44, CD54, MHC Class I and B7H1 on MP2 (A) or PL12 (B) were determined using staining with PE-conjugated antibodies followed by flow cytometric analysis. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and mean channel fluoresence intensities in each histograom. One of minimum of three representative experiments is shown.

Fig S2A.



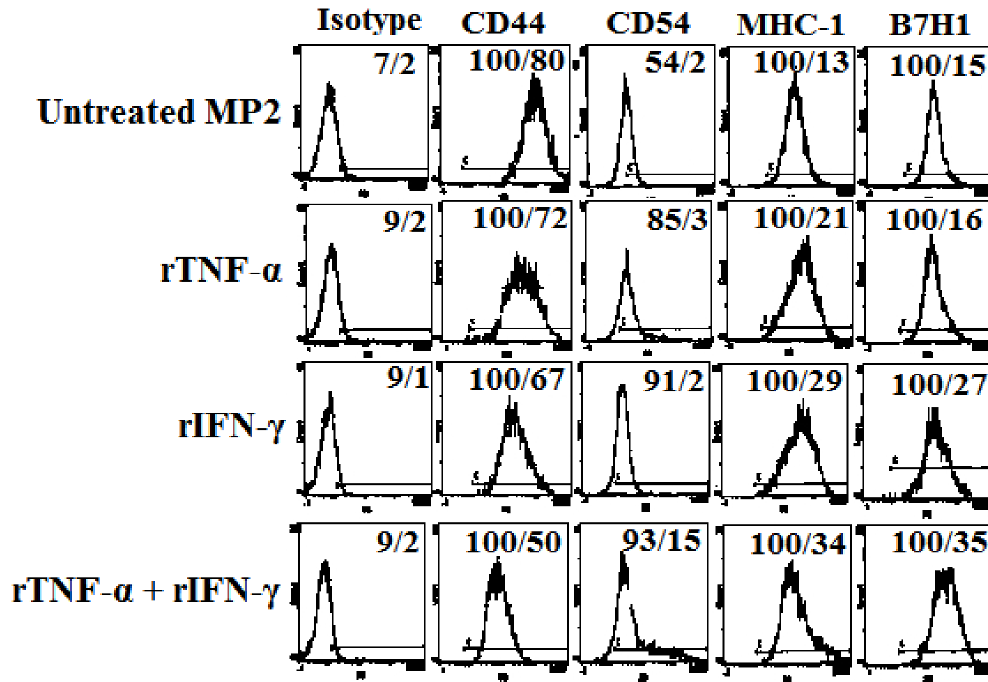
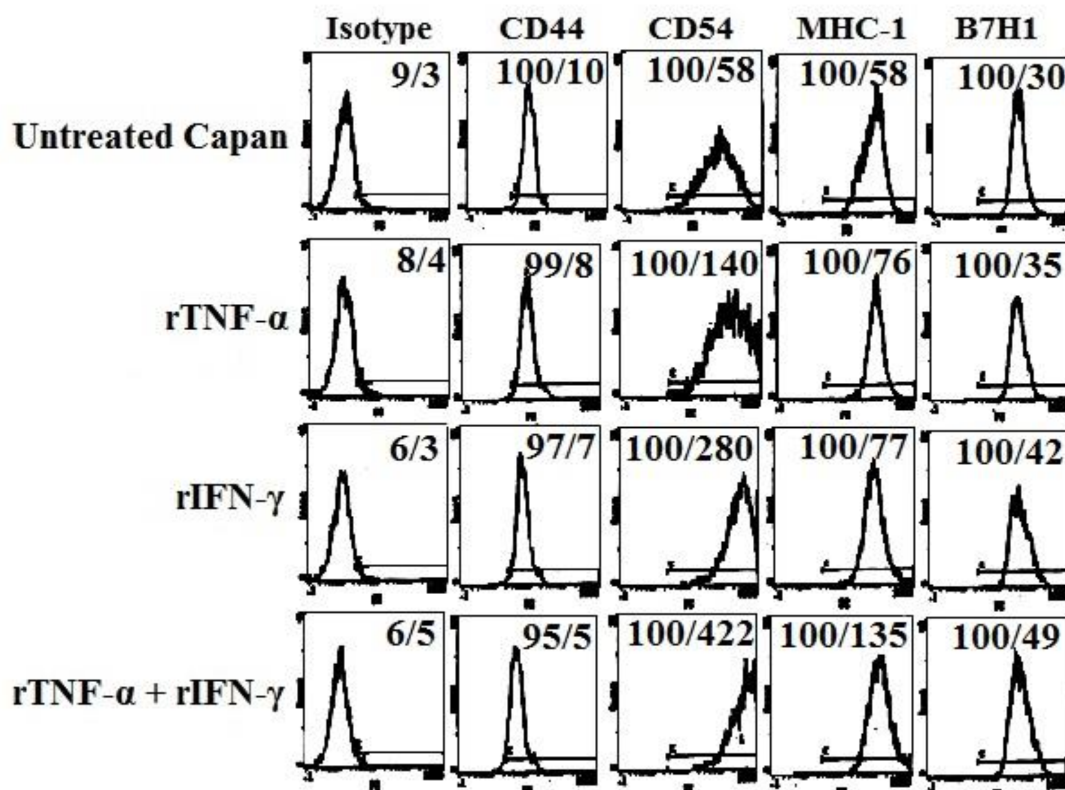


Fig. S2B



CHAPTER 7

Conditional COX2 knock out in mouse monocytes augments the cytokine production and cytotoxic function of Natural Killer cells

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Running Title: Myeloid COX2 knock out increases NK cells functions

Keywords: COX2, NK, IFN-γ, cytotoxicity, LPS

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Abstract

Cyclooxygenase (COX), which exists in two isoforms, COX-1 and COX-2, is responsible for the conversion of arachidonic acid to prostaglandins. COX-2 can be induced by pro-inflammatory cytokines and growth factors and studies have shown that aberrant expressions of COX-2 and its enzymatic product have strong influences in the development and progression of many cancer cell types. Previous studies have demonstrated that Nonsteroidal Anti-Inflammatory Drug (NSAID), which directly blocks the activities of COX, can reduce the growth rate, number and size of tumors. It has been demonstrated that Natural Killer (NK) cells obtained from cancer patients are "anergized" with a significant reduction in their ability to mediate cytotoxicity. Since COX-2 has been shown to be overexpressed and NK cells are inactivated in many tumor types, we aim to investigate the effects of COX-2 on NK cells in an *in vivo* system utilizing conditional knockout of COX-2 in myeloid cells. In this paper we show that NK cells obtained from COX-2 knockout animals have inherently higher cytotoxic function than cells from wildtype animals. We also demonstrate that COX-2 knockout monocytes, and not T cells, cultured with NK cells can heighten the NK mediated cytotoxicity and IFN-γ production. The addition of Lipopolysaccharide (LPS) causes significant level of split anergy in NK cells which results in a loss of NK cell mediated lysis and an increase in IFN-γ by NK cells.

Introduction

The prostanoids, which include prostaglandins, prostacyclins and thromboxanes, modulate several important physiological and pathophysiological processes such as gastric mucosal integrity [278, 279], vasodilation [280, 281], allergic response [282, 283], platelet adhesion and aggregation [284, 285], wound healing [286-288], water balance [289] and much more. Cyclooxygenase (COX) is the key enzyme required to convert arachidonic acid to prostaglandin H2 to generate prostanoids. COX exists in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues at a consistent level and dysregulation results in gastric damage, bleeding and ulceration [290, 291]. Under normal, healthy circumstances, the COX-2 activity is low and upon induction by growth factors and pro-inflammatory cytokines, the protein expression can be up- or down-regulated within hours [292, 293].

The involvement of COX-2 in tumor development and progression has been demonstrated in numerous cancer types. Over expression of COX-2 in human gastric cancer associates with metastasis and depth of invasion which negatively correlates with survival rate of patients [294-296]. Increased expression of COX-2 is observed in breast cancer as compared to surrounding normal tissues [297]. Piroxicam, a Nonsteroidal Anti-Inflammatory Drug (NSAID) which blocks COX activity, was shown to reduce the growth rate of human cultured breast cancer cells [298]. The administration of a different NSAID, Sulindac sulfide, resulted in a significant decrease in the number of cancer incident within the experimental group and number of tumors per animal was observed in rats with mammary carcinoma [299]. A study by Narko et al. demonstrated that with the use of a selective COX-2 inhibitor, Celecoxib, the number of tumors, size, multiplicity of mammary tumors were reduced in mice with COX-2 overexpression

[300]. The use of Sulindac sulfide in a clinical trial has also demonstrated its efficacy in reducing the number and size of colorectal polyps that can eventually become cancerous in patients with familiar adenomatous polyposis (FAP) [301]. A complementary mouse model of FAP in humans has shown to have reduced number of spontaneous polyposis with either a selective COX-2 inhibitor or by the deletion of the COX-2 gene [302]. COX-2 deletion in pdx1+ pancreatic progenitor cells significantly delays the development of pancreatic ductal adenocarcinoma in mice [303]. These data collectively suggests that COX-2 plays an important role in the progression of cancer expansion and ectopic expression may result in tumor development.

Natural Killer (NK) cells are lymphocytes that arise from the bone marrow and are capable of mediating direct natural cytotoxicity and Antibody-Dependent Cellular Cytotoxicity (ADCC). NK cells are identified by the expression of CD16 and CD56 and the lack of CD3 on the cell surface. NK cells mediated immediate cytotoxicity against a variety of malignant tumors, virally infected cells, as well as healthy untransformed undifferentiated cells [18, 304]. Many mechanisms have been proposed for the functional inactivation of NK cells, such as decreased CD16 and its associated zeta chains [305], over-expression of Fas-ligand and the loss of mRNA for granzyme B [176]. It has been previously demonstrated that NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [146, 147, 183, 306]. Reports have also shown that prostaglandin E2 (PGE2), which is regulated by COX-2, greatly compromises the capability of NK cells to exert cytotoxicity against tumor cells, produce cytokines and migrate [307, 308]. Immune suppression seen in NK cells could be avoided by the inhibition of PGE2 or the PGE2 receptor, which was shown to greatly diminish metastasis [307].

Our laboratory has coined the term “split anergy” as a phenomenon in which NK cells suffer a significant loss of cytotoxicity with an augmented ability to secrete cytokines [17, 41-43, 46, 255, 309]. Split anergy could be initiated by the receptor triggering of CD16, NKp46, but not NKp30 or NKp44, toll-like receptors and interaction with both healthy and transformed stem-like cells, other immune effectors such as fibroblasts and myeloid-derived suppressor cells [45, 310-312] (manuscript submitted). Our laboratory has extensive data demonstrating that the addition of lipopolysaccharide (LPS) as well as both gram-negative and –positive bacteria to NK cell culture can initiate split anergy in NK cells (manuscript in prep).

Several cancer types such as breast [313], lung [314], gastric [315], melanoma [316] and head and neck [317] demonstrate constitutive activation of Nuclear Factor kappa B (NFkB) and have shown to be a critical contributor to the development and metastasis of these cancers. The utilization of an IkB super-repressor of NFkB in HEp2 cells, a cell line commonly used as an oral tumor model, blocked tumor-induced NK cell death and significantly increased the function of NK cells which correlated with an increased expression of CD69, an early activation antigen on NK cells. By inhibiting NFkB from translocating into the nucleus, an augmented proliferation and increased secretion of IFN-γ by NK cells were observed [158]. Furthermore, targeted inhibition of NFkB in both the intestinal epithelial cells and the myeloid cells were shown to decrease the numbers as well as the size of tumor cells [318]. The addition of Sulindac to HEp2 cells was shown to inhibit NFkB and increase the functions of NK cells and dendritic cells [151].

STAT3, another important transcription factor crucial for cell growth, differentiation and apoptosis, has been shown to be phosphorylated in HER2-overexpressing human breast tumors

which associates with tumorigenesis and drug resistance [319]. In addition, STAT3 was shown to directly upregulates toll like receptors (TLR)-2 and in turn promotes gastric tumorigenesis *in vivo* [320]. By blocking STAT3 in hepatocellular carcinoma cells, an increase in NK cell mediated cytotoxicity was observed. It has also been reported that inactivation of STAT3 favors T cells and NK cells mediated anti-tumor response against breast cancer [321] and colorectal cancer cell line [162]. These data collectively suggest that the inhibition of important transcription factors, such as NFkB and STAT3, results in an increased cytotoxic function of NK cells and may contribute to the elimination of tumors. This paper aims to elucidate the connection between COX-2 and NK cells and to understand the role of COX-2 in NK cell mediated cytotoxicity in an *in vivo* system.

Materials and Methods

Mice

Myeloid cell specific *Cox-2* conditional knockout mice were used for this study [322]. All animals were kindly provided by Dr. Harvey Herschman (UCLA Department of Molecular and Medical Pharmacology). Animal experiments were carried out with Animal Research Committee approval at UCLA.

Cell lines, reagents, and antibodies

RPMI 1640 supplemented with 10% Fetal Bovine serum (FBS) was used for the cultures of human NK cells and monocytes and mouse NK cells, T cells monocytes and dendritic cells. RPMI 1640 supplemented with 10% FBS was also used to culture mouse T cell lymphoma (YAC-1). ST63 cells were cultured in DMEM supplemented with 10% FBS. Primary mouse tongue keratinocytes (MOK) were cultured in keratinocyte serum free medium containing 0.1% gentamicin and 1% antibiotic-antimycotic. COX2 wildtype and COX2 knock out Mouse Embryonic Fibroblasts (MEF) were kind gifts from Dr. Harvey Herschman and the cells were cultured in DMEM supplemented with 10% FBS [322]. Oral Squamous Cancer Stem cells (OSCSCs) were isolated from the tongue tumors of the patients at UCLA and cultured in RPMI 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1.4% antibiotic antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine, 0.2% gentamicin (Gemini Bio-Products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, PA). Human Dental Pulp Stem Cells (DPSCs) were isolated as previously described [323] and they were cultured in DMEM complete medium supplemented with 2% FBS and 1% penicillin and streptomycin

(Gemini Bio-Products, CA). Lipopolysaccharides (LPS) were purchased from Sigma-Aldrich (St. Louis, MO) and a final concentration of 20ng/mL was used. IL-4 and GM-CSF were purchased from Biolegend (San Diego, CA) and used to differentiate purified monocytes into dendritic cells. The mouse and human NK cells, T cells and monocyte purification kits were obtained from Stem Cell Technologies (Vancouver, Canada). Recombinant IL-2 was obtained from NIH- BRB. Antibody for CD16 was purchased from eBioscience (San Diego, CA).

Purification of human NK cells and monocytes

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors and all the procedures were approved by the UCLA-IRB. NK cells from healthy donors were isolated as described before [137]. Briefly, peripheral blood lymphocytes were obtained after Ficoll-hypaque centrifugation and purified NK cells were negatively selected by using an NK cell isolation kit (Stem Cell Technologies, Vancouver, Canada). The purity of NK cell population was found to be greater than 90% based on flow cytometric analysis of anti-CD16 antibody stained cells. The levels of contaminating CD3+ T cells remained low, at 2.4%±1%, similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures. The adherent subpopulation of PBMCs was detached from the tissue culture plates and monocytes were purified using isolation kit obtained from Stem Cell Technologies (Vancouver, Canada). Greater than 95% purity was achieved based on flow cytometric analysis of CD14 antibody stained monocytes.

Mouse NK cells, T cells, monocytes and dendritic cell cultures

All animal work performed was based on the guidelines established and approved by

UCLA Office of Animal Research Oversight (Protocol: 2009-125-01). Single cell preparations of mouse splenocytes were used to negatively select for mouse NK cells using mouse NK isolation kit purchased from Stem Cell Technologies (Vancouver, Canada). The purity of mouse NK cells were greater than 90% based on staining with PE conjugated NK1.1 and CD49b antibodies. NK cells were treated with IL-2 ($1X10^4$ U/million NK cells) for 7 days before the cells were used for experiments. T cells were purified using mouse T cell isolation kit purchased from Stem Cell Technologies (Vancouver, Canada). Bone marrow cells were isolated by flushing femurs with PBS supplemented with 2% heat-inactivated FBS. Murine monocytes were then purified from bone marrow cells using monocyte isolation kit obtained from Stem Cell Technologies (Vancouver, Canada). The purity of monocytes was greater than 90% based on staining with PE conjugated anti-CD14 antibody. To differentiate mouse dendritic cells from purified monocytes, IL-4 (20ng/mL) and GM-CSF (20ng/mL) were added to monocytes for 7 days.

ELISA

Single ELISAs were performed as described previously [137]. To analyze and obtain the cytokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines provided by the manufacturer.

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [151]. Briefly, different numbers of purified NK cells were incubated with ^{51}Cr–labeled target cells. After a 4 hour incubation period the supernatants were harvested from each sample and counted for released

radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}}$$

LU $30/10^6$ is calculated by using the inverse of the number of effector cells needed to lyse 30% of tumor target cells X100.

Statistical analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

Results

Targeted inhibition of COX-2 in bone marrow derived monocytes from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice increased NK cell cytotoxicity

Purified NK cells obtained from spleens of control mice (*Cox-2$^{flox/flox}$ LysM$^{+/+}$*) and those with targeted knock down of COX-2 gene in myeloid cells (*Cox-2$^{flox/flox}$ LysM$^{Cre/+}$*) were left untreated or treated with IL-2 for 7 days before the NK cells were used in a standard ^{51}Cr release assay against YAC-1 cells (A), Mouse Embryonic Fibroblasts (MEFs) (B) and ST63 cells (C). As shown in figure 1A, NK cells purified from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice lysed YAC-1 cells significantly more than NK cells from control mice which had no/low cytotoxicity. Untreated NK cells from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice were also able to lyse YAC-1 cells more than NK cells from control animals, although the cytotoxicity levels were significantly lower than IL-2 treated cells. In addition, IL-2 treated NK cells from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice mediated high cytotoxicity against MEFs (B) and ST63 cells (C) as compared to the control animals.

NK cells obtained from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice cultured with autologous monocytes mediated significantly higher level of cytotoxicity than control animals and those without monocytes.

Purified NK cells from control mice and *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice were cultured with or without bone marrow derived purified monocytes for 6 days before the cells were used in a standard 4 hour ^{51}Cr release assay against YAC-1 (A) MOK (B). As shown in Fig. 2A, IL-2 treated NK cells from control animals cultured with monocytes mediated slightly higher cytotoxicity compared to those NK cells cultured alone without monocytes. The difference in

cytotoxicity is more apparent in *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals. NK cells purified from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ mice and cultured with autologous monocytes lysed YAC-1 cells significantly more (4.4 fold) compared to NK cells cultured alone without monocytes. Untreated NK cells with or without monocytes mediated little or no cytotoxicity against YAC-1 cells. NK cells cultured with monocytes from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals also exhibited 1.5 fold increase in cytotoxicity against MC38 cells as compared to wildtype NK cells cultured with monocytes (B).

NK cells purified from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals cultured with autologous monocytes produce significantly higher IFN-γ compared to NK cell cultured alone without monocytes

Purified NK cells obtained from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals were cultured with or without autologous monocytes for 6 days and after which the supernatant was collected and the level of IFN-γ produced by NK cells were measured with specific ELISA. Untreated NK cells were used as controls and as shown in figure 3, no IFN-γ was produced as expected. IL-2 treated NK cells from both wildtype and *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals produced minimal levels of IFN-γ although *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals produced slightly higher levels of IFN-γ. Significant levels of IFN-γ could be observed when NK cells and monocytes from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ mice were co-cultured whereas no increase in IFN-γ secretion was seen with control animals.

Monocytes from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$animals cultured with autologous NK cells, and not NK cells from control animals, enhance the NK cell mediated cytotoxicity and IFN-γ secretion

NK cells purified from either wildtype or *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals were co-cultured with wildtype monocytes or monocytes with the COX-2 gene knocked out. NK cells and monocytes were co-incubated for 6 days before the NK cells were used as effector cells in a standard 4 hour ^{51}Cr release assay against YAC-1 cells (Fig. 4A) and the supernatants were removed at the end of the co-culture period and the levels of IFN-γ were measured with specific ELISA kit (Fig. 4B). As shown previously in figures 1 and 2 and here, mouse NK cells require several days of IL-2 stimulation to acquire cytotoxic function as compared to Human NK cells which only takes a few hours (Fig. 4A). The cytotoxic function of NK cells obtained from wildtype animals remains unchanged when cultured with either wildtype monocytes or COX-2 knockout monocytes (Fig. 4A). On the other hand, NK cells derived from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals mediated significantly higher levels of lysis against YAC-1 cells when cultured with monocytes with COX-2 gene knocked out and not monocytes from wildtype animals (Fig. 4A).

IL-2 treated NK cells from both wildtype and *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals secreted no detectable IFN-γ (Fig. 4B). Significant amounts of IFN-γ were detected only when NK cells were co-incubated with monocytes for 6 days. Co-culture between monocytes and NK cells both obtained from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$*animals resulted in significant amounts of IFN-γ as compared to NK cells co-culture with monocytes from wildtype animals. NK cells from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals cultured with wildtype monocytes were also able to trigger more IFN-γ than both NK cells and monocytes were from the control animals (Fig. 4B).

Co-culture with monocytes, and not T cells, increases the cytotoxic function of NK cells and IFN-γ secretion

NK cells, T cells and monocytes were purified from either control animals or *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals. NK cells were treated with IL-2 and cultured alone, with T cells or monocytes for 6 days. Afterwards, T cells and monocytes were removed from culture and complete removal was confirmed with microscopy and NK cells were used as effector cells against wildtype and COX-2-/- MEF in a standard ^{51}Cr release assay (Fig. 5A). The cytotoxic function of NK cells from control mice was minimal against both wildtype and COX-2$^{-/-}$ MEF and the addition of either T cells or monocytes also from control mice did not increase the cytotoxicity. NK cells co-cultured with monocytes obtained from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals increased the cytotoxicity of NK cells significantly, while the addition of T cells from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals with NK cells did not have an effect on the cytotoxicity (Fig. 5B). The addition of T cells to NK cell culture also did not affect the levels of IFN-γ secreted by NK cells, whereas monocytes triggered significantly more IFN-γ production by the NK cells.

COX-2 gene deletion in Mouse Embryonic Fibroblasts (MEF) resulted in a significant susceptibility to NK cell mediated lysis

Purified NK cells obtained from spleens of control mice and *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ mice were treated with IL-2 for 7 days and cultured with or without monocytes. Afterwards, complete removal of monocytes was confirmed with microscopy and NK cells were used as effectors cells in a standard ^{51}Cr release assay against wildtype and COX-2 knock out MEF. As shown in figure 6, NK cells from controls animals mediated minimal levels of cytotoxicity against COX-2$^{-/-}$ MEF and complete resistance was observed by the wildtype MEF. The pre-incubation with monocytes

did not alter the cytotoxic function of the NK cells from control animals against MEF. NK cells obtained from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals cultured with autologous monocytes have heightened cytotoxic function against COX-2$^{-/-}$ MEF as well as wildtype MEF which is resistance to NK cells cultured with monocytes from control animals.

Monocytes derived dendritic cells from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals are more susceptible to NK cell mediated cytotoxicity than dendritic cells from control animals

Since COX-2 gene deletion in mouse embryonic cells rendered the cells to become more susceptible to NK cell mediated lysis, we next wanted to test if COX-2 deletion in dendritic cells would also result in increased sensitivity to NK cells (Fig. 7). Dendritic cells were differentiated from purified monocytes with the addition of IL-4 and GM-CSF for 7 days. Fully differentiated dendritic cells from control or *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals were labeled ^{51}Cr and used as targets in a standard ^{51}Cr release assay against IL-2 treated NK cells alone or pre-incubated with monocytes. As predicted, dendritic cells differentiated from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ monocytes were more sensitive to NK cell mediated lysis as compared to dendritic cells differentiated from control monocytes. Dendritic cells derived from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals were highly susceptible to IL-2 treated NK cells alone and even more so with the addition of monocytes to NK cells. *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$monocytes with NK cells induced the highest lysis of *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ dendritic cells as compared to dendritic cells from control animals.

Lipopolysaccharide induces split anergy in NK cells obtained from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals as evident by a decrease in cytotoxicity and an increase in IFN-γ secretion by NK cells

Purified NK cells were obtained from control animals and treated with IL-2 in the presence of monocytes and lipopolysaccharide (LPS) for 7 days and used in a standard ^{51}Cr release assay against YAC-1 cells (Fig. 8A) or MEF (Fig. 8C). At the end of the co-culture period, the supernatants were removed and the level of IFN-γ was measured by ELISA (Fig. 8B). The addition of LPS to NK cells cultured with monocytes from control animals resulted in a complete shutdown of the NK cells' ability to lyse YAC-1 cells (Fig. 8A) while it increased the amount of IFN-γ secreted by NK cells (Fig. 8B). The effects of split anergy induced by LPS could also be observed, even more prominently so, with NK cells cultured with monocytes obtained from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals. The cytotoxic function of the NK cells was completely abolished and it triggered the highest amount of IFN-γ secreted by NK cells (Figs. 8A and C). Monocyte derived dendritic cells from either control or *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals were used as targets against NK cells. As shown in figure 8C, the addition of LPS to NK cells cultured with monocytes from control animals resulted in NK cell anergy and NK cells lose their cytotoxic function against both wildtype and *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* dendritic cells. The anergic effect of LPS is evident when it was added to co-culture of NK cells with monocytes from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals and the cytotoxic function decreased by 7 fold as compared to without LPS (Fig. 8B).

Split anergy induced by LPS also occurs in Human NK cells

The addition of LPS, as well as strains of gram positive and gram negative bacteria (manuscript in prep), to human NK cells cultures causes significant amounts of split anergy. As demonstrated in figure 9A, the addition of LPS to either untreated or IL-2 treated NK cells resulted in a inhibition of cytotoxicity by NK cells against OSCSCs. Although the addition of

LPS caused a loss of cytotoxic function in NK cells, there was a significant increase in the secretion of IFN-γ when NK cells were cultured alone or in the presence of monocytes. The highest IFN-γ secretion was seen when IL-2 or IL-2+ anti-CD16mAb treated NK cells were cultured with monocytes and LPS (Fig. 9B).

Figure legends

Fig. 1 Increased NK cell cytotoxicity observed in NK cells derived from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$*mice with targeted inhibition of COX-2 in bone marrow derived monocytes

NK cells obtained from either wildtype or *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals were left untreated or treated with IL-2 (1X10^4 units/million) for 7 days before the cells were used as effector cells against YAC-1 cells in a standard 4 hour ^{51}Chromium release assay. IL-2 treated NK cells were activated as described in Figure 1A and used to target Mouse Embryonic Fibroblasts (MEF) (B) or ST63 (C) in a standard 4 hour ^{51}Chromium release assay. The lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100.

Fig. 2 NK cells obtained from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$*mice cultured with autologous monocytes lyse YAC-1 and MOK cells significantly more than NK cells without monocytes

NK cells obtained from wildtype or *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice were left untreated or treated with IL-2 (1X10^4 units/million) in the presence or absence of autologous monocytes for 7 days. Afterwards, the cytotoxic function of NK cells was determined using a standard 4 hour ^{51}Chromium release assay (A). NK cells derived from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice were activated with IL-2, as described in Figure 1A, and cultured with monocytes from either wildtype or *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals for 7 days and NK cell mediated cytotoxicity was determined using a standard 4 hour ^{51}Chromium release assay. The lytic units 30/10^7 cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100.

Fig. 3 NK cells cultured with monocytes from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice secrete high levels of IFN-γ as compared to NK cells cultured alone

NK cells from wildtype or *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice were left untreated, treated with IL-2 ($1X10^4$ units/million) or treated with IL-2 ($1X10^4$ units/million) in the presence of autologous monocytes for 7 days. Afterwards, the supernatant was removed from the co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs.

Fig. 4 Monocytes from *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* animals enhance the cytotoxic function of autologous NK cells and induce high levels of IFN-γ secretion

Wildtype or *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* derived NK cells were activated with IL-2 ($1X10^4$ units/million) and cultured with either wildtype or *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* monocytes for 7 days. Afterwards, the cytotoxic function of NK cells was determined using a standard 4 hour ^{51}Chromium release assay. The lytic units $30/10^7$ cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100 (A). Supernatants from NK cell cultures were harvest after co-incubation with monocytes and the levels of IFN-γ secretion were determined using specific ELISAs. Monocytes from wildtype and *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* were used as control.

Fig. 5 Monocytes, and not T cells, enhances the cytotoxic function of NK cells and secretion of IFN-γ

NK cells obtained from wildtype or *Cox-2$^{flox/flox}$ LysM$^{Cre/+}$* mice were treated with IL-2 ($1X10^4$ units/million) for 7 days in the presence of autologous T cells or monocytes. Afterwards, NK cells were used as effectors against wildtype MEF or MEF with specific COX-2 deletion.

The cytotoxic function of NK cells was determined using a standard 4 hour ^{51}Cr release assay. The lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100 (A). NK cells were treated as described in figure 5A and cultured with autolgoous T cells or monocytes for 7 days and afterwards the supernatant was removed from the co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs (B).

Fig. 6 MEF with COX-2 gene deletion are susceptible to NK cell mediated cytotoxicity

IL-2 treated (1X10^4 units/million) NK cells from wildtype or *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ mice were cultured with autologous monocytes for 7 days and used as effector cells against wildtype or COX-2$^{-/-}$ MEFs in a standard 4 hour ^{51}Chromium release assay. The lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100.

Fig. 7 Monocytes derived dendritic cells from *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals were more sensitive to NK cell mediated cytotoxicity than monocytes derived dendritic cells from control animals

IL-2 treated (1X10^4 units/million) NK cells obtained from wildtype animals were cultured with monocytes from wildtype animals or *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals for 7 days before the cells were used as effector cells in a standard 4 hour ^{51}Chromium release assay. Monocytes derived dendritic cells from wildtype or *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ mice were prepared as described in Materials and Methods section and used as target cells. The lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100.

Fig. 8 The addition of LPS to NK cell cultured with or without monocytes or dendritic cells induced split anergy in NK cells which resulted in significant inhibition of NK cell cytotoxicity but increased IFN-γ secretion

IL-2 treated ($1X10^4$ units/million) NK cells obtained from wildtype animals were cultured with monocytes from wildtype animals or *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals for 7 days and then treated with or without LPS (20ng/mL) for an additional day. Afterwards, NK cells were used as effector cells in a standard 4 hour ^{51}Chromium release assay against YAC-1 cells (A) or monocytes derived dendritic cells from wildtype or *Cox-2*$^{flox/flox}$ *LysM*$^{Cre/+}$ animals (B). The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100. NK cells were treated as described in Figure 8A and after an overnight treatment with LPS, the supernatant was removed from the co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs (C).

Fig. 9 Induction of split anergy mediated by LPS was observed in Human NK cells resulting in a loss of their cytotoxic function but gain the ability to secrete high levels of IFN-γ, especially in the presence of autologous monocytes or dental pulp stem cells

Human NK cells were purified from healthy donors and were left untreated or treated with IL-2 (1000 units/mL) in the presence or absence of LPS (20ng/mL) for 24-48 hours. Afterwards, the cytotoxicity against YAC-1 cells was assessed using a standard 4 hour ^{51}Chromium release assay. Percent cytotoxicity was obtained at different effector to target ratio and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the tumor cells X100 (A). Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml)

in the presence of monocytes and/or LPS (20ng/ml) for 18-24 hours. NK cells were added to OSCSCs (B) or DPSCs (C) at a 0.1 to 1 ratio (NK cells to OSCSCs or DPSCs) and after an overnight incubation, the supernatants were removed from the co-cultures and the levels of IFN-γ cytokine were measured with specific ELISA. Purified NK cells were treated as described in Figure 9B and added to monocytes alone, monocytes treated with LPS (20ng/mL), monocytes cultured with DPSCs or a combination of monocytes, DPSCs and LPS (20ng/mL) for 18-24 hours. Afterwards, the supernatants were removed from each of the co-cultures and the levels of IFN-γ cytokine were measured with specific ELISA (D).

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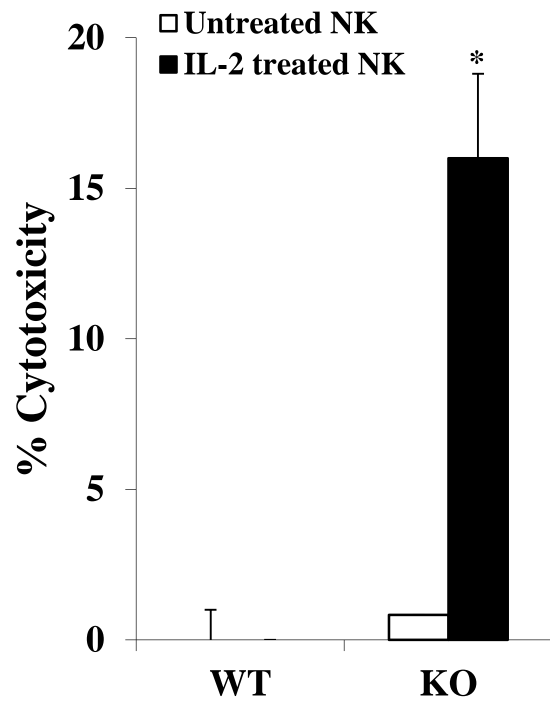
Fig. 1A



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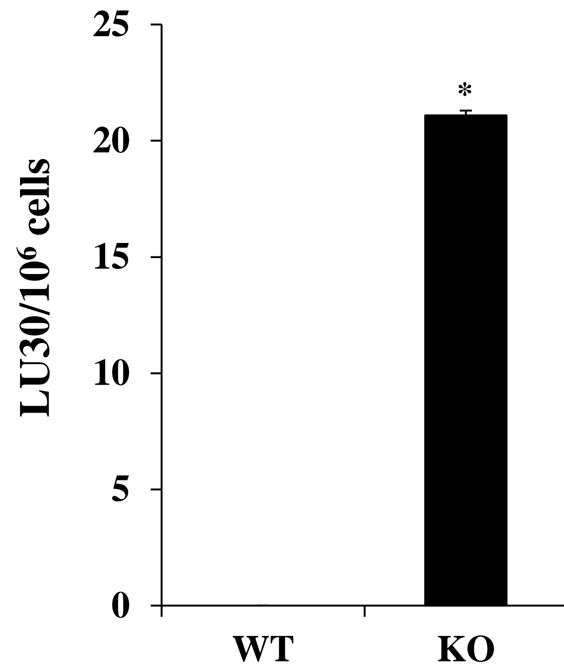

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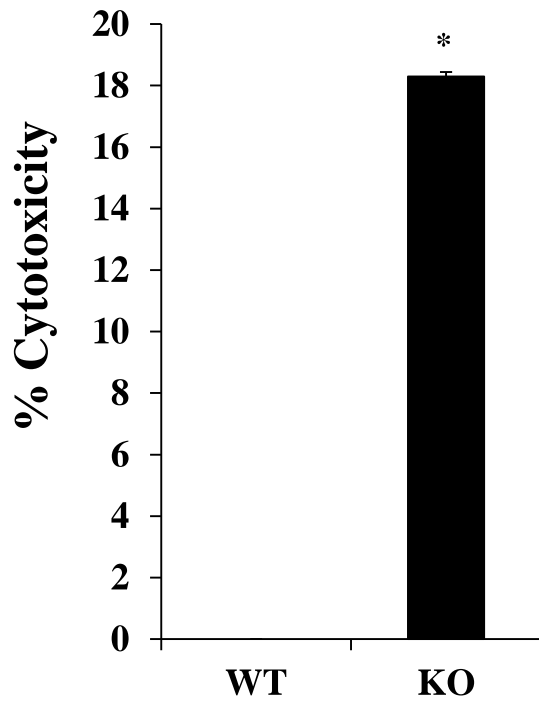
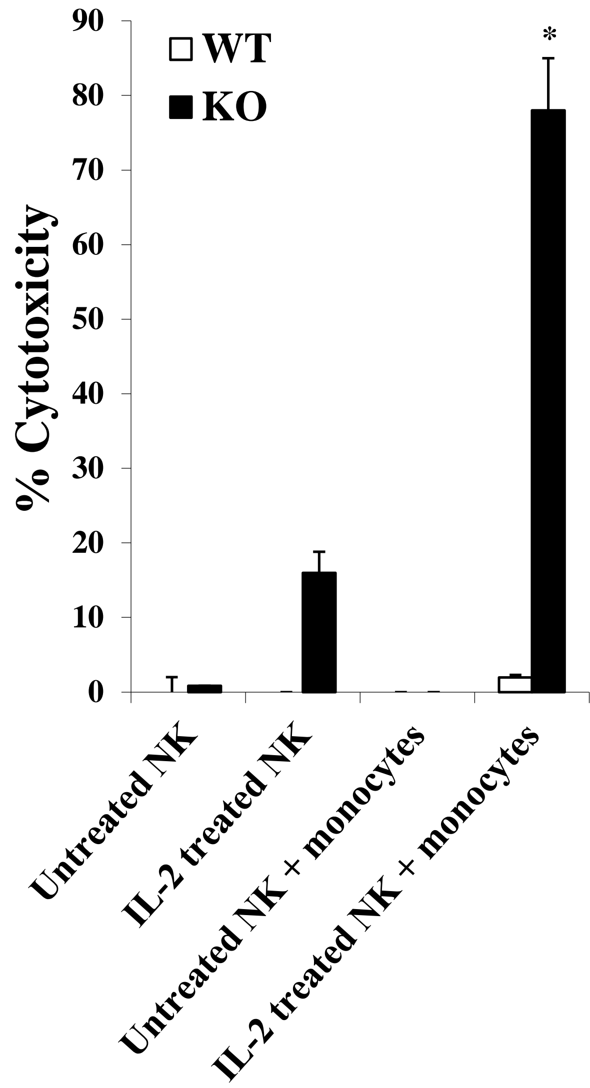


Fig. 2A



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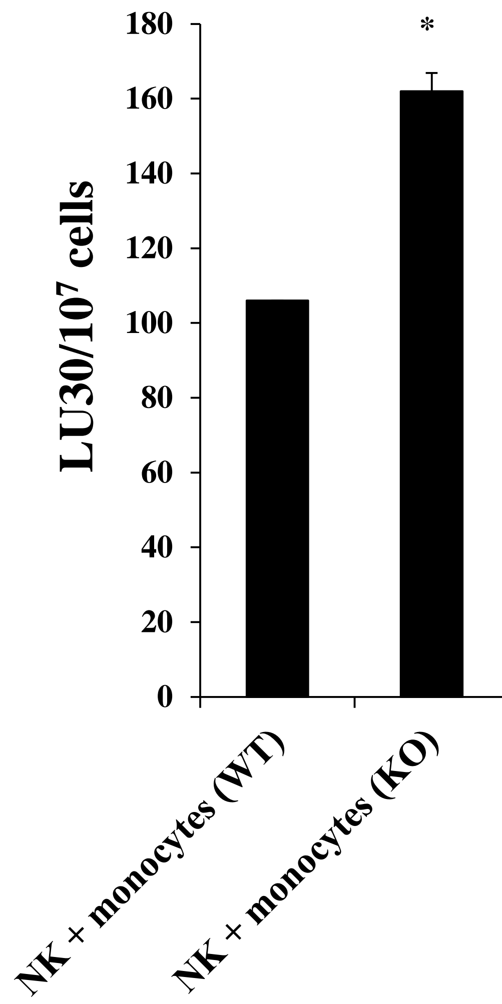

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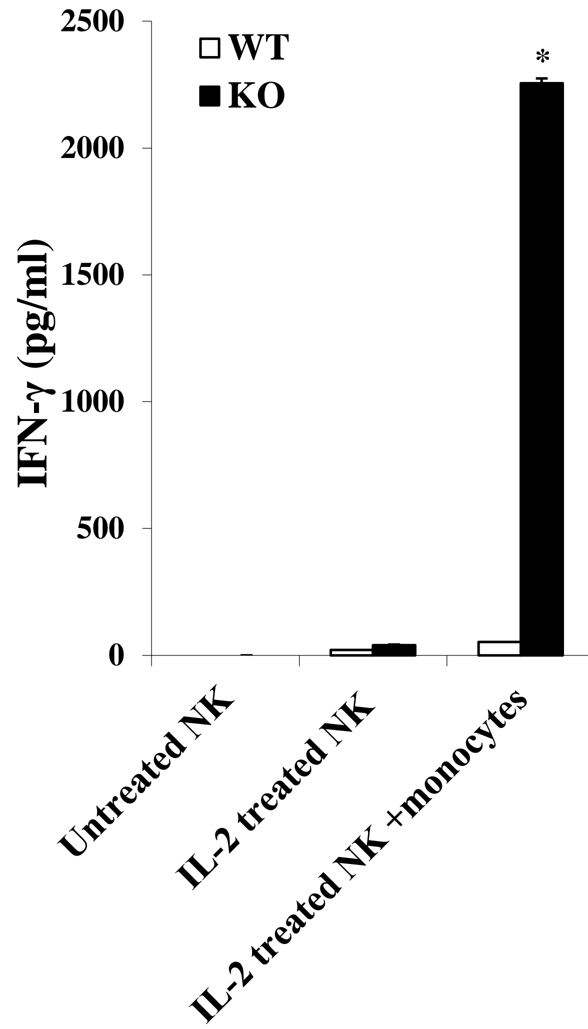

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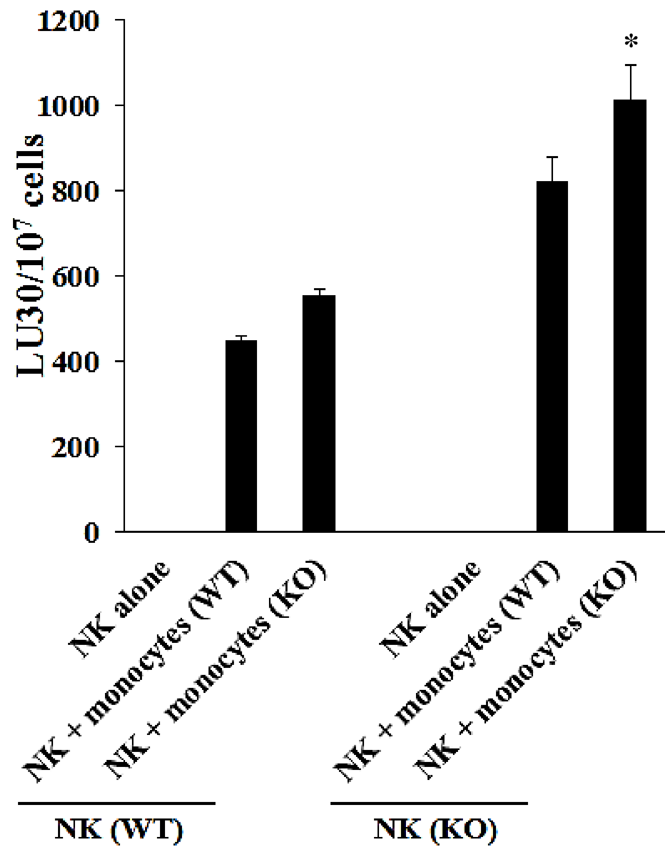

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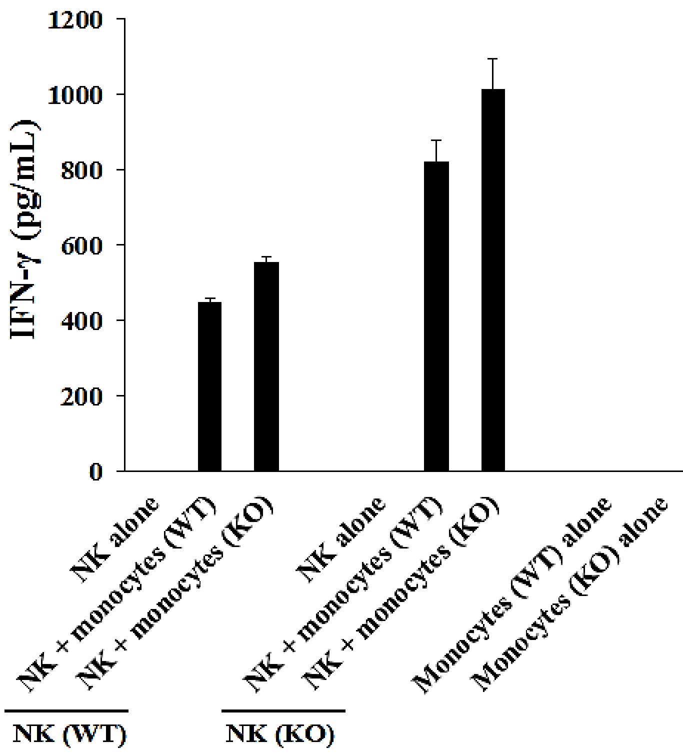
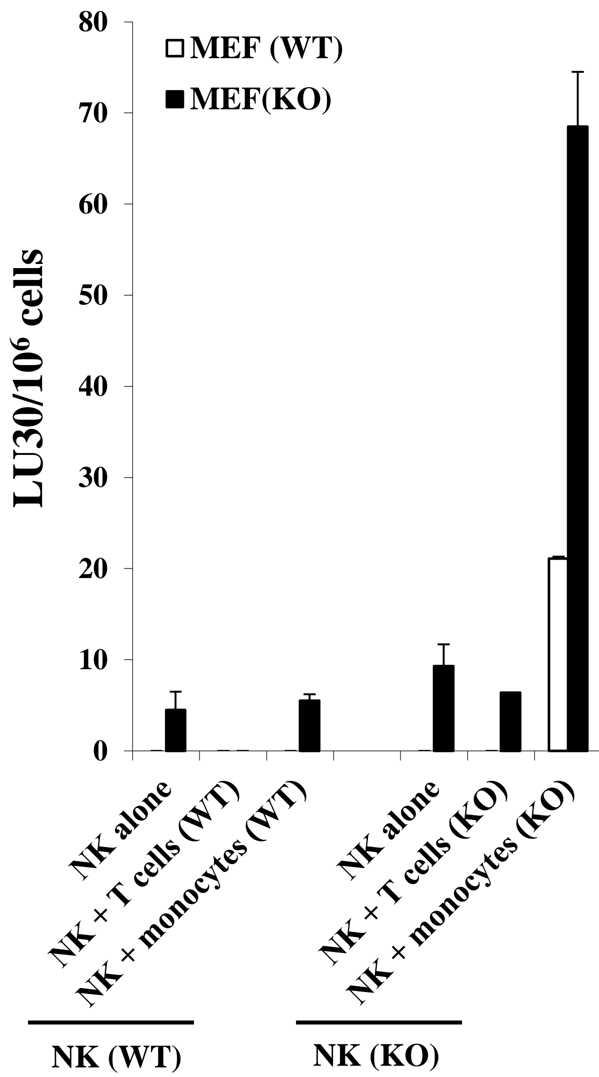


Fig. 5A



B.

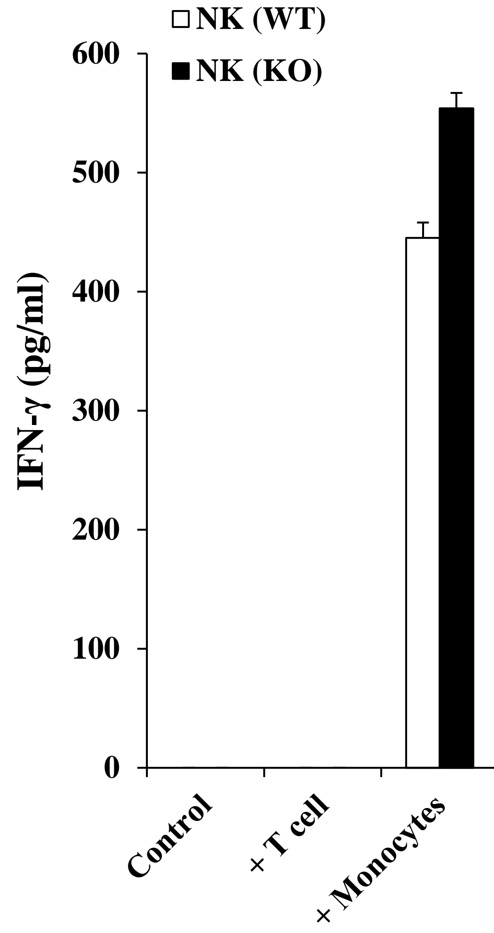


Fig. 6

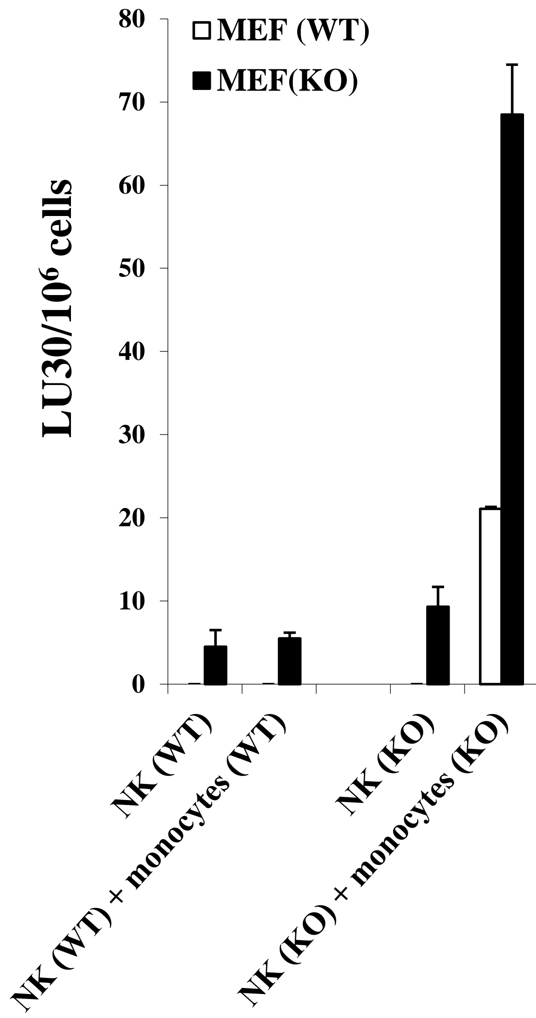


Fig. 7

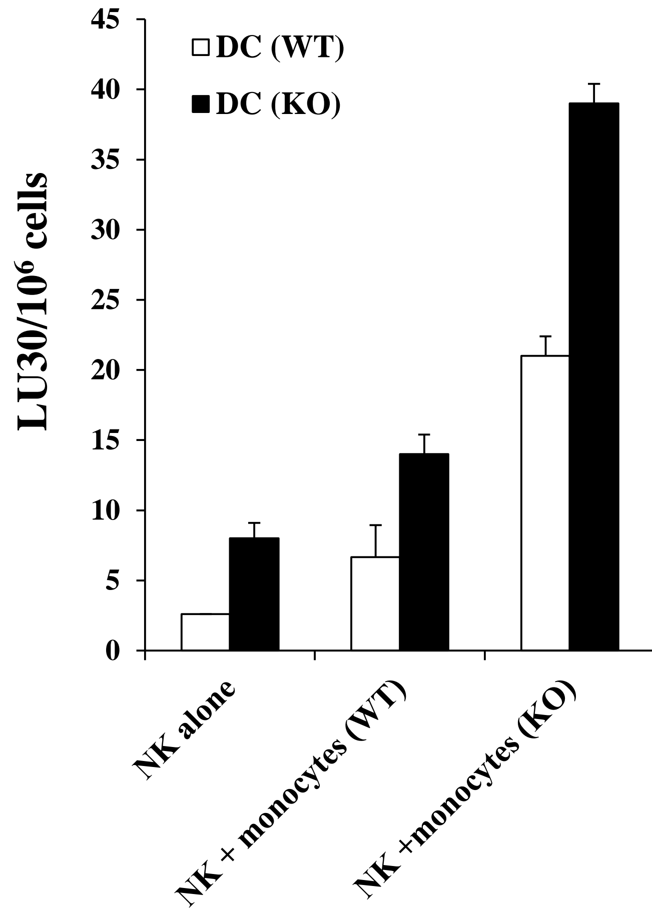
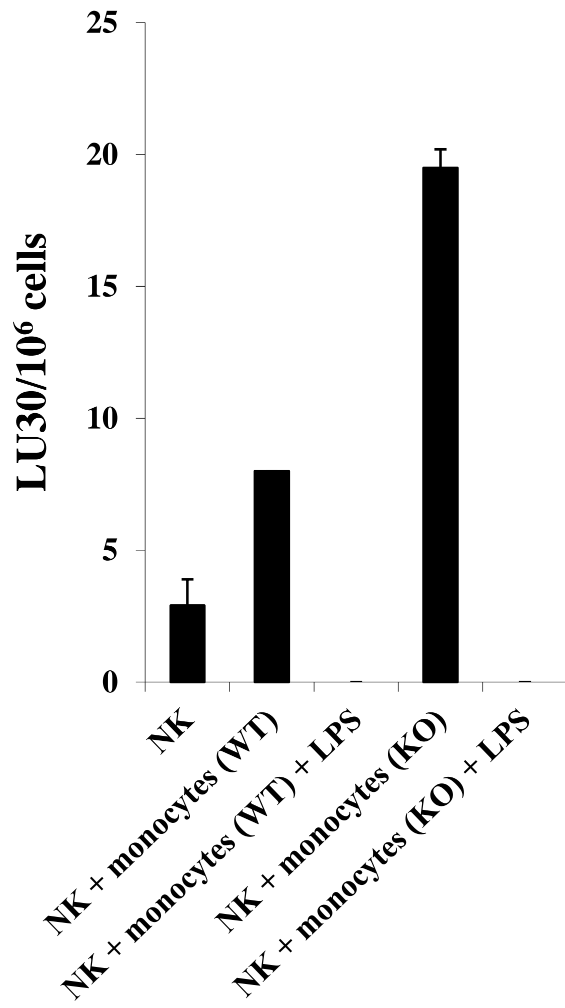


Fig. 8A



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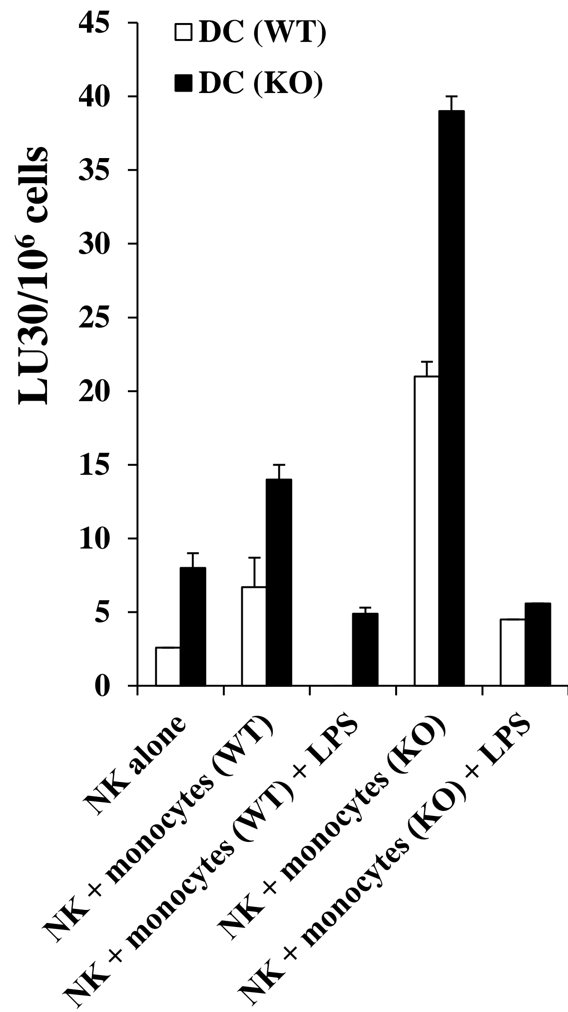


Fig. 8C

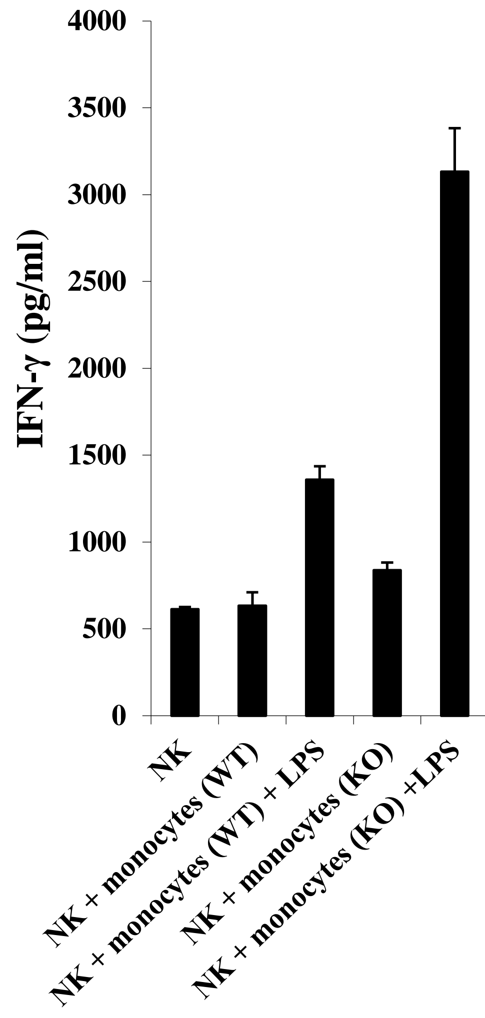


Fig. 9

A.

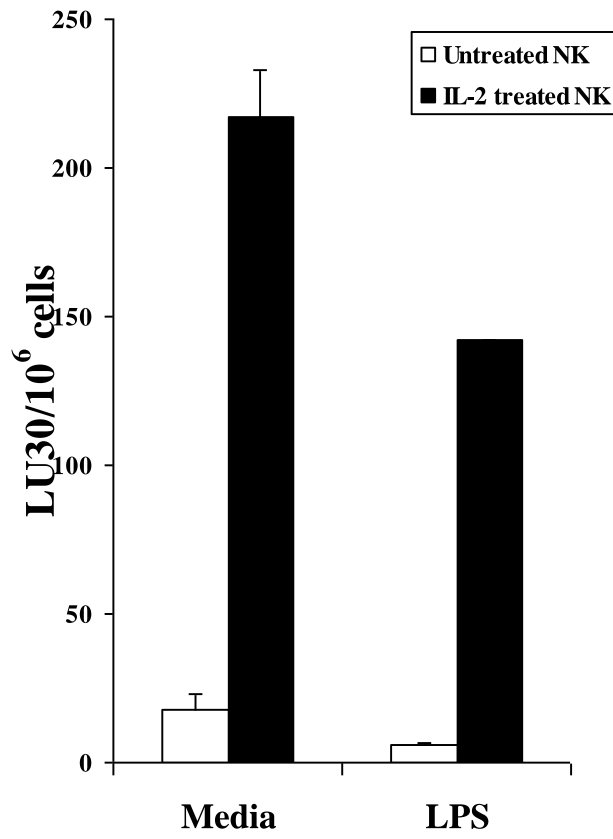


Fig. 9B

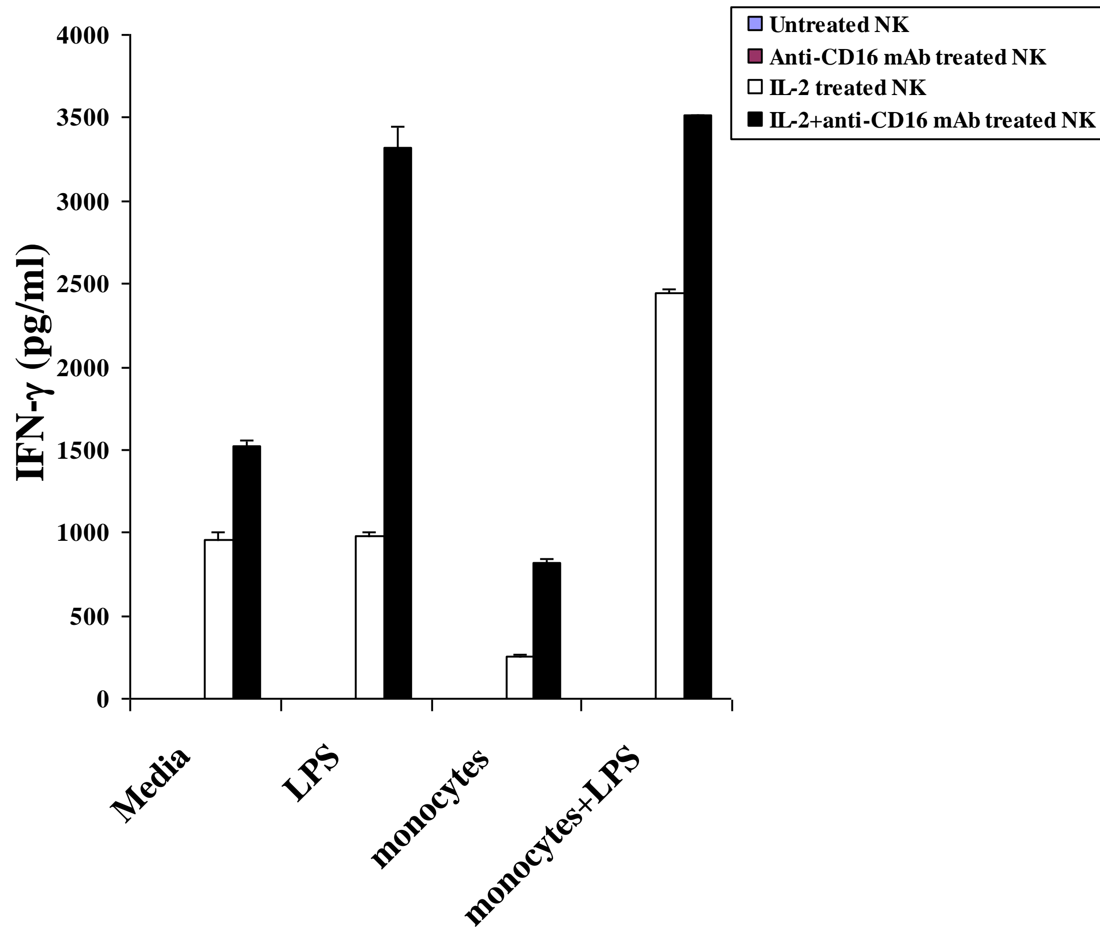


Fig. 9C

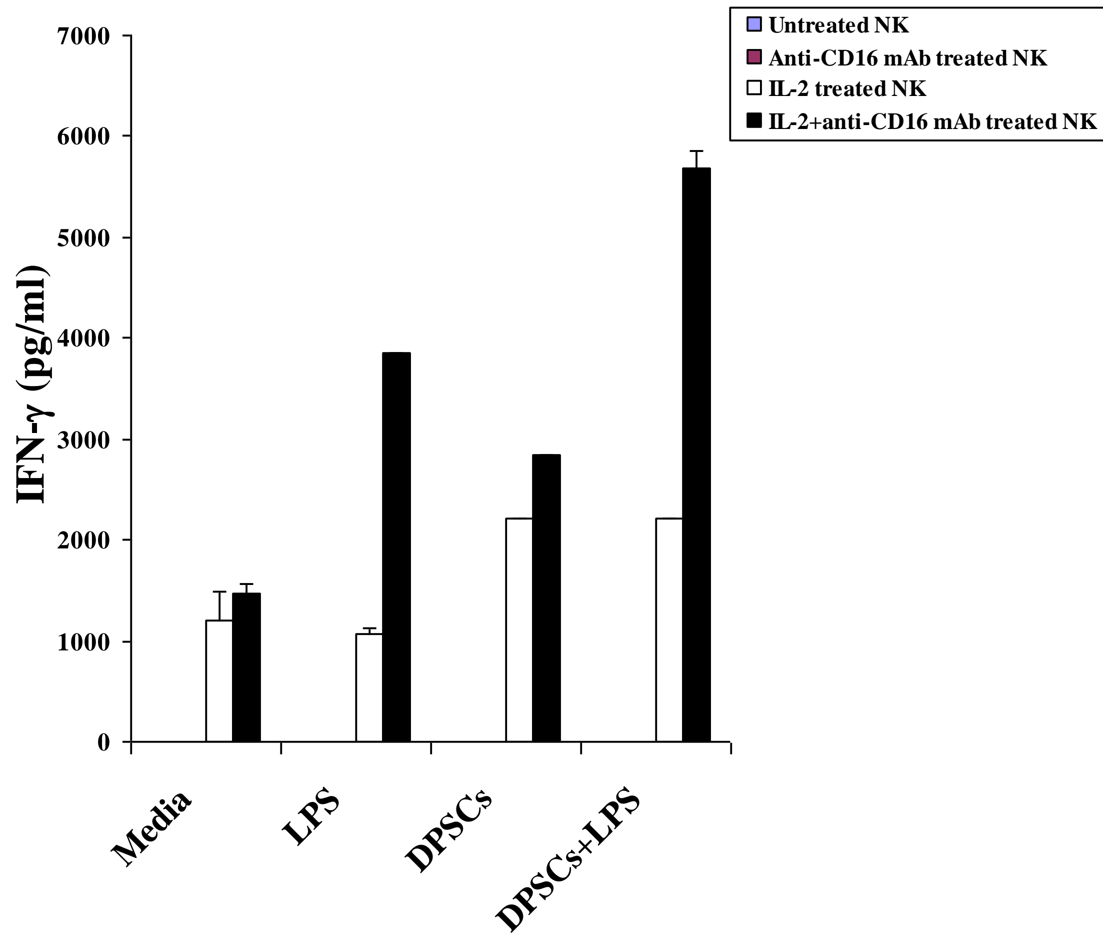
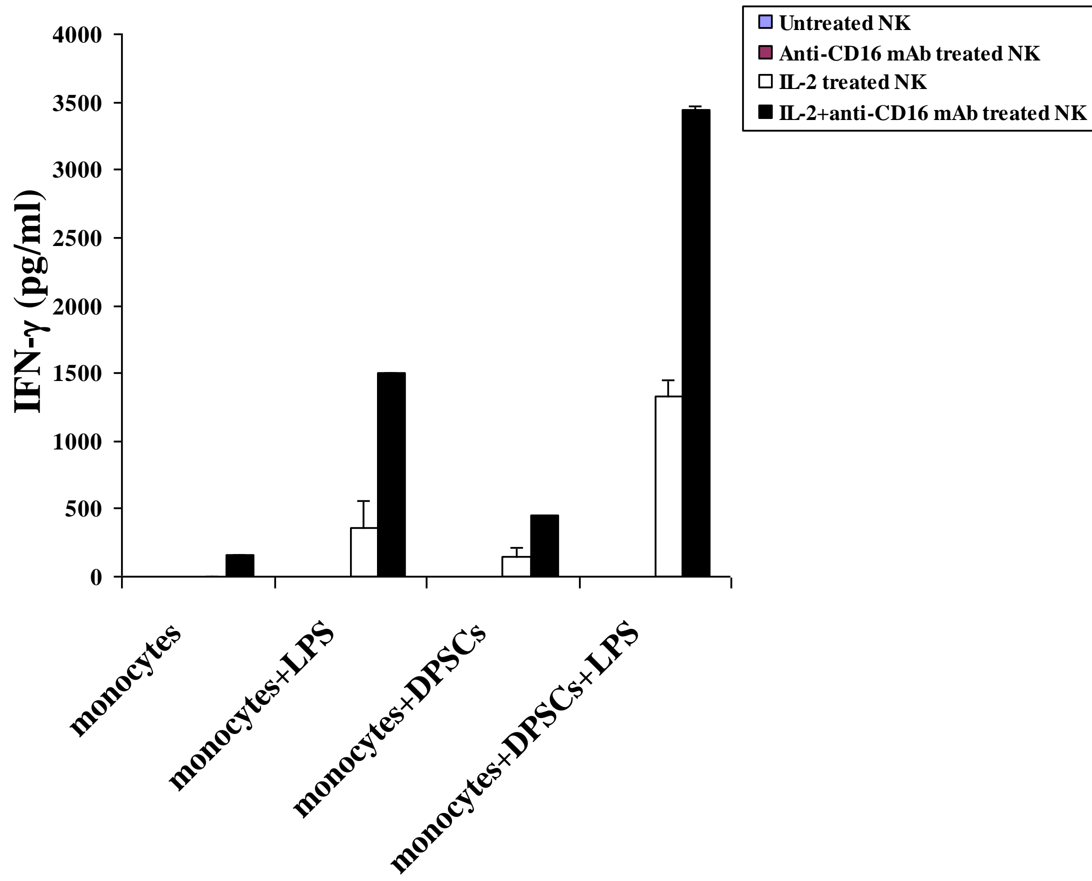


Fig. 9D



CHAPTER 8

Osteoclasts as key subsets of immune effectors modulating the function of Natural Killer cells; Role in bisphosphonate driven differentiation

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Abstract

The aim of this study is to establish osteoclasts as key immune effectors capable of modulating the function of Natural Killer (NK) cells and to determine the effect of nitrogen-containing bisphosphonates Zometa and Alendronate and non-nitrogen containing Etidronate in maintaining a pro-inflammatory microenvironment during interaction with NK cells. Bisphosphonates, particularly those of Zolendronic acid and Alendronate trigger significant levels of pro-inflammatory cytokines and chemokines from osteoclasts, and the levels synergistically rises when cultured with NK cells. Nitrogen containing Bisphosphonates mediate significant dose dependent release of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β whereas they inhibit the anti-inflammatory IL-10 secretion by osteoclasts. The profiles of 27 cytokines, chemokines and growth factors released from osteoclasts were significantly different from Dendritic (DC) cells and M1 macrophages but resembled those of untreated monocytes and M2 macrophages. The surface expression of CD14, CD33, CD54, CD44, CD11b, MHC class I and II and B7H1 were significantly increased when osteoclasts were treated with Bisphosphonates. All three bisphosphonates decreased pit formation by osteoclasts. Treatment of osteoclasts with Zometa and much less with Alendronate was capable of inhibiting NK cell cytotoxicity whereas it induced significant secretion of cytokines and chemokines in the cultures of NK cells with osteoclasts. NK cells were able to lyse osteoclasts much more than their precursor cell monocytes and this correlated with the decreased expression of MHC class I and CD54 expression on osteoclasts. These results suggest that zometa treated osteoclasts may remain viable in the microenvironment for a prolonged period of time during interaction with NK cells providing continuous secretion of pro-inflammatory cytokines and chemokines in the absence of anti-inflammatory cytokine IL-10 resulting in the chronicity of inflammation.

Introduction

The bone resorptive and remodeling function of osteoclasts have been known for a long time, however, it is only recently that their function in the regulation of immune function has gained popularity [324]. Osteoclasts are known to initiate normal bone remodeling during bone growth, tooth eruption and fracture healing and mediate bone loss in pathologic conditions such as osteoarthritis and osteoporosis. Osteoclasts are multinuclear giant cells derived from myeloid lineage [325, 326].

Bisphosphonates have become the treatment of choice for a variety of bone diseases in which excessive osteoclastic activity is one of the underlying pathological effects governing the disease, including Paget's disease of the bone, metastatic and osteolytic bone disease, and hypercalcaemia of malignancy, as well as osteoporosis. Etidronate was the first bisphosphonate to be used in humans. Currently there are at least eleven bisphosphonates which have been registered for various clinical applications in different countries. It was not until the 1990s that bisphonsphonate biochemical actions were elucidated.

Bisphosphonates are classified as non-nitrogen containing bisphosphonates such as Etidronate or clodronate, and nitrogen-containing bisphonates, such as zolendronic acid and Alendronic acid which inhibit key enzymes of the mevalonate/cholesterol biosynthetic pathway. The major enzyme target for bisphosphonates is farnesyl pyrophosphate synthase (FPPS) [327]. Inhibition of FPPS prevents the biosynthesis of isoprenoid compounds notably farnesol and geranylgeraniol that are required for the post-translational prenylation of small GTP-binding

proteins such as rab, rho and rac, which are essential for intracellular signaling events within osteoclasts [327].

Bisphosphonates affect osteoclast-mediated bone resorptive activity in a variety of ways including osteoclast recruitment, differentiation, and apoptosis [328-332]. Characteristic morphological feature of bisphosphonate-treated osteoclasts is the lack of a ruffled border, the region of invaginated plasma membrane facing the resorptive cavity. Bisphosphonates were also shown to disrupt the cytoskeleton of the osteoclast. [333]. It is widely accepted that BPs exert their major effect on mature osteoclasts, however, Kimachi *et al* [334], suggested that nitrogen-containing bisphosphonates not only inhibit mature osteoclasts but also prevent osteoclast precursors from differentiating and migrating towards inflammatory osteolytic lesions. It was also shown that bisphosphonates inhibit in a dose-dependent manner the formation of osteoclast-like cells in long-term cultures of human bone marrow cells [335].

Osteonecrosis of the jaw (ONJ) is a severe bone disease that affects the jaw bones, the maxilla and the mandible. ONJ is commonly associated with bisphosphonate therapy and is widely known as bisphosphonate-associated osteonecrosis of the jaw. The clinical manifestations of ONJ vary significantly from asymptomatic small fistulation to painful swelling with extensive bone exposure leading to pathological bone fracture [336-338].

Oral mucosa contains effectors of both innate and adaptive immunity and they are uniquely situated in the connective tissue area to form a barrier which could potently respond to injury and infection and guide healing of the mucosal tissues [237-239, 250]. Oral mucosa

contains a number of immune effectors including both CD8+ and CD4+ T cells, B cells, monocyte/macrophages and Natural Killer (NK) cells in addition to γδ T cells and NKT cells [339, 340]. Our previous studies also demonstrated significant numbers of NK cells in the oral blood as compared to peripheral blood of healthy as well as patients with periodontal disease (manuscript submitted). Increased numbers of NK cells in chronic periodontitis patients and in peri-tumoral and intra-tumoral infiltration in normal oral mucosa, leukoplakia, actinic cheilitis, lip squamous cell carcinoma , non-metastatic and metastatic oral cavity squamous cell carcinoma has been reported previously [339, 340].

It has been well documented that Natural killer (NK) cells participate in the clearance of virus-infected and transformed cells, as well as healthy stem cells [18, 341]. The function and role of NK cells in bone remodeling are not well understood. IFN-γ, produced by both NK cells and Th1 lymphocytes, has been shown to inhibit osteoclastogenesis *in vitro* [342]. However, the *in vivo* effects of IFN-γ on bone tissue are less clear since often provide a contrasting effect when compared to *in vitro* studies [343, 344]. TNF-α, another key cytokine produced by NK cells, can increase RANKL expression and RANKL dependent osteoclastogenesis [345-347]. NK cells have also been identified within inflamed synovial fluid and express RANKL and M-CSF which during their interaction with monocytes can trigger the formation of osteoclasts in a process that is RANKL and M-CSF dependent [348].

As indicated above the role of osteoclasts in bone remodeling and regulation is well established, in contrast, their role as a member of the immune repertoire with a significant role in regulation of both innate and adaptive immune cell function have not been elucidated and is the

subject of this paper. Although the role of monocytes and Dendritic cells in the regulation of NK cell function have received considerable attention previously, no or very few reports have shown the effect of osteoclasts on the function of NK cells. In this paper we demonstrate that osteoclasts are potent activators of NK cell function and, indeed, their effect is more potent than monocytes in regulating cytotoxicity and secretion of cytokines and chemokines. In addition, bisphosphonate treated osteoclasts modulate the function of NK cells in such a way which may establish chronic inflammation leading to the pathologies observed in ONJ patients.

Materials and Methods

Cell Lines, Reagents, and Antibodies

Alpha-MEM medium (Life Technologies, CA) supplemented with 10% FBS and penicillin-streptomycin (Gemini Bio-Products, CA) was used to culture human osteoclasts. Human M-CSF (Biolegend, CA) and soluble RANKL (PeproTech, NJ) were dissolved in alpha-MEM and stored at -20°C. Zometa, Alendronate and Etidronate were purchased from UCLA Ronald Reagan Pharmacy. Fluorescent Zometa analogs were synthesized via a linker strategy (Hokugo et al. 2013). PE conjugated IgG1 and 2b, PE-CD14, PE-CD11b, PE-CD124, PE-B7H1, PE-CD15, PE-CD33, PE-CD44, PE-CD54, PE-MHC-I and PE-MHC-II were all purchased from Biolegend, CA.

Purification of peripheral blood monocytes and generation of osteoclasts

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors and all the procedures were approved by the UCLA-IRB. Peripheral blood mononuclear cells (PBMCs) were obtained after Ficoll-hypaque centrifugation. PBMCs were cultured onto the tissue culture plate for 1 hour after which the adherent subpopulation of PBMCs was detached from the tissue culture plates and the monocytes were purified using isolation kits obtained from Stem Cell Technologies (Vancouver, Canada). Greater than 95% purity was achieved for each subset based on flow cytometric analysis of CD14. Monocytes were cultured in alpha-MEM medium containing 25ng/mL M-CSF and Rank Ligand (25ng/mL). Medium was refreshed every 3 days with alpha-MEM containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days.

Purification of human NK cells

PBMCs from healthy donors were isolated as described before [137]. Briefly, peripheral blood lymphocytes were obtained after Ficoll-hypaque centrifugation and purified NK cells were negatively selected by using an NK cell isolation kit (Stem Cell Technologies, Vancouver, Canada). The purity of NK cell population was found to be greater than 90% based on flow cytometric analysis of anti-CD16 antibody stained cells. The levels of contaminating CD3+ T cells remained low, at 2.4%±1%, similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures.

TRAP staining

Osteoclasts were detached from tissue culture plate and seeded in 96-well plate at $3X10^4$ cells/well for 18-24 hours. Afterwards the cells were rinsed twice with 1X PBS and fixed with 10% formaldehyde for 5 minutes at room temperature. The cells were then rinsed three times with 1X PBS and incubated with Chromogenic Substrate solution (Primary Cell, Co., Japan) for 30 minutes or until stained TRAP is clearly seen. Finally, cells were then rinsed with deionized water to neutralize the reaction and images were taken with Leica DMI 6000B inverted microscope.

Pit resorption assay

Purified Human osteoclasts were generated from healthy donor's monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. Afterwards, osteoclasts were rinsed, detached from tissue culture plates and seeded at $1X10^4$ cells/well in 24

well plate pre-coated with synthetic carbonate apatite (Cosmo Bio Co, Japan) for 7 days. After the incubation period, culture medium was removed and cells were rinsed with 5% sodium hypochlorite for 5 minutes. The cells were then washed with water and photographed using Leica DMI 6000B inverted microscope.

ELISA

ELISAs for IFN-γ measurement were performed as described previously [137]. To analyze and obtain the cytokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines.

Multiplex Cytokine Array kit

Fluorokine MAP cytokine multiplex kits were purchased from Life Technologies and the procedures were conducted as suggested by the manufacturer. To analyze and obtain the cytokine concentration a standard curve was generated by threefold dilution of recombinant cytokines provided by the manufacturer. Analysis was performed using MAGPIX (Life Technologies, CA).

Surface Staining

Staining was performed by labeling the cells with antibodies as described previously [137, 142, 201].

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [193]. Briefly, different numbers of purified NK cells were incubated with ^{51}Cr–labeled tumor target cells. After a 4 hour

incubation period the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}}$$

LU $30/10^6$ is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X100.

Statistical analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

Results

Phenotypic and functional characterization of osteoclasts purified from human monocytes

Human osteoclasts were generated using purified monocytes treated with Rank Ligand and M-CSF as described in the Materials and Methods section. Osteoclasts purified from monocytes at day 21 were positive for TRAP staining (Fig. 1). The analysis of cytokines, chemokines and growth factors using multiplex cytokine array demonstrated a gradual increase in the secretion of IL1RA, IL2R, IL12 cytokines and MIP-1a, MIP-1b and Rantes chemokines whereas a decrease in IL-6 cytokine secretion can be observed from day 2 to day 21 of differentiation of monocytes to osteoclasts (Table 1). Increased detection of IL-15 and IFN-α but not IFN-γ was also observed (Table 1). The levels of MCP-1 and IL-8 remained significantly high at all-time points tested. No significant secretion of IL-1b, IL-2, IL-4, IL-5, IL-7, IL-13, IL-17 and Eotaxin at the time points and concentration tested (Table 1). Therefore, osteoclasts have the ability to secrete inflammatory cytokines and chemokines which can play important roles in differentiation and tissue remodeling.

Since there was a gradual decrease in IL-6 secretion from day 2 to 21 we compared the levels of IL-6 decrease to the release of anti-inflammatory cytokine IL-10 (Fig. 2). In contrast to gradual decrease in IL-6 secretion from day 3 to day 16 of culture, IL-10 secretion in osteoclast precursors exhibited a gradual increase from day 3 to day 16th (Fig. 2). Therefore, there was an

inverse modulation of IL-6 and IL-10 during differentiation of osteoclasts from monocyte precursors.

Irradiated PBMCs increased IL-6 secretion but inhibited resorptive activity of the osteoclasts

We then determined the effect of irradiated PBMCs on IL-6 and IL-10 secretion by day 21 differentiated osteoclasts. Addition of irradiated autologous PBMCs to osteoclasts significantly increased the secretion of IL-6 but it had marginal effect on the secretion of IL-10 (Fig. 3B and 3C). Addition of irradiated PBMCs to osteoclasts abrogated completely the resorptive activity of osteoclasts (Fig. 3D). In contrast serum prepared from the autologous donor neither had augmenting effect on IL-6 secretion nor had an effect on the resorptive function of osteoclasts indicating that PBMC-osteoclast interaction is likely the mechanism to augment IL-6 secretion and inhibit the resorptive activity of the osteoclasts (Fig. 3).

Differential induction of cytokines, chemokines and growth factors by monocytes, M1 and M2 macrophages, dendritic cells and Osteoclasts

The profile and amounts of cytokines and chemokine secretion in osteoclasts resembled those of the freshly purified monocytes and cultured M2 macrophages, and were greatly distinct from M1 macrophages and Dendritic cells (Table 2). M1 macrophages demonstrated the highest secretion of cytokines followed by the Dendritic cells which had lower overall secretion for the majority of cytokines tested, although there were some exceptions such as IL-1Ra which was

higher from Dendritic cells (Table 2). The amounts of cytokines were largely similar between freshly isolated monocytes, M2 macrophages and Osteoclasts with Osteoclasts having the lowest secretion (Table 2). Interestingly, the levels of chemokines were high in all the subsets, M1 macrophages having the highest for the MIP-1a and MIP-1b and the lowest for MCP-1 (Table 2). IL-8 secretion was the highest from M1 macrophages and M2 macrophages, and monocytes had the next highest secretion, whereas Dendritic cells and Osteoclasts secreted lower amounts (Table 2). Secretion of Rantes was the lowest for the M1 macrophages and higher in the other subsets (Table 2). Monocytes, M2 macrophages and Osteoclasts secreted highest levels of MCP-1 and IP-10 when compared to DCs or M1 macrophages (Table 2). Osteoclasts had the lowest amounts of growth factor secretion whereas M1 macrophages had the highest with the exception of GM-CSF where they secreted the least (Table 2). Overall, these results indicated that the profiles of cytokine, chemokine and growth factor secretion of Osteoclasts resemble to the monocytes and M2 macrophages.

Uptake and the pro-inflammatory effect of nitrogen-containing Zometa and Alendronate and non-nitrogen containing Etidronate on Osteoclasts

To determine the effect of bisphosphonates on Osteoclasts, first we determined the specific uptake of fluorescently labeled Zometa by Osteoclasts (Fig. 4). As shown in the figure 4 Osteoclasts which were taken up zometa appeared red under the microscope since zometa was labeled with red florescence. We then determined the effect of all three bisphosphonates on cell viability. As shown in Fig. 5 there was a dose dependent increase in cell death when nitrogen containing Zometa and Alendronate were added to Osteoclasts, with Zometa having higher

toxicity than Alendronate (Fig. 5). The non-nitrogen containing Etidronate did not mediate cell death at any concentration (Fig. 5). Both nitrogen-containing Zometa and Alendronate but not Etidronate were able to induce secretion of IL-6 at the level of 1μM and the levels significantly decreased with the 25-100 μM concentration of Zometa. Alendronate at the levels of 100 μM exhibited significant decrease in IL-6 secretion when compared to 1-25μM levels (Fig. 6A). Etidronate at all different concentrations were not able to induce secretion of IL-6. In contrast to elevated secretion of IL-6 by nitrogen containing bisphosphonates, the levels of anti-inflammatory cytokine IL-10 was severely suppressed at the concentrations of 1-50 μM (Fig. 6B). Etidronate at all concentrations had the ability to increase or retain the secretion of IL-10 by osteoclasts (Fig. 6B).

Since the highest increase in IL-6 secretion was observed at 1μM, we then determined the ability of lower concentrations of bisphosphonates to induce IL-6 secretion at the range of 10 nM to 1μM (Fig. 7). The results demonstrated a dose and time dependent increase in IL-6 secretion by Zometa and Alendronate, with Zometa having higher ability to induce IL-6 (Fig. 7A and 7B). Etidronate had no or minimal effect on the secretion of IL-6 at all different concentrations (Fig. 7A and 7B). Since both Zometa and Alendronate were found to induce cell death at higher concentrations, we then normalized the levels of IL-6 secretion based on live cells. The results indicated that Zometa exhibited the highest induction followed by Alendronate, with Etidronate having no or minimal activity (Fig. 7C and 7D). When the levels of IL-6 secretion normalized based on the live cells at the concentrations of 1-50 μM similar results to those obtained with the lower concentrations of Zometa and Alendronate were obtained, Zometa having the highest

effect on the secretion of IL-6, followed by Alendronate and Etidronate did not change the levels at any concentration (Fig. 7E and 7F). Both Zometa and Alendronate inhibited IL-10 secretion at the concentrations of 1-50 μM (Fig. 8). When the amounts were normalized based on the live cells, Zometa demonstrated the highest increase per live cell (Fig. 8B and 8D). The inhibitory effect of Zometa and Alendronate at the lower concentrations was not remarkable, however, still per live cell basis Zometa induced the highest secretion of IL-10, followed by Alendronate and Etidronate having the least activating effect on IL-10 secretion (Fig. 8A and 8B). The inhibitory effect of Zometa and Alendronate were dose and donor dependent (Fig. 8).

In addition, to IL-6 and IL-10 secretion, the levels of TNF-α (Fig. 9) and IL-1β were also determined after bisphosphonate treatment. Dose dependent increase in TNF-α (Fig. 9) and IL-1β secretion was also observed by Zometa followed by Alendronate and no secretion by Etidronate. Overall, these data demonstrated the ability of both Zometa and Alendronate but not Etidronate to induce pro-inflammatory cytokines, whereas they had inhibitory effect on the release of anti-inflammatory IL-10 since the release of this cytokine was greatly affected by the decreased viability of the cells, even though Zometa had enhancing effect on the secretion of IL-10 per live cell basis.

Decreased pit numbers and size by Bisphosphonate-treated Osteoclasts

The resorptive activity of the osteoclasts was determined after treatment with Zometa, Alendronate and Etidronate. Although dose dependent decrease in the resorptive activity of osteoclasts could be seen by the treatment with the three bisphosphonates, Zometa exerted the

most severe inhibition (Fig. 11B). Both the numbers of the pits and the size of the pits were affected by the treatment with the three bisphosphonates (Fig. 11).

Comparison of cell surface receptor expression between Osteoclasts, monocytes, Dendritic cells and macrophages

When cell surface receptor expression was compared between Osteoclasts and freshly isolated autologous monocytes, a significant down-modulation of all the cell surface receptors were observed (Fig. 12). Profound decreases in CD14, CD11b, CD44, MHC-class I and II and CD54 were observed on the surface of osteoclasts as compared to freshly isolated autologous monocytes (Fig. 12). However, when monocytes were cultured in media in the absence of Rank ligand and M-CSF and the levels compared to Osteoclasts, the differences between monocytes and osteoclasts substantially decreased (Fig. 13). The expression levels of CD14, CD11b B7H1 and CD54 increased and CD44 decreased, whereas no differences for MHC class I and II, CD33, CD15, CD124 could be seen (Fig. 13). In contrast, the expression of all the cell surface receptors was significantly increased on the surface of macrophages as compared to either monocytes or osteoclasts (Fig. 13). DCs expressed higher levels of CD11b, CD54, MHC class II, CD33, B7H1 and CD44 and lower levels of CD14 and MHC class I when compared to monocytes and osteoclasts (Fig. 13). When the surface expression of day 8 differentiated Osteoclasts were compared with day 21, a slight increase in CD14, CD11b, CD33, and B7H1 and a decrease in MHC class II were noted. It is of note that even though mean channel florescence intensity of CD14 and CD44 were increased, the percentages of the cells expressing these surface receptors were decreased (Fig. 13). Addition of combination of IFN-γ and TNF-α

decreased CD44 and increased CD14, CD54, MHC class II, CD33, CD124 and B7H1 whereas no change in CD11b, MHC class I, CD15 were noted (Fig. 13).

Zometa modulated surface receptor expression on Osteoclasts

Treatment of Osteoclasts with Zometa increased all the cell surface receptors at lower concentration of Zometa which correlated with the increased cytokine induction (Fig. 14). At higher concentration of Zometa there was less increase in the cell surface receptors (Fig. 14). Comparison between Zometa treated Osteoclasts and those treated with the supernatants prepared from the activated NK cells demonstrated higher induction of cell surface receptors by Zometa with the exception of CD54 where supernatant treated Osteoclasts had higher induction (Fig. 15). Of note both CD14 and CD44 expression were significantly down-modulated by NK supernatant treated Osteoclasts (Fig. 15).

Osteoclasts are targets of NK cells and induce significant IFN-γ secretion by the NK cells

Since osteoclasts express lower levels of MHC class I on the surface they may be targets of NK cell lysis. To determine whether osteoclasts similar to monocytes are targets of NK cells, untreated, IL-2 treated and IL-2 in combination with anti-CD16 treated NK cells were used in cytotoxicity assays against Osteoclasts. As shown in Fig. 16A, both untreated and IL-2 treated NK cells were able to lyse osteoclasts although the levels of IL-2 treated NK cells were significantly higher than the untreated NK cells. Addition of anti-CD16 mAb triggering antibody with IL-2 inhibited IL-2 induced NK cell cytotoxicity (Fig. 16A). To compare NK cell

cytotoxicity against osteoclasts and monocytes, untreated and IL-2 treated NK cells were used in cytotoxicity assay. As shown in Fig. 16B NK cells lysed osteoclasts much more than monocytes. In addition, both monocytes and osteoclasts were able to induce significant secretion of IFN-γ by the IL-2 treated NK cells, albeit the levels were higher when NK cells were cultured with osteoclasts than monocytes. NK cells treated with IL-2 in combination with anti-CD16 mAb triggered significant release of IFN-γ, and the combination with monocytes or osteoclasts were unable to increase beyond the amount which was induced by IL-2 in combination with anti-CD16mAb.

Zometa treated Osteoclasts are resistant to NK cell mediated cytotoxicity

We then determined the cytotoxic activity of NK cells against bisphosphonate treated osteoclasts and OSCSCs. IL-2 treated NK cells lysed untreated osteoclasts and the treatment with zometa induced resistance in osteoclasts against NK cell cytotoxicity. Zometa at 500 nM caused more resistance to NK cell mediated cytotoxicity than at 1μM (Figs. 17A-17B). The resistance to cytotoxicity is also seen with Alendronate but at much lower levels (Fig. 17A). To determine whether the ability to induce resistance in NK cells is specific for osteoclasts, we treated OSCSCs for 15-30 min and used in cytotoxicity assay against NK cells. Treatment of OSCSCs with zometa also induced resistance against NK cytotoxicity, as seen in Fig 17C. The effect of Zometa treated osteoclasts on NK cells is similar to that induced by IL-2+anti-CD16mAb treated NK cells in which NK cytotoxicity is suppressed whereas there is significant induction of cytokine secretion by the NK cells (split anergy).

NK cells secrete significant levels of inflammatory cytokines and chemokines in culture with Zometa treated Osteoclasts

We next determined the effect of bisphosphonates when NK cells were cultured with either zometa or Alendronate treated osteoclasts. As shown in Table 3 Zometa treated osteoclasts triggered significantly higher induction of cytokines and chemokines in the co-cultures with NK cells, and the effect was higher when compared to Alendronate treated Osteoclasts (Fig. 18A), whereas Etidronate had no enhancing effect (supplemental figure). As expected, based on surface expression, osteoclasts triggered cytokine and chemokine secretion significantly more than monocytes (Fig. 18 and Table 3). IL-2 treated NK cells triggered significantly higher release of IL-6, IFN-g, IL-10, IL-8,MCP-1,MIP-1a and MIP-1b in the cultures with osteoclasts than monocytes (Table 3). Zometa treated osteoclasts upregulated secretion of cytokines and chemokines 2-6 fold higher for IL-6, IFN-g, MIP1a and MIP-1b by IL-2 and IL-2+anti-CD16mAb treated NK cells when compared to untreated osteoclasts (Table 3 and Fig. 18). The levels of IL-8 and MCP-1 secretion were very high and plateaued in the cultures of NK cells with osteoclasts (Table 3). Both Zometa and Alendronate treated osteoclasts demonstrated decreased secretion of IL-10 in the cultures with NK cells when compared to untreated osteoclasts (Fig. 18C). Zometa treated osteoclasts secreted higher levels of IL-18 when compared to monocytes, the levels significantly increased when cultured with NK cells (Fig. 18E).

Discussion

Phenotypic and functional characteristics of osteoclasts treated with and without two nitrogen containing Zometa and Alendroante and one non-nitrogen containing bisphosphonate Etidronate were determined in this paper. Osteoclasts were generated from their precursor cells monocytes and differentiated with Rank Ligand and M-CSF. Initial characterization indicated that during differentiation with Rank L and M-CSF osteoclasts gradually increased the secretion of a number of chemokines and cytokines from day 2 to day 21 of culture, and the profiles of secretion was similar to those of M2 macrophages and monocytes than DCs or M1 macrophages. In comparison to all other subsets, osteoclasts, in general, secreted lower amounts of cytokines, however, they secreted substantial amounts of chemokines (Table 2). Interestingly, secretion of IL-6 by osteoclasts decreased whereas the IL-10 secretion gradually rose from day3 of differentiation to day16, and even though cultures of osteoclasts with PBMCs triggered significant secretion of IL-6 it did not have an effect on the secretion of IL-10. Osteoclasts cultured with PBMCs lost the ability to induce pit formation. Similar to PBMCs, Zometa and Alendronate but not Etidronate treated osteoclasts triggered dose dependent secretion of IL-6 whereas they inhibited the secretion of IL-10. Indeed, our in vivo experiments with Zometa injected mice during and after tooth extraction demonstrated significantly higher secretion of IL-6 by osteoclasts when determined in in situ immunohistochemical analysis of oral gingival mucosa indicating clear agreement between our in vitro and in vivo experiments (Manuscript submitted). The dose dependent increase in IL-6 secretion by zometa was evident when Osteoclasts were treated with 10nM-1μM, and at higher concentration of zometa from 1μM-

100μM a dose dependent decrease in the secretion of IL-6 could be observed which related to the ability of zometa to induce functional suppression and/or cell death in osteoclasts since after normalization based on the live cells an increase in IL-6 secretion could be observed in doses of 1μM-50μM. Zometa induced increase of IL-6 was higher when compared to Alendronate whereas Etidronate demonstrated no ability to induce IL-6 secretion. In contrast, IL-10 secretion was not changed from doses 10nM-1μM, and at higher concentration of Zometa and Alendronate but not Etidronate a dose dependent suppression of IL-10 secretion could be observed, however, this could be due to the ability of zometa and Alendronate to induce cell death at high concentrations since after normalization based on the live osteoclasts a slight increase in IL-10 secretion in Zometa and much less with Alendronate could be seen. Zometa was also able to induce TNF-α and IL-1β from osteoclasts. The increase in inflammatory cytokines induced by Zometa and to a lesser extent by Alendronate correlated with the inability of Zometa and Alendronate treated osteoclasts to retain their resorptive activity since both the number and size of the pits formed on the resorptive plates were decreased. Interestingly, even though Etidronate did not induce inflammatory cytokines it was able to decrease the ability of osteoclasts to resorb hydroxyapatite significantly.

To determine whether zometa had the ability to modulate surface receptors on osteoclasts we first analyzed a number of key cell surface receptors on osteoclasts and compared it to monocytes, macrophages and DCs. As can be seen in Fig. 12 Osteoclasts had significantly down-modulated many of the cell surface receptors, notably, MHC class I and II, CD14, CD11b and CD54. There was 4-25 fold decrease in the expression of surface receptors and the most decrease

was seen for MHC class I (8 fold) and II (25 fold) expression on osteoclasts. Considering the size of the osteoclasts such a decrease in surface receptors is quite substantial and it may have significant physiological consequences for the activation of immune inflammatory cells (please see below).

Since osteoclasts were compared to freshly isolated monocytes, we next determined the surface receptors when both osteoclasts and monocytes were cultured for 8 days and compared the expression to monocytes treated with IFN-γ and LPS and Dendritic cells generated from monocytes treated with GM-CSF and IL-4. Monocytes cultured for 8 days also down-modulated their surface receptors and the levels of expression remained lower when compared to either 8 day or 21 day cultured osteoclasts for CD14, CD11b and CD54. The levels of MHC class I and II did not change substantially, however, lower amounts of MHC class II were seen on 21 day osteoclast culture. The surface expressions on osteoclasts were quite different from either macrophages or DCs. Activation of osteoclasts with IFN-γ and TNF-α up-regulated the majority of surface receptors, however, the increase never reached to the levels obtained on the surface of macrophages. These experiments suggested that monocytes in the periphery may be less activating for innate immune cells such as NK cells since they retain higher levels of key surface expression such as MHC class I, whereas once they move to the tissues and down-modulate their surface receptors they may become more activating. Indeed, this may be one reason why NK cells in peripheral blood remain relatively quiescent, even in the presence of competent NK cytotoxic machinery.

Treatment of osteoclasts with Zometa up-regulated surface receptors significantly and this increase was comparable or even higher when osteoclasts were treated with culture supernatants from NK cells and monocytes treated with sonicates of gram positive bacteria in which we had obtained the highest increase in surface receptors for cells such as OSCSCs and DPSCs (data not shown).

In our previous studies we determined that the stage of maturation and differentiation of the healthy untransformed stem cells, as well as transformed tumorigenic cancer stem cells, is predictive of their sensitivity to NK cell lysis. In this regard we have shown that OSCSCs, which are stem-like oral tumors, are significantly more susceptible to NK cell mediated cytotoxicity; whereas, their differentiated counterpart OSCCs is significantly more resistant [18]. In addition, hESCs and hiPSCs, as well as a number of other healthy normal stem cells such as hMSCs and hDPSCs, were found to be significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts [18]. Based on these results, we proposed that NK cells may play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact (Tseng et al, "in press"). In addition, we have shown previously that CD14+HLADR- monocytes can condition NK cells to lose cytotoxicity and gain the ability to secrete inflammatory cytokines [19, 236-239]. The signals received from the stem cells or monocytes alter the phenotype of NK cells and cause NK cells to lose cytotoxicity and change into cytokine producing cells. These alterations in NK cell effector function is found to ultimately aid in driving differentiation of surviving, healthy, as well as transformed stem cells [19, 236-239]. Differentiation of stem cells and their resistance to NK cell mediated cytotoxicity correlated with significant increase in the expression of MHC class I, CD54, B7H1 surface

expression in a number of healthy and tumor stem cell models, and it was blocked by the addition of the combination of anti-TNF-a and anti-IFN-g which restored NK cell cytotoxicity and blocked the increased expression of above-mentioned surface markers in addition to inhibition of cytokine and chemokine secretion (manuscript submitted). Since Zometa increased MHC class I, CD54 and B7H1 on osteoclasts we reasoned that it may behave as a differentiation agent capable of decreasing NK cell mediated cytotoxicity. Indeed, treatment of osteoclasts with Zometa and much less with Alendronate was able to inhibit NK cell cytotoxicity significantly. In addition, NK cells were able to lyse osteoclasts much more than freshly isolated monocytes and this correlated with the decreased expression of MHC class I and CD54 expression.

In contrast to the decrease in cytotoxicity, Zometa mediated dose dependent increase in cytokine secretion such as TNF-α and IFN-γ in the co-cultures of NK cells with osteoclasts (Tseng et al, "in press"). As mentioned above, TNF-a and IFN-g secreted by the NK cells synergistically increase differentiation of stem cells resulting in their resistance to NK cell mediated cytotoxicity and substantial decrease in cytokine and chemokine secretion by the NK cells cultured with differentiated cells (Tseng et al, "in press"). Decrease in NK cell cytotoxicity by Zometa can be observed after 30 min. of treatment of cells with Zometa which is quite fast. These results suggest that zometa treated osteoclasts may remain viable in the microenvironment for a prolonged period of time and continuously trigger high levels of cytokines and chemokines resulting in the chronicity of inflammation. Indeed, under the conditions where supernatants from the NK cells induce differentiation in stem cells such as DPSCs or OSCSCs, there is a significant inhibition of both cytotoxicity and cytokine and chemokine secretion, however, in the presence of Zometa, even though NK cell cytotoxicity is blocked, cytokine secretion continues at

a very high level which may be the reason why complete wound closure does not occur after tooth extraction. In addition, Zometa treated osteoclasts may provide continuous NK cell stimulation by the increased production and synergistic functions of NK activating cytokines such as IL-18, IL-15, IL-12 and IFN-α which we have shown to be secreted by the osteoclasts. This possibility is under investigation in our laboratory and is the subject of a future report. Both Zometa and Alendronate but not Etidronate are able to increase cytokine and chemokine secretion by the NK cells in the co-cultures of NK cells with osteoclasts.

The significance and function of Monocyte/macrophages and Dendritic cells in driving an effective immune response has been known for decades, however, their close relative, osteoclasts were primarily known for their function during bone turn-over and remodeling. Our studies impart greater significance to this subset as potentially another subset of the immune system, and place them in the ranks of Monocyte/macrophages and DCs in regulating the function of innate immunity.

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Table legends

Table 1. Production of cytokines, chemokines and growth factors by Osteoclasts.

Human monocytes purified from healthy donor's PBMCs were differentiated into osteoclasts in culture with medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. Culture medium was harvested on days 2, 6, 10, 14 and 21 and the levels of cytokine and chemokine production were measured using multiplex cytokine array kit.

Table 2. Production of cytokines, chemokines and growth factors by monocytes, Dendritic cells, M1 and M2 macrophages and Osteoclasts.

Highly purified Human monocytes were left untreated or treated with different stimuli as described below. Supernatants were harvested from each sample on the indicated days and the levels of cytokines and chemokines were measured using multiplex cytokine array kit.

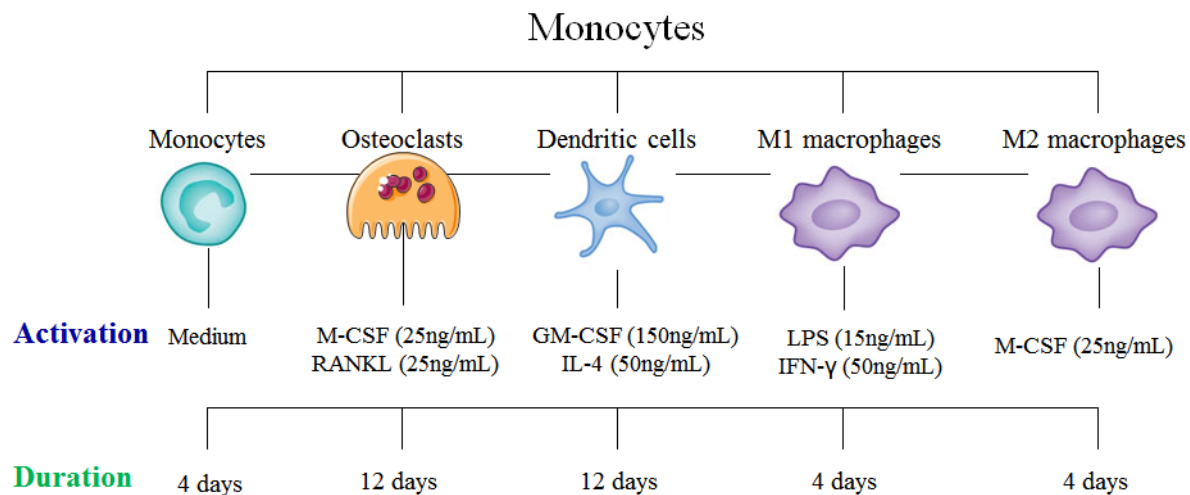


Table 3. Production of cytokines, chemokines and growth factors in cultures of NK cells with monocytes and Osteoclasts.

Human monocytes purified from healthy donor's PBMCs were differentiated into osteoclasts in culture with medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. After 72 hours of treatment of monocytes and cultured osteoclasts with bisphosphonates at 1uM concentration, treated monocytes and osteoclasts were washed extensively with fresh media and cultured with untreated, IL-2 treated and IL-2 + anti-CD16mAb treated NK cells. Supernatants from the co-cultures of monocytes or osteoclasts with NK cells were harvested after 24 hours of incubation and the levels of cytokine and chemokine production were measured using multiplex cytokine array kit.

Figure legends

Fig. 1. Osteoclasts generated from monocytes *in vitro* are TRAP positive.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. After the differentiation period, cells were rinsed with 1X PBS, detached from tissue culture plate and seeded in 96-well plate at $3X10^4$ cells/well for 24 hours. Afterwards, the cells were fixed with 10% formaldehyde and stained with Chromogenic Substrate. Images were taken with Leica DMI 6000B inverted microscope.

Fig. 2. Gradual decrease in IL-6 and increase in IL-10 production during osteoclast differentiation from monocytes.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 16 days. Culture medium was collected at various time points and the levels of IL-6 (A) and IL-10 (B) produced by osteoclasts were measured using specific ELISAs.

Fig. 3. Irradiated autologous PBMCs significantly increased the secretion of IL-6 by Osteoclasts, but had marginal effect on IL-10 production and inhibited resorptive activity of osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) with and without autologous donors serum (3%) or with irradiated PBMCs (30 grey) at 1:1 ratio of osteoclast to PBMCs for 21 days before they were subjected to TRAP staining as described in Fig. 1 (A). The supernatants from each culture condition were then harvested and the levels of IL-6 (B) and IL-10 (C) secreted by osteoclasts

were analyzed using specific ELISAs. The Osteoclasts were then detached from the tissue culture plate and seeded at $1X10^4$ cells/well in 24 well plates pre-coated with carbonate apatite for 7 days. After the incubation period, culture medium was removed and cells were rinsed with 5% sodium hypochlorite for 5 minutes. The cells were then washed with water and photographed using Leica DMI 6000B inverted microscope (D).

Fig 4. Uptake of fluorescently labeled Zometa by Osteoclasts.

Purified Human osteoclasts were generated from healthy donor's monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. After the differentiation period, osteoclasts were rinsed with 1X PBS, detached from the tissue culture plate and seeded at a density of $0.3X10^5$ cells/well in 24 well plate. After an overnight incubation, the cells were treated with Zometa conjugated with *5 - Carboxy - X - rhodamine* (5uM) for 24 hours and the image was taken with Leica DMI 6000B microscope.

Fig 5. Nitrogen-containing Zometa and Alendronate, but not Etidronate, were able to induce death in Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. After the differentiation period, osteoclasts were rinsed with 1X PBS, detached from tissue culture plate and seeded at $1.5X10^4$ cells/well in 24 well plate for 24 hours. Cells were then treated with Zometa, Alendronate or Etidronate at 1, 5, 10, 25, 50 and 100uM for 6 days. The cells were then rinsed with 1X PBS, stained with propidium iodide and analyzed by flow cytometry.

Fig 6. Pro-inflammatory effect of nitrogen-containing Zometa and Alendronate and non-nitrogen containing Etidronate on Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. After the differentiation period, osteoclasts were rinsed with 1X PBS, detached from tissue culture plate and seeded at $1.5X10^4$ cells/well in 24 well plate for 24 hours. Cells were then left untreated or treated with Zometa, Alendronate or Etidronate at 1, 5, 10, 25, 50 and 100uM for 6 days. Supernatant was removed on the day of experiment and the levels of IL-6 (A) and IL-10 (B) were determined with specific ELISAs.

Fig 7: Increased IL-6 secretion by Zometa and Alendronate but not Etidronate treated Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 12 days. Osteoclasts at a concentration of $2X10^5$/ml were treated with 10nM-1□M concentrations of nitrogen-containing Zometa and Alendronate and non-nitrogen containing Etidronate as indicated in the figure for the duration of 3 days (A) and 6 days (B), after which the supernatants were harvested and subjected to a specific ELISA for IL-6. The results obtained in Fig. 7A and 7B were then normalized based on 10^3 live osteoclasts for the 3 day (C) and 6 day (D) incubation. Osteoclasts at a concentration of $2X10^5$/ml were treated with 1uM-50uM concentrations of nitrogen-containing Zometa and Alendronate and non-nitrogen containing Etidronate, after which the supernatants were harvested and subjected to a specific ELISA for IL-6. The amount of IL-6 secreted in the supernatants were then normalized based on 10^3 live osteoclasts for the duration of 3 day (E) and 6 day (F) incubation.

Fig 8: IL-10 secretion by Zometa and Alendronate and Etidronate treated Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 12 days. Osteoclasts at a concentration of $2X10^5$/ml were treated with 10nM-1uM concentrations of nitrogen-containing Zometa and Alendronate and non-nitrogen containing Etidronate as indicated in the figure for the duration of 3 days (A), after which the supernatants were harvested and subjected to a specific ELISA for IL-10. The results obtained in Fig. 8A were then normalized based on 10^3 live osteoclasts for the 3 day (B) incubation. Osteoclasts at a concentration of $2X10^5$/ml were treated with 1uM-50uM concentrations of nitrogen-containing Zometa and Alendronate and non-nitrogen containing Etidronate as indicated in the figure for the duration of 3 days (C) , after which the supernatants were harvested and subjected to a specific ELISA for IL-10. The amount of IL-10 secreted in the supernatants were then normalized based on 10^3 live osteoclasts for the duration of 3 day (D) incubation.

Fig 9: Increased TNF-α secretion by Zometa and Alendronate but not Etidronate treated Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 12 days. Osteoclasts at a concentration of $2X10^5$/ml were treated with different concentrations of nitrogen-containing Zometa and Alendronate and non-nitrogen containing Etidronate as indicated in the figure for the duration of 4 days (A), after which the supernatants were harvested and subjected to a specific ELISA for TNF-α. The amount of TNF-α secreted in the supernatants obtained in 9A were then normalized based on 10^3 live osteoclasts (B).

Fig 10: Increased IL-1β secretion by Zometa and Alendronate but not Etidronate treated Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 12 days. Osteoclasts at a concentration of $2X10^5$/ml were treated with different concentrations of nitrogen-containing Zometa and Alendronate and non-nitrogen containing Etidronate as indicated in the figure for the duration of 4 days, after which the supernatants were harvested and subjected to a specific ELISA for IL-1β.

Fig 11: Decreased size and the numbers of pits by Zometa, Alendronate, and Etidronate treated Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 12 days. Osteoclasts at a concentration of $2.5X10^5$/ml were treated with different concentrations of nitrogen-containing Zometa and Alendronate and non-nitrogen containing Etidronate in carbonate-apatite coated plate as described in figure 2, after which, osteoclasts were washed using 5% bleach, and both the pictures (A) the number of pits counted (B) using microscopy at 5X magnification.

Fig 12: Significant levels of decrease in CD14, CD11β, CD44, MHC-class I and II and CD54 expression were observed on the surface of osteoclasts as compared to freshly isolated autologous monocytes.

Surface expression of Monocytes and Osteoclasts was analyzed by flow cytometry. Osteoclasts were generated from monocytes and cultured in medium containing M-CSF

(25ng/mL) and RANKL (25ng/mL) for 21 days. 1 X10^4 Monocytes and osteoclasts were used to stain and analyze for the surface expression of CD14, CD11b, CD124, B7H1, CD15, CD33, CD44, CD54, MHC-I, and MHC-II.

Fig 13: Comparison of surface expression of Monocytes, Macrophages, Dendritic cells, and Osteoclast.

Monocytes were cultured with RPMI for 8 days. Macrophages were generated from monocytes cultured in medium containing LPS (15ng/ml) and rh-IFN-γ (50 ng/ml) for 8 days. Dendritic cells were generated from monocytes, cultured in medium containing GM-CSF (150 ng/ml) and IL-4 (50 ng/ml) for 8 days. Osteoclasts were generated from monocytes cultured in medium containing M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 8 days and 21 days. Osteoclasts at day 20 of differentiation were treated with rh-IFN-γ (10ng/ml) and rh-TNF-α (20ng/ml) overnight. 1 X10^4 cells were used to analyze the surface expression of, CD14, MHC-I, MHC-II, CD11b, CD44, CD54, and CD133.

Fig 14: Zometa modulated surface receptor expression on Osteoclasts. Surface expression of Zometa treated osteoclasts were analyzed by flow cytometry.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts were then treated with Zometa at 500 nM and 5 μM. After 4 days of incubation, surface expression of CD14, CD44, CD54, B7H1, MHC-I, MHC-II, and CD11b were determined by flow cytometric analysis.

Fig 15: Osteoclasts treated with supernatants from activated NK cells or Zometa demonstrated higher induction of cell surface receptors.

Surface expression of Zometa treated osteoclasts were compared to NK supernatant treated osteoclasts. Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts were treated with zometa for 5 days and supernatant from NK cells and monocyte co-culture for 2 days. CD54, MHC-I, MHC-II, CD44, CD14 and CD11b expression were determined by flow cytometric analysis.

Fig. 16: Osteoclasts are susceptible to NK cells mediated cytotoxicity and induce significant IFN-γ secretion by the NK cells.

Highly purified NK cells ($1X10^6$ cells/ml) were left untreated, treated with IL-2 (1000 units/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours before they were added to ^{51}Cr labeled autologous osteoclasts at various effector to target ratios. NK cell mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay (A). NK cells were purified and left untreated or treated with IL-2 (1000 units/ml) for 18 hours and then added to ^{51}Cr labeled autologous monocytes or autologous osteoclasts at various effector to target ratios. NK cell mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay (B). Purified NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18 hours before the cells were added to autologous monocytes or osteoclasts at an effector to target ratio of 1:1 of NK cells to monocytes or osteoclasts. After an overnight incubation, the supernatants were removed and the level of secreted IFN-γ is measured using specific ELISA (C).

Fig. 17: Zometa treated OSCSCs are more resistant to NK cells mediated cytotoxicity.

(A) Highly purified NK cells ($1X10^6$ cells/ml) were left untreated, treated with IL-2 (1000 units/ml), treated with anti-CD16mAb (3 ug/mL), and with combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours before they were added to ^{51}Cr labeled OSCSCs at various effector to target ratios. OSCSCs were pre-treated with nitrogen-containing BP, Zometa and Alendronate and non-nitrogen containing BP, Etidronate for 30 mins at 1μM concentration. NK cell mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay. Zometa treated OSCSCs were more resistant to NK cell mediated cytotoxicity. (B) Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days, followed by the treatment with nitrogen-containing BP, Zometa (1μM and 500nM) and Alendronate (500nM) for 4 days. Highly purified NK cells ($1X10^6$ cells/ml) were left untreated, treated with IL-2 (1000 units/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours before they were added to ^{51}Cr labeled autologous osteoclasts at various effector to target ratios. NK cell mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay. Zometa treated hOC were more resistant to NK cell mediated cytotoxicity. (C) Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days, followed by the treatment with nitrogen-containing BP, Zometa (1μM and 500nM) for 4 days. Highly purified NK cells ($1X10^6$ cells/ml) were left untreated, treated with IL-2 (1000 units/ml), treated with anti-CD16mAb (3 ug/mL) and with combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours before they were added to ^{51}Cr labeled autologous osteoclasts at various effector to target ratios. NK cell mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay.

Fig. 18: Zometa treated hOC secrete higher IFN-γ, when co-cultured with IL-2 treated and combination of IL-2 and anti-CD16mAb treated NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts at a concentration of $3 \text{ X } 10^5$ ml were treated with 1μM of nitrogen-containing BP, Zometa and Alendronate. After 72 hours of treatment, BP were washed off, and fresh media was added. Purified NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18 hours before the cells were added to autologous monocytes or osteoclasts at an effector to target ratio of 1:3 of NK cells to monocytes or osteoclasts. After an overnight incubation, the supernatants were harvested and subjected to a specific ELISA for and IL-6 (A) , IFN-γ (B) and IL-10 (C).

Human monocytes purified from healthy donor's PBMCs, were differentiated into osteoclasts in culture with alpha-MEM medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. Seeded in 6 well plate, keeping 0.2 million cells per well, treated with BP at 1uM concentration. After 12 hour incubation, media was replaced with fresh media. Untreated NK cells (1 million/mL), IL-2 (1000 U/mL) treated NK and combination of IL-2 (1000 U/mL) and anti-CD16mAb (3 ug/mL), were added in 1:2. effector to target ratio. Supernatant were harvested after 24 hours from co-culture and level of IFN-γ secretion was determined using specific ELISA (D). Osteoclasts and NK cells were treated as described in Fig. 18A and the supernatants were harvested and subjected to a specific ELISA for IL-18 (E).

Table 1

Concentration (pg/mL)					
	Day 2	**Day 6**	**Day 10**	**Day 14**	**Day 21**
IL-1RA	680.21	673.11	3168.01	4897.2	4342.09
IL-2R	30.7	30.7	65.33	156.18	212.19
IL-12	19.8	7.8	17.25	23.56	32.06
IL-15	58.53	71.99	162.6	78.49	51.51
IFN-alpha	90.08	107.54	125.92	105.93	68.78
IFN-gamma	0	0	0	0	0
IL-6	108.12	14.35	58.17	33.92	15.58

Concentration (pg/mL)					
	Day 2	**Day 6**	**Day 10**	**Day 14**	**Day 21**
MIP-1 alpha	39.71	43.28	639.82	1931.8	5070.89
MIP-1 beta	116.41	109.21	1914.16	3907.9	5168.19
RANTES	271.49	54.85	63.02	238.27	432.49
MCP-1	>6100	>6100	>6100	>6100	3627.89
IL-8	20001.6	24988.27	22072.3	0	22267
MIG	0	2.15	27.67	12.39	14.25
IP-10	15.87	23.73	55.62	19.48	12.82
Eotaxin	0	0	0	0	0

Table 2

	IL-1b	IL-1Ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-9	IL-10	IL-12p70	IL-13	IL-15	IL-17	IFN-γ	TNF-α
Monocytes	8.09	159.16	12.84	2.75	0	293.42	2.51	11.73	21.01	23.96	0	16.59	42.34	0	25.9
DCs	10.25	9397.28	21.91	NA	4.02	686.75	3.78	11.3	86.89	6	4.7	17.98	72.05	0	46.76
M1 macrophages	>1952	4433.97	35.23	6.42	0	32200	13.54	14.63	1080.91	73.15	12.27	0	70.46	NA	710.12
M2 macrophages	14.76	433.73	20.06	3.7	4.02	395.1	8.25	18.21	32.89	33.21	3.75	20.05	76.01	0	43.68
Osteoclasts	0	91.34	11.06	2.12	0	46.38	0	9.53	13.55	10.42	0	21.47	31.55	0	24.19

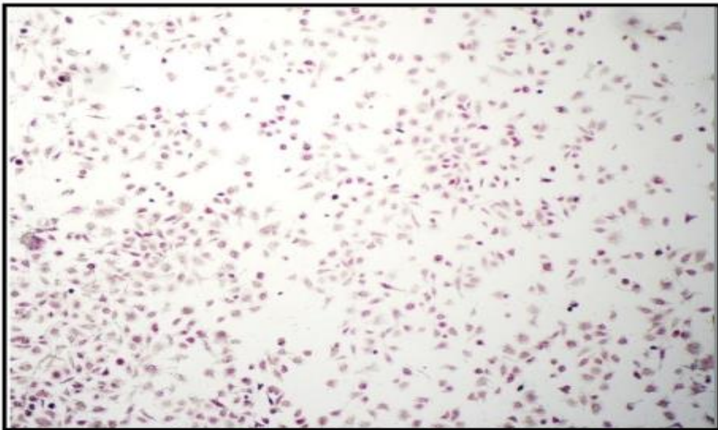
	MCP-1	MIP-1α	MIP-1β	RANTES	Eotaxin	IL-8	IP-10
Monocytes	1151.65	17.51	205.34	>1890.95	0	7209.76	618.56
DCs	110.77	21.42	146.5	>1890.95	0	3110.04	174.96
M1 macrophages	93.68	206.93	2099.65	672.6	33.79	22261.52	481.04
M2 macrophages	3001.59	19.83	297.83	>1890.95	0	27886	1877.6
Osteoclasts	2133.02	16.56	186.84	1559.69	0	3873.57	1285.13

	Basic FGF	VEGF	PDGF-BB	G-CSF	GM-CSF
Monocytes	0	151.04	281.15	66.34	84.74
DCs	0	19.4	324.82	85.95	NA
M1 macrophages	0	995.54	337.5	9348.9	52.7
M2 macrophages	0	250.87	424.7	81.29	62.03
Osteoclasts	0	76.05	117.12	31.25	90.28

Table 3

	IL-6	IFN-γ	IL-10	MCP-1	IL-8	MIP-1α	MIP-1β
Media	7	1	0	56	7	1039	0
Untreated NK	24	1	0	999	1514	942	375
IL-2 treated NK	54	4	0	1252	3638	1039	586
IL-2 + anti-CD16mAb treated NK	252	25	0	3818	16969	5803	20368
Monocytes	106	4	0	239	11217	1377	375
Monocytes + Untreated NK	99	2	0	264	10426	1135	163
Monocytes + IL-2 treated NK	136	4	10	1232	11125	1232	374
Monocytes + IL-2 + anti-CD16mAb treated NK	424	33	10	28195	21638	1619	4229
hOC	41	4	14	31691	16607	1377	2129
hOC + Untreated NK	63	4	23	29258	16544	1715	2185
hOC + IL-2 treated NK	535	4	24	38655	19349	1908	5680
hOC + IL-2 + anti-CD16mAb treated NK	3866	57	82	38916	23766	6114	61555
hOC + ZA	196	4	14	37820	18589	1425	2157
hOC + ZA + Untreated NK	312	6	14	38159	20986	1715	2538
hOC + ZA + IL-2 treated NK	3267	12	20	39332	22631	4294	11587
hOC + ZA + IL-2 + anti-CD16mAb treated NK	11605	80	31	38748	24420	6105	38411

Fig. 1



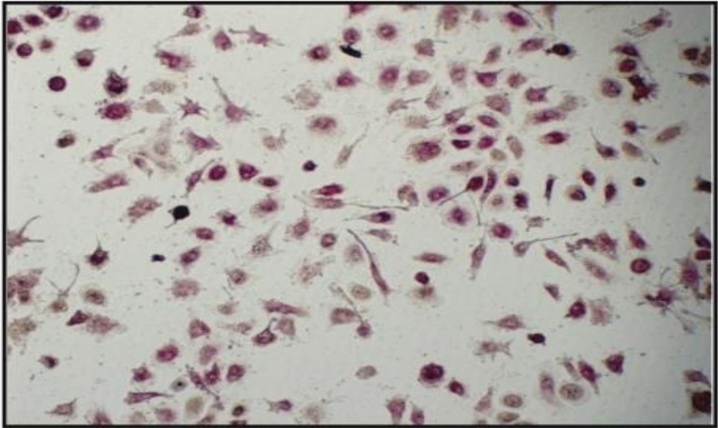


Fig. 2A

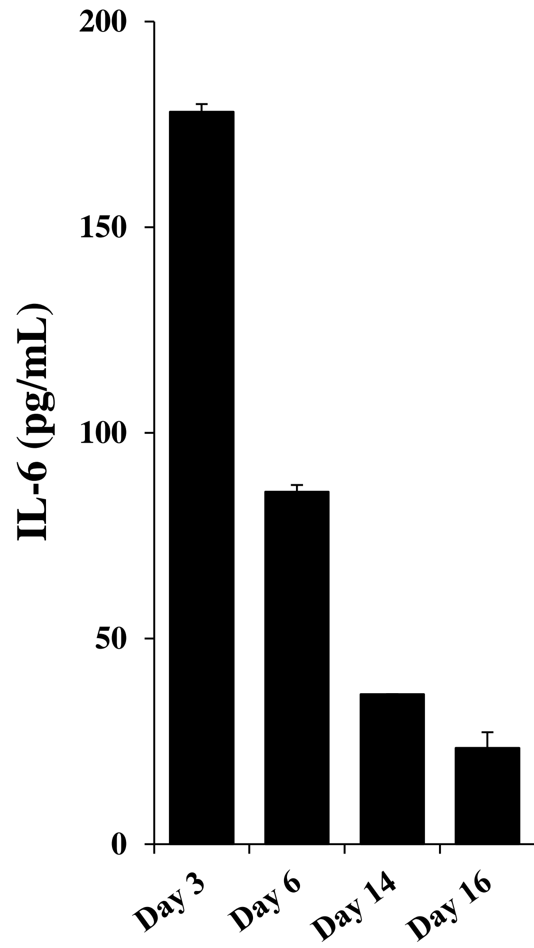


Fig. 2B

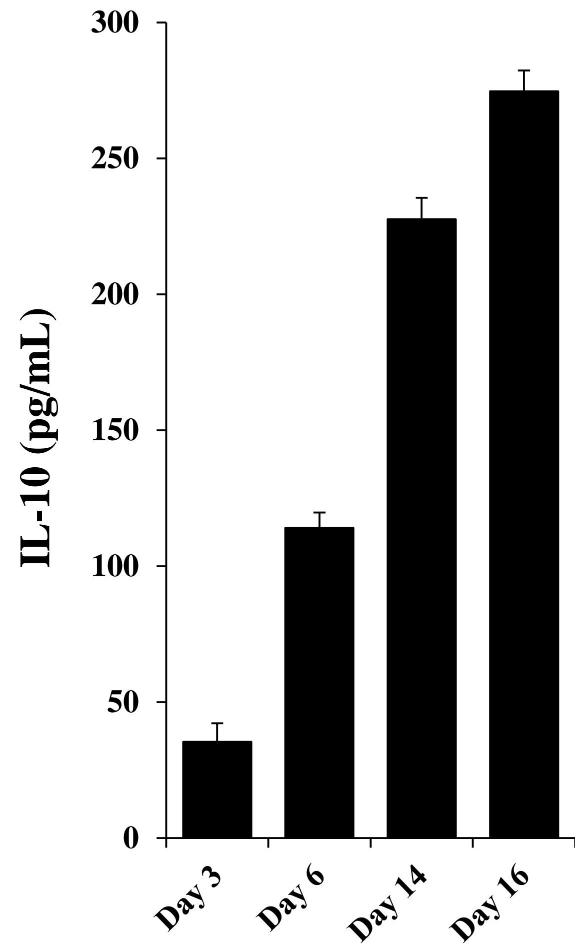


Fig. 3A

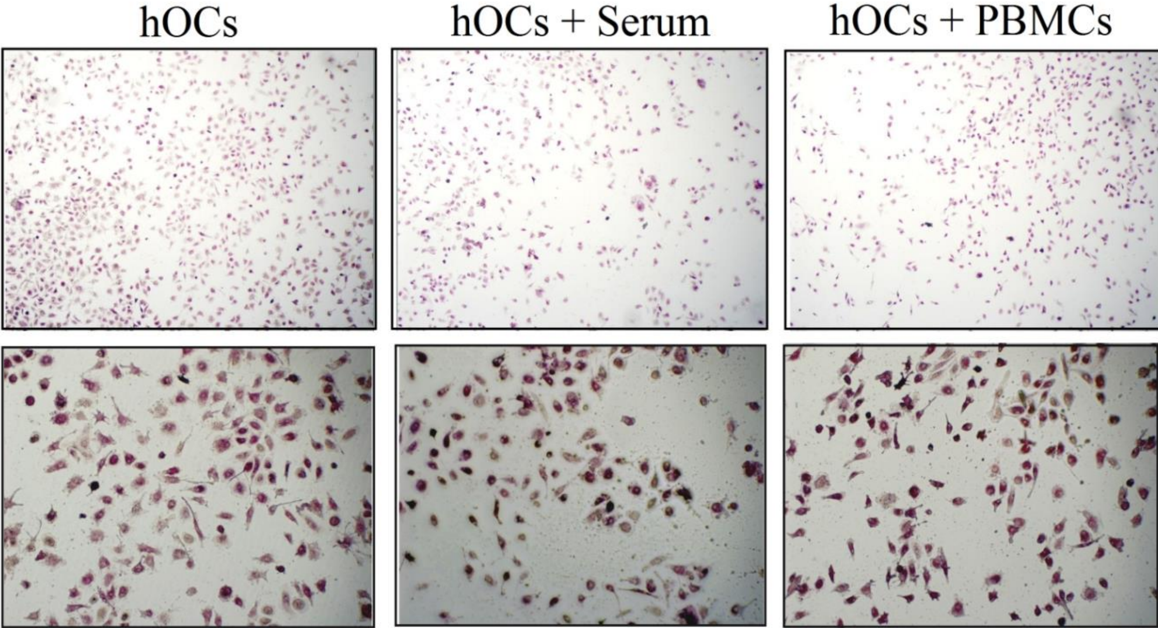


Fig. 3B

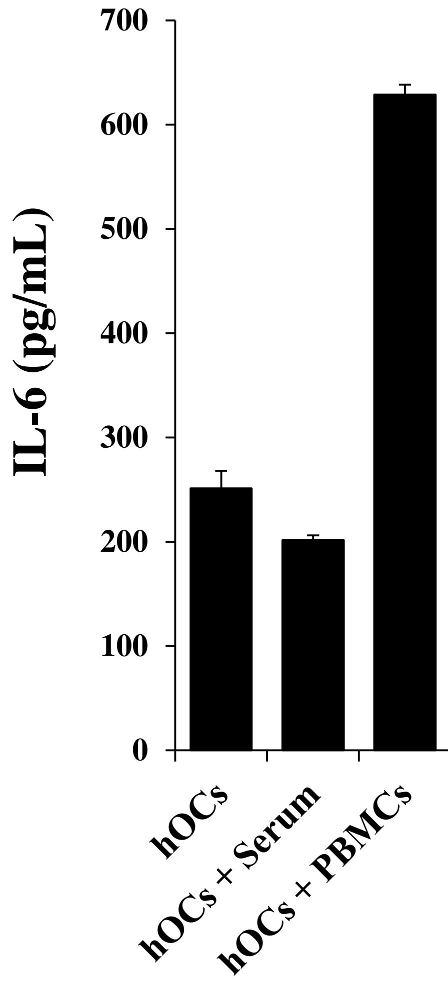


Fig. 3C

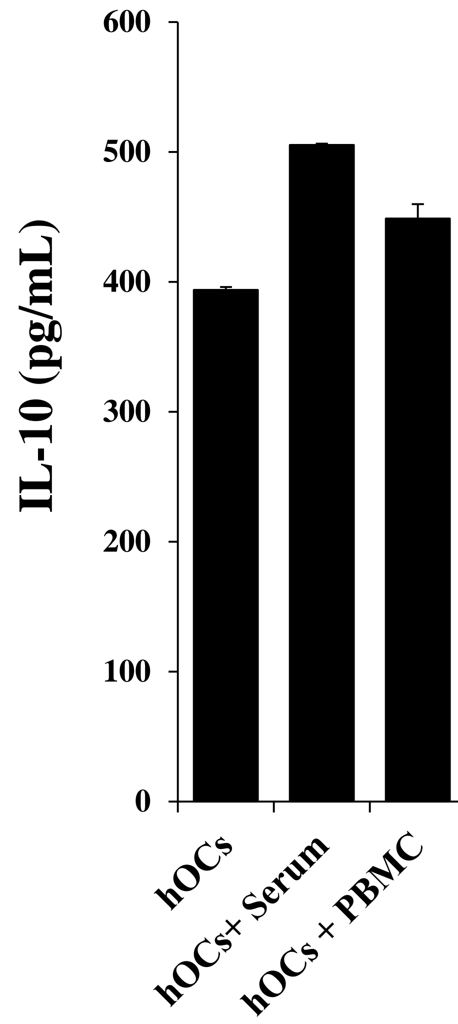


Fig. 3D

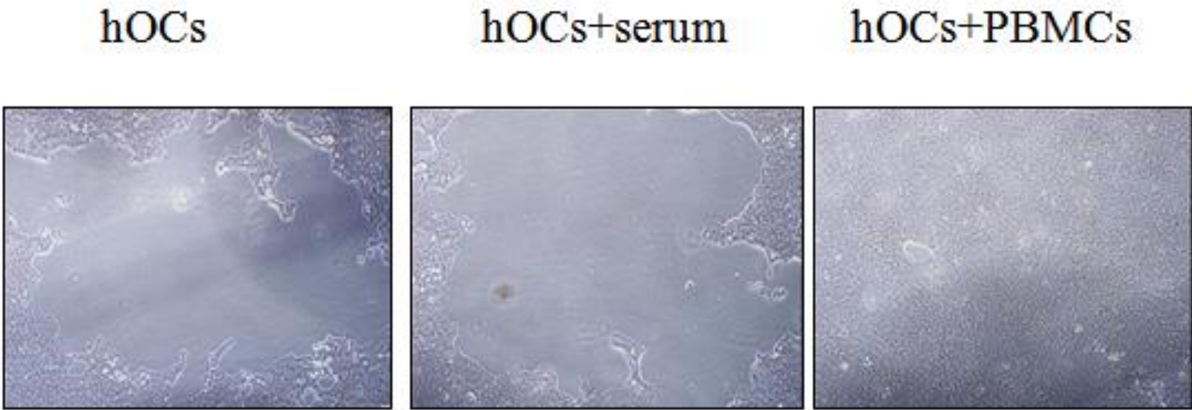


Fig. 4

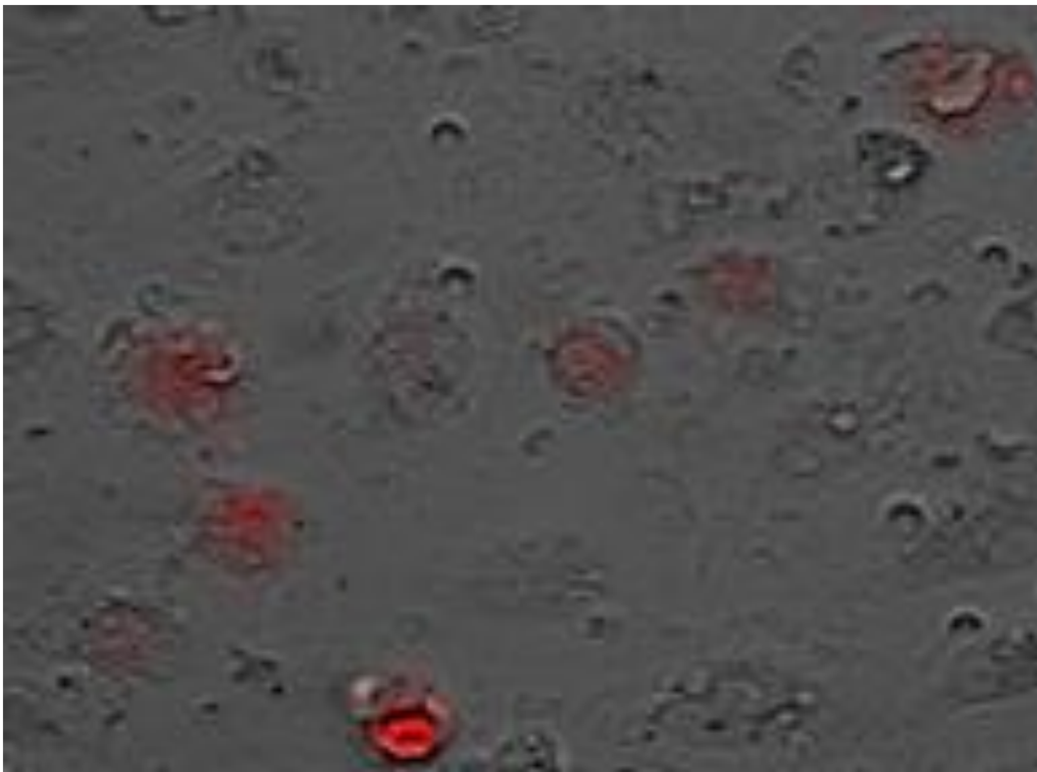
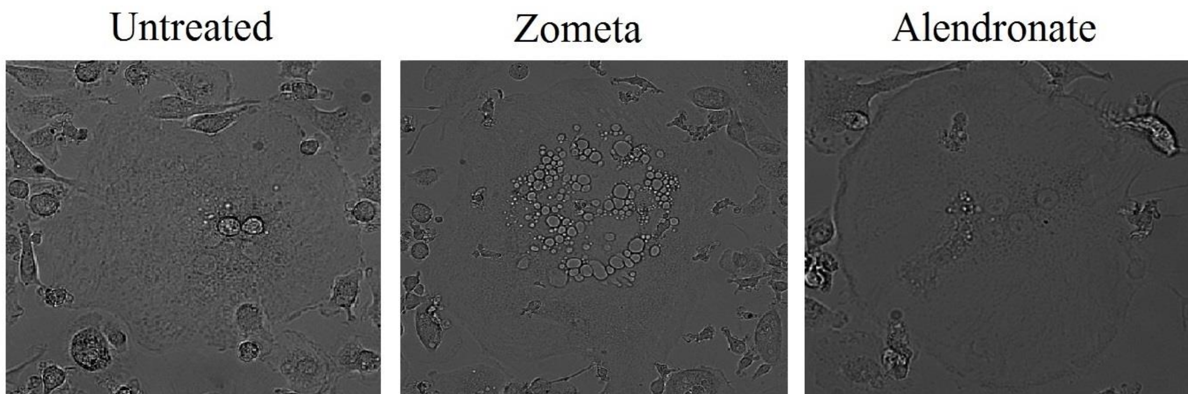


Fig. 5



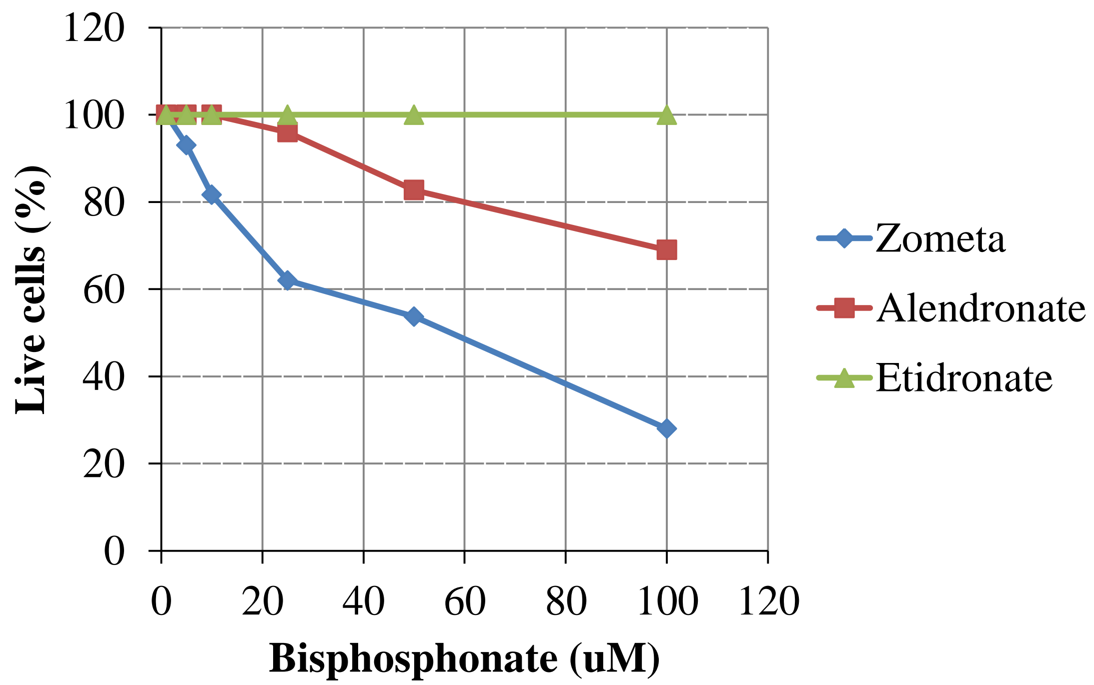


Fig. 6A

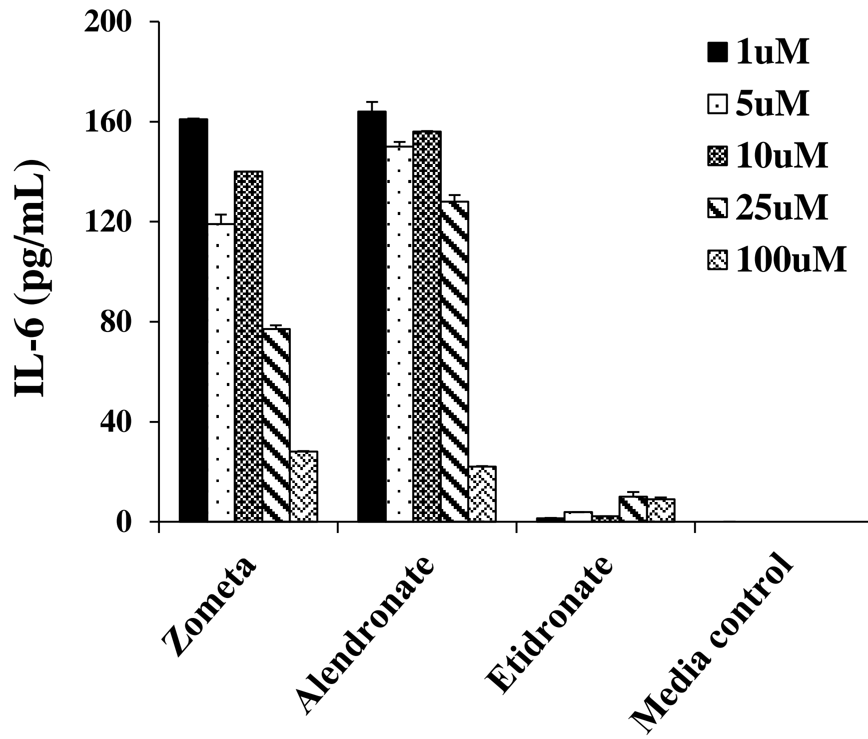
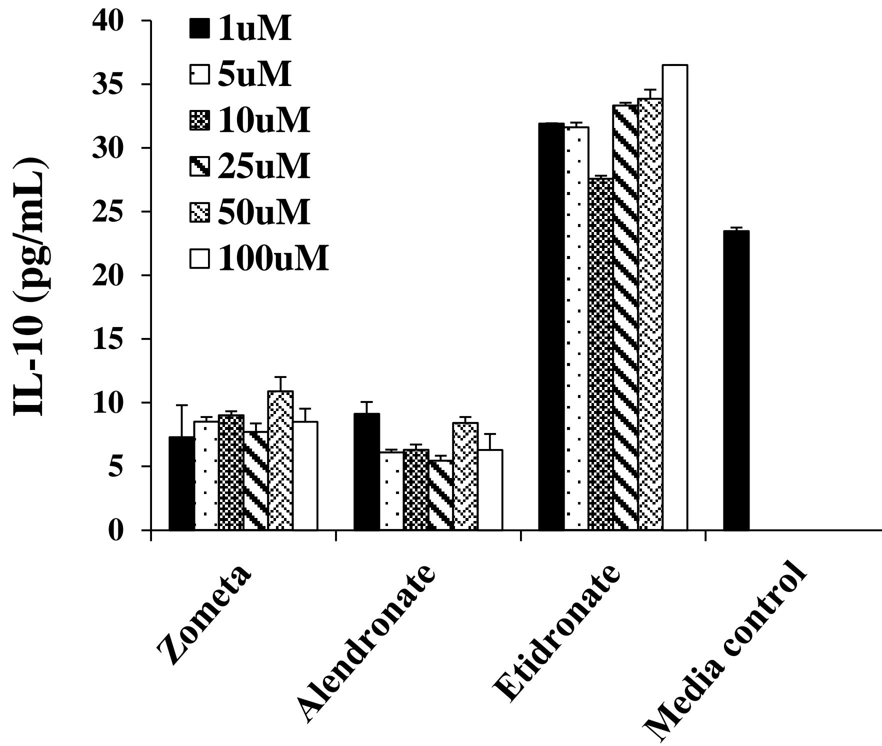


Fig. 6B



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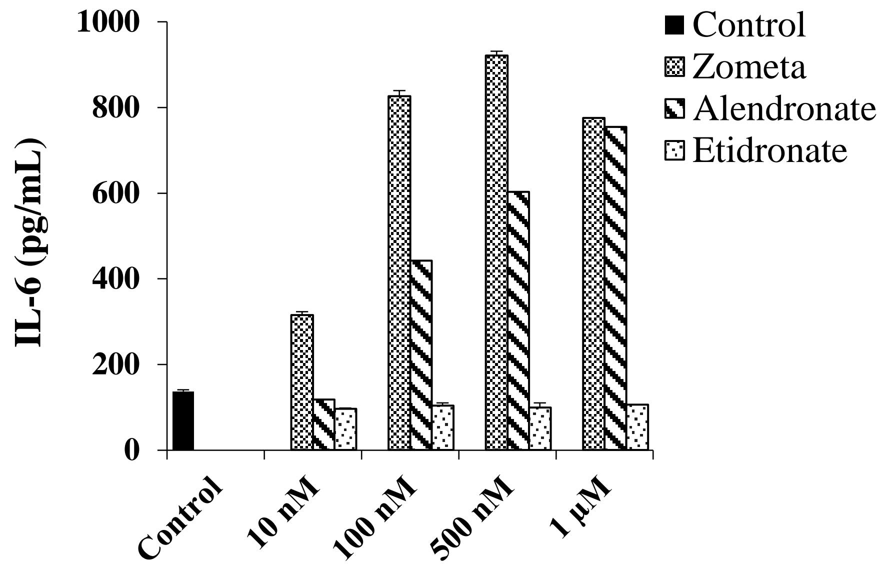

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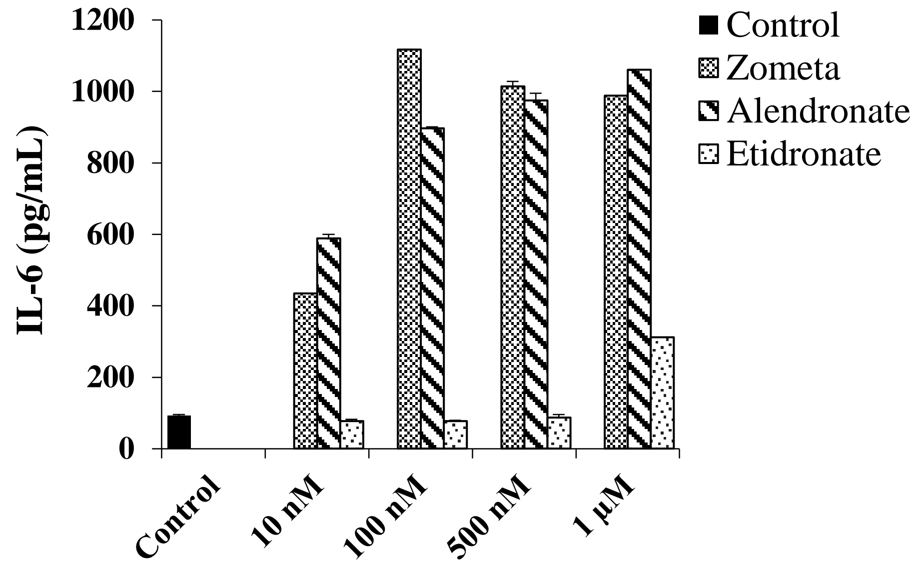
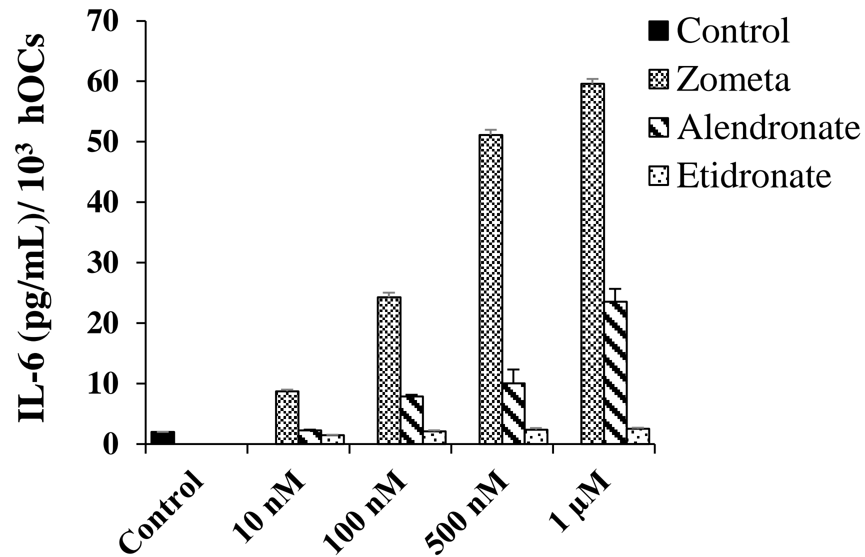


Fig. 7C



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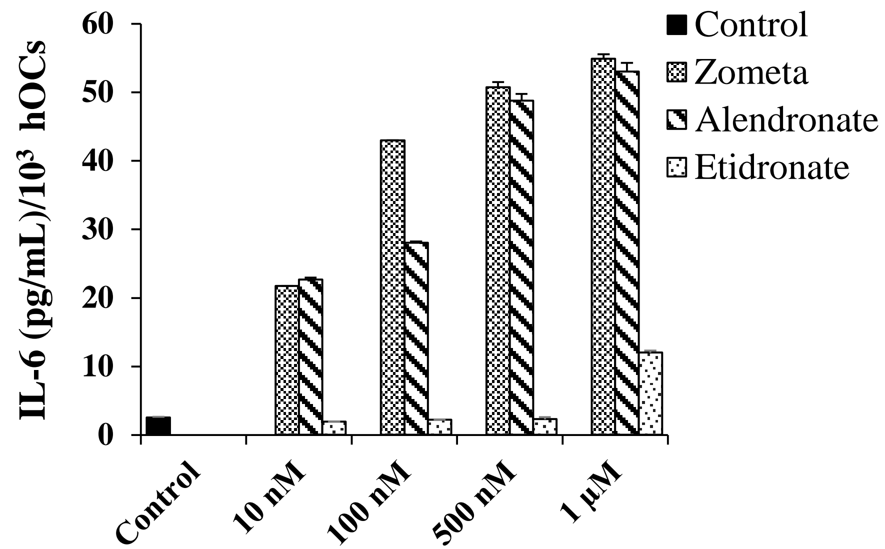

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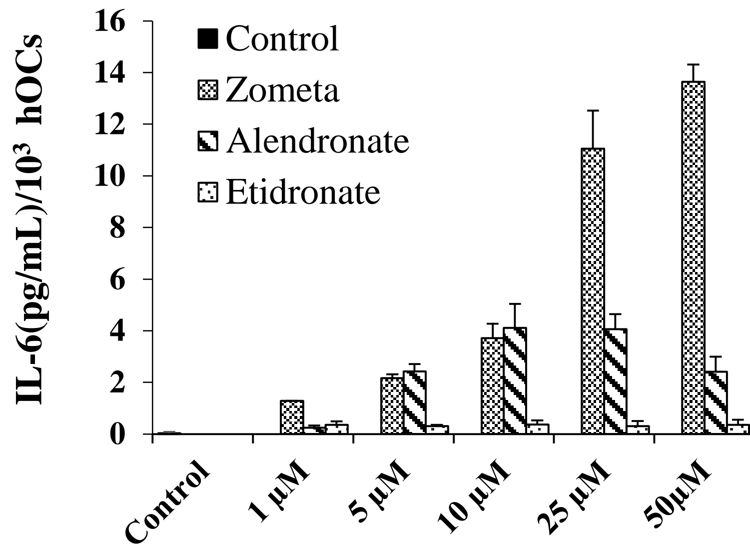

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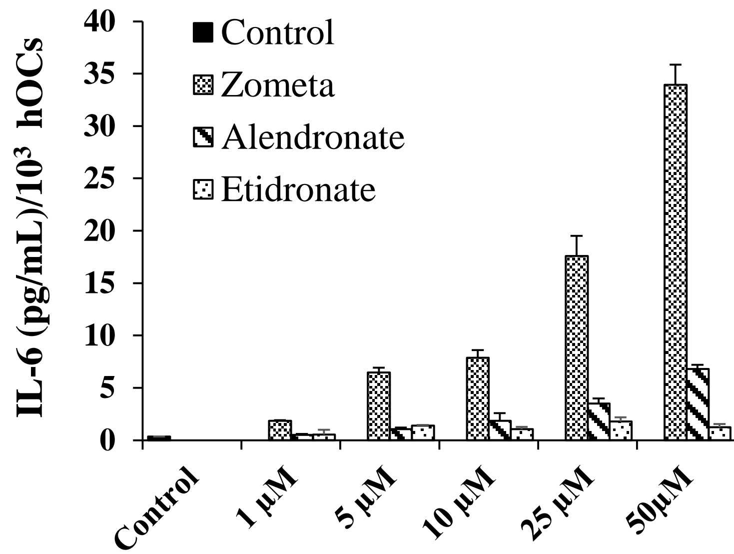
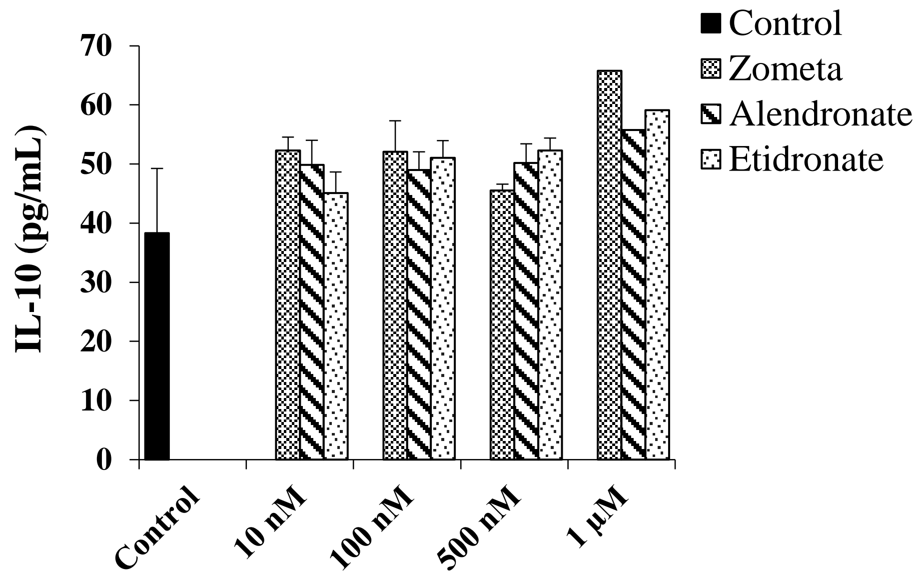


Fig. 8A



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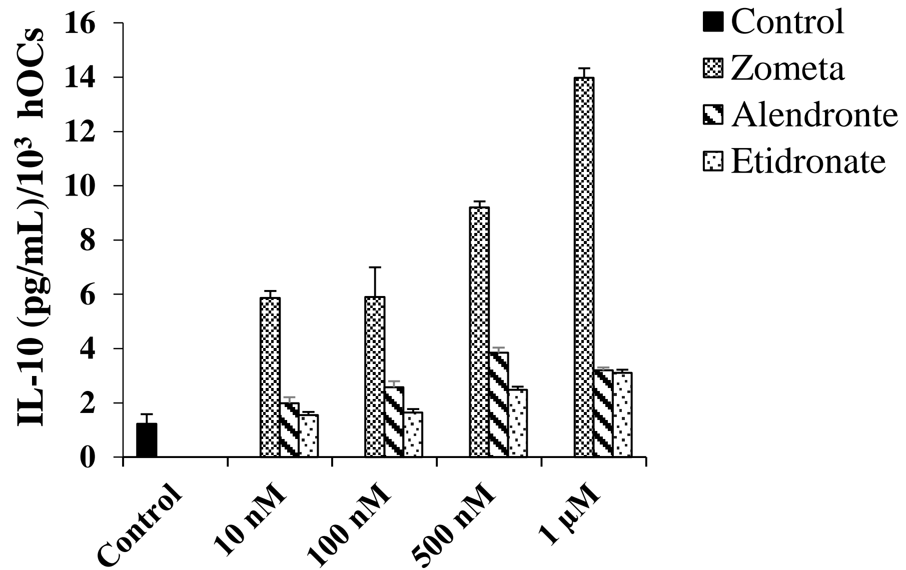
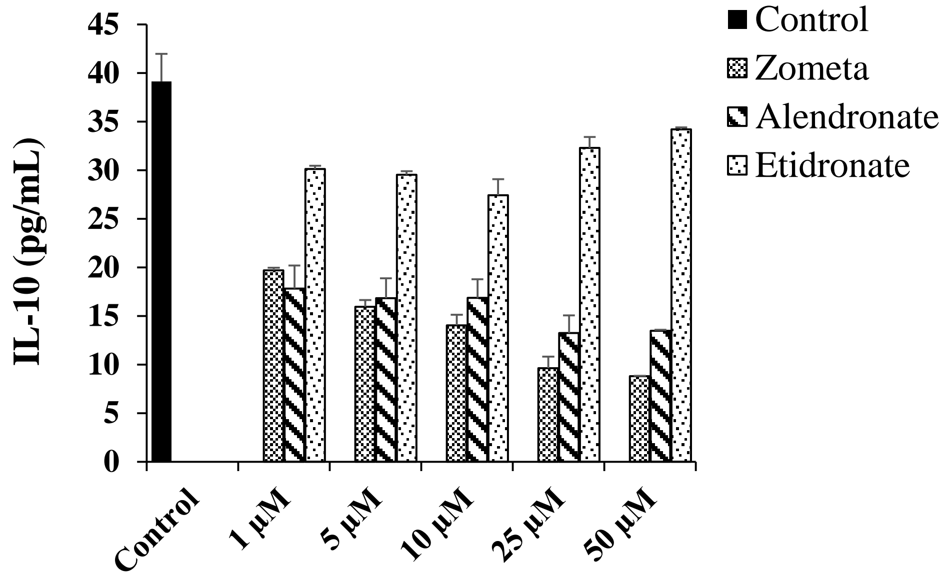


Fig. 8C



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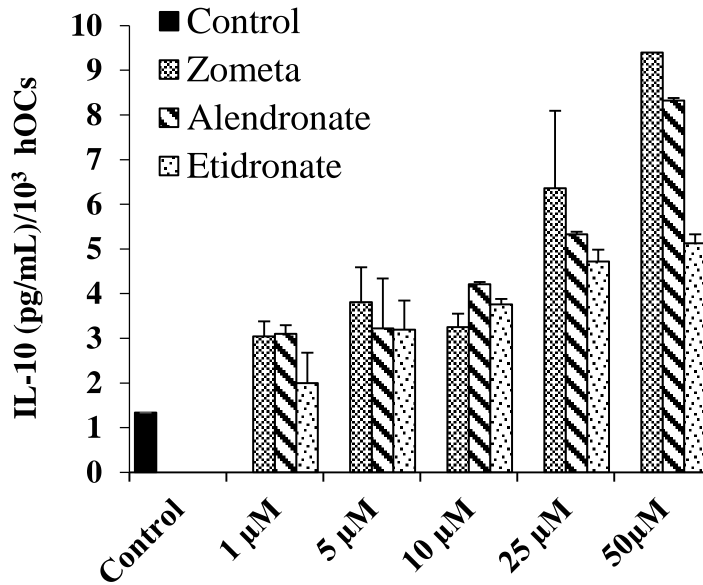


Fig. 9A

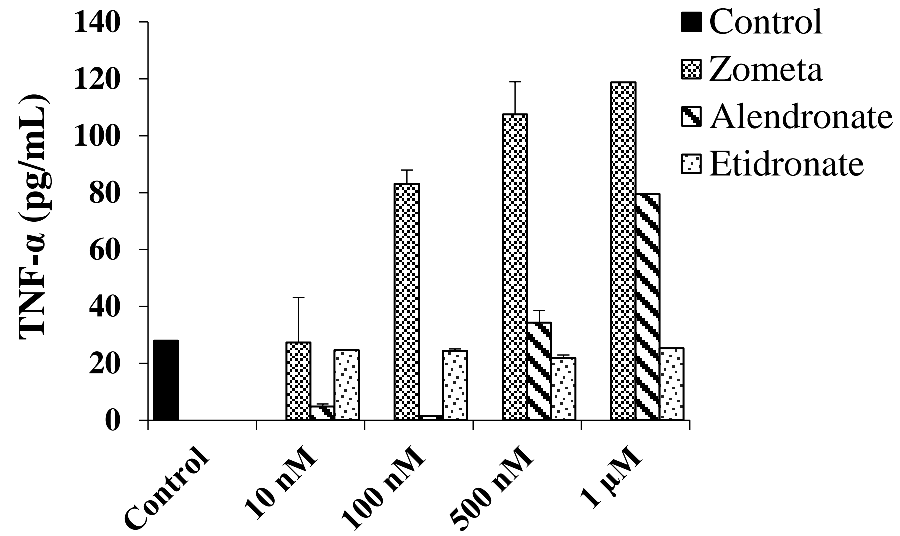


Fig. 9B

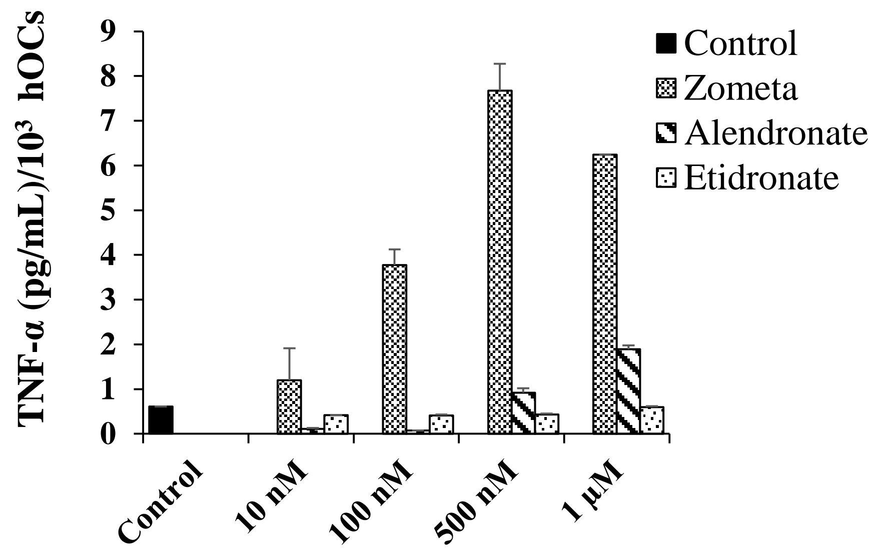
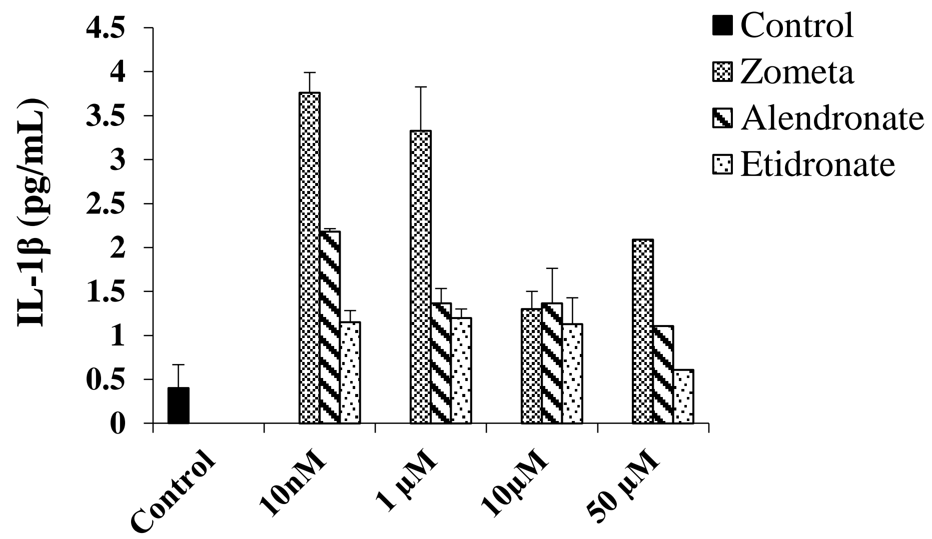


Fig. 10



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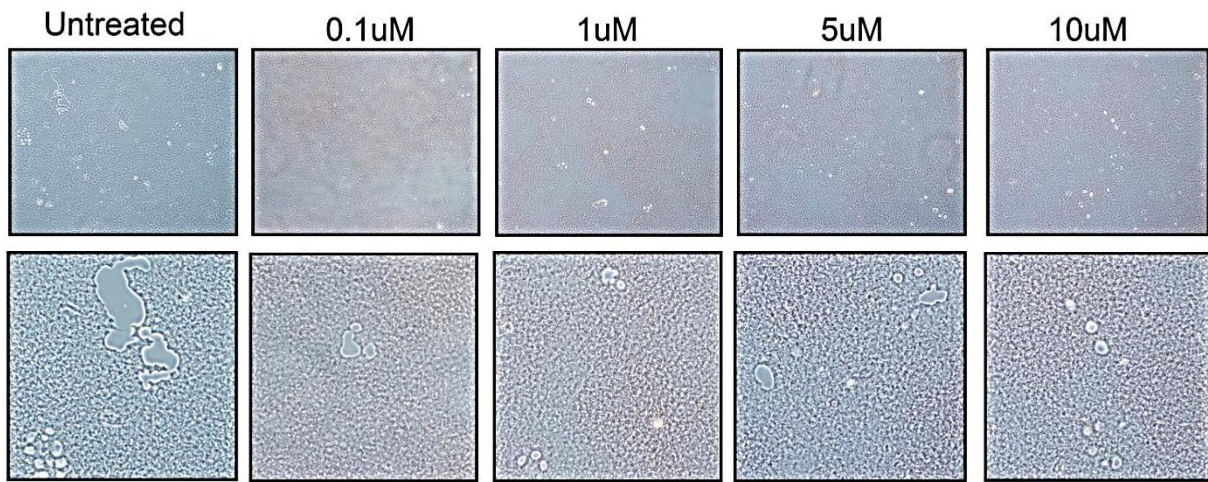


Fig. 11B

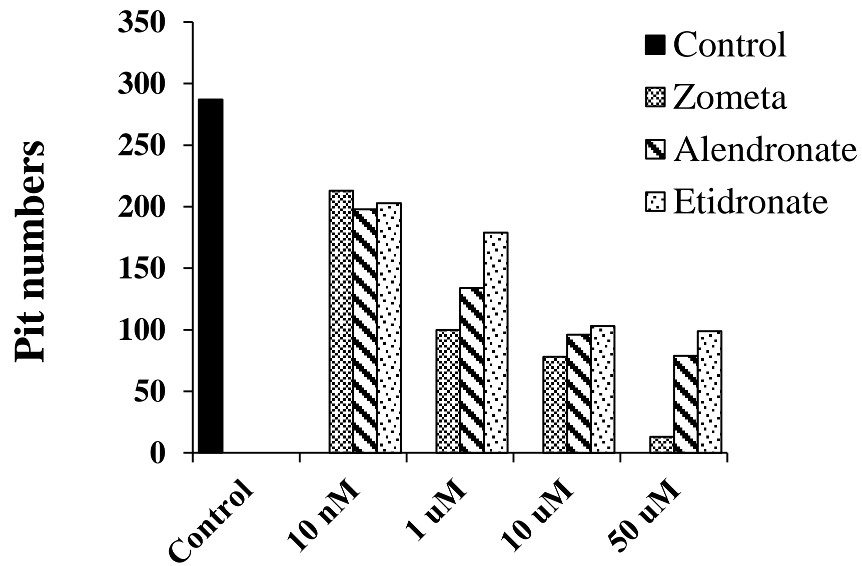


Fig. 12

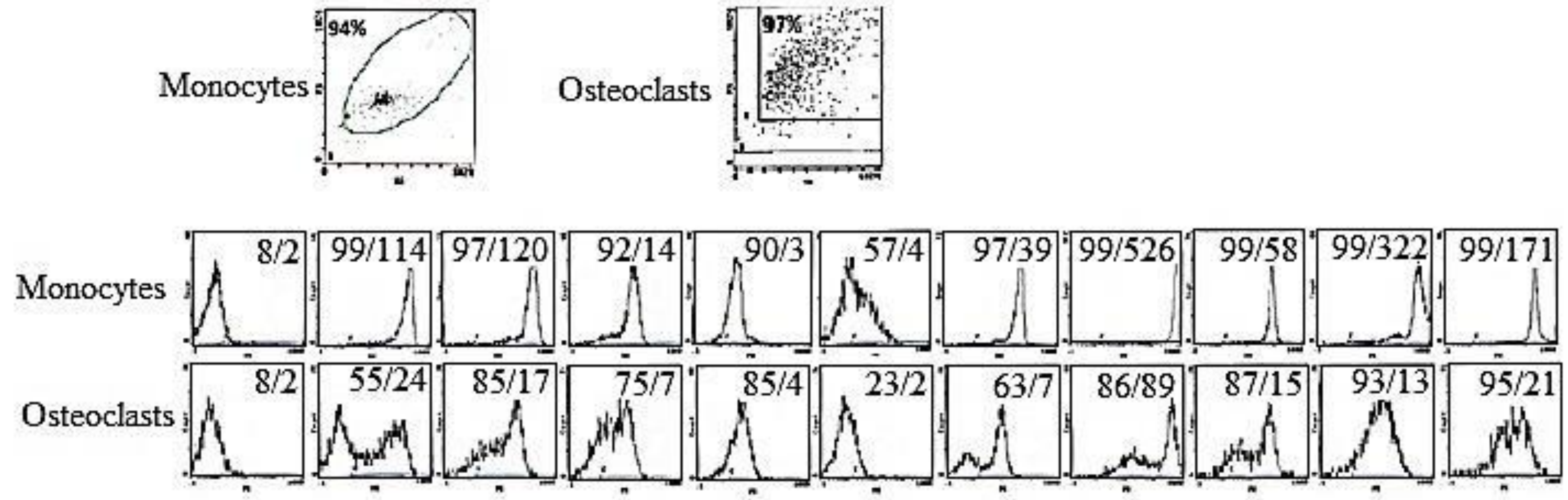


Fig. 13

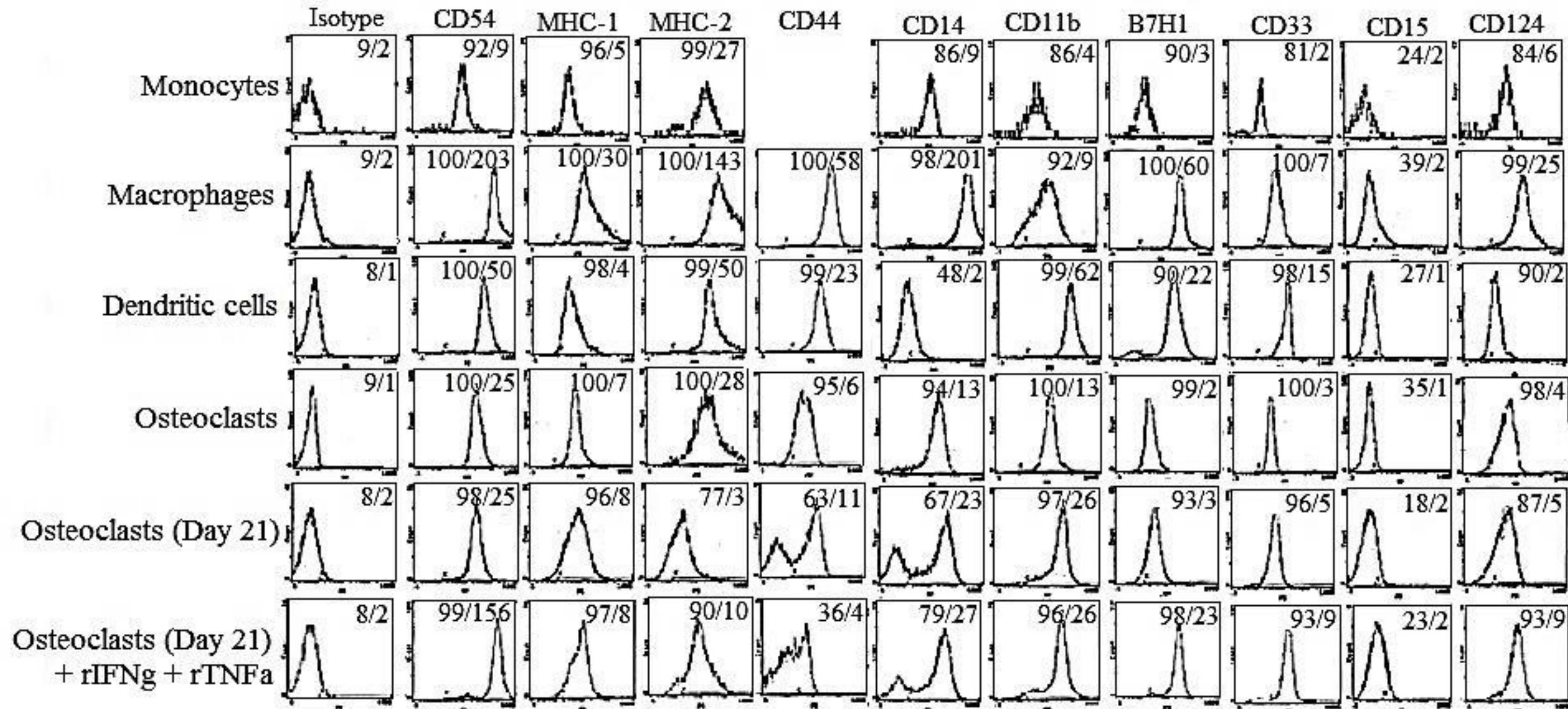


Fig. 14

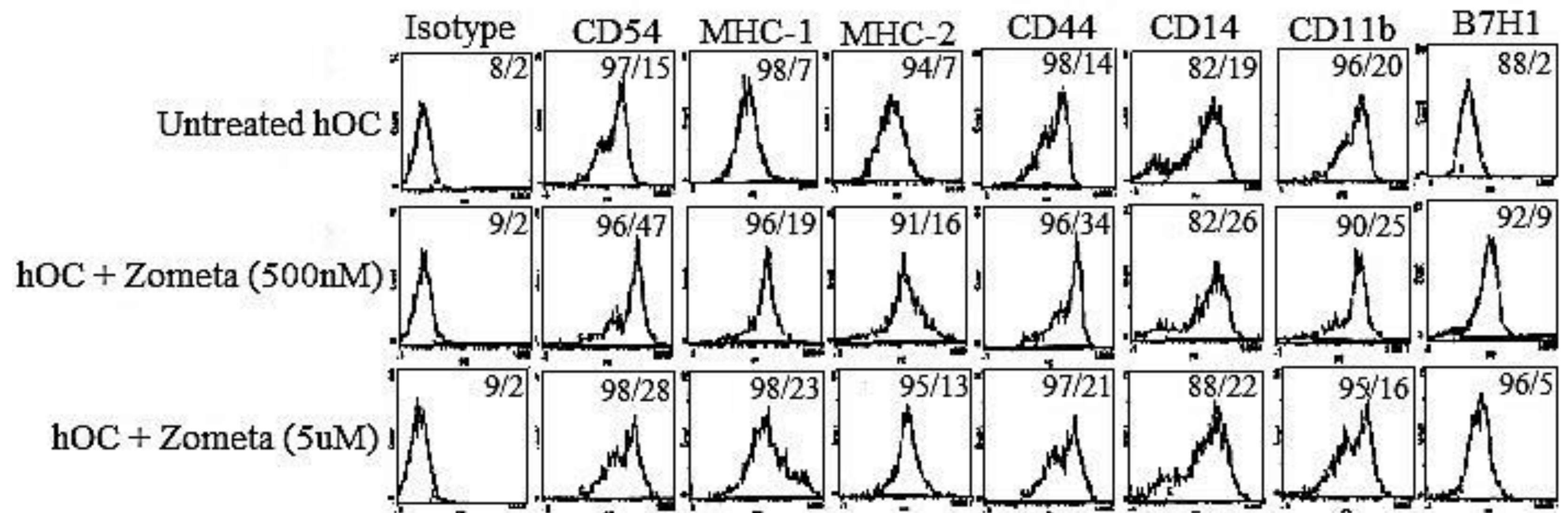


Fig. 15

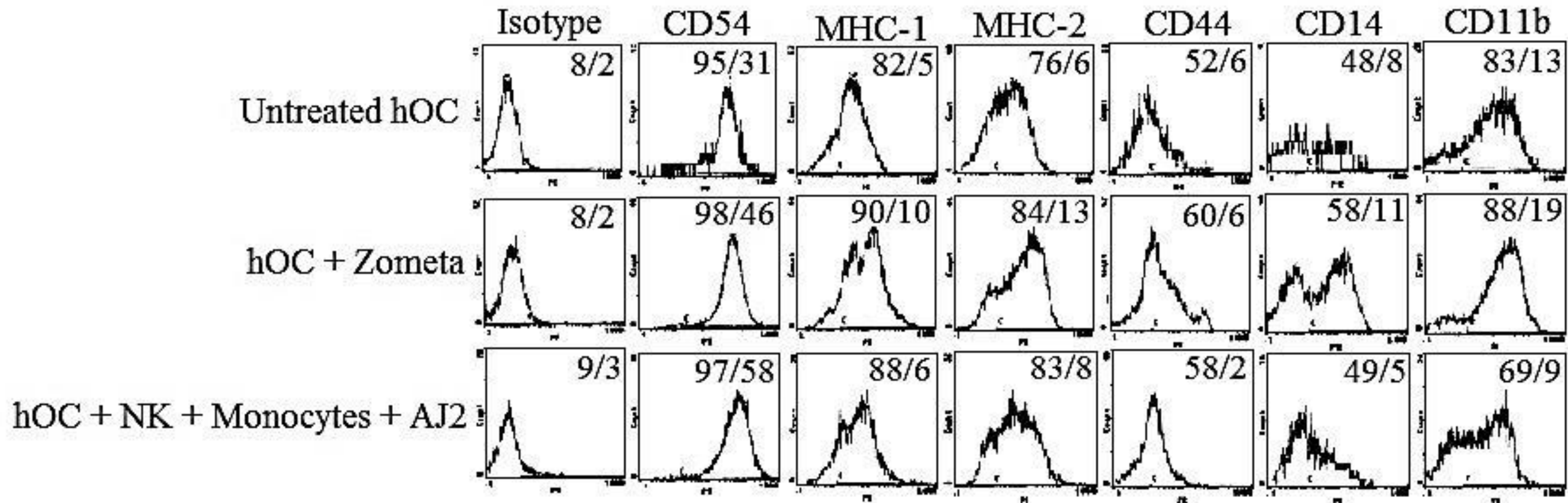
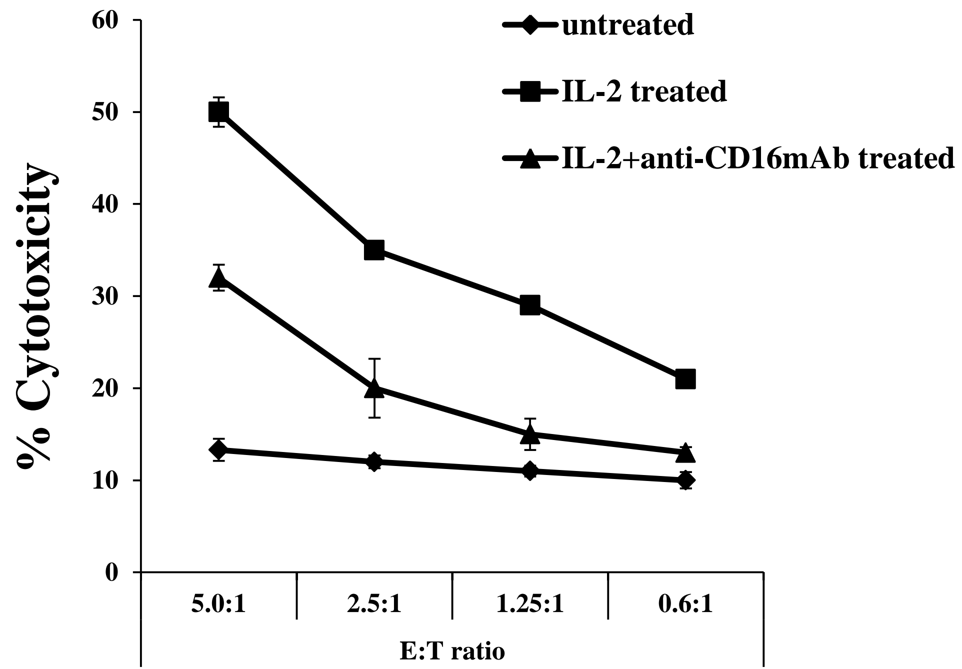


Fig. 16A



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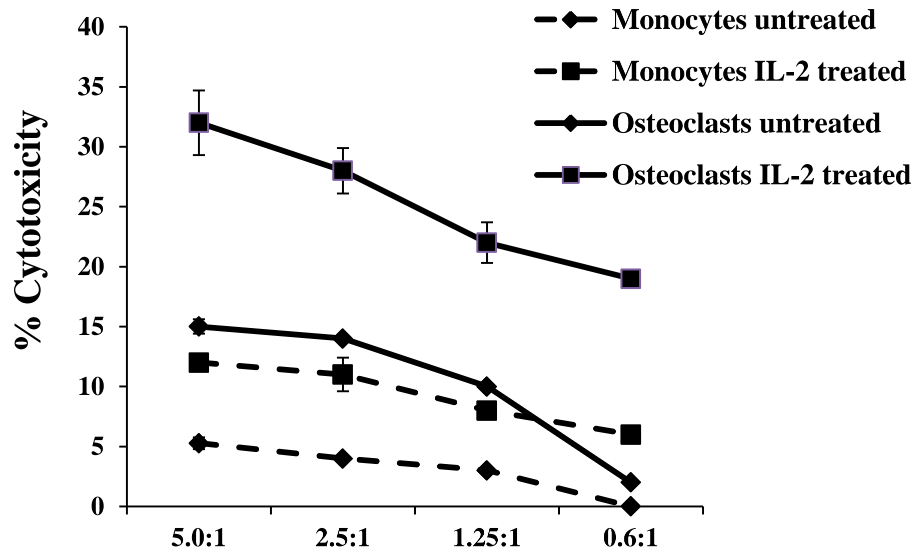


Fig. 16C

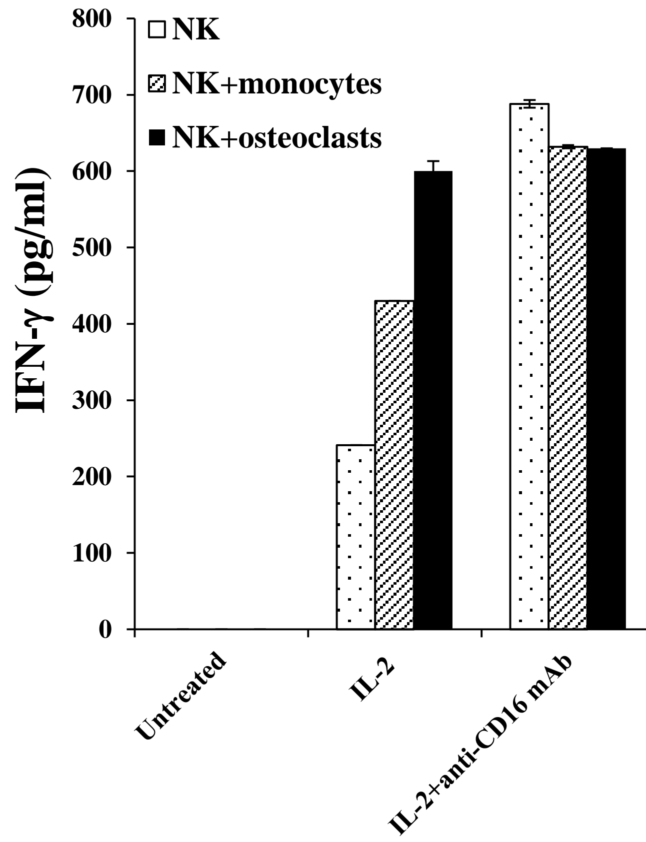


Fig. 17A

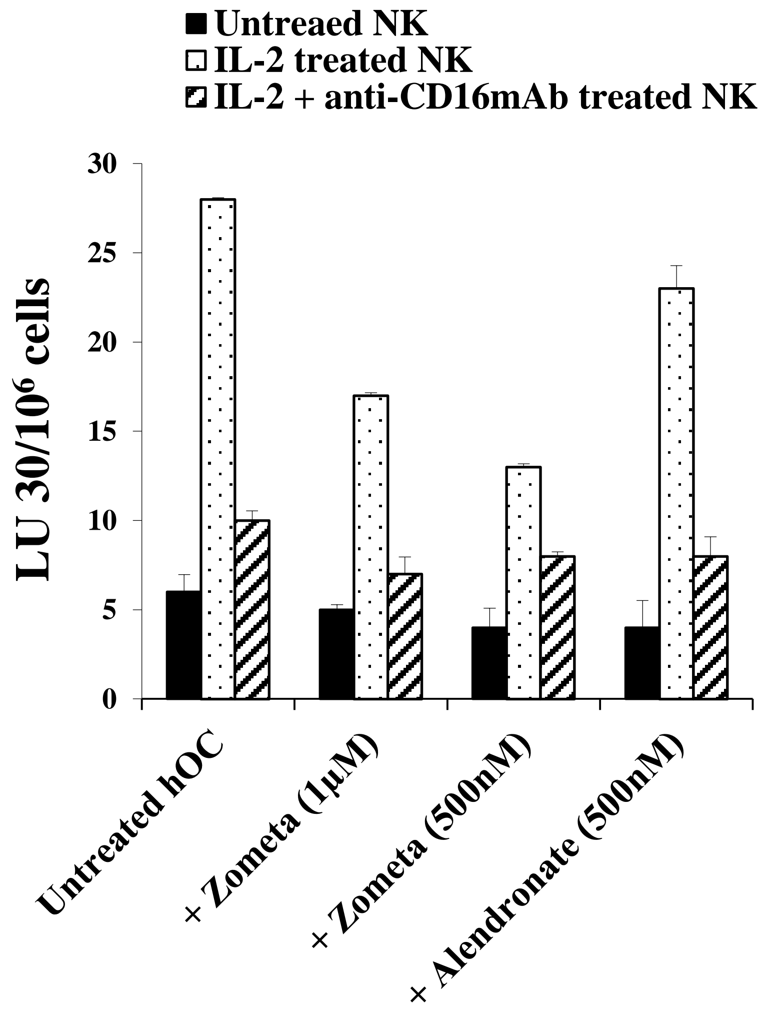
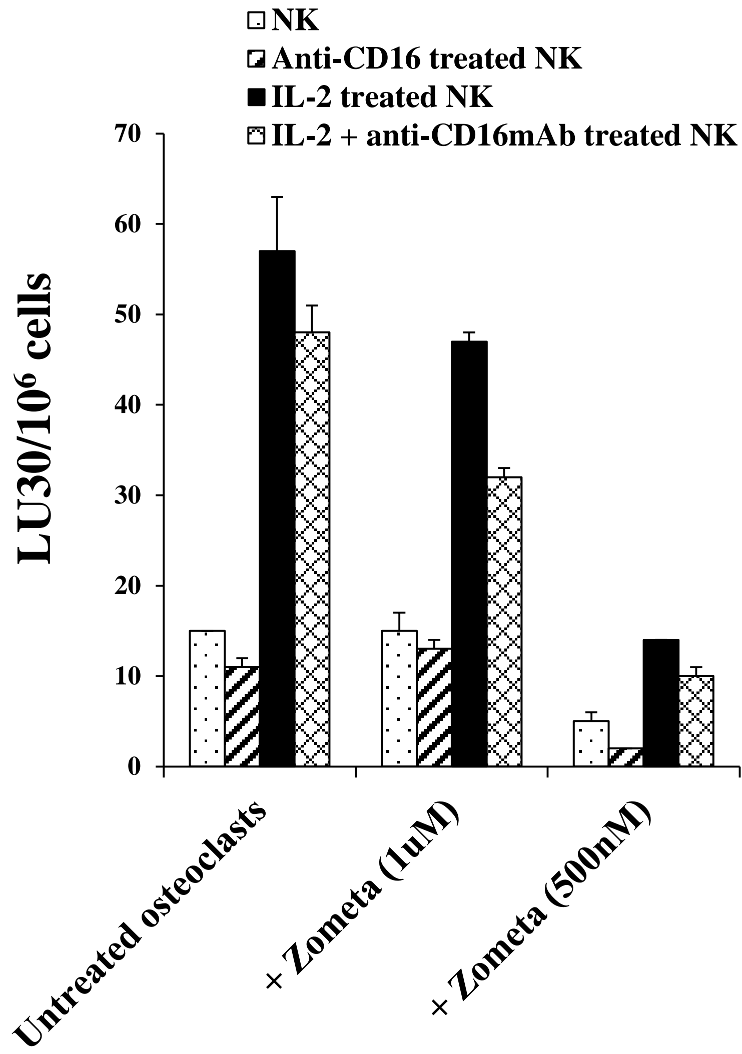


Fig. 17B



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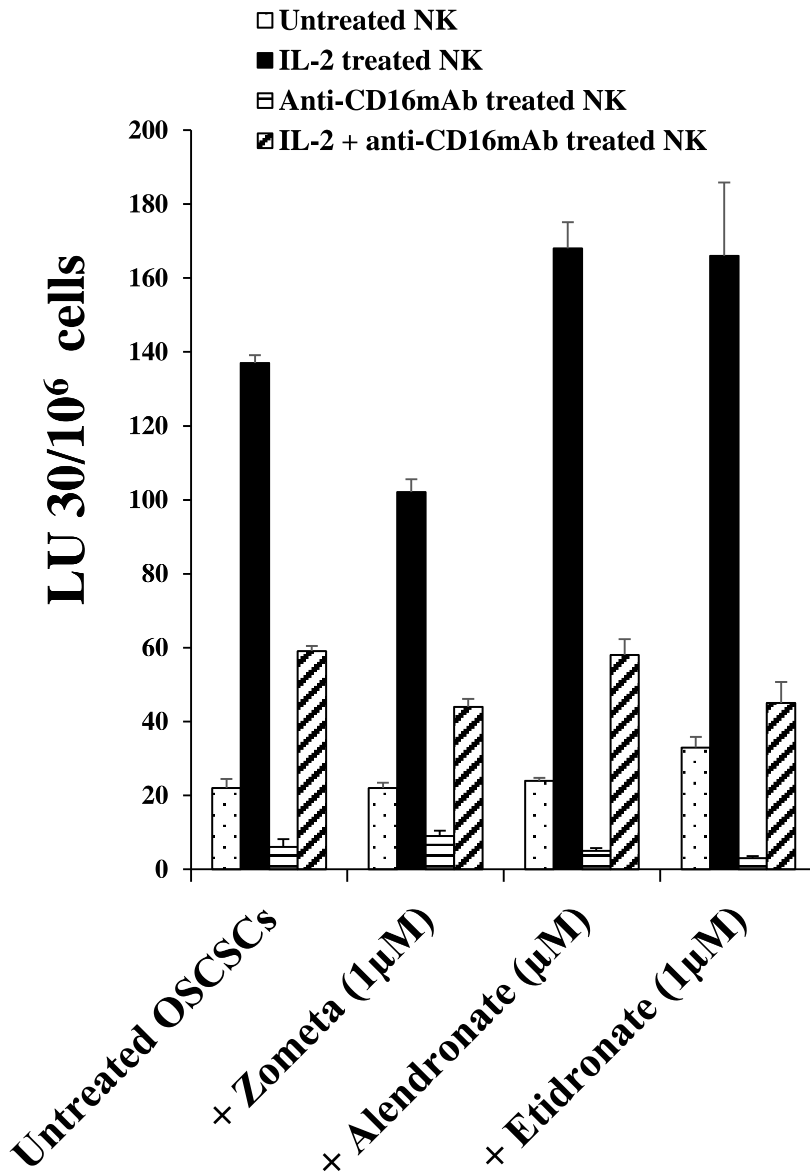

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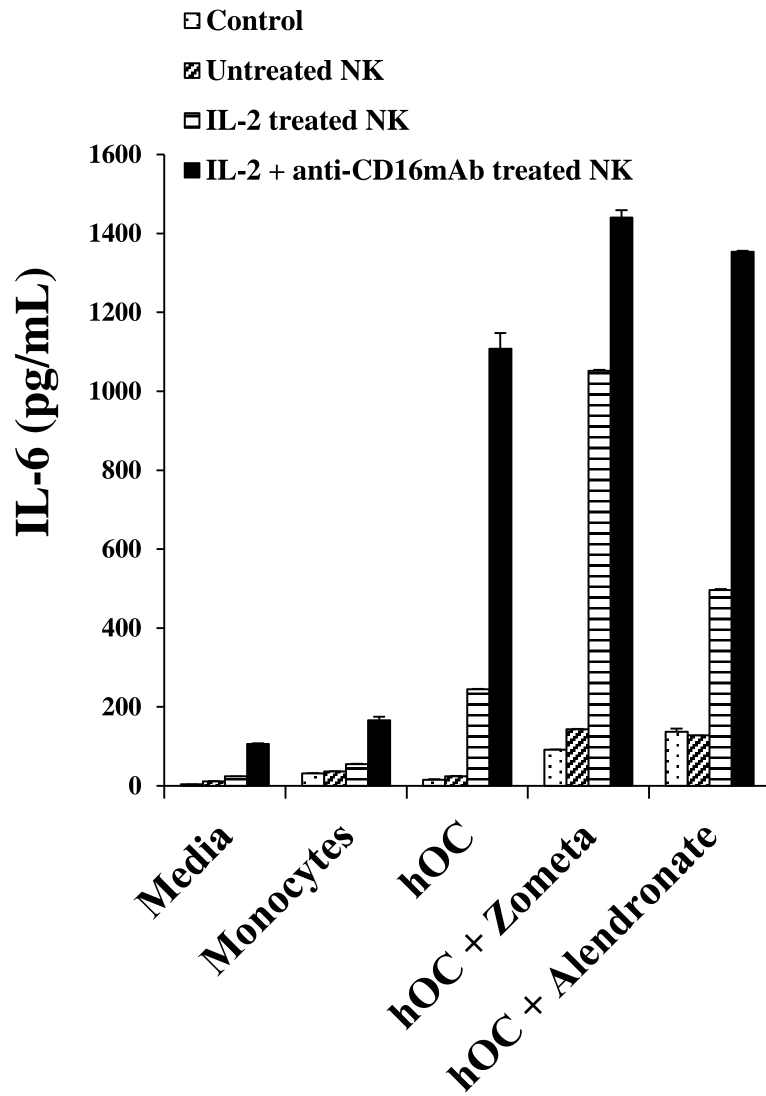


Fig. 18B

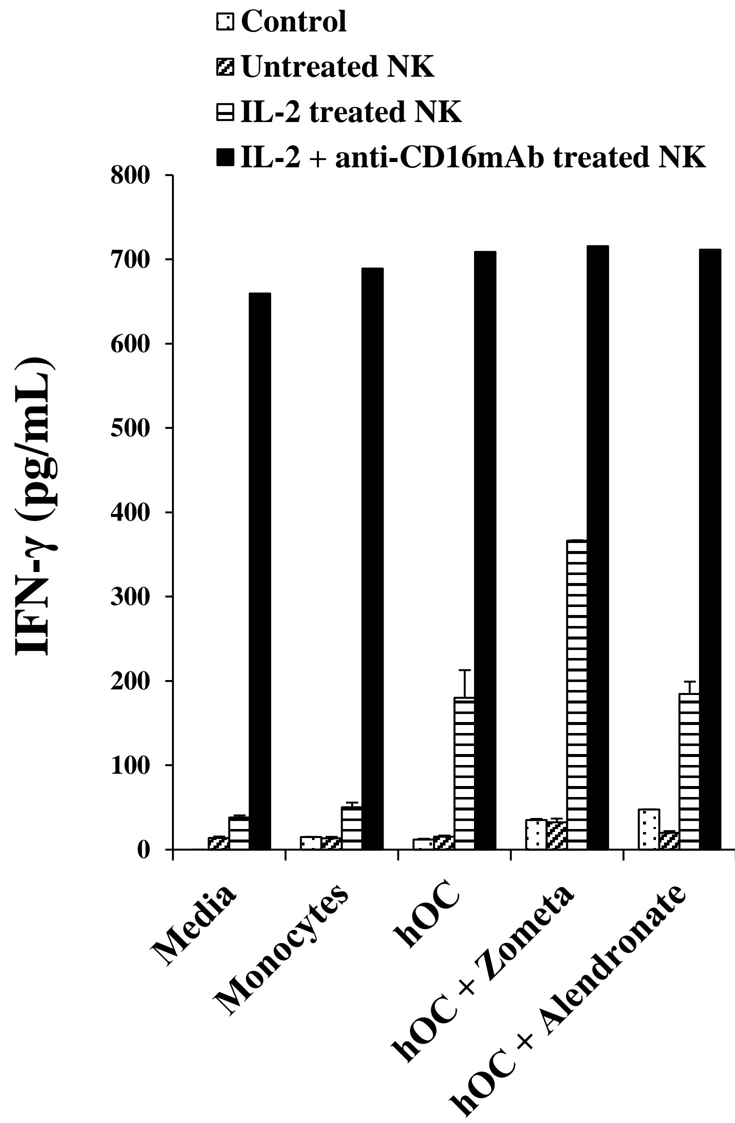


Fig. 18C

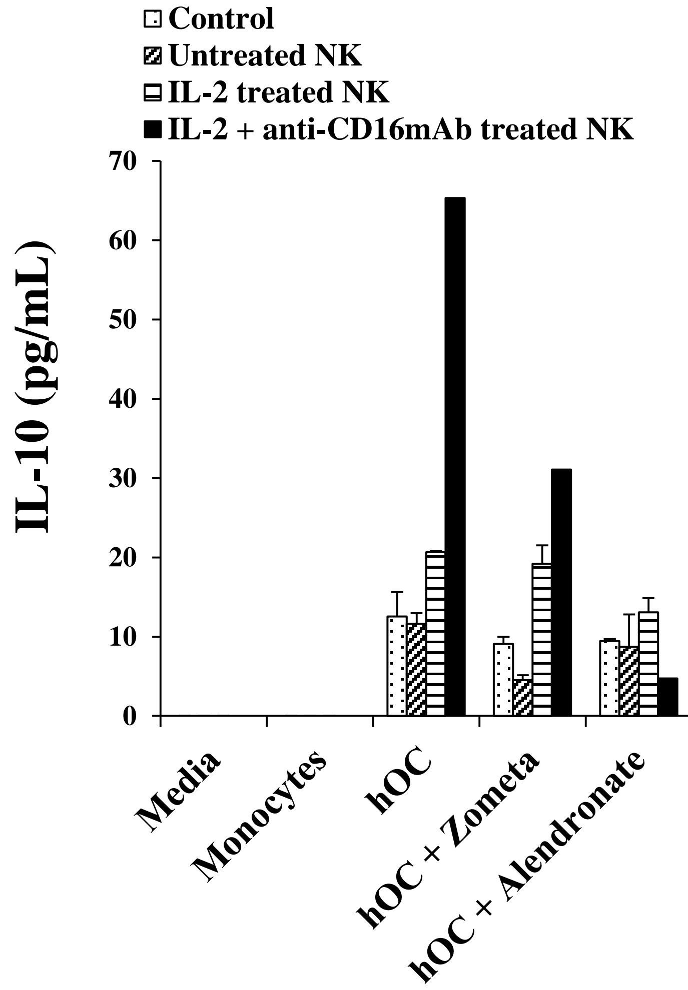


Fig. 18D

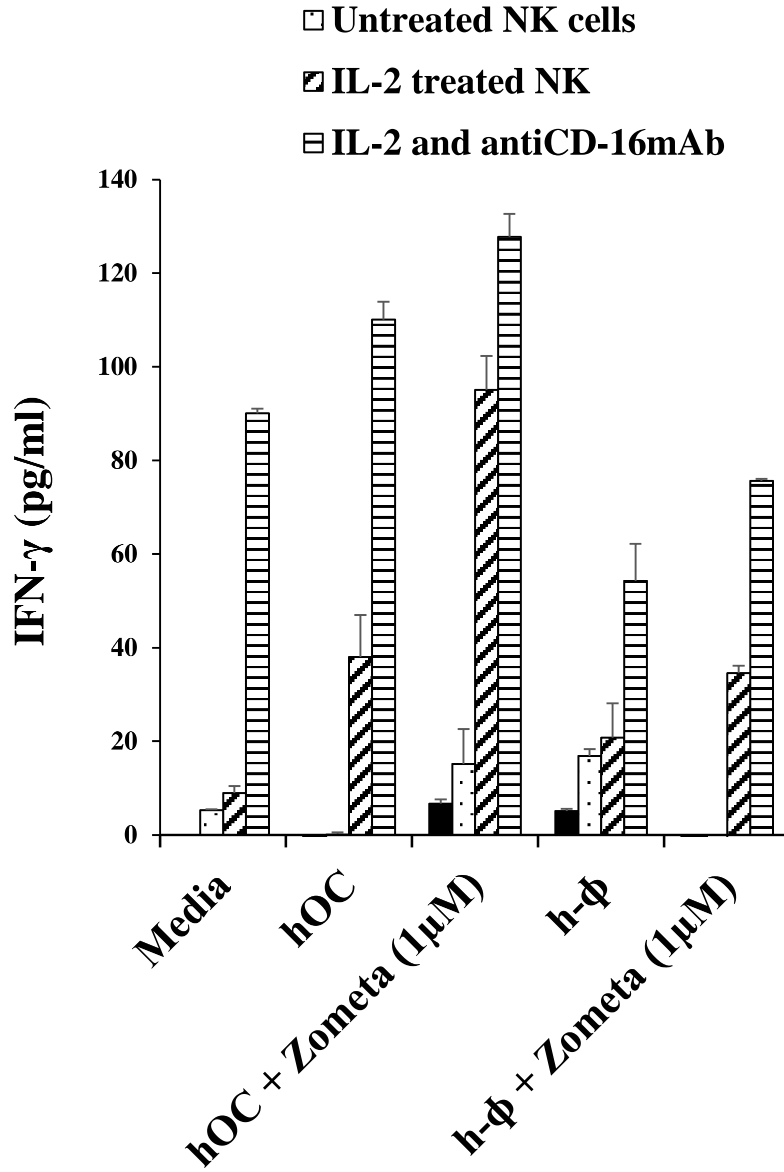
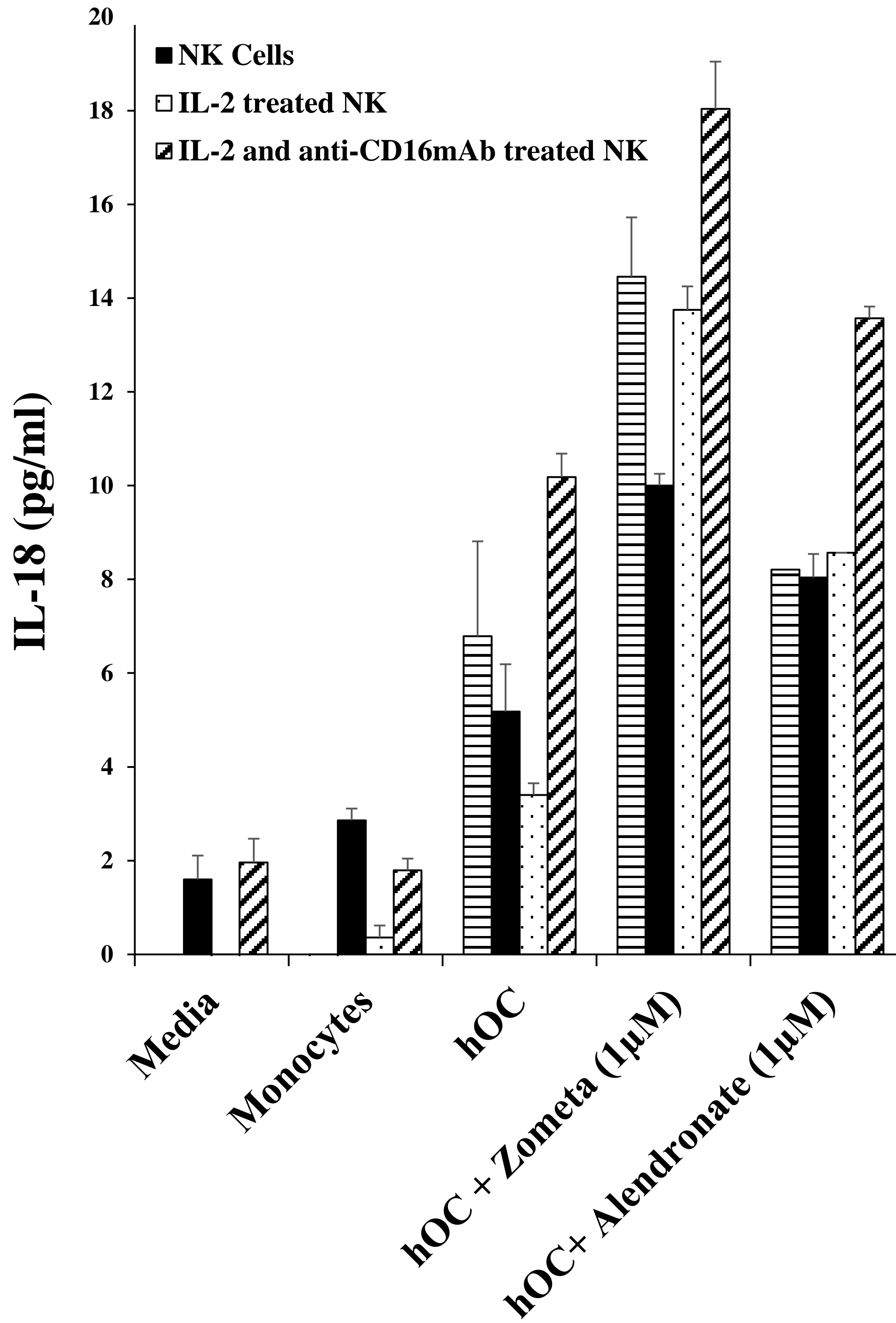


Fig. 18E



CHAPTER 9

Regulation of split anergy in natural killer cells through inhibition of cathepsin C by cystatin F

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Abstract

Freshly isolated primary NK cells induce apoptosis in differentiated Oral Squamous Carcinoma Cells (OSCCs) and Oral Squamous Carcinoma Stem Cells (OSCSCs), while anti-CD16 antibody and monocytes induce functional split anergy of NK cells by decreasing the cytotoxic function of NK cells and increasing the release of IFN-γ. We demonstrate that the decreased expression of mature form of aminopeptidases cathepsins C and H, which regulate the activation of effector granzymes in NK cells, correlate with lower cytotoxic function after triggering the CD16 receptor. Lower levels of mature cathepsins C and H can be a result of impaired expression and processing from their precursor forms and their activity can be further decreased by high level of monomeric, N-terminally truncated form of a cysteine protease inhibitor cystatin F, which co-localised with both enzymes in endosomal/lysosomal vesicles in NK cells. Our study provides a molecular mechanism target cells may induce to neutralize the cytotoxic effect of NK cells.

Introduction

Natural killer (NK) cells are large granular lymphocytes that mediate natural immunity against variety of tumor and virally infected cells [349]. Although NK receptors for target cell recognition have not been elucidated, many functional properties of NK cells have been reported. For instance, NK cells have been shown to lose cytotoxic function following their interaction with target cells [350, 351]. The inactivation of cytotoxicity is not linked to the signaling for proliferation function and secretion of cytokines by NK cells [352]. This response termed ″split anergy″, have been observed by subsets of NK cells, NK_{DC} (NK cells dissociated from tumor conjugates) and NK_{C} (NK cells not dissociated from the tumor conjugate). Whereas NK_{DC} responded to IL-2 activation for cytotoxicity, they were unresponsive to IL-2 mediated induction of proliferation or secretion of cytokines. In contrast, NK_{C} showed an inverse response namely, they did not respond to IL-2 activation for cytotoxicity, but proliferated and secreted cytokines [352, 353]. Treatment of NK cells with IL-2 and anti-CD16 antibody also induced split anergy [144, 354, 355]. Furthermore, IL-2 rescued anti-CD16 antibody mediated apoptosis induced in a subset of NK cells [355]. Inhibition of NK cell cytotoxic function and induction of NK cell death were more potent when NK cells were treated with anti-CD16 antibody [355, 356]. It is therefore evident that anti-CD16 signaling for NK cell loss of cytotoxicity is not associated with ADCC and it is likely that the increase in signaling load on NK cells results in decreased cytotoxicity and increased secretion of cytokines.

Many factors responsible for the tumor associated suppression of NK cell cytotoxicity have been identified, including the over-expression of Fas-ligand, and down-regulation of granzyme B [357] and CD16 receptor and its associated zeta chain [358-360]. However, the mechanisms by which tumor cells contribute to the induction of NK suppression are complex and have not been fully elucidated. Several human cancer cell types exhibit constitutively activated nuclear factor kappa B (NFκB) [189]. NFκB activity in tumor cells was shown to have inhibitory effect on NK cell function as inhibition of NFκB functional activity by the upstream super-repressor IκB increases the activation of NK cell cytotoxicity [361, 362]. It was also known that certain subsets of the myeloid arm of the immune system, such as monocytes, induce resistance in tumor cells and suppress NK cell mediated cytotoxicity [227, 363, 364].

Cysteine cathepsins are lysosomal cysteine peptidases that are involved at different levels of the innate and adaptive immune responses [365-367]. Amino-dipeptidase cathepsin C functions as a key enzyme in the activation of granule serine peptidases, granzymes A and B, in cytotoxic T lymphocytes and NK cells [368]. Granule exocytosis includes the release of a pore-forming protein perforin and activated granzymes, into the synapse formed between the killer and the target cell [369, 370]. Granzyme B is the most potent pro-apoptotic molecule in the granule-mediated apoptosis of target cells [371]. It is synthesized as a pro-granzyme B with an inhibitory dipeptide and upon reaching the secretory lysosomes it is activated by cathepsin C [372, 373]. Although cathepsin C generates the majority of granzyme B activity, some studies support alternative mechanisms for processing and activation of granzyme B. For

instance, lymphocytes derived from patients with congenital deficiency of cathepsin C (Papillon-Lefèvre syndrome) contain active granzyme B and kill target cells with the efficiency similar to healthy controls [374]. Similarly, the lymphocytes from cathepsin C-null mice expressed reduced but still appreciable granzyme B activity and kill target cells almost as efficiently as wild-type mice [375]. An alternative pro-granzyme B convertase was suggested to be cathepsin H; however, lymphocytes deficient in both cathepsins C and H are still able to generate active granzyme B, indicating additional enzymes involved in progranzyme processing [376].

The activity of cysteine cathepsins is controlled by their endogenous inhibitors, cystatins. Cystatins comprise a superfamily of evolutionarily related proteins, each consisting of at least one inhibitory domain of 100–120 amino acid residues [377-379]. Type I cystatins, or stefins, are cytosolic proteins, type II cystatins are predominantly secreted from the cells, whereas type III cystatins, the kininogens, are multifunctional proteins found in blood and other body fluids [380]. Cystatins are considered to be typical emergency inhibitors, trapping proteases escaped from the endosomes/lysosomes or cells in stable, proteolytically inactive complexes [379] and are thus generally considered not to regulate protease activity within the endosomal/lysosomal pathway. However, cystatin F is an exception. Although it is a secretory type II cystatin, it is present intracellularly to a much greater degree than other type II cystatins [381] and furthermore, it is localized in endosomal/lysosomal vesicles [382, 383] due to mannose 6-phosphate targeting pathway [384]. Cystatin F is produced in cells as a disulphide-linked dimer [385] inactive as an inhibitor of cysteine cathepsins [382]. *In vitro*,

unusually strong reducing conditions are needed to dissociate dimer to monomer [382]. However, the truncation of the N-terminal region to Lys35, presumably by cathepsin V (Maher 2014) significantly enhances the monomerization and also changes the inhibitory properties of the monomer [386]. Intact monomeric cystatin F was shown to bind tightly cysteine endopeptidases such as cathepsins L, F, K and V, less tightly cathepsins S and H, but not exopeptidases cathepsins B, X and C [382, 387, 388]. By N-terminal truncation, cystatin F becomes a strong inhibitor of cathepsin C [386] but weaker inhibitor of cathepsin S, whereas the inhibitory potential towards cathepsin H was only slightly increased.

In this study we investigated the relation between expression and activity of cathepsins C and H, their endogenous inhibitor cystatin F and cytotoxicity of primary NK cells. First, we demonstrated that primary NK cells are capable of lysing different target tumor cells and that lysis can be blocked by anti-CD16 antibody. The effect of monocytes on NK cells is similar to that of anti-CD16 antibody or target mediated induction of split anergy in NK cells. We showed that triggering the CD16 antigen on NK cells decreased the expression of mature cathepsins C and H, affecting the activation of effector granzymes. At the same time the level of cystatin F increased, additionally suppressing cathepsins C and H activity.

Materials and Methods

Cell lines and Reagents

RPMI 1640 supplemented with 10% FBS was used to culture human NK cells and human PBMCs. NK-92 cells were obtained from ATCC and were maintained in RPMI 1640 supplemented with 12.5% Fetal Bovine Serum (FBS), 12.5% Horse Serum and 100 u/mL IL-2. UCLA oral squamous carcinoma stem cells (OSCSCs) and differentiated UCLA oral squamous carcinoma cells (OSCCs) were isolated from freshly resected tongue tumors, and were cultured in RPMI 1640 supplemented with 10% FBS. Human mesenchymal stem cells (hMSCs) were purchased from Clonetics and cultured with the basal medium provided by the manufacturer. Human Dental Pulp Stem cells (hDPSCs) were isolated as described previously [198] and they were cultured in complete DMEM supplemented with 10% FBS. Recombinant IL-2 was obtained from NIH-BRB. The ELISA kit for IFN-γ was purchased from R&D Systems (Minneapolis, MN).

Antibodies and proteins

Rabbit anti-cystatin F polyclonal antibody, recombinant cistatin F and mouse 1D10 anti-cathepsin H Mab was prepared by our group in Ljubljana as reported [382, 388]. Rabbit N1 antibody against N-terminal of cystatin and Δ15N cystatin F were prepared at Dundee University, Dundee, UK, (Hamilton et al., 2008) . Goat anti-cathepsin C pAb was from R&D Systems (Minneapolis, MN), mouse anti-GAPDH mAb was from Invitrogen and rabbit anti-CRK-L pAb was from Sigma. The dye identifying the lysosomes, LysoTracker, was from Molecular Probes (Eugene, OR). Secondary labelled antibodies goat anti-rabbit Alexa Fluor 488 and donkey anti-goat Alexa Fluor

555 were from Molecular Probes (Eugene, OR). Antibodies to CD16 were purchased from biosciences (San Diego, CA). FITC anti-human/mouse Granzyme B antibody was purchased from Biolegend (San Diego, CA).

Purification of NK cells and monocytes

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors and all the procedures were approved by the UCLA-IRB. PBMCs and NK cells from healthy donors were isolated as described [352]. Briefly, peripheral blood lymphocytes were obtained after Ficoll-hypaque centrifugation and purified NK cells were negatively selected by using NK cell isolation kit (Stem Cell Technologies, Vancouver, Canada). The purity of NK cell population was found to be greater than 90% based on flow cytometric analysis of anti-CD16 antibody labelled cells. The levels of contaminating CD3+ T cells remained low, at 2.4%±1%, similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures. The adherent subpopulation of PBMCs was detached from the tissue culture plates and monocytes were purified using isolation kit obtained from Stem Cell Technologies (Vancouver, Canada). Greater than 95% purity was achieved based on flow cytometric analysis of CD14 antibody labeled monocytes.

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described [362]. Briefly, target cells were either co-cultured or not with irradiated (10 Gy) monocytes for 24–48 hours before they were labeled with ^{51}Cr for 1 hour, after which they were washed and added to

different numbers of NK cells. After 4 hour incubation, the supernatants were harvested from each sample and counted for released radioactivity using the γ counter. The purified NK cells were either left untreated or treated with anti-CD16 mAb (3 µg/ml), IL-2 (1000 u/ml), or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3 µg/ml) for 24–48 hours before they were added to ^{51}Cr labeled target cells. The percentage of cytotoxicity was calculated as follows:

% Cytotoxicity = (experimental cpm – spontaneous cpm)/ (total cpm – spontaneous cpm)

LU 30/10^6cells were calculated using the inverse of the number of effector cells needed to lyse 30% of target cells x100.

ELISA and Multiplex assays

Single ELISAs were performed as described previously [137]. Fluorokine MAP cytokine multiplex kits were purchased from R&D Systems (Minneapolis, MN) and the procedures were conducted as suggested by the manufacturer. To analyze and obtain the cytokine and chemokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines provided by the manufacturer. Analysis was performed using the Star Station software.

Intracellular stain and flow cytometry

NK cells were fixed and permeabilized with Fixation Buffer and Permeabilization Buffer purchased from Biolegend (San Diego, CA) and the procedures were conducted as instructed by the manufacturer.

Western blot

1 x 10^6 cells/ml were treated with IL-2 (1000 U/ml), anti-CD16 antibody (3 µg/ml), or a combination of IL-2 (1000 U/ml) and anti-CD16 antibody (3 µg/ml) for 4 h. NK92 cells were cultured in complete RPMI 1640 medium. Cells were washed twice and lysed by the lysis buffer (150 mM NaCl, 1% (v/v) Triton X-100, 2 mM EDTA, 100 mM citrate buffer pH 5.5) with the addition of 2 mM phenylmethylsulfonyl fluoride (Sigma), 10 µg/ml leupeptin (ICN, Aurora, OH), 2 u/ml aprotinin (ICN, Aurora, OH) for 20 min on ice. The samples were then centrifuged at 10,000 rpm for 10 min at 4°C to remove the nuclei, the supernatants were removed and the levels of proteins quantified by the Bradford method. The lysates were boiled in 4 x SDS sample buffer and reduced with the addition of 200 mM DTT. Samples were loaded onto 12% SDS-PAGE and gels immunoblotted to a Immobilon-P membranes (Millipore, Billerica, MA). The membranes were blocked with 5% non-fat milk in PBS plus 0.1% Tween-20 for 1 hour and incubated in predetermined dilutions of primary antibodies overnight at 4°C. Membranes were then incubated corresponding horseradish peroxidase-conjugated secondary antibody. Blots were developed by enhanced chemiluminiscence (ECL-purchased from Pierce Biotechnology, Rockford, IL). Density of protein bands was analysed by ImageJ . Relative density values were calculated by dividing the percent values of each sample by the percent value of the contol (untreated NK). Further, adjusted denstity values were

obtained by scaling the relative density values for the samples by the relative density values of the corresponding loading control bands for each lane.

Confocal immunofluorescence microscopy

NK-92 cells (1 x 10^5) were centrifuged with cytospin (Cytofuge) for 6 min at 1000 rpm onto glass cover slides. Before labelling, cells were allowed to recover for 15 min and then fixed with 4% paraformaldehyde for 45 min and permeabilized by 0.1% Triton X-100 in PBS, pH 7.4, for 10 min. Nonspecific staining was blocked with 3% BSA in PBS, pH 7.4, for 1 h. Lysosomes were labelled by adding LysoTracker to the cells for 2 min before fixation with 4% paraformaldehyde, as recommended by Molecular Probes. All primary antibodies were added after nonspecific blocking. After 2 h incubation with primary antibody, cells were washed with PBS and treated with fluorophore-labelled secondary antibody for 1 h. After a final wash with PBS, the ProLong antifade kit (Molecular Probes) was used to mount coverslips on glass slides. Controls were run in the absence of primary antibodies. Fluorescence microscopy was performed using a Carl Zeiss LSM 510 confocal microscope with 63x Plan-Apochromat Oil DIC objective, N.A. 1.4. Alexa Fluor 488, LysoTracker, or Alexa Fluor 555 was excited with an argon (488 nm) or He/Ne (543 nm) laser, and emission was filtered using narrow-band LP 505–530 nm (green fluorescence) and LP 560 nm (red fluorescence) filters, respectively. Images were analyzed using Carl Zeiss LSM image software 3.0.

Total RNA isolation and real time PCR

1×10^6 cells/ml were treated with IL-2 (1000 U/ml), anti-CD16 antibody (3 μg/ml), or a combination of IL-2 (1000 U/ml) and anti-CD16 antibody (3 μg/ml) for 12 h. NK-92 cells were cultured in complete RPMI 1640 medium. Cells were washed twice with PBS, pH 7.4 and total RNA was extracted from the cells using the RNeasy Mini kit (Qiagene, Valencia, CA). 200 ng of total RNA from each sample was then used for reverse transcription of mRNA to cDNA using QuantiTect Reverse Transcription kit (Qiagene, Valencia, CA). In the second step, we performed real time PCR using SYBR Green PCR master mix (Invitrogen, CA). 10 ng per well of cDNA was used and the total volume of the reaction was 20 μl. The premix of forward and reverse primers for IFN-γ gene was purchased (Invitrogen, CA). Primer sequences for the reference gene RPLP0 were provided by Primer Bank database (http://pga.mgh.harvard.edu/cgi-bin/primerbank/) and synthesized by GenoMechanix (Gainesville, FL). The PCR reactions were performed in the iCycler (Bio-Rad Laboratories, Hercules, CA) using a PCR program of activation step for 10 min at 93°C, followed by 50 cycles of amplification, with each cycle consisting of a denaturation step at 95°C for 45 s, followed by an annealing step at 55°C for 30 s and an extension step at 72°C for 1 min. We also performed a melting curve analysis (temperature range 55–95°C) to check for the formation of primer-dimers and production of nonspecific products. Each reaction was performed in triplicate and threshold cycles (CT) were calculated using the second derivative of the reaction. The CT of each gene was normalized against that of RPLP0, which showed no regulation by IL-2 and anti-CD16 antibody treatment. Fold changes were determined using the −ΔΔCT method. Controls without template cDNA were performed to ensure that amplification of products was specific.

Statistical analysis

An unpaired, two-tailed Student t-test was used to analyze the differences between the samples. A p-value of <0.05 was considered statistically significant.

Results

Anti-CD16 antibody inhibited NK cell mediated lysis of primary human differentiated Oral Squamous Carcinoma Cells (OSCCs) and carcinoma stem cells (OSCSCs), however, induction of NK cell anergy by anti-CD16 mAb resulted in a significant secretion of IFN-γ by the NK cells

As shown in a number of previous studies [19, 144, 355] and in this report, anti-CD16 mAb treatment induced anergy as well as death of NK cells, thereby inhibiting NK cell cytotoxicity against different target cells. In this study, two types of patient derived tumors, differentiated Oral Squamous Carcinoma Cells (OSCCs) and Oral Squamous Carcinoma Stem Cells (OSCSCs), were tested for their sensitivity to NK cell mediated cytotoxicity and ability to induce IFN-γ secretion by the NK cells. Treatment of NK cells with anti-CD16 mAb significantly decreased cytotoxicity against both tumor types (Figs. 1A, 1C). The addition of IL-2 and anti-CD16 also significantly inhibited the NK cell cytotoxicity against both tumor types when compared to IL-2 activated NK cells (Figs. 1A, 1C). Untreated or anti-CD16 mAb treated NK cells did not secrete IFN-γ when co-cultured with any of the tumor cell populations but did so when treated with IL-2 and with IL-2 in combination with anti-CD16 mAb (Figs. 1B, 1D). In addition, both types of tumor cells triggered significant secretion of IFN-γ from IL-2 and anti-CD16 mAb treated NK cells when compared to IL-2 treated NK cells (Figs. 1B, 1D).

Monocytes protected primary human differentiated OSCCs and OSCSCs against NK cell mediated cytotoxicity and induced significant secretion of IFN-γ by the NK cells

The addition of monocytes to primary human differentiated OSCCs or OSCSCs prior to cytotoxicity assay inhibited the NK cell mediated lysis of OSCCs (Fig. 2A) or OSCSCs (Fig. 2C). Significant inhibition of NK cell cytotoxicity by monocytes can be observed against untreated or IL-2 treated NK cells against both tumor types (Figs. 2A, 2C). These data indicate that monocytes protect differentiated OSCCs and OSCSCs against NK cell mediated lysis.

As expected IL-2 treated NK cells when co-cultured with OSCCs or OSCSCs secreted moderate amounts of IFN-γ (Figs. 2B, 2D). The addition of anti-CD16 mAb in combination with IL-2 to NK cells cultured with OSCCs or OSCSCs increased secretion of IFN-γ when compared to IL-2 alone treated NK cells (Figs. 2B, 2D). Monocytes added to IL-2 alone or IL-2 and anti-CD16 mAb treated NK cells in the presence of OSCCs or OSCSCs synergistically increased the levels of secreted IFN-γ compared to NK cells without monocytes (Fig. 2B, 2D).

Monocytes prevented NK cell mediated lysis of healthy human mesenchymal stem cells (hMSCs) and human dental pulp stem cells (hDPSCs); however, the addition of monocytes induced significant secretion of IFN-γ by the NK cells in the presence and absence of stem cells.

To determine whether monocytes decrease sensitivity of healthy stem cells to NK cell mediated cytotoxicity similarly to oral tumor cells, we chose human mesenchymal stem cells (hMSCs) and human dental pulp stem cells (hDPSCs). The addition of monocytes to hMSCs or hDPSCs prior to cytotoxicity assay significantly inhibited the NK cell mediated lysis of hMSCs (Fig. 3A) or hDPSCs (Fig. 3C). These data indicate that, in addition to tumor cells, monocytes protect also healthy hMSCs and hDPSCs against NK cell mediated lysis.

Similarly as observed for OSCCs and OSCSCs in the previous experiment, IL-2 alone and IL-2 and anti-CD16 mAb treated NK cells when co-cultured in the presence of hMSCs or hDPSCs secreted increased amounts of IFN-γ compared to untreated cells. Monocytes again synergistically increased the levels of secreted IFN-γ (Figs. 3B, 3D). These results indicated that, in addition to tumor cells, monocytes increased IFN-γ in co-cultures of NK cells with hMSCs or hDPSC.

Lack of cytotoxic function of NK-92 cells and decreased secretion of IFN-γ, GM-CSF, IL-6, but a significant increase in IL-10 production

NK-92 is a NK cell line, negative for surface antigen CD16 and dependent on the presence of recombinant IL-2, therefore with similar characteristics than IL-2 and anti-CD16 treated NK cells. To determine the cytotoxicity of NK-92 cells, we compared the lysis of K562 cells by NK-92 cells and untreated, IL-2 treated and IL-2 and anti-CD16 antibody treated NK-92 cells in the ^{51}Cr cytotoxicity assay. Treatment of NK cells with IL-2 increased the cytotoxicity of NK-92 cells against K562 cells, whereas the addition of

anti-CD16 mAb significantly decreased he cytotoxicity ($p<0.05$) (Fig. 3). Treatment of IL-2 + anti-CD16mAb in primary NK cells resulted in a significant increase in production of IFN-γ (Fig. 4A), TNF-α (Fig. 4B), GM-CSF (Fig. 4C) and IL-6 (Fig. 4E). The levels of secretion synergistically increase when IL-2+anti-CD16mAb treated NK cells were co-incubated with OSCSCs. The treatment of IL-2 in primary NK cells although also resulted in an increase in cytokine secretion, the levels were lower compared to IL-2+anti-CD16mAb treated primary NK cells (Fig. 4A, 4B, 4C and 4E). Whereas, NK92 cells treated with either IL-2 or IL-2+anti-CD16mAb did not result in a change in the secretion of all three abovementioned cytokines. However, NK92 cells were shown to produce secrete significantly higher levels of IL-10 in both IL-2 and IL-2+anti-CD16mAb treated NK cells. The level of IL-10 secretion was the highest when NK92 cells were treated with IL-2+anti-CD16mAb in the presence of OSCSCs (Fig. 4D).

Colocalization of cystatin F with cathepsins C and H and LysoTracker in NK-92 cells

The localization of cystatin F as well as its potential targets cathepsins C and H was determined in NK-92 cells. They all showed a vesicular staining, corresponding to lysosomal/endosomal vesicles. The presence of cystatin F in lysosomes was confirmed by colocalization with lysosomal marker LysoTracker (Fig. 6A). Cystatin F partially colocalizes with cathepsin C (Fig. 6B) and cathepsin H (Fig. 6C), demonstrating both enzymes as targets for inhibitory action in NK-92 cells. From Fig. 6D it is evident that cathepsins C and H are colocalized practically in the same vesicles.

The monomeric cystatin Fin NK-92 cells is in truncated form that permits inhibition of cathepsins C and H

As shown previously, the sequence of the cellular monomeric cystatin F isolated from U937 cells was N-terminally truncated by 15 residues to Lys35 and only truncated but not full-length/intact monomeric cystatin F was an inhibitor of cathepsin C [386]. Truncated form of cystatin F inhibits also cathepsin H, to the similar extent as its full length/intact form. To demonstrate whether cystatin F is similarly processed in NK-92 cells, we separated the dimeric and monomeric forms of cystatin F by non-reducing SDS-PAGE and used two different antibodies for cystatin F, anti-cystatin F pAb that recognizes intact and truncated cystatin F and an antibody specific for the 15-residue N-terminal sequence of cystatin F that recognizes only intact cystatin F. We found that antibody raised against the whole cystatin F reacted with dimeric and monomeric cystatin F (Fig. 7, left panel) whereas the antibody raised against N-terminal sequence of cystatin F reacted with dimeric but not with monomeric cystatin F (Fig. 7, right panel). These results indicate, that in NK-92 cells both, monomeric and dimeric form of cystatin F is present and that the monomeric form is N-terminally truncated.

Decreased mature cathepsins C and H and increased pro-cathepsin C expression following addition of anti-CD16 antibody to the NK cells in the presence or absence of IL-2

To assess the involvement of cathepsins C and H in the loss of cytotoxic function of primary NK cells we compared its protein levels in untreated, IL-2 treated and IL-2

and anti-CD16 antibody treated NK cells (Fig. 7) In the left panel (Fig. 7A) the higher band (~ 55 kDa) corresponds to the molecular mass of pro-cathepsin C [389] and lower band (~ 24 kDa) to the heavy chain of mature cathepsin C [390]. Right panel (Fig. 7B) represents the mature form of cathepsin H. We found that treatment of NK cells with anti-CD16 antibody with or without IL-2 significantly decreased the levels of the heavy chain of mature cathepsin C and increased the levels of pro-cathepsin C when compared to untreated and IL-2 treated NK cells, respectively (Fig. 8). For cathepsin H mature form the difference is less significant, the lowest level can be observed in NK cells treated with IL-2 and anti-CD16 antibody. These results demonstrate that CD16 receptor may regulate the expression and/or processing of cathepsin C and cathepsin H. The levels of both cathepsins are in line with lower cytotoxicity of NK cells, caused by anti-CD16 mAb treatment.

Increased cystatin F expression following addition of anti-CD16 antibody to the NK cells in the presence or absence of IL-2

To determine the impact of cystatin F on the activity of cathepsins C and H and thus on cytotoxic function of primary NK cells we compared its protein levels between untreated, IL-2 treated and IL-2 and anti-CD16 antibody treated NK cells. On SDS-PAGE under reducing conditions, we analysed the content of truncated monomeric cystatin F and found 1.72-fold increase of its level in anti-CD16 treated NK cells when compared to untreated NK cells. The level of cystatin F remained similar in IL-2 and IL-2 and anti-CD16 treated NK cells (2.35 vs. 2.22) (Fig. 8A). Multiple bands correspond to differentially glycosylated forms as observed also in previous studies.

On SDS-PAGE under non-reducing conditions we analysed the content of dimeric cystatin F in pre-treated NK cell lysates. We found 1.39-fold increase of dimeric cystatin F level in anti-CD16 treated NK cells when compared to untreated NK cells. The addition of anti-CD16 antibody to IL-2 treated NK cells caused 1.2-fold increase of dimeric cystatin F compared to IL-2 treated NK cells (Fig. 9B). Overall, these data indicate that increased expression of cystatin F may attenuate the protease activity in anti-CD16 treated NK cells in the presence or absence of IL-2 and thus contribute to the lower cytotoxic function of primary NK cells.

Treatment of NK cells with anti-CD16 antibody to the NK cells in the presence or absence of IL-2 abolishes the expression and processingof granzyme B

To confirm the relationship between cytotoxicity, cathepsin C and H expression and activity and cytatin F levels in NK cells, we determined the granzyme B, a final executive molecule in NK cell cytotoxicity in in untreated, IL-2 treated and IL-2 and anti-CD16 antibody treated NK cells. Our results show that the granzyme B protein level is drastically decreased in NK cells, treated with anti-CD16 mAb resulting in decreased cytotoxicity of NK cells against target cells (Fig 10).

Discussion

We studied the interaction of primary NK cells with primary oral tumor cells, mesenchymal and dental pulp derived stem cells after the treatment with IL-2, anti CD16 antibody and monocytes. Primary NK cells were cytotoxic to tumor and stem cells, IL-2 significantly increased cytotoxicity, whereas anti-CD16 antibody decreased it triggering split anergy of NK cells. Opposite to cytotoxic primary NK cells the NK-92 cells are anergized NK cells that produce cytokines, such as IFN-γ and IL-10, in the absence of cytotoxicity. We showed that aminopeptidases cathepsins C and H are involved in CD16 antigen signaling for the loss of NK cell cytotoxicity. Decreased cathepsins C and H activities in less cytotoxic NK cells may occur due to lower processing of their propeptides into active enzymes, and increased expression of endogenous inhibitor, cystatin F.

Target-mediated inactivation of NK cell cytotoxicity correlated with target cell sensitivity to NK cell cytotoxicity. As reported, K562, an NK sensitive tumor, caused loss of NK cell cytotoxicity, while NK resistant tumors, such as RAJI cells had less impact on loss of NK cell cytotoxicity [352, 353]. Target cell inactivated NK cells express $CD16^{-}CD56^{dim/-}CD69^{+}$ phenotype [352, 353]. Decreased CD16 receptor expression and NK cell cytotoxic function were seen in several cancer patients [358, 359]. As expected, we showed here that IL-2 treated NK cells lysed differentiated Oral Squamous Carcinoma Cells (OSCCs) and carcinoma stem cells (OSCSCs) at much higher degree as untreated NK cells. In addition, anti-CD16 antibody treated NK cells

lost their cytotoxic function against OSCCs and OSCSCs. Moreover, significant induction of IFN-γ secretion could be observed in supernatants of co-cultures of either IL-2 or IL-2 and anti-CD16 antibody treated NK cells with OSCCs or OSCSCs. We have previously shown that the triggering of CD16 on untreated or IL-2 treated NK cells resulted in decreased expression of CD16 receptors and a great loss of cytotoxicity in NK cells [141, 352-354, 391]. Induction of apoptosis through CD16 receptor is not limited to NK cells, and has been observed also on apoptotic of polymorphonuclear neutrophils [392-394]. The precise mechanism is not known yet, however it includes induced DNA fragmentation and apoptosis in a subset of NK cells, pointing on the action of caspases in this process [141, 352-354]. Cell death of NK cells was shown to be regulated by endogenously secreted TNF-α from the NK cells, however the addition of either anti TNF-α antibody or TNF-binding protein only partially blocks anti-CD16-induced apoptosis of NK cells [354], indicating the existence of alternative mechanisms.

In agreement with our previous studies [18, 19], we showed that monocytes cultured with hMSCs, hDPSCs, OSCCs or OSCSCs caused a significant decrease in NK cell mediated cytotoxicity. Decreased lysis of stem cells was partially due to a competitive lysis of monocytes by the NK cells. [19]. However, when stem cells were co-cultured with monocytes and sorted to remove the monocytes before cytotoxicity assay, NK cell mediated lysis was still inhibited, indicating that interaction of monocytes with stem cells can also provide resistance of stem cells against NK cell cytotoxicity [19]. Both the total populations of monocytes and CD16- subsets were capable of inducing inhibition of NK cell cytotoxicity against stem cells [19]. Furthermore, this effect was not

specific to monocytes, since the addition of T or B cells to stem cells also induced resistance of stem cells against NK cell mediated killing, pointing on increased resistance of stem cells after interaction with different immune effectors [19]. Here we demonstrate that monocyte-induced decrease in NK cell lysis of hMSCs, hDPSCs, OSCCs or OSCSCs was paralleled with a significant induction of IFN-γ secretion, as observed also in our previous reports [18, 19]. We could observe induction of IFN-γ secretion when hMSCs or hDPSCs were cultured with IL-2 treated NK cells alone, but the highest increase was seen when NK cells were cultured with hMSCs or hDPSCs in the presence of monocytes. Therefore, although decreased killing of stem cells by the NK cells could be observed in the presence of monocytes, synergistic secretion of IFN-γ by the NK cells in the presence of monocytes and stem cells could be observed, indicating an inverse effect regarding cytotoxicity and IFN-γ secretion (split anergy). Mechanisms, by which immune effectors induce split anergy are not known yet. Our previous finding indicated that monocytes protected oral tumors via NFκB dependent and independent ways. The addition of IL-2 [355] or monocytes [19] to anti-CD16 antibody treated NK cells prevented NK cell apoptosis. However, similarly to anti-CD16 antibody mediated effect on IL-2 treated NK cells, monocytes too can shield target cells from killing by increasing the secretion of IFN-γ by the NK cells while decreasing the cytotoxic function of NK cells. It is therefore likely that mechanism of split anergy caused by engagement of CD16 antigen on NK cells is similar to that induced by monocytes.

Anti-CD16 antibody which induces functional split anergy in NK cells inhibited NK cell mediated cytotoxicity against differentiated cancer cells OSCCs and cancer stem

cells OSCSCs in the presence of significant IFN-γ production by the NK cells. These results further point on functional split anergy of NK cell line NK-92, which does not express surface antigen CD16 and is IL-2 activated. We have shown that, like in IL-2 and anti-CD16 antibody treated NK cells, in NK-92 cells the cytotoxicity is decreased and the expression of IFN-γ and IL- is elevated. We therefore suggest that NK-92 is not appropriate system for measuring the NK cytotoxic function however it will simulate the secretion of cytokines.

Cathepsin C is synthesized as pro-enzyme with molecular mass of 55 kDa, which is processed into mature cathepsin C [395]. Mature cathepsin C consists of a part of the pro-region, also known as residual pro-part, and of the catalytic region [395-397]. The catalytic part is cleaved into a heavy and light chain of 24 kDa and 6 kDa, respectively and the active enzyme exists as a tetramer [390, 395]. We show lower expression of heavy chain of mature cathepsin C (with molecular weight around 24 kDa) in the NK cells with low cytotoxicity, treated with anti-CD16 antibody or a combination of IL-2 and anti-CD16 antibody than in non-treated or IL-2 treated NK cells. The results also demonstrate that triggering of CD16 antigen on NK cells impaired the processing of pro-cathepsin C, to mature cathepsin C and thus induced accumulation of pro-cathepsin C. Activation of CD16 receptor may attenuate the mechanism of granzyme-mediated cytotoxicity, in which the activation of granzyme B is dependent on active cathepsin C [368, 373]. Cathepsin H is an additional convertase of pro-granzyme B. The processing of its propeptide is an autocatalytic, multistep process proceeding from an inactive 41 kDa pro-form, through a 30 kDa intermediate form, to the 28 kDa mature form (Rojnik et

al., 2012). Mature 28 kDa form contains an additional octapeptide, termed the mini-chain, which originates from the propeptide and is bound to the mature enzyme by a disulphide bond and is essential for the aminopeptidase activity of cathepsin H (Vasiljeva et al., 2003). By confocal microscopy we identified cathepsins C and H in practically the same endosomal/lysosomal vesicles in NK-92 cells confirming previously suggested functional redundancy of these two enzymes in granzyme B maturation. In different primary NK cells we showed that triggering of CD16 receptor induces split anergy of NK cells thereby decreasing the cytotoxic function and increasing the IFN-γ release. Triggering of CD16 receptor also impaired the processing of pro-cathepsins C and H, which may unable effector granzyme B activation and cytotoxic function by the NK cells. Indeed, our results show that the level of granzyme B is significantly decreased in NK cells treated by anti-CD16 antibody. As shown in our previous study, anti-CD16 antibody also decreased the levels of mRNA for preforin, granzyme A and granzyme B [355].

Cystatin F possesses some unique features among human type II cystatins [387, 388, 398]. It shares only 35% sequence identity with other members, has a 6 amino acid extension at the N terminus and is one of only two glycosylated type II cystatins [388]. In contrast to other family members, it has two additional cysteines (Cys26 and Cys63) and forms intermolecular disulphide bonds with another cystatin F molecule [388, 399]. Cystatin F expression is limited to immune cells such as monocytes, T cells, natural killer (NK) cells and dendritic cells (DCs) [387, 388, 400, 401]. The 29-fold increase in gene expression for cystatin F was shown in human NK cells compared to $CD8^+$ T

lymphocytes [401]. Cystatin F is present intracellularly in active monomeric and inactive dimeric forms, whereas secreted cystatin F is predominantly an inactive dimer [382, 385]. It was shown that in promonocyte U937 cells, a large proportion of the cystatin F resides in the endosome/lysosome-like vesicles [381, 382] as a truncated monomeric form, whereas in ER and Golgi apparatus inactive dimeric form was predominant [386]. Similarly, our results demonstrate lysosomal localization of cystatin F in NK-92 cells. Moreover, the monomeric fraction of cystatin F in NK-92 cells is in a truncated form that is able to inhibit cathepsins C and H. Similarly, the primary NK cells also possess truncated monomeric cystatin F besides inactive dimer. Cathepsin C has been suggested as the main target of cystatin F in cytotoxic cells such as T cells, NK cells, neutrophils and mast cells [368, 386, 402]. Partial co-localiazation of cystatin F and cathepsin C we determined in NK-92 cells supports this thesis. Additionally, increased expression of cystatin F in primary NK cells with reduced cytotoxicity favors its potential to regulate cathepsin C activity and cytotoxic function. Cathepsin H represents another target for inhibition by cystatin F. Truncated monomeric cystatin F inhibits cathepsin H in a nanomolar range (Ki 8.3±4.7 nM, Magister et al. 2012) and may prevent on this way the generation of active granzyme in vesicles or cells lacking the activity of cathepsin C. Cystatin F is able to inhibit cathepsin H also as an intact monomer, however, in this study we did not determine any intact monomeric form in cell lysates of NK cells. Increased levels of cystatin F in NK cells could be as a result of accelerated synthesis and/or internalization of secreted cystatin F. For the latter it has been shown that extracellular dimeric cystatin F could be internalized and then activated in endosomal/lysosomal vesicles within the cells [403]. One may speculate that target cells may secrete inactive

cystatin F which after internalization and activation inhibits cathepsins C and H and down-regulates NK cell cytotoxicity.

Overall, our results demonstrate that the induction of functional split anergy of NK cells by anti-CD16 antibody or monocytes changes the levels of cathepsins C and H, key molecules involved granzyme activation and consequently in granule-mediated apoptosis of target cell. Their activities are further impaired by higher levels of monomeric cystatin F, a cysteine protease inhibitor, capable to act in the endosomal/lysosomal vesicles in cytotoxic cells. Further studies are needed to clarify the whole mechanism and to define the contribution of other related proteases in these processes.

Figure legends

Figure 1: Lysis of OSCCs (Oral Squamous Carcinoma Cells) and OSCSCs (Oral Squamous Carcinoma Stem Cells) by untreated and IL-2 treated NK cells are inhibited by anti-CD16 antibody, however, the same treatment induced significant secretion of IFN-γ by the NK cells

NK cells ($1x10^6$/ml) were left untreated or treated with IL-2 (1000 units/ml), anti-CD16 mAb (3 μg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 μg/ml) for 12-24 hours before they were added to ^{51}Cr labeled OSCCs (A) or OSCSCs (C) at different effector to target (E:T) ratios. NK cell cytotoxicities were determined at different E:T ratio, and the lytic units $30/10^6$ cells were calculated using inverse number of NK cells required to lyse 30% of the tumor cells x 100. NK cells were treated as described above and each NK sample (1×10^5/ml) was added to OSCCs (B) or OSCSCs (D) at an effector to target ratio 1:1. After an overnight culture, supernatants were removed from the co-cultures and the levels of IFN-γ secretion were determined using specific ELISA. One of three representative experiments is shown in this figure.

Figure 2: Monocytes protect primary differentiated oral squamous carcinoma cells (OSCCs) and oral squamous carcinoma stem cells (OSCSCs) against NK cell mediated cytotoxicity, but significantly augment the secretion of IFN-γ in co-cultures of NK, monocytes and carcinoma cells

OSCCs (A) or OSCSCs (C) at $1x10^6$ cells/plate were co-cultured with the irradiated monocytes (10 Gy) (monocytes: carcinoma cells ratio of 1:1) for 24–48 hours

before they were removed from the plates, washed and labeled with ^{51}Cr and used as targets in the cytotoxicity assays against NK cells. The NK samples were either left untreated or treated with anti-CD16 mAb (3 µg/ml), IL-2 (1000 u/ml), or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3 µg/ml) for 24–48 hours before they were added to ^{51}Cr labeled OSCCs or OSCSCs at different effector to target (E:T) ratios. Supernatants were removed after 4 hours of incubation and the released radioactivity counted by a γ counter. % cytotoxicities were determined at different E:T ratio, and LU30/10^6 cells were calculated using the inverse of the number of effectors needed to lyse 30% of the carcinoma cells x 100. One of three representative experiments is shown in this figure. OSCCs (B) or OSCSCs (D) (1x10^5 cells/well) were co-cultured with and without irradiated monocytes at 1:1 OSCCs or OSCSCs to monocytes for 24–48 hours before untreated or IL-2 (1000 u/ml) pre-treated or anti-CD16 mAb (3 µg/ml) pre-treated, or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3 µg/ml) pre-treated NK cells at 1:1:1 NK:monocyte:carcinoma cells ratios were added. NK cells were pre-treated as indicated for 24–48 hours before they were added to the co-cultures. After 24–48 hours of the addition of NK cells the supernatants were removed from the cultures and the levels of IFN-γ secretion were determined using a specific ELISA. One of five representative experiments is shown in this figure.

Figure 3: NK 92 cells do not mediate cytotoxicity

1 x 10^6 NK cells/ml were treated with IL-2 (1000 u/ml), anti-CD16 antibody (3 µg/ml), or a combination of IL-2 (1000 u/ml) and anti-CD16 antibody (3 µg/ml) for 18-24 hrs after which the primary human NK cells and NK-92 cells were added to ^{51}Cr

labeled OSCCs and OSCSCs. NK cell cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the target cells x 100.

Figure 4: NK 92 cells do not secrete significant IFN-γ, TNF- α and GM-CSF whereas they secrete IL-6 and IL-10

Highly purified NK cells were treated as described in Figure 1A and cultured with OSCSCs at an effector to target ratio of 0.1 to 1 for 24 hours. Afterwards, the supernatant was removed from the co-cultures and the levels of IFN-γ (A), TNF- α (B), GM-CSF (C), IL-10 (D) and IL-6 (E) were determined using ELISAs in a multiplexed format using Luminex technology.

Figure 5: Blocking IL-10 in NK 92 cells does not increase cytotoxicity or IFN-γ secretion

Purified primary NK cells and NK92 cells were left untreated or treated with IL-2 (1000 units/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml). NK92 cells treated IL-2 + anti-CD16mAb were also treated with anti-IL-10 (10ug/ml). After an overnight incubation, primary human NK cells and NK-92 cells were added to ^{51}Cr labeled OSCSCs. NK cell cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay, and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of the target cells x 100 (A). Primary NK cells and NK92 were treated as described in figure 5A. Afterwards, the supernatant from each of the NK

cell samples was removed and the levels of IFN-γ (A) and IL-10 (B) were determined using specific ELISAs.

Figure 6: Immunofluorescence confocal microscopy: colocalisation of cystatin F with LysoTracker, and cathepsin C and H in NK-92 cells

(A) Colocalization of cystatin F with LysoTracker, (B) Colocalization of cystatin F with cathepsin C, (C) Colocalisation of cystatin F with cathepsin H, (D) Colocalization of cathepsins C and H. Samples were labelled with primary antibodies as indicated on micrographs: anti-cystatin F pAb (A,B,C), anti-cathepsin C pAb (B,D), anti-cathepsin H mAb. Red colour in A originates from labeling with LysoTracker. Green colour originates from Alexa Fluor 488-labelled secondary antibodies. Red colour in B, C, D originates from Alexa Fluor 555-labelled secondary antibodies. Before merging the confocal images, signals for red and green fluorescence were adjusted to comparable levels. The yellow colour indicates co-localization of two labelled antigens. In A, B, C differential interference contrast (DIC) images are shown. In D, right image, the colocalization of both cathepsins is represented by the pixels above the threshold in both channels on the contour plot and on the merged image (white color).

Figure 7: N-terminally truncated monomeric cystatin F is present in NK-92 cells

Lysates from NK-92 cells were separated by non-reducing SDS-PAGE and blots were probed with antibodies against either whole cystatin F (anti-cys F Ab, left panel) or a 15 residue N-terminal peptide (anti-N-terminal of cysF Ab, right panel). Reactivities

with recombinant intact (dimeric) cystatin F (intact cysF) and recombinant truncated (monomeric) Δ15N cystatin F (Δ15N cysF) are also shown.

Figure 8: Cathepsin C (A) and cathepsin H (B) expression is inhibited following addition of anti-CD16 antibody to the NK cells in the presence of IL-2

1 x 10^6 NK cells/ml were treated with IL-2 (1000 u/ml), anti-CD16 antibody (3 μg/ml), or a combination of IL-2 (1000 u/ml) and anti-CD16 antibody (3 μg/ml) for 4 h. Lysates from NK cells were separated by reducing SDS-PAGE and blots were probed with antibodies against cathepsin C (A) and cathepsin H (B). GAPDH staining was used to show equivalent protein loading.

Figure 9: The levels of monomeric and dimeric cystatin F are increased following addition of anti-CD16 to the NK cells in the presence or absence of IL-2

1 x 10^6 NK cells/ml were treated with IL-2 (1000 U/ml), anti-CD16 antibody (3 μg/ml), or a combination of IL-2 (1000 U/ml) and anti-CD16 antibody (3 μg/ml) for 4 h. Lysates from NK cells were separated by reducing (A) and non-reducing (B) SDS-PAGE and blots were probed with antibodies against cystatin F. Reactivity with truncated Δ15 monomeric cystatin F (A) and intact dimeric cystatin F (B) is also shown. CRK-L staining was used to show equivalent protein loading. One of three representative experiment is shown. Adjusted denstity values were calculated by dividing the relative density of each sample lane by the relative density of the loading control for the same lane.

Sample	NK	NK IL-2	NK+anti-CD16mAb	NK+IL2+anti-CD16mAb
Adjusted density for dimeric cystatin F	1	2.31	1.39	2.79
Adjusted density for monomeric cystatin F	1	2.35	1.72	2.22

Figure 10: CD16 triggering in primary NK cells inhibits Granzyme B

Purified NK cells were treated as described in Figure 1A and each NK sample ($1X10^5$/ml) was added to OSCSCs at an effector to target ratio 0.1:1. After an overnight culture, the supernatants were removed from the co-cultures and the levels of Granzyme B (A) and Granulysin (E) secretions were determined using specific ELISA. OSCSCs were seeded at $1X10^5$ cells/well in 12 well plate for 18 hours before highly purified, treated as described in Figure 1 A, was added to OSCSCs at an effector to target ratio of 5:1. After an overnight incubation, NK cells were fixed, permeabilized and stained, as described in the Materials and Methods section, with PE-conjugated anti-human CD56 and FITC-conjugated anti-human Granzyme B. The intracellular expressions were assessed with flow cytometric analysis (B). Isotype control antibody was used as control. Overlay of isotype, IL-2 treated NK cells and IL-2+anti-CD16mAb treated NK cell histograms were analyzed by FlowJo software (C). Primary NK cells and NK92 cells ($1X10^6$ cells) were left untreated or treated with anti-CD16mAb (3ug/ml), IL-2 (1000 units/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 24 hours before the cells were fixed, permeabilized and stained as described in the Materials and Methods section. The intracellular expression of Granzyme B was analyzed by flow cytometry (D).

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Fig. 1
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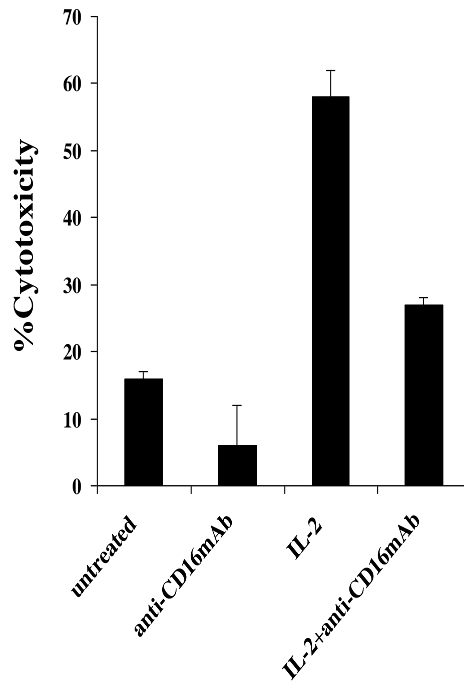

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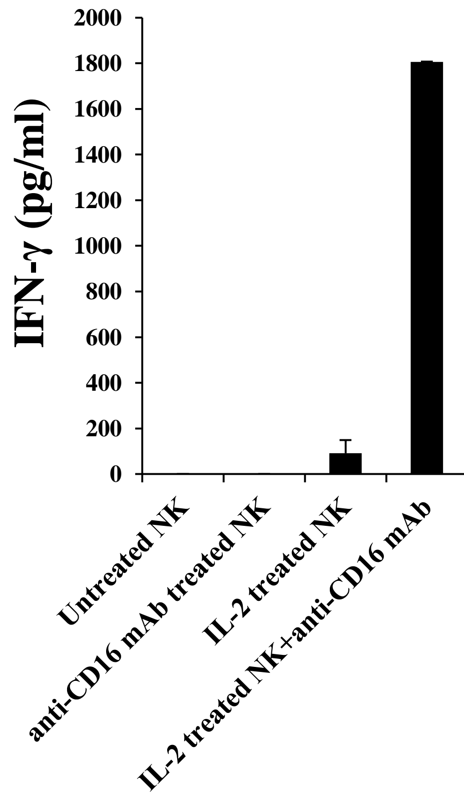

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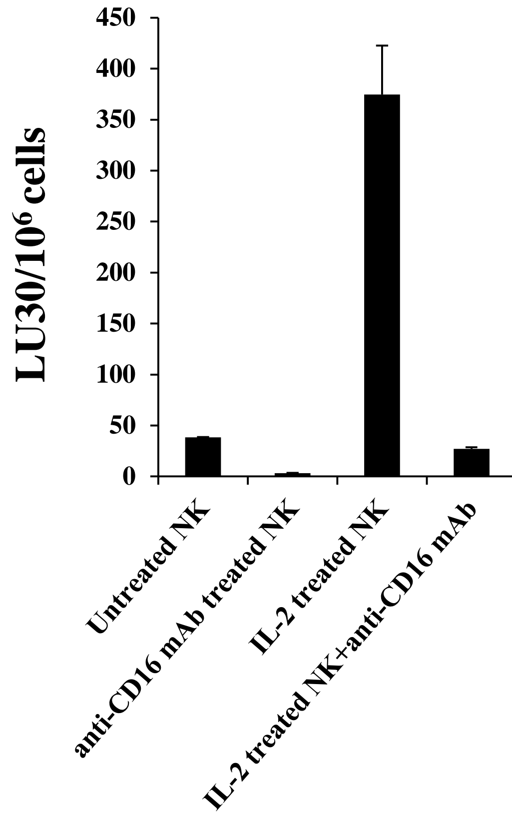

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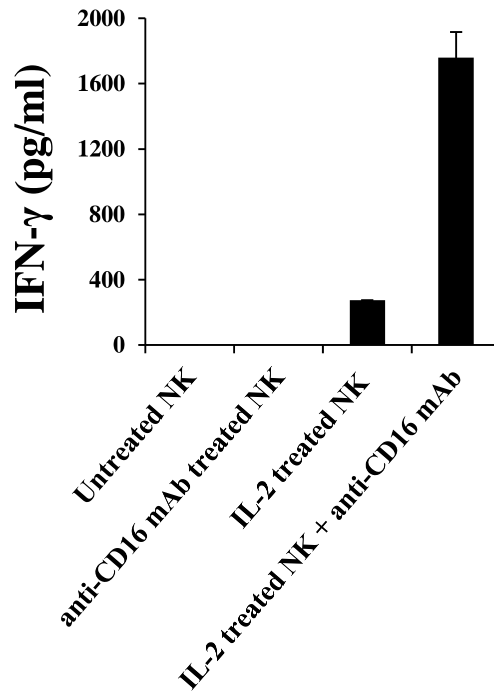

Fig. 2
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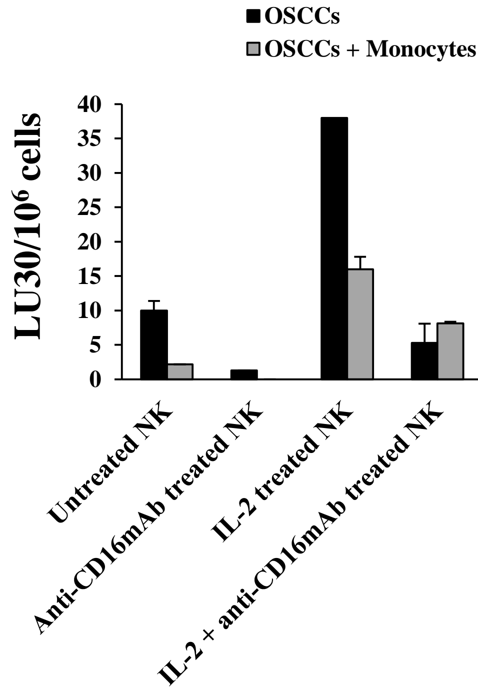

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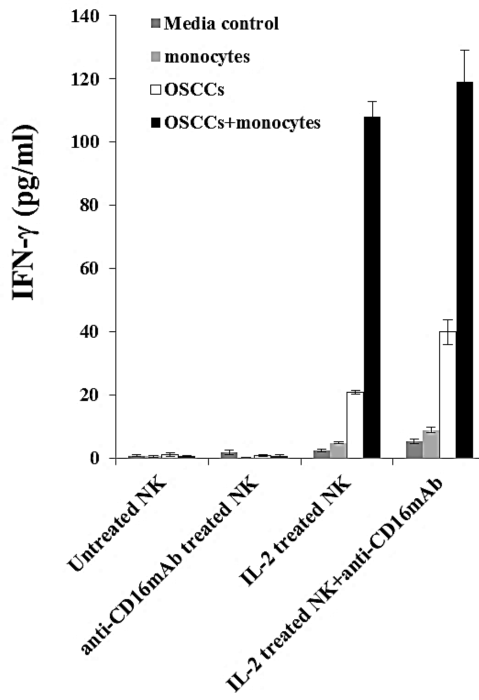

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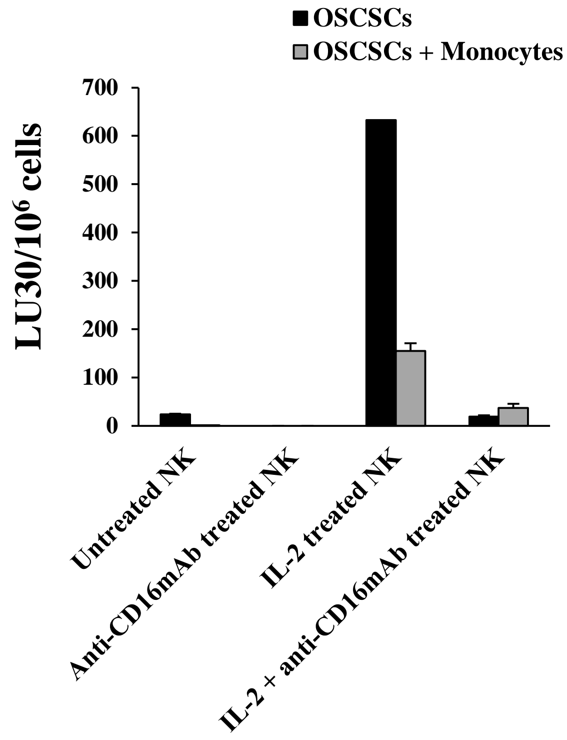


D.

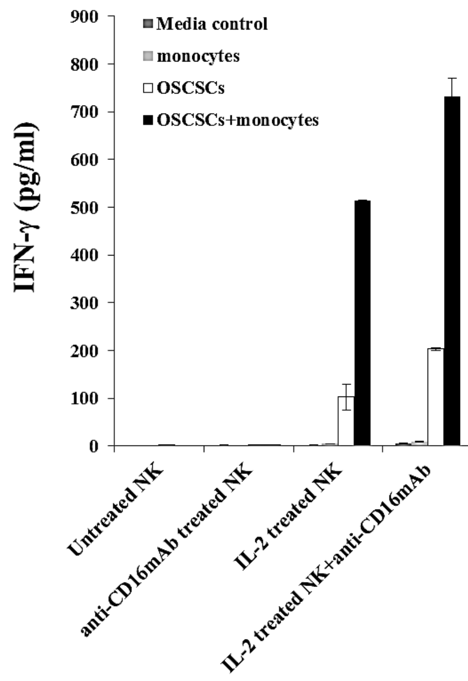


Fig. 3

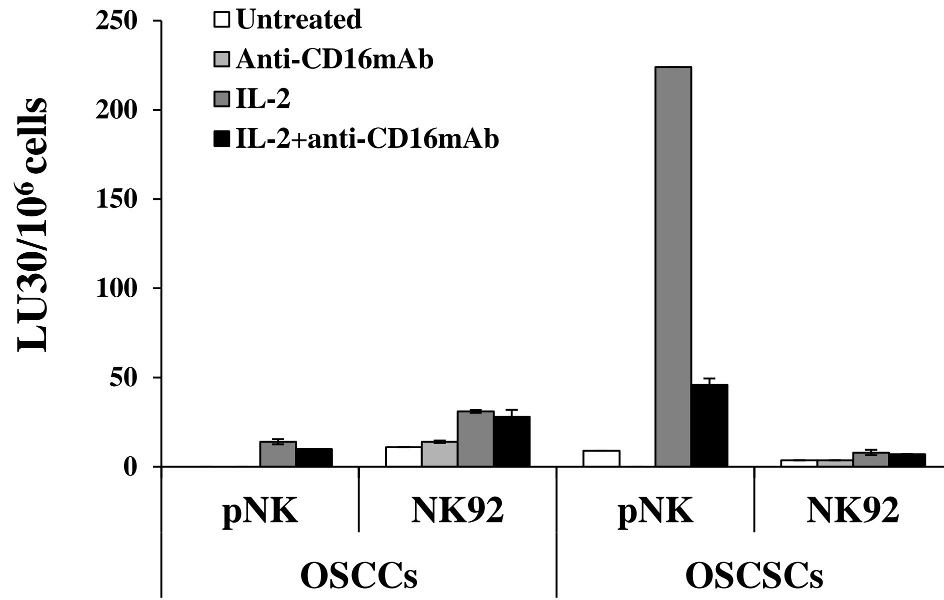


Fig. 4A

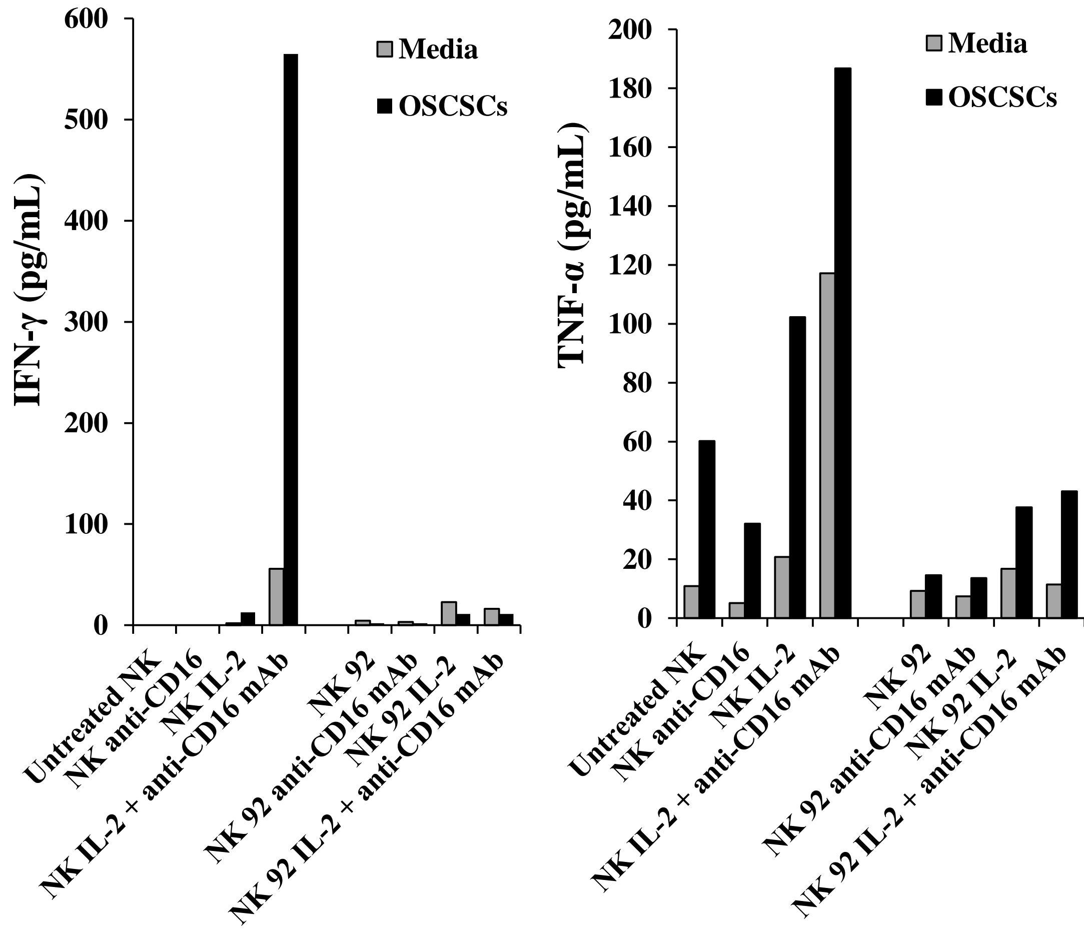


Fig. 4C

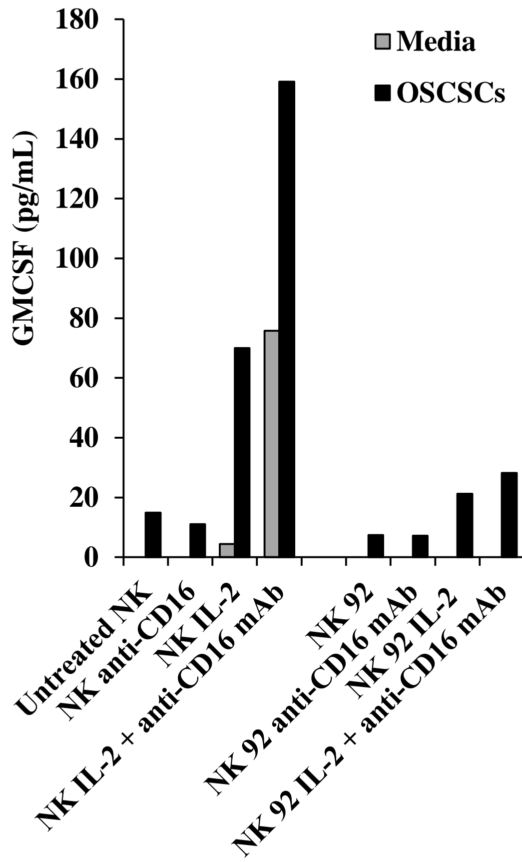


Fig. 4D

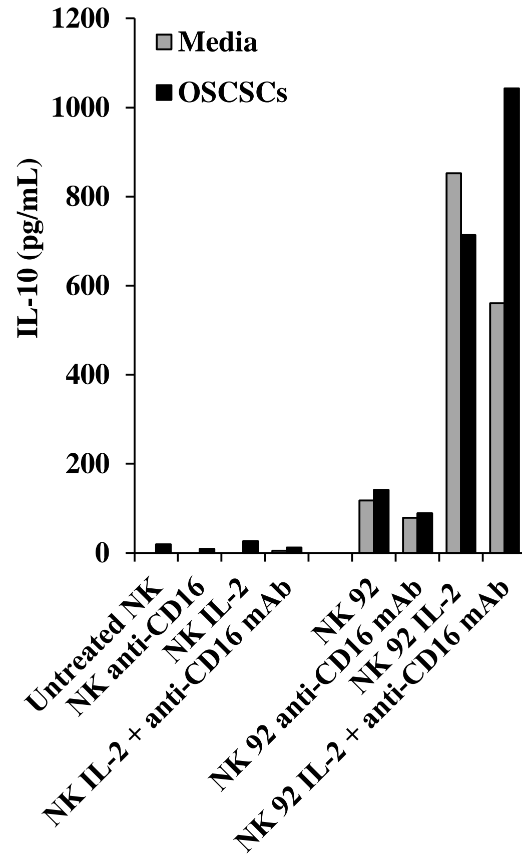


Fig. 4E

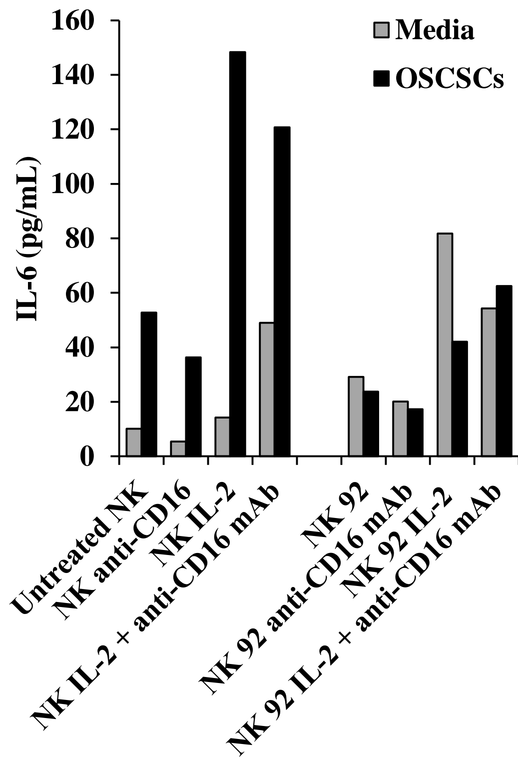


Fig. 5A

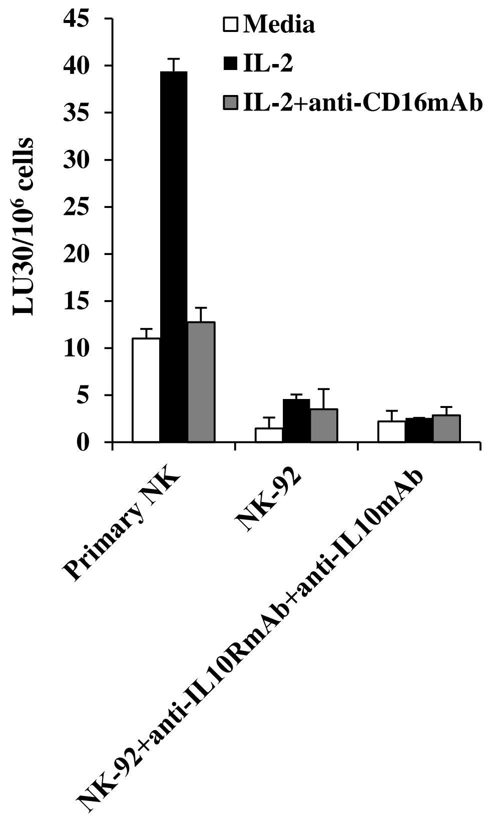


Fig. 5B

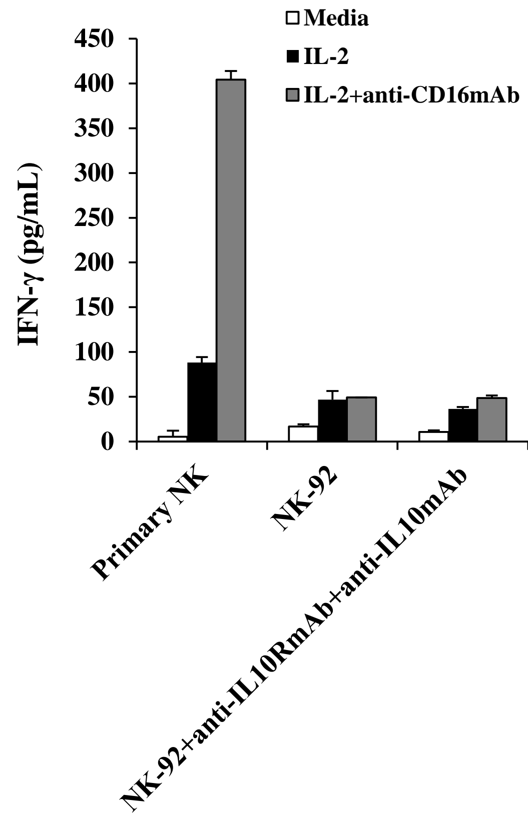


Fig. 5C

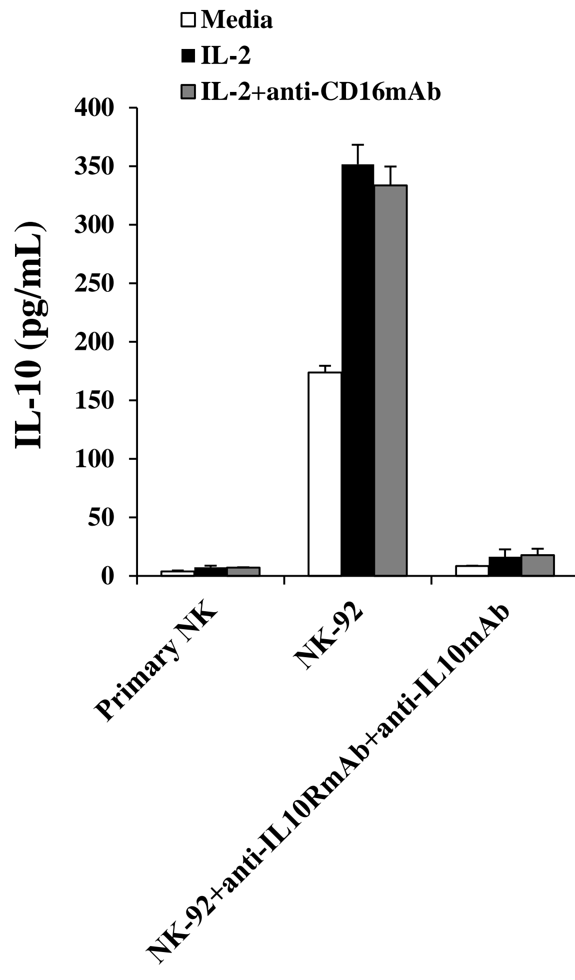


Fig. 6

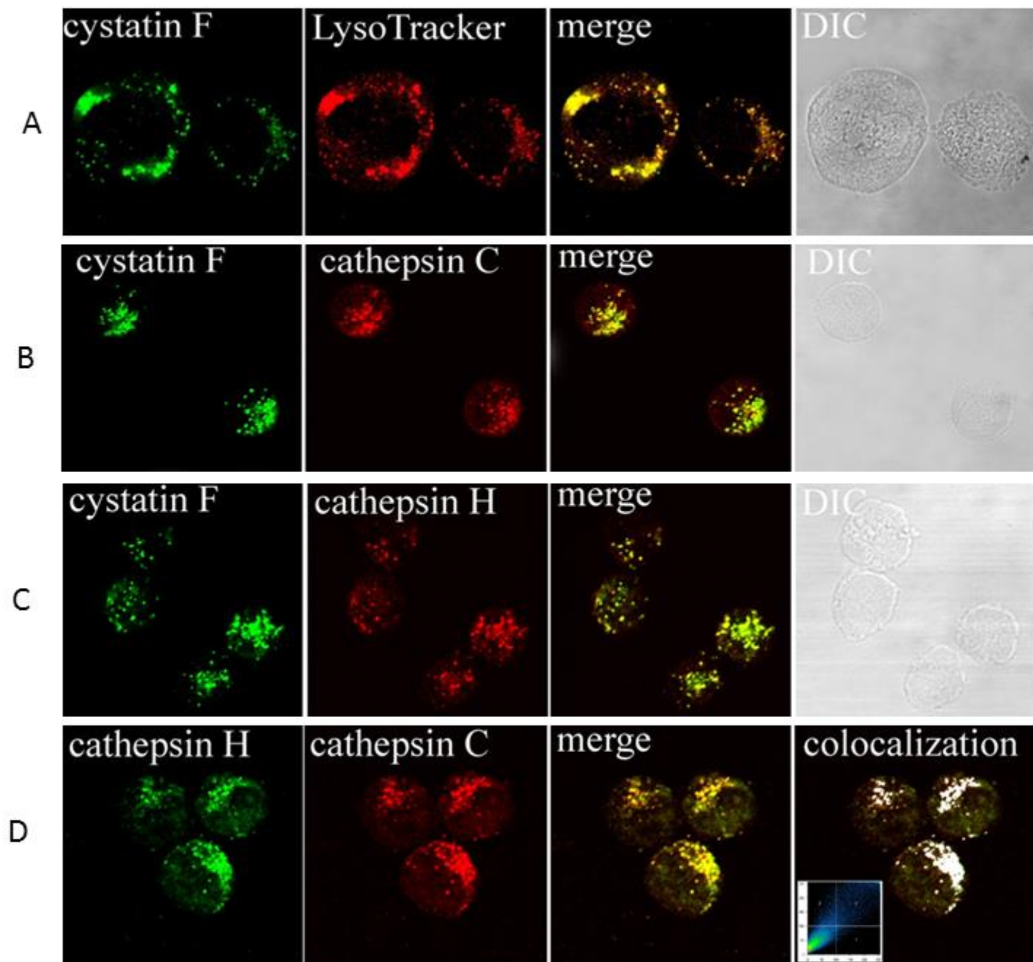


Fig. 7

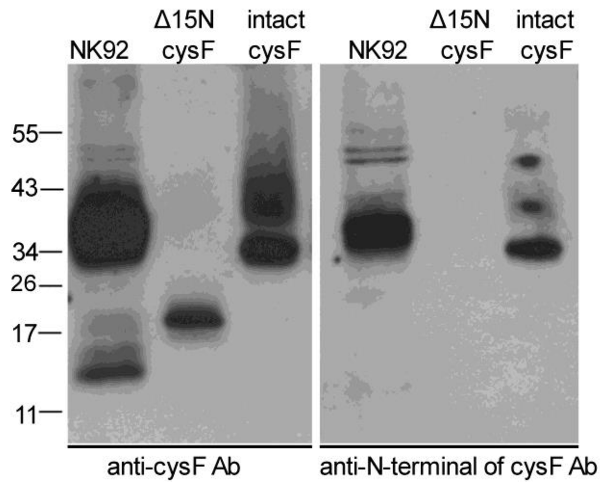


Fig. 8

A.

B.

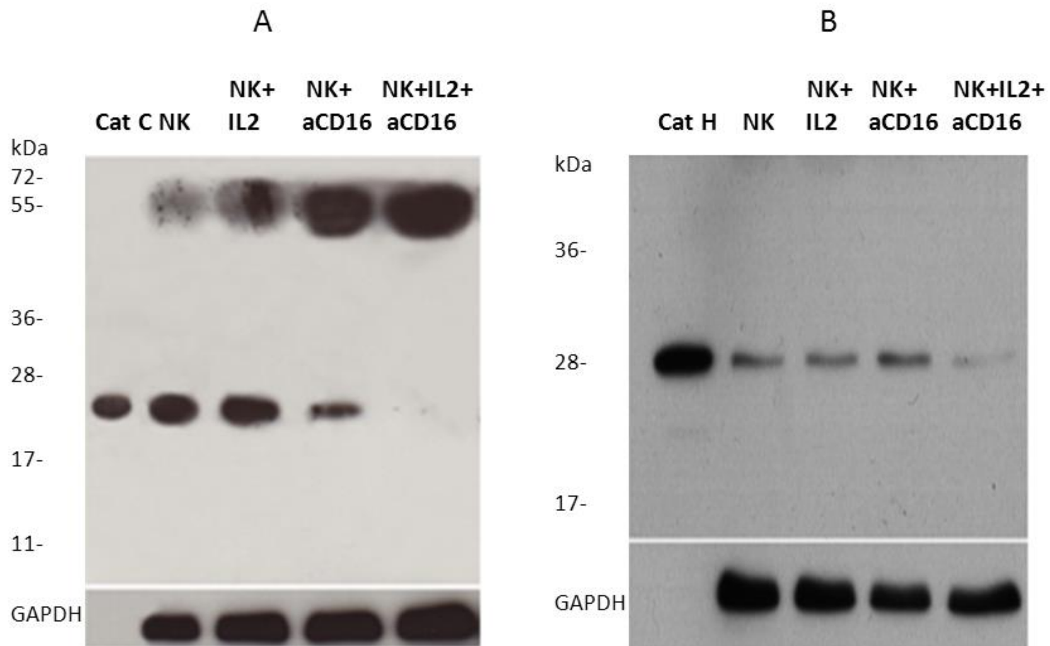


Fig. 9

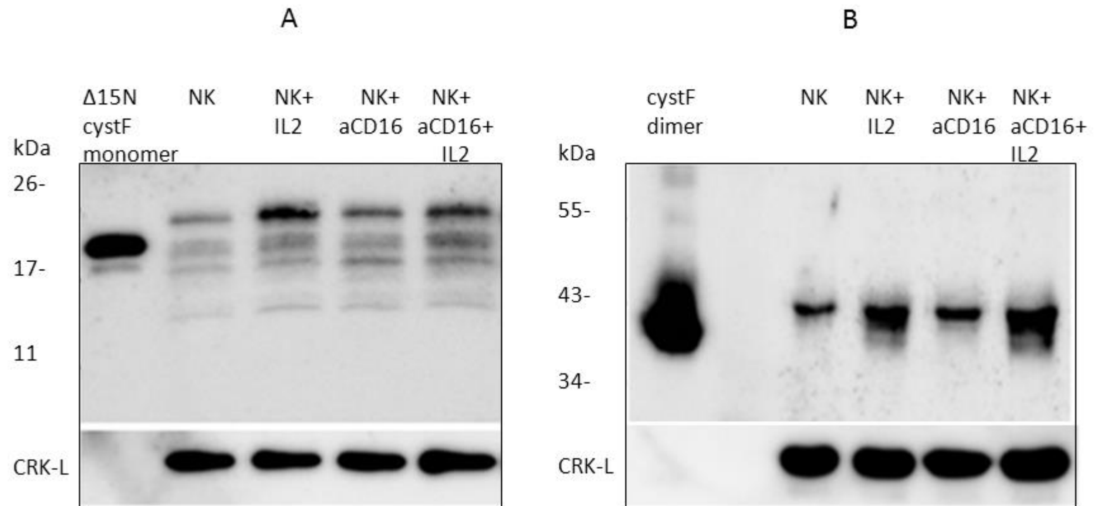
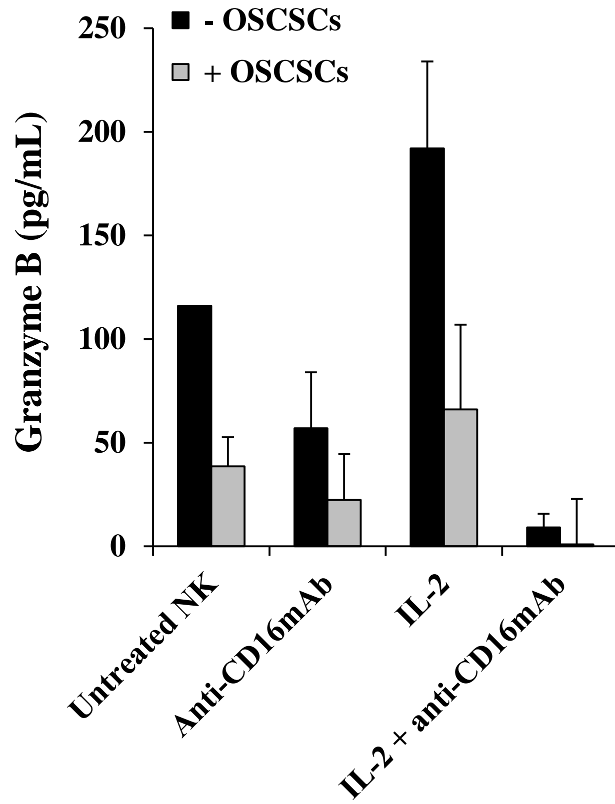


Fig. 10

A.



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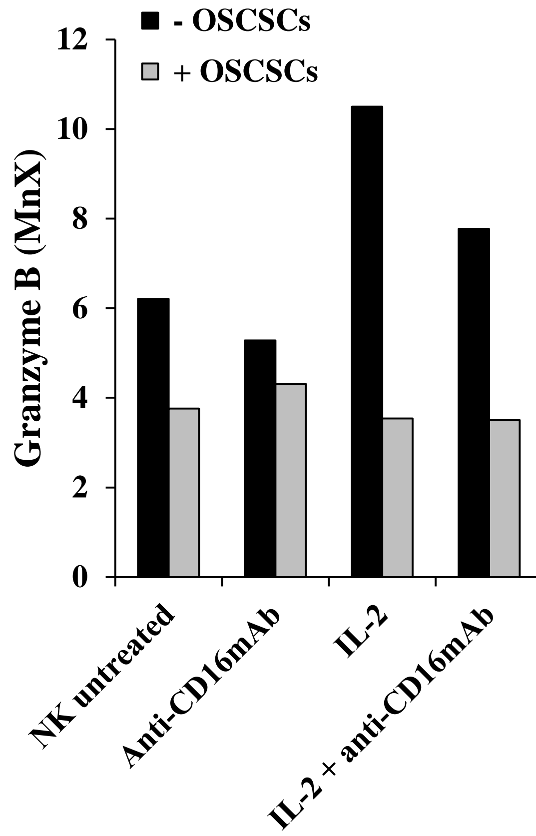

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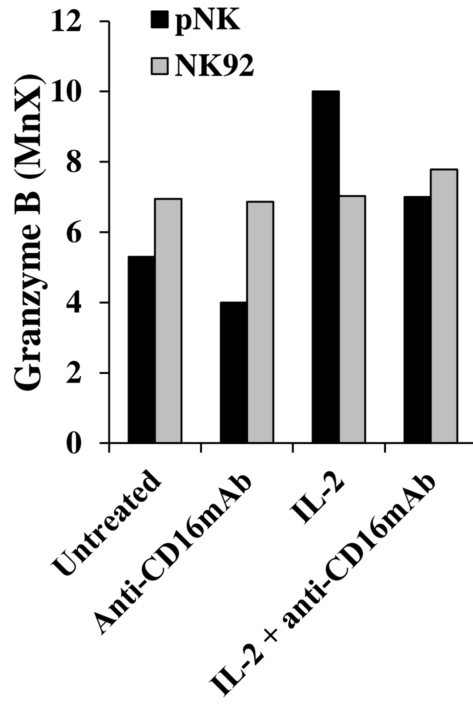

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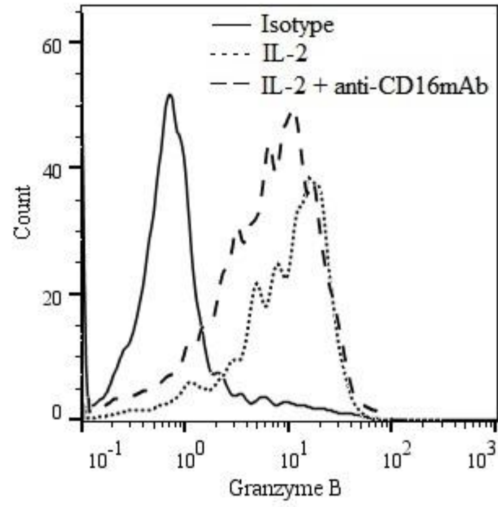

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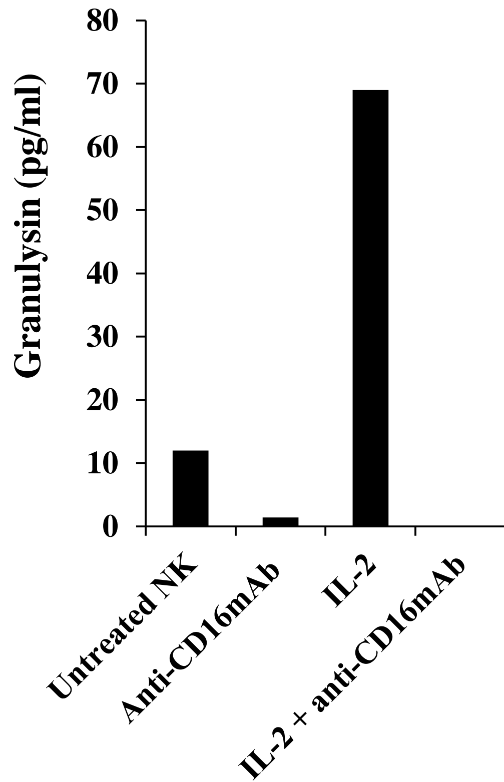

DISCUSSION

1) NK cell preferentially target cancer stem cells; Role of monocytes in protection against NK mediated lysis of cancer stem cells

(Current Drug Delivery, 2012)

2) Potential rescue, survival and differentiation of cancer stem cells and primary non-transformed stem cells by monocyte-induced split anergy in NK cells

(Cancer Immunology Immunotherapy, 2012)

3) Tumor induced inactivation of NK cell cytotoxic function; Implication in growth, expansion and differentiation of cancer stem cells

(Journal of Cancer, 2011)

4) Dual functions of NK cells in selection and differentiation of stem cells; Role in regulation of inflammation and regeneration of tissues

(Journal of Cancer, 2013)

5) NK cells as effectors of selection and differentiation of stem cells: Role in resolution of inflammation

(Journal of Immunotoxicology, 2013)

6) Tumor microenvironment may shape the function and phenotype of NK cells through the induction of split anergy and generation of regulatory NK cells

(Chapter 15, “The Tumor Microenvironment”, 2013)

Natural Killer cells preferentially target cancer stem cells; Role of monocytes in protection against NK cell mediated lysis of cancer stem cells

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Abstract

Mounting effective anti-tumor immune responses by cytotoxic effectors is important for the clearance of tumors. However, accumulated evidence suggests that the cytotoxic function of immune effectors is largely suppressed in the tumor microenvironment by a number of distinct effectors and their secreted factors. The aims of this review are to provide a rationale and potential mechanism for immunosuppression in cancer, and to demonstrate the significance of such immunosuppression in cellular differentiation and tissue regeneration in pathological conditions, and progression of cancer. We have recently shown that increased NK cell function was seen when they were cultured with primary oral squamous carcinoma stem cells (OSCSCs) as compared to their more differentiated oral squamous carcinoma cells (OSCCs). In addition, human embryonic stem cells (hESCs), Mesenchymal Stem Cells (hMSCs), dental pulp stem cells (hDPSCs) and induced pluripotent stem cells (hiPSCs) were significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts or parental cells from which they were derived. We have also reported that inhibition of differentiation or reversion of cells to a less-differentiated phenotype by blocking NFκB or targeted knock down of COX2 augmented NK cell function significantly. Total population of monocytes and those depleted of CD16(+) subsets were able to substantially prevent NK cell mediated lysis of OSCSCs, MSCs and DPSCs. Taken together, our results suggest that stem cells are significant targets of the NK cell cytotoxicity. The concept of split anergy in NK cells and its contribution to tissue repair and regeneration and in tumor resistance and progression will be discussed in this review.

Therefore, patients with cancer may benefit from repeated allogeneic NK cell transplantation at the site of the tumor for specific elimination of cancer stem cells.

Introduction

There is ample evidence for the role of effective immune cell surveillance in the prevention of cancer. Resolution of oral non-Hodgkins lymphoma in a renal allograft recipient was observed after the reduction of immunosuppressive therapy [404]. In a liver transplant recipient rapid progression of oral leukoplakia to carcinoma was observed after immunosuppression [405]. Furthermore, neoplasias of tongue and lip have been widely described in renal transplant patients [406-409], and finally induction of oral cavity cancers was second to liver cancer in patients after bone marrow transplantation [410]. These results indicated the significance of effective immunosurveillance in prevention of malignancies of the oral cavity. Further corroboration is provided by the high occurrence of both virally and non-virally associated lymphomas and Kaposi's Sarcomas in patients with HIV-1 infection [411]. In addition, there is substantial evidence that indicates that immune responses are inhibited by oral tumors, and this may largely be responsible for their induction and progression. This review will focus on the emerging new roles of NK cells in regulation of numbers, resistance and differentiation of cancer stem cells as well as healthy untransformed stem cells. In addition, the significance and role of anergic NK cells, which have largely been ignored or under-appreciated, will be discussed in the process of differentiation and resistance of oral tumors. Thus, we will also focus on the physiological role of NK cells in shaping the size and differentiation of stem cells.

Immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, however, neither underlying physiological significance nor the exact mechanisms by which

immunosuppression occurs are well understood. A number of factors responsible for the suppression of NK cell cytotoxicity in humans has been previously identified [175-180]. It is shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [181-184]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [185-187]. In contrast, interaction of NK cells with the resistant tumors does not lead to suppression of NK cell cytotoxicity [137].

Many mechanisms have been proposed for the functional inactivation of tumor associated NK cells including the over-expression of Fas ligand, the loss of mRNA for granzyme B [176] and decreased CD16 and its associated zeta chain [188].

Many metastatic tumor cells exhibit constitutively elevated NFκB activity [189]. Increased NFκB activity is shown to have a causal relationship to neoplastic transformation, and uncontrolled cell growth in many cell types [189]. Human solid tumors exhibit constitutively activated NFκB [189].

We have previously shown that NK resistant primary oral epithelial tumors demonstrate higher nuclear NFκB activity and secrete significant levels of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF), Interleukin (IL)-1β, IL-6 and IL-8 [190, 412]. Moreover, the addition of Non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit NFκB have the ability to reverse immunosuppression induced by a tobacco-specific carcinogen [413] in addition to their well established ability to decrease oral

dysplasia as well as induction of overt cancer in transgenic animals [191]. In agreement, we have previously demonstrated that inhibition of NFκB by Sulindac treatment of tumor cells increases functional activity of NK cells [192, 193]. In addition, targeted inhibition of NFκB in skin epithelial cells resulted in the induction of auto-immunity and inflammation [153].

The significance and exact mechanism by which NFκB nuclear function in oral tumors modulate and shape the function of key interacting immune effectors is starting to unravel. We have previously shown that inhibition of NFκB by the IκB super-repressor in HEp2 tumors, or in primary OSCCs, or in non-tumorigenic oral keratinocytes (HOK-16B) leads to a significant increase in cytotoxicity and secretion of IFN-γ by the human NK cells [192, 193]. However, the underlying significance and the physiological relevance of NFκB modulation in tumors or in primary cells responsible for the alteration of NK cell cytotoxic function is just being understood. It is clear that the objective in cancer is to enhance the function of cytotoxic immune effectors, while in auto-immunity and inflammation the aim is to inhibit immune effector function. Therefore, dissection of the underlying mechanisms of immune activation when NFκB is modulated in the cells might help design strategies to target each disease accordingly. Indeed, targeted inhibition of NFκB function in both the intestinal epithelial cells and myeloid cells was previously shown to result in a significant decrease in size and numbers of the tumor cells [194].

In this report we review the previous studies from our laboratory and other studies regarding the factors and mechanisms involved in immunosuppression observed in cancer. Furthermore, we will provide evidence which indicates that the stage of maturation and differentiation of the healthy untransformed stem cells, as well as transformed tumorigenic cancer stem cells, is predictive of their sensitivity to NK cell lysis. In this regard we have previously demonstrated that OSCSCs, which are stem-like oral tumors, are significantly more susceptible to NK cell mediated cytotoxicity; whereas, their differentiated counterpart OSCCs is significantly more resistant [412]. In addition, hESCs and hiPSCs, as well as a number of other healthy normal stem cells such as hMSCs and hDPSCs, were found to be significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts [412]. Based on these results, we proposed that NK cells may play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact. In order to drive differentiation, however, NK cells first need to receive signals from stem cells or those which have disturbed or defective capabilities to differentiate. In addition, CD14+HLADR- monocytes, fibroblasts or Tumor associated macrophages can condition NK cells to support differentiation of the cells. These signals alter the phenotype of NK cells and cause NK cells to lose cytotoxicity and change into cytokine producing cells. These alterations in NK cell effector function will ultimately aid in driving differentiation of a minor population of surviving, healthy, as well as transformed stem cells. In cancer patients since the majority of NK cells have lost cytotoxic activity, they may eventually contribute to the progression of cancer by not only driving the differentiation of tumor cells but more importantly, by allowing the growth and expansion of cancer stem cells.

Immunosurveillance in the Prevention of Cancer

The theory of immunosurveillance was initially set forth by Burnet [414] to indicate that the key thymus dependent effectors were responsible for eliminating developing cancers [415, 416]. However, the opponents of the immunosurveillance theory strongly criticized the concept primarily due to the lack of data showing elevated susceptibility to cancer in nude mice which had T-cell defects [417, 418]. New data obtained from severely immunocompromised STAT1-/- and RAG-/- mice which have defects in both the innate and adaptive immune effector functions have revived the concept of immunosurveillance, and highlighted the significance of both innate and adaptive immune responses in prevention of cancer [419-422].

Since then, the concept of immunosurveillance has expanded to include immunoediting as an important mechanism for the development of cancer [421]; [422]. It was suggested that cancer immunoediting comprises of three phases: elimination, equilibrium and escape [422]. Elimination represents the classical concept of immunosurveillance. However, during equilibrium and escape, the interaction and cross signaling between immune effectors and tumor cells shape the cells by progressive generation of tumors capable of gradual inactivation and death of immune effector cells. The final stages of cancer development may result in the induction of less immunogenic tumors in the presence of fewer immune effectors capable of lysing the tumors. Thus, the pressure exerted by tumor cells and immune effectors on each other may eventually shape the environment to benefit invading tumors. Similarly, certain elements of such

interactions are also observed during the interaction of NK cells with healthy non-transformed human stem cells in which case the three phases of interaction may include elimination which marks the decrease in the size of stem cells due to the selection of stem cells by the NK cells, equilibrium which denotes the conditioning of NK cells to lose cytotoxicity and support maturation and differentiation of stem cells, and finally the resolution phase which denotes elimination of anergized NK cells and differentiation of selected stem cells. Similarities and differences between these phases in cancer and during stem cell maturation will be discussed below.

Immune Responses in Head and Neck Cancer

Head and neck cancers represent approximately 6% of all new cancers in the United States. Despite many advances in surgical procedures and the availability of new chemotherapeutic agents and optimized radiotherapeutic procedures, survival of patients with head and neck cancers has not improved in the last forty years There is substantial evidence which indicates that immune responses, which should otherwise suppress or eliminate oral cancer cells, are inhibited by properties and functions of oral cancers. NK cells and cytotoxic T-cells, which play crucial effector functions in the host defense against neoplasia, are functionally inactivated in oral cancers [137, 141, 142, 192, 193, 349, 423]. Regressing tumor grafts of oral origin contain significantly larger numbers of functional NK and T-cells than those associated with the primary tumors [424], while patients with metastasis of head and neck cancers have low NK and T-cell activity [425]. Spontaneous apoptosis of circulating peripheral blood T-cells and decreased frequencies

of key peripheral blood dendritic cell subsets, attributable to the presence of tumors, are important indicators of immune cell paralysis in head and neck cancers [426].

Immunotherapy with cytokines or adaptively transferred effector cells was found to be ineffective in the treatment of head and neck cancers [427-429], although limited success in immunotherapy of metastatic melanomas or renal cell carcinomas has previously been reported [430]. The reason for this failure of known immunotherapeutic modalities in many cancers including head and neck cancers is poorly understood. It has been hypothesized that a widespread paralysis of cytotoxic effectors residing inside the inflammatory infiltrate of advanced cancer patients is the main reason for poor prognosis [431, 432]. Furthermore, freshly isolated tumor infiltrating lymphocytes are not cytotoxic to autologous tumor cells and show a significantly reduced clonogenicity [182-184, 433]. Functional paralysis of cytotoxic cells have also been reported in a variety of other cancers, notably breast [177, 178, 434, 435], renal [175], and colon cancers [176]. More importantly, depletion of cytotoxic effectors in the tumor milieu has an unfavorable outcome for survival in cancer patients [434, 436-438]. Indeed, a significantly shorter survival rate is reported for colorectal carcinoma patients with little or moderate NK cell infiltration as compared to those with extensive infiltration [439]. A five year survival advantage was also seen with higher CD3 positive tumor infiltrating T-cells than with lower T-cell count in the carcinoma of uterine cervix [438]. In primary cutaneous melanoma a five year survival rate for high tumor associated lymphocyte infiltrate was 77%, with medium lymphocyte infiltrate 53%, and for tumors with absent tumor

infiltrating lymphocytes (TILs) 37% [437] suggesting an important function for NK and CTLs in cancer cell rejection.

Defects in NK, T-cells and DCs have been reported in oral cancer patients. Signaling abnormalities, spontaneous apoptosis and reduced proliferation of circulating T-cells, DCs and TILs have also been reported in patients with oral cancers [426, 440]. The percentage of myeloid-derived LIN-DR+CD11c+ DCs is significantly lower in head and neck cancer patients compared to healthy controls [426]. A decrease in the number of DCs in patients was related to the presence of tumor cells since the numbers of myeloid-derived DCs returned to normal levels when the tumors were excised in patients with head and neck cancers [426].

Mechanisms of Immunosuppression by Tumor Cells

Many mechanisms have been proposed for the functional inactivation of tumor associated lymphocytes [175-179]. Soluble products derived from renal cell carcinoma inhibit proliferative capacity of T-cells infiltrating human tumors due to a downregulation of Janus kinase 3 (Jak 3), p56 (lck), p59 (fyn) and zap 70 [175]. Expression of Fas ligand by many human tumor cells including oral tumors has been hypothesized to be a major cause of lymphocyte depletion in the tumor microenvironment [179]. In mice, tumor induced immunosuppression has been associated with a decreased expression of the zeta-chain of the T-cell receptor and the loss of mRNA for granzyme B [176]. Indeed, as observed in mice, the frequency of TCR-zeta positive and granzyme positive lymphocytes are decreased in advanced stage head and neck carcinomas, and the

restoration of expression during in vitro stimulation suggests the presence of tumor derived suppressive factors [176]. Decreased CD16 and its associated zeta chains are also observed in tumor infiltrating NK cells of patients with cancer [188]. The relative lack of IFN-γ and granulocyte-macrophage colony stimulating factor (GM-CSF), rather than a deficiency of IL-2 by tumor infiltrating lymphocytes (TILs) in breast cancer, has been hypothesized to be a mechanism for impaired immune function [177]. Moreover, the breast cancer associated antigen DF3/MUC1, has been shown to induce apoptosis of activated human T-cells [178]. Overall, both secreted factors and direct cell-cell contact during the interaction of immune effectors with the tumor cells were shown to be responsible for the suppression of immune effector function.

Key Immunosuppressive factors induced in the tumor microenvironment

Prostaglandin E2 (PGE2)

Immunosuppression linked to enhanced PGE2 synthesis has been documented in many human cancers [441]. Freshly excised human head and neck cancers demonstrated elevated levels of PGE2, transforming growth factor- β1 (TGFβ1) and interleukin-10 (IL-10) secretion which are known to upregulate the expression of Killer Immunoglobulin-like Receptors (KIRs) on the surface of NK, T-cells and DCs and block immune effector function [442]. Furthermore, metastatic head and neck cancers released higher levels of above-mentioned inhibitory factors and lower levels of immune activating factors IFN-γ and IL-2 than did their corresponding primary tumors [442]. PGE2 overproduction in the tumor microenvironment was also shown to lead to dendritic cell (DC) abnormalities

[443]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as Sulindac, which inhibit PGE2 production by blocking NFκB-induced cyclooxygenase (COX)-2 production, can reverse immunosuppression induced by a tobacco-specific carcinogen [413], and delay the onset and severity of oral cancer in transgenic animals [191]. In agreement with these observations, we have previously demonstrated that Sulindac treatment of tumor cells increases functional activity of NK cells [193].

Interleukin (IL)-6

IL-6 is secreted constitutively by oral squamous carcinomas [209], and is found to be elevated in oral cancer patients [444]. IL-6 is known to interfere with IFN-γ signaling by inducing Th2 differentiation via activation of NFAT and secretion of IL-4, which subsequently inhibits Th1 polarization via STAT3-induced expression of Suppressor Of Cytokine Signaling (SOCS)-1 in CD4+ T-cells [210]. In support of a role for IL-6 in mediating immune evasion of tumor cells, Menetrier-Caux et al showed that conditioned medium from human renal cell carcinoma cell lines blocked the differentiation of CD34+ bone marrow cells into immature DCs, and this inhibitory effect could be blocked with antibodies against either IL-6 or granulocyte colony stimulating factor (G-CSF) [445]. Furthermore, in other studies, recombinant IL-6 alone could block the differentiation of CD34+ bone marrow-derived cells to functional DCs, supporting a model in which tumor-derived IL-6 enhances cancer progression by impairing host anti-tumor immunity [445].

Vascular Endothelial Growth Factor (VEGF) and Granulocyte Monocyte Colony Stimulating Factor (GM-CSF)

Other factors which have been implicated in immunosuppression in cancers are angiogenic factors such as VEGF [446] and cytokines such as G-CSF and GM-CSF. The finding that neutralizing antibodies to VEGF or GM-CSF could partially reverse the inhibitory effects of tumor cell supernatant on DC maturation demonstrated that these factors could interfere with DC differentiation and function [446]. These results suggested that there may be a strong selection pressure for cells that produce one or more of these factors, because of their ability to avoid immune detection and destruction [445].

Increased numbers of immature DCs were found in the peripheral blood of cancer patients with elevated levels of circulating VEGF [446]. Accordingly, when similar concentrations of VEGF to those found in cancer patients were injected in mice the number of immature myeloid cells and immature DCs were increased in their peripheral blood. Therefore, increased secretion of VEGF in the tumor microenvironment may prevent maturation and differentiation of DCs and contribute to poor anti-tumor immune responses in cancer patients.

Immunosuppression Mediated by Direct Cell-Cell Contact.

Contact-dependent immune suppression can occur by engagement of MHC class I molecules on CD8+CD28- suppressor cells with immunoglobulin-like transcript (ILT2 and ILT4) inhibitory receptors on DCs. Blocking of both MHC Class I and ILTs by specific antibodies can reverse immunosuppression [447]. Similarly binding of c-type

lectin receptors or Killer Immunoglobulin-like Receptors (KIRs) to MHC Class I ligands inhibit NK cell function [448-451]. In addition, the expression of co-stimulatory molecules, such as B7H1 on tumor cells and inhibitory DCs and T-cells can inhibit T-cell activation and proliferation [452].

Immunosuppressive effectors in tumor microenvironment

The tumor microenvironment consists of a number of heterogeneous cell populations with ability to suppress and limit the function and survival of cytotoxic immune effectors. Patients with cancer often bear high numbers of immature CD14+HLADR- monocytes [227, 453]. Tumor associated Macrophages (TAMs) have previously been shown to significantly influence and limit immune activation in the tumor microenvironment [454, 455]. In addition, Myeloid Derived Suppressor Cells (MDSCs) which are comprised of a number of distinct cell populations of myeloid origin and whose roles in immunosuppression are now well established in both animal and human cancer models have received significant attention in the recent years [453]. T-cell dysfunction is shown to be induced by MDSCs by the secretion of IL10, TGF-β, reactive oxygen species (ROS), arginase and Nitric Oxide synthase (NOS). T-regulatory (Treg) and DC regulatory (DCreg) cells were also shown to have significant immunosuppressive roles in the tumor microenvironment [453]. Perhaps one of the most interesting recent observations regarding immunosuppressive effectors is the identification of Cancer Associated Fibroblasts (CAFs) and Mesenchymal Stem Cells (MSCs) as two potential tumor promoters. Fibroblasts from tumor tissues demonstrate an activated phenotype and have the ability to secrete many immunosuppressive factors such as TGF-β and VEGF

among other factors [456]. We have also found that fibroblasts, as well as MSCs and CD14+HLA-DR- monocytes irrespective of their surface expression of CD16 are significantly more susceptible to NK cell mediated cytotoxicity [205], therefore, these cells may condition NK cells to become anergic (please see below). Indeed, in OSCCs the majority of recruited immune effectors are usually found in the connective tissue area where, through cell-cell interaction with the immunosuppressive cells listed above, can inhibit the cytotoxic function of NK cells, leading to increased cytokine secretion capability of the NK cells resulting in differentiation and resistance of oral epithelial tumors (Fig. 1) (please see below).

Role of transcription factors in tumor resistance

Although each one of the factors indicated above can in part be responsible for the resistance of tumors, previous data obtained from different laboratories [193, 211] indicated that targeting transcription factors may decrease resistance and increase sensitivity of tumors to immune mediated cytotoxicity.

NFκB in Cancer

Many tumor cells exhibit constitutively elevated NFκB activity [189]. Human leukemias and lymphomas as well as human solid tumors exhibit constitutively activated NFκB in the nucleus [189]. However, although previous studies have attributed a significant role for NFκB in oncogenesis and tumor progression, relatively fewer studies have been conducted to explore the significance of elevated NFκB function in tumor cells in the modulation of immune effector function against the tumor cells [192, 193].

Blocking NFκB in oral tumors increases NK cell function.

We have shown previously that NFκB nuclear function in a primary Oral tumor OSCCs and an established tumor line, HEp-2 cells previously used as an oral tumor model [193, 457-459], modulates and shapes the function of interacting immune effectors [192, 193]. It is believed that HEp-2 cells are Hela contaminants since cells of these lines are shown to contain Hela marker chromosomes (ATCC). Since knock down of NFκB was shown to increase the function of immune inflammatory cells in diverse cell types (please see below) it is not surprising to find similar patterns of immune activation in both oral and non-oral derived cell lines, even in those which have been derived from contaminants such as Hela cells. In addition, the majority if not all cells increase NFκB during their activation and differentiation, a knock down of NFκB is likely to revert the cells, irrespective of their cellular origin, to their less differentiated phenotypes resulting in the potential activation of the immune effectors in order to aid in their differentiation [412]. Thus, inhibition of NFκB by an IκB super-repressor in HEp-2 cell line (Hep2-$I\kappa B_{(S32AS36A)}$) or in primary oral tumors resulted in a significant level of activation of human NK cell cytotoxic function and increased IFN-γ secretion. Similarly, inhibition of NFκB by Sulindac increased the functional activation of NK and enhanced anti-tumor cytotoxic activity [192, 193]. Similar experiments to those performed with HEp-2 and OSCC tumors were also conducted using immortalized but non-tumorigenic human oral keratinocytes (HOK-18). Inhibition of NFκB in HOK-18 also resulted in an increased function of interacting NK cells [412].

NFκB Activation in Tumor Cells Alters the Cytokine/Chemokine Profiles of NK Cells

Inverse modulation of IFN-γ and IL-6 cytokine secretion was seen in co-cultures of NK cells with NFκB knock down OSCCs and HEp2- $I\kappa B_{(S32AS36A)}$ cells indicating that blocking NFκB in these cells serves to switch the balance from Th2 type responses to more of a Th1 type response [192, 412]. Moreover, HEp2- $I\kappa B_{(S32AS36A)}$ cells produced large amounts of pro-inflammatory chemokines MCP-1 and Rantes suggesting that inhibition of NFκB in tumor cells may recruit NK and T cells to the tumor microenvironment [192].

NFκB inhibition in mice and humans activate immune inflammatory functions

Targeted deletion of IKK-β which inhibits NFκB function in epidermis of mice has previously been shown in one study to lead to inflammatory skin manifestations similar to that seen in patients with Incontinentia Pigmenti (IP) [153]. Elevated levels of cytokines and chemokines have also been demonstrated in the epidermis of patients and animals with Iκκγ and Iκκβ deletions [153, 460].

Blocking of NFκB function by deleting Iκκ-β in intestinal epithelial cells dramatically decreased the rate of tumor formation without affecting the size of the tumors in colitis-associated cancer model [461, 462]. Moreover, deleting Iκκ-β in myeloid cells in the same model system resulted in a decrease in tumor size. These studies also underscore the significance of a cross talk between different subsets of

immune effectors with the epithelial cells in induction and progression of the intestinal tumors.

Mice with a keratinocyte-specific deletion of Iκκ−β demonstrated decreased proliferation of epidermal cells and developed TNF-α dependent inflammatory skin disease [153]. In contrast, in other studies in which NFκB function was blocked in dermal keratinocytes by a mutant IκB-α an increased proliferation and hyperplasia [463] and eventual development of cutaneous squamous cell carcinomas of skin were seen if mice were allowed to survive and reach adulthood [464, 465]. In contrast to the results obtained in epidermis, blocking NFκB with a mutant IκB-α super-repressor in HEp2 cells did not change or moderately decreased the rate of tumor cell growth when compared to vector-alone transfected HEp2 cells (unpublished observations). It is of interest to note that in these studies with diverse functional outcomes in keratinocytes, blocking TNF-α function resulted in the prevention of both the neoplastic transformation and the inflammatory skin disease [153, 465] . Thus, it appears that TNF-α is the primary factor mediating the pathological processes in both of these studies. Elevated numbers of immune inflammatory cells recruited to the site of epidemis are likely responsible for the increased secretion of TNF-α. Indeed, we have demonstrated that synergistic induction of TNF-α could be observed when NFκB knock down oral tumors were cultured with either PBMCs or NK cells [192].

NK cells lyse cancer stem cells, as well as hESCs, hiPSCs, hMSCs and hDPSCs but not their differentiated counterparts

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed when NK cells were co-incubated with OSCSCs which released significantly lower levels of GM-CSF, IL-6 and IL-8 and demonstrated decreased expression of phospho-Stat3, B7H1 and EGFR, and much lower constitutive NFκB activity when compared to differentiated OSCCs [412]. More importantly, OSCSCs expressed CD133 and CD44^{bright} oral stem cell markers [412]. Increase in IFN-γ secretion was correlated with a decrease in secretion of IL-6 in co-cultures of NK cells with OSCSCs as compared to those co-cultured with OSCCs. Therefore, from these results a specific profile for differentiated NK resistant oral tumors emerged which demonstrated increased GM-CSF, IL-6 and IL-8 secretion in the context of decreased IFN-γ secretion during their interaction with the NK cells. In contrast, co-cultures of cancer stem cells with NK cells demonstrated increased IFN-γ in the context of lower GM-CSF, IL-6 and IL-8 secretion [205, 412]. In addition, three brain tumor stem cells which were previously characterized [259, 466, 467] were found to be significantly more susceptible to NK cell mediated cytotoxicity when compared to their differentiated counterparts which were significantly more resistant (manuscript submitted). Since OSCSCs and brain stem cells were significantly more susceptible to NK cell mediated cytotoxicity we reasoned that healthy, non-transformed primary stem cells may also be susceptible to NK cell mediated cytotoxicity. We demonstrated previously that NK cells lysed hMSCs, hDPSCs, hESCs and hiPSCs significantly. All different types of stem cells became resistant to NK cell mediated cytotoxicity once they were differentiated [412]. In addition, higher sensitivity of hiPSCs to NK cell mediated lysis was also observed when compared to parental line from which they were derived. Taken together these results indicated that undifferentiated cells were

targets of NK cell cytotoxicity. Thus, the stage of differentiation of the cells is predictive of their susceptibility to NK cell mediated cytotoxicity.

Targeting NFκB revert the cells to more of an undifferentiated phenotype and increases NK cell mediated cytotoxicity against Oral tumors.

Since the degree of differentiation in the cells is predictive of their sensitivity to NK cell mediated cytotoxicity, we reasoned that blocking NFκB in the cells may dedifferentiate and consequently revert the cells to more an of undifferentiated phenotype, resulting in their increased susceptibility to NK cell mediated cytotoxicity. Indeed, blocking NFκB in oral tumors was also found to increase CD44 surface receptor expression, which is one of the hallmarks of stem cells (manuscript in prep). In addition, the profiles of cytokines secreted in the co-cultures of NK cells or PBMCs with NFκB knock down tumors resembled those secreted in the co-cultures of NK cells with OSCSCs or healthy stem cells [192, 205, 412]. Since tumorigenic and non-tumorigenic human oral keratinocytes acquire sensitivity to NK cell mediated lysis when NFκB is inhibited, it is likely that this phenomenon is not specific to cancer, and it may occur during other pathological conditions. Indeed, when human primary monocytes are differentiated to dendritic cells they too became more resistant to NK cell mediated cytotoxicity [412]. Moreover, knock down of COX2 in primary monocytes [412], or in mouse embryonic fibroblasts (manuscript in prep), resulted in the reversion or de-differentiation of the monocytes and fibroblasts respectively, and the activation of NK cell cytotoxicity. Indeed, it is likely that any disturbance in cellular differentiation may

predispose the cells to NK cell mediated cytotoxicity. Since STAT3 is an important factor that is increased during differentiation, blocking STAT3 is also critical in the activation of immune effectors [211]. In support of a critical role of STAT3 in immune evasion of tumor cells in humans, we and others have recently shown that glioblastoma multiforme (GBM) tumors display constitutive activation of STAT3 (Cacalano and Jewett, unpublished observation) [160], and poorly induce activating cytokines and tumor-specific cytotoxicity in human peripheral blood mononuclear cells (PBMCs) and NK cells (unpublished observations). Ectopic expression of dominant-negative STAT3 in the GBM cells increased lysis of the tumor cells by the immune effectors and induced production of IFN-γ by the interacting immune effectors (manuscript in prep).

Since NFκB is shown to regulate IL-6 secretion in OSCCs, HOK-16B and HEp2 cells and secreted IL-6 in tumors is known to activate STAT3 expression and function, increases in NFκB nuclear function should in turn induce STAT3 activation and result in a significant resistance of tumors to NK cell mediated cytotoxicity. Therefore, targeting STAT3 or signaling pathways upstream of STAT3, such as NFκB, may dedifferentiate the cells and predispose the cells to NK cell mediated cytotoxicity.

Sensitive but not resistant tumors cause anergy in NK cells

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity and induce cell death in a small subset of NK cells [136, 137]. On the other hand NK resistant tumors such as RAJI cells induce no or much lower levels of anergy or loss of NK cell cytotoxicity [136, 137]. Furthermore, following NK cell

cultures with sensitive tumor-target cells overnight, the target binding NK cells undergo phenotypic and functional changes. Target cell inactivated NK cells express CD16-CD56dim/-CD69+ phenotype [136, 137]. This phenotype has also been observed in several disease manifestations including HIV infection [468]. Significant downmodulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in several cancer patients including those of the oral and ovarian cancer patients [138, 139]. In addition, downregulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [140]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [140]. These observations suggested that CD16 receptors may play an important role in target cell induced loss of NK cell cytotoxicity. Indeed, CD16:Ig fusion proteins are shown to bind to a variety of tumor-target cells indicating the existence of specific ligands for CD16 receptors on tumor cells [469]. Furthermore, we have previously shown that the triggering of CD16 on untreated and IL-2 treated NK cells was found to result in downmodulation of CD16 receptors and in a great loss of cytotoxicity in NK cells. In addition, a subset of NK cells was programmed to undergo cell death [136, 137, 141, 142]. Cell death of NK cells was shown to be regulated, in part, by endogenously secreted TNF-α from the NK cells [142]. Previous studies by other groups have also shown that IL-2 activated NK cells undergo cell death following cross-linking of the CD16 receptor [470, 471]. Thus, we have coined the term “split anergy” for the responses observed by the NK cells after their interaction with sensitive target cells or after the triggering of CD16 receptors by the antibody in combination with IL-2 treatment. Indeed, three subpopulations of NK cells; namely Free,

Binder and Killer NK cells with varying degrees of loss of cytotoxicity were identified after the formation of conjugates with K562 targets [141, 201, 472-474]. Free cells which did not bind or form conjugates with target cells were not inactivated, or exhibited the least inactivation of cytotoxicity, whereas both Binder and Killer subsets exhibited significant loss of cytotoxicity [141, 201, 472-474]. Inactivation of Killer NK cells were somewhat less than the Binder NK cells since they killed their respective targets and thus they were inactivated less than the Binder NK cells which remained bound to their target cells for longer time and therefore, were inactivated the most and had the least ability to mediate cytotoxicity [141, 201, 472-474]. Indeed, when NK cells were dissociated from the NK-K562 conjugates (NK_{DC}) and then treated with IL-2, a subset of NK cells responded to IL-2 activation for cytotoxicity, however, they were less responsive to IL-2 mediated induction of proliferation or secretion of cytokines. In contrast, those which remained conjugated with the tumor cells (NK_C) did not respond to IL-2 activation for cytotoxicity, but did proliferate significantly and secreted large amounts of cytokines [136, 137]. Treatment of NK cells with IL-2 and anti-CD16mAb also induced split anergy by significantly decreasing the NK cell cytotoxicity while increasing the cytokine secretion capabilities of NK cells [142-145]. Furthermore, IL-2 rescued anti-CD16 mAb mediated apoptosis induced in a subset of NK cells [142, 475]. Loss of cytotoxicity in NK cells was exacerbated when NK cells were either treated with $F(ab)'_2$ fragment of anti-CD16 mAb or treated with a combination of MHC-Class I and anti-CD16 mAb, while the same treatments resulted in an increased secretion of cytokines [143, 145]. These results suggested that receptor signaling on NK cells in the presence of IL-2 is likely to result in a decrease in NK cell cytotoxicity while increasing secretion of

cytokines by the NK cells. Therefore, three distinct functional outcomes could be observed in NK cells which have either interacted with sensitive tumor-target cells or treated with anti-CD16 mAb in the presence of IL-2 treatment, namely; 1-Loss of cytotoxicity, 2-gain in the ability to secrete cytokines and 3- death in a subset of NK cells.

Split anergy in NK cells is induced by total monocytes and those depleted of CD16+ subsets of monocytes

A significant decrease in NK cell mediated cytotoxicity could be observed when MSCs and DPSCs were cultured with either viable or irradiated monocytes before they were exposed to IL-2 treated NK cells. Interestingly, significant lysis of MSCs and DPSCs by untreated NK cells was also significantly and reproducibly blocked by the addition of monocytes [205]. To determine whether CD16- subset of monocytes were also able to inhibit the cytotoxic function of NK cells in a 3 way interaction with the stem cells we sorted this subset of the monocytes and used both the unsorted, and those sorted to remove CD16+ subsets (CD16-) in a 3 way killing assay with the NK cells. Both the total populations of monocytes and CD16- subsets were capable of inducing inhibition of NK cell cytotoxicity against stem cells [205]. We then determined whether decreased lysis of stem cells by NK cells was due to a competitive lysis of monocytes by the NK cells. We confirmed that monocytes were also lysed by the NK cells significantly. Furthermore, when we co-cultured stem cells with monocytes and removed the monocytes from the stem cells we could still observe significant inhibition of NK cell mediated lysis. This argues against the protection of stem cell lysis by NK cells solely by

competitive lysis of monocytes. Therefore, even though lysis of monocytes by the NK cells may in part contribute to the prevention of NK cell lysis of stem cells, interaction of monocytes with stem cells can also provide resistance of stem cells against NK cell cytotoxicity. Decrease in NK cell lysis of MSCs and DPSCs was paralleled with a significant induction of IFN-γ. Indeed, when MSCs or DPSCs were cultured with IL-2 treated NK cells alone we could observe significant induction of IFN-γ secretion. However, the highest increase was seen when NK cells were cultured with MSCs or DPSCs in the presence of monocytes. Therefore, although decreased killing of stem cells by the NK cells could be observed in the presence of monocytes, synergistic secretion of IFN-γ by the NK cells in the presence of monocytes and stem cells could be observed, indicating an inverse relationship between cytotoxicity and IFN-γ secretion (split anergy). This was similar to the profiles which we had seen when NK cells were treated with IL-2 and anti-CD16 antibody in which significant decrease in cytotoxicity of NK cells was observed in parallel with increased secretion of IFN-γ (split anergy) [142].

Tumor microenvironment may shape the function and phenotype of the NK cells

The above observations prompted us to speculate regarding the significance of interaction of monocytes with NK cells and stem cells. It is plausible that monocytes may serve as shields against NK cell lysis of stem cells. Similar to anti-CD16 antibody mediated effect on IL-2 treated NK cells, monocytes too can shield stem cells from killing by the NK cells by increasing the total IFN-γ release by the NK cells and decreasing the cytotoxic function of NK cells (split anergy), resulting in an increased protection and differentiation of stem cells. Indeed, monocytes also increased TNF-α, IL-

6 and VEGF secretion in the co-cultures of stem cells with NK cells which could further augment the induction of NFκB and increased differentiation of stem cells. The shielding effect of monocytes could be a more generalized function of other effectors since NK cells can also target fibroblasts and to a much lesser extent the T and B cells [205]. It would be interesting to see whether NK cells will also be able to significantly lyse MDSCs or PMNs. This may have significant implications regarding the role of NK cells not only in limiting inflammation, but also the significance of other immune effectors limiting the cytotoxic function of NK cells against stem cells raising the secretion of key cytokines for speedy and optimal differentiation of stem cells during inflammation. This is precisely what is observed in cancer patients in whom global decrease in NK, cytotoxic T cells and monocytes have all been reported [180].

Potential role of anergized NK cells in differentiation and regeneration of tissues

Even though conditioning of NK cells to support differentiation of stem cells is discussed in the context of tumors, induction of split anergy in NK cells, we believe, is an important conditioning step responsible for the repair of tissues during pathological processes irrespective of the type of pathology. In tumors, since the generation and maintenance of cancer stem cells is higher, the majority if not all of the NK cells may be conditioned to support differentiation and repair of the tissues. As such, the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble that of the anergic NK cells, i.e., decreased NK cell cytotoxicity, acquisition of CD16-/dimCD56-/dimCD69+ phenotype and augmented ability to secrete inflammatory cytokines (Fig. 2). Of course, the degree of decrease in NK cell cytotoxicity may be

directly proportional to the load of cancer stem cells. Therefore, our results suggest two very important functions for the NK cells. One function is to limit the number of stem cells by selecting those with a greater potential for differentiation for the repair of the tissues and second to support differentiation of the stem cells and subsequent regeneration of the tissues.

To achieve these tasks NK cells have to acquire two different phenotypes and be conditioned to carry out both functions successfully. CD16+CD56+/dimCD69- subsets of NK cells are cytotoxic and will mediate cytotoxicity depending on which sensitive targets they will encounter first. In respect to the oral squamous cell carcinomas since the majority of immune effectors can be found at the connective tissue area the chances are that they may first encounter and interact with either the other immune effectors or the effectors of connective tissue such as fibroblasts. However, there is also the possibility that NK cells may also first encounter the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they too can become anergized. Surprisingly, allogeneic CTLs were also found to target Glioblastoma stem-like cells and not their differentiated counterparts (Veronique Quillien, personal communication) (Fig.2). By eliminating a subset of stem cells or after their interaction with other immune inflammatory cells or effectors of connective tissue NK cells could then be in a position to support differentiation of selected population of stem cells since they will be conditioned to lose cytotoxicity, induce cytokine and growth factor secretion and gain the CD16-/dimCD56dim phenotype (Fig. 2). In vivo physiological relevance of above-mentioned observations could be seen in a subpopulation of NK cells in peripheral blood,

uterine and liver NK cells which express low or no CD16 receptors, have decreased capacity to mediate cytotoxicity and are capable of secreting significant amounts of cytokines [213, 214]. In addition, 70% of NK cells become CD16 dim or negative immediately after allogeneic or autologous bone marrow transplantation [213]. Since NK cells lose their cytotoxic function and gain in cytokine secretion phenotype and down modulate CD16 receptors after their interaction with tumor cells or the stem cells [137, 142], it is tempting to speculate that in vivo identified CD16- NK cells and in vitro tumor induced CD16- NK cells have similar developmental pathways since they have similar if not identical functional and phenotypic properties.

The ultimate proof of concept in support of this model was recently obtained in our laboratory. We observed that anergized NK cells were directly responsible for the increased differentiation and resistance of a number of different stem cells including cancer stem cells against cytotoxic effectors (manuscript submitted). In addition, we now have evidence which supports the notion that the induction of anergy in NK cells is an active process which is induced by the triggering of CD16 receptors on the NK cells and is not due to degranulation and exhaustion of cytotoxic granules (manuscript in prep).

Our work collectively suggests that anergized NK cells are as important as the non-anergized NK cells in their effector functions. NK cells are not only important for the removal and shaping of the size of the stem cells but also their differentiation and regeneration of new tissues. The task of NK cells in this regard goes above and beyond their most appreciated function of being the effectors of first line defense against viral

infection and malignancies. They too can be effectors of differentiation and tissue regeneration.

Conclusion

Recent advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between the immune effectors and the tumor cells. Effectors of the immune system are known to shape tumor cells (immunoediting) and select for cancers with reduced immunogenicity and enhanced capacity to actively induce immunosuppression. However, the same effector mechanisms are likely responsible for the selection of healthy stem cells with enhanced capacity to induce immunosuppression for the ultimate goal of the regeneration of damaged or disturbed tissues. Much work has been done to identify strategies by which tumor cells evade the immune system. Altered expression of MHC molecules which block recognition and activation of T and NK cells are examples of these mechanisms. In addition, tumor cells induce T and NK cell apoptosis, block lymphocyte homing and activation, and dampen macrophage and dendritic cell function by releasing immunosuppressive factors such as Fas, VEGF, IL-6, IL-10, TNF-α, GM-CSF and IL-1β. However, the same effector functions are also important in tissue repair mechanisms induced by the immune effectors. Furthermore, progress has been made in identification of the upstream mechanisms which control the expression of immunosuppressive factors in tumor cells. Two key control elements, NFκB and STAT3 were identified and shown to coordinately regulate the production of multiple tumor-derived immunosuppressive molecules and play a pivotal role in tumor cell immune suppression. The pathways interacting and possibly even amplifying each other underscore the potential for these two signaling modules to repress immune responses. One model for NFκB-STAT3-

mediated immunosuppression suggests that NFκB-induced IL-6 expression activates STAT3 in tumor cells and modulates the production of inhibitory cytokines and chemokines resulting in decreased T cell infiltration and activation. However, the same mechanisms are likely to be important for normal tissue regeneration and induction of resistance to NK and T cell mediated cytotoxicity.

Based on the work presented in this review we suggest that NK cells may have two significant functions; one that relates to the removal of stem cells that are either defective or disturbed or in higher numbers than are needed for the regeneration of damaged tissue. Therefore, the first task is to select stem cells that are competent and are able to achieve the highest ability to regenerate tissues. The second important task for NK cells is to support the differentiation of the selected cells after altering their phenotype to cytokine secreting cells (Fig. 2). NK cells may be conditioned to support differentiation of stem cells either by interacting with NK sensitive immune effectors or by effectors of connective tissue as well as with the stem cells. This process will not only remove cells that are damaged and have flaws in the differentiation process, but also it will ensure the regeneration of damaged or defective tissues, while aiding in the resolution of the inflammatory processes. Therefore, processes in which suboptimal differentiation and regeneration of the tissues occurs, a chronic inflammatory process may be established causing continual tissue damage and recruitment of stem cells and NK cells.

The inability of patient NK cells to contain cancer stem cells due to flooding of NK cells by proliferating cancer stem cells and conversion of NK cells to cytokine

secreting cells may likely be one mechanism by which cancer may progress and metastasize. Therefore, there should be two distinct strategies by the NK cells to eliminate tumors, one which targets stem cells and the other which targets differentiated cells. In theory this should be achieved by the use of antibodies to surface receptors that are highly expressed on differentiated cells, however, we have also found that NK cell Antibody Dependent Cellular Cytotoxicity (ADCC) is also lower against differentiated tumors when compared to their respective stem cells (manuscript submitted). Therefore, since a great majority of patient NK cells have modified their phenotype to support differentiation of the cells, they may not be effective in eliminating the cancer stem cells. Therefore, cancer stem cells may accumulate and eventually result in the demise of the patient. These patients may therefore, benefit from repeated allogeneic NK cell transplantation at the site of the tumor for elimination of cancer stem cells.

Figure Legend

Fig. 1 Immune inflammatory cells are mainly concentrated in the connective tissue area right beneath the epithelial layer of OSCC.

The slides from OSCC were prepared and stained with H&E. Significant infiltration of immune effectors right beneath the epithelial layer can be seen in the connective tissue area where the immune inflammatory cells are likely to condition NK cells to lose cytotoxicity and to support differentiation of epithelial cells. Epithelial dysplasia with small microinvasive islands can also be seen in the slide.

Fig. 2 Schematic representation of hypothetical model of oral cancer stem cell differentiation by NK cells and monocytes

Interaction of cancer stem cells or primary stem cells with monocytes and NK cells results in the loss of NK cell cytotoxicity due partly to the induction of resistance of cancer stem cells by monocytes and indirectly by monocytes serving as targets of NK cells, thus serving as a shield which protects the stem cells from lysis by the NK cells. Loss of NK cell cytotoxicity by monocytes and gain in secretion of IFN-γ results in a significant induction of transcription factors, cytokines and growth factors in stem cells and differentiation of stem cells.

Figure 1

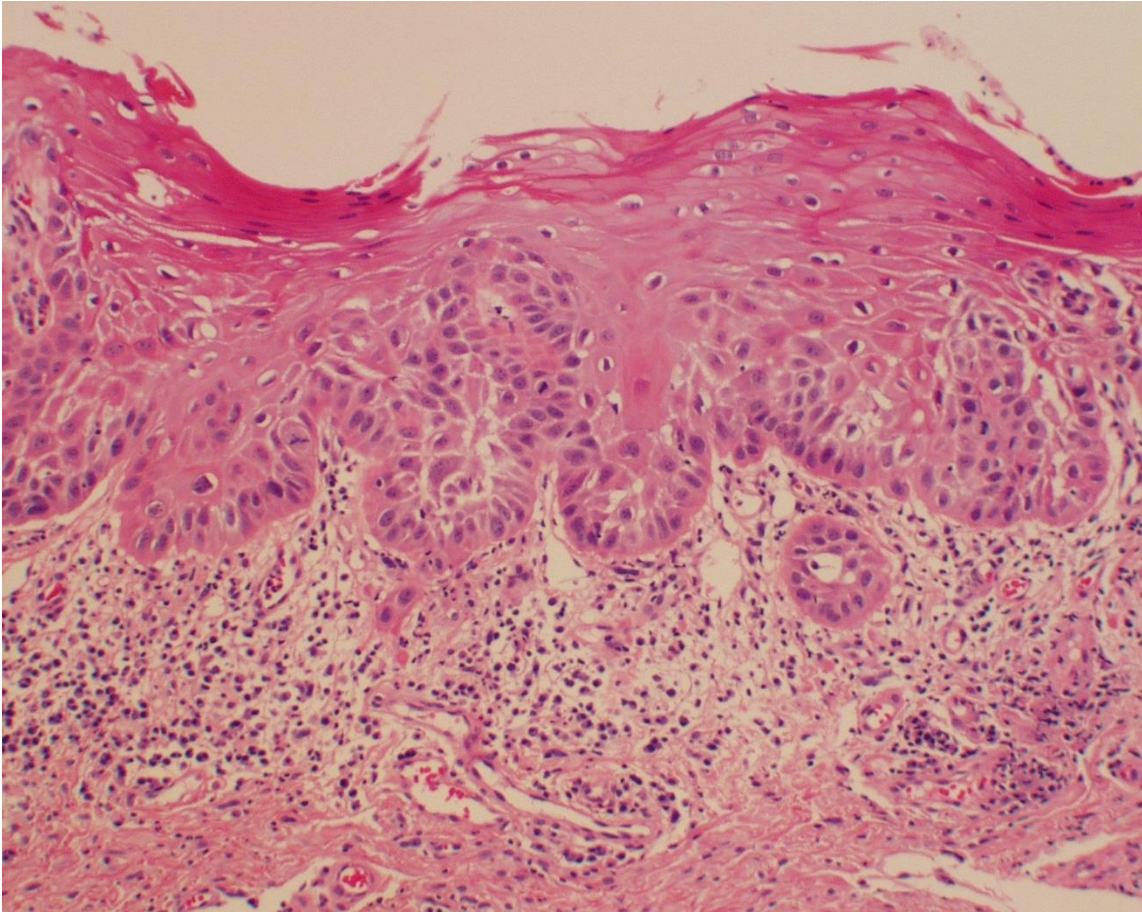
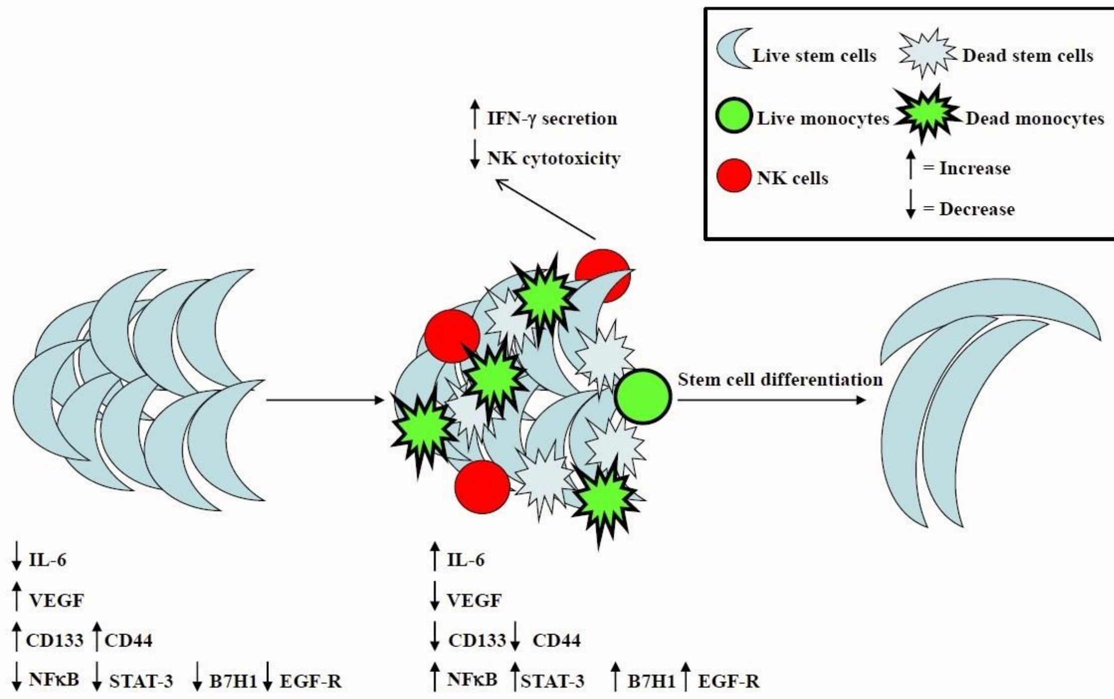


Figure 2



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Potential Rescue, Survival and Differentiation of Cancer Stem Cells and primary non-transformed stem cells by monocyte induced split anergy in Natural Killer cells

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Abstract

Cytotoxic function of NK cells is largely suppressed in the tumor microenvironment by a number of distinct effectors and their secreted factors. The aims of this review are to provide a rationale and a potential mechanism for immunosuppression in cancer and to demonstrate the significance of such immunosuppression in cellular differentiation and progression of cancer. We have recently shown that NK cells mediate significant cytotoxicity against primary oral squamous carcinoma stem cells (OSCSCs) as compared to their more differentiated oral squamous carcinoma cells (OSCCs). In addition, human embryonic stem cells (hESCs), Mesenchymal Stem Cells (hMSCs), dental pulp stem cells (hDPSCs) and induced pluripotent stem cells (hiPSCs) were all significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts or parental cells from which they were derived. We have also reported that inhibition of differentiation or reversion of cells to a less-differentiated phenotype by blocking NFκB significantly augmented NK cell function. Total population of monocytes and those depleted of CD16(+) subsets were able to substantially suppress NK cell mediated lysis of OSCSCs, hMSCs and hDPSCs. Overall, our results suggest that stem cells but not their differentiated counterparts are significant targets of the NK cell cytotoxicity. The concept of split anergy in NK cells and its contribution to cell differentiation, tissue repair and regeneration and in tumor resistance and progression will be discussed in this review.

Introduction

Immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, however, neither underlying physiological significance nor the exact mechanisms by which immunosuppression occurs are well understood. A number of factors responsible for the suppression of NK cell cytotoxicity in humans have been identified previously [175-180, 476]. It is shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [181-184]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [185-187]. In contrast, interaction of NK cells with the resistant tumors leads to a much lower suppression of NK cell cytotoxicity when compared to those dissociated from the NK sensitive target cells [136, 137]. Many mechanisms have been proposed for the functional inactivation of tumor associated NK cells including the over-expression of Fas ligand, the loss of mRNA for granzyme B [176] and decreased CD16 and its associated zeta chain [188].

In this report we review the previous studies from our laboratory regarding the factors and mechanisms involved in NK cell immunosuppression observed in cancer, and furthermore we discuss the emerging view from our laboratory which indicates that the stage of maturation and differentiation of healthy untransformed stem cells as well as transformed tumorigenic cancer stem cells is predictive of their sensitivity to NK cell lysis. In this regard we have previously demonstrated that OSCSCs, which are stem-like

oral tumors, are significantly more susceptible to NK cell mediated cytotoxicity; whereas, their differentiated counterpart OSCCs is significantly more resistant [412]. In addition, hESCs and hiPSCs, as well as a number of other healthy normal stem cells such as hMSCs and hDPSCs were found to be significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts [412]. Based on these results, we propose that NK cells play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact. To be conditioned to drive differentiation NK cells may have to first receive signals either from healthy stem cells or those which have disturbed or defective capabilities to differentiate. In addition, NK cells by targeting other immune inflammatory cells or fibroblasts in the tumor microenvironment may become conditioned to lose cytotoxicity and gain cytokine producing phenotype before they can aid in differentiation of oral squamous cancer stem cells. These alterations in NK cell effector function will ultimately aid in driving differentiation of a population of surviving healthy as well as transformed stem cells. In cancer patients since the majority of NK cells have lost cytotoxic activity, they may eventually contribute rather than halt the progression of cancer by not only driving the differentiation of tumor cells but more importantly, by allowing the growth and expansion of the pool of cancer stem cells.

Immunosuppressive effectors in tumor microenvironment

The tumor microenvironment consists of a number of heterogeneous cell populations with ability to suppress and limit the function and survival of cytotoxic

immune effectors. Patients with cancer often have higher numbers of immature monocytes serving as Myeloid Derived Suppressor Cells (MDSCs) expressing CD14+HLADR- phenotype [227, 453, 477, 478]. Tumor associated Macrophages (TAMs) were previously shown to significantly influence and limit immune activation in the tumor microenvironment [454, 455]. In addition, MDSCs which are comprised of a number of distinct cell populations of myeloid origin and whose roles in immunosuppression have received significant attention in recent years are major cells capable of suppressing the cytotoxic function of T and possibly NK cells [453]. T cell dysfunction is shown to be induced by MDSCs by the increased secretion of IL-10, TGF-β, induction of reactive oxygen species (ROS), and increased expression of arginase-1 and inducible nitric oxide synthase (iNOS). T regulatory (Treg) and DC regulatory (DCreg) cells were also recently shown to have significant immunosuppressive roles in the tumor microenvironment [453]. Perhaps one of the most intriguing observations regarding the immunosuppressive effectors is the identification of Cancer Associated Fibroblasts (CAFs) and Mesenchymal Stem Cells (MSCs) as two potential tumor promoters. Fibroblasts from tumor tissues demonstrate an activated phenotype and have the ability to secrete many immunosuppressive factors such as TGF-β and VEGF [456]. We have also found that undifferentiated fibroblasts, as well as MSCs and CD14+HLA-DR- monocytes irrespective of their surface expression of CD16 are significantly more susceptible to NK cell mediated cytotoxicity [205], therefore, these cells may condition NK cells to become anergic (please see below). Indeed, in oral epithelial tumors the majority of recruited immune effectors are usually found in the connective tissue area where through cell-cell interaction with the immunosuppressive cells such as fibroblasts,

monocytes-macrophages and to a lesser extent T and B cells [205] can condition NK cells to lose cytotoxicity and gain in cytokine secretion capabilities, resulting in differentiation and resistance of oral epithelial tumors (please see below).

NK cells are conditioned in the tumor microenvironment to drive differentiation

The theory of immunosurveillance was postulated by Burnet [414] to indicate that the key thymus dependent effectors were responsible for clearance of tumors [415, 416]. However, since then the concept of immunosurveillance has been expanded to include immunoediting as an important mechanism for the development of cancer [421, 422]. It was suggested that cancer immunoediting comprises of three phases: elimination, equilibrium and escape [422]. Elimination represents the classical concept of immunosurveillance. However, during equilibrium and escape the interaction and cross signaling between the immune effectors, the tumor cells, and perhaps the effectors of the connective tissue in the tumor microenvironment may result in the generation of tumors which are capable of gradual inactivation and death of the immune effector cells. The final stages of cancer development may result in the induction of less immunogenic tumors in the presence of fewer immune effectors capable of lysing the tumors. Thus, pressures exerted by the tumor cells and immune effectors may eventually shape the microenvironment for the growth, expansion and invasion of tumors. Similarly, certain elements of such interactions may also be observed during the interaction of NK cells with healthy non-transformed human stem cells in which case the three phases of interaction may include elimination which marks the decrease in the numbers of stem cells due to the selection of stem cells by the NK cells, induction of tolerance or anergy

which denotes the conditioning of NK cells by the stem cells and/or by the other effectors of microenvironment to lose cytotoxicity and support maturation and differentiation of stem cells, and finally the resolution phase which denotes the elimination of anergized NK cells and generation of less immunogenic differentiated cells. Similarities and differences between these phases in cancer and during stem cell maturation will be discussed below.

NK cells lyse cancer stem cells, as well as hESCs, hiPSCs, hMSCs and hDPSCs but not their differentiated counterparts

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed when NK cells were co-incubated with OSCSCs which released significantly lower levels of GM-CSF, IL-6 and IL-8 and demonstrated decreased expression of phospho-Stat3, B7H1 and EGFR, and much lower constitutive NFκB activity when compared to differentiated OSCCs [412]. More importantly, OSCSCs expressed CD133 and $CD44^{bright}$ oral stem cell markers [412]. Increase in IFN-γ secretion was correlated with a decrease in secretion of IL-6 in co-cultures of NK cells with OSCSCs as compared to those co-cultured with OSCCs. Therefore, from these results a specific profile for differentiated NK resistant oral tumors emerged which demonstrated increased GM-CSF, IL-6 and IL-8 secretion in the context of decreased IFN-γ secretion during their interaction with the NK cells. In contrast, co-cultures of cancer stem cells with NK cells demonstrated increased IFN-γ in the context of lower GM-CSF, IL-6 and IL-8 secretion [205, 412]. In addition, three brain tumor stem cells which were previously characterized [259, 466, 467] were found to be significantly more susceptible to NK cell mediated cytotoxicity when

compared to their differentiated counterparts which were significantly more resistant (manuscript submitted). Since OSCSCs and brain stem cells were significantly more susceptible to NK cell mediated cytotoxicity we reasoned that healthy, non-transformed primary stem cells may also be susceptible to NK cell mediated cytotoxicity. We demonstrated previously that NK cells lysed hMSCs, hDPSCs and hESCs significantly. All different types of stem cells became resistant to NK cell mediated cytotoxicity once they were differentiated [412]. In addition, higher sensitivity of hiPSCs to NK cell mediated lysis was also observed when compared to parental line from which they were derived. Increased lysis of cancer stem cells or non-transformed healthy stem cells may be attributed to the use of allogeneic NK cells, however, our previous work using autologous NK cells exhibited similar levels of cytotoxicity against hDPSCs when compared to lysis by allogeneic NK cells [205]. Taken together these results indicated that undifferentiated cells were targets of both allogeneic and autologous NK cells. Thus, the stage of differentiation of the cells is predictive of their susceptibility to NK cell mediated cytotoxicity.

Blocking NFκB may revert the oral tumors to more of an undifferentiated phenotype resulting in an increase in NK cell mediated cytotoxicity.

Since the degree of differentiation in the cells is predictive of their sensitivity to NK cell mediated cytotoxicity, we reasoned that blocking NFκB in the cells may de-differentiate and subsequently revert the cells to more of undifferentiated phenotype, resulting in their increased susceptibility to NK cell mediated cytotoxicity. Indeed, blocking NFκB in oral tumors was found to increase CD44 surface receptor expression,

which is one of the hallmarks of stem cells (manuscript in prep). In addition, we have shown previously that NFκB nuclear function in a primary Oral tumor OSCCs and in a non-tumorigenic oral cells (HOK-16B) as well as in an established tumor line, HEp-2 cells known to be Hela contaminant [193, 457-459], modulates and shapes the function of interacting NK cells [192, 193]. It is believed that HEp-2 cells are Hela contaminants since cells of this line are shown to contain Hela marker chromosomes (ATCC). Since knock down of NFκB was shown to increase the function of immune inflammatory cells in diverse cell types (please see below) it is not surprising to find similar patterns of immune activation in both oral and non-oral derived cell lines, even in those which have been derived from contaminants such as Hela cells. In addition, since the majority, if not all cells, increase NFκB during their activation and differentiation, a knock down of NFκB is likely to revert the cells, irrespective of their cellular origin, to a less differentiated phenotype resulting in the potential activation of the immune effectors in order to aid in their differentiation [412]. Similarly, inhibition of NFκB by Sulindac increased the functional activation of NK and enhanced anti-tumor cytotoxic activity [192, 193]. Inverse modulation of IFN-γ and IL-6 cytokine secretion was seen in co-cultures of NK cells with NFκB knock down OSCCs, HOK-16B and HEp2 cells indicating that blocking NFκB in these cells serves to switch the balance from Th2 type responses to more of a Th1 type response [192, 412].

In agreement with our studies, targeted deletion of IKK-β in epidermis of mice has previously been shown in one study to lead to inflammatory skin manifestations [153]. Elevated levels of cytokines and chemokines have also been demonstrated in the

epidermis of patients and animals with Iκκγ and Iκκβ deletions [153, 460]. Mice with a keratinocyte-specific deletion of Iκκ–β demonstrated decreased proliferation of epidermal cells and developed TNF-α dependent inflammatory skin disease [153]. In contrast, in other studies in which NFκB function was blocked in dermal keratinocytes by a mutant IκB-α an increased proliferation and hyperplasia [463] and eventual development of cutaneous squamous cell carcinomas of skin were seen if mice were allowed to survive and reach adulthood [464, 465]. It is of interest to note that in these studies with diverse functional outcomes in keratinocytes, blocking TNF-α function resulted in the prevention of both the neoplastic transformation and the inflammatory skin disease [153, 465]. Elevated numbers of immune inflammatory cells recruited to the site of epidermis are likely responsible for the increased secretion of TNF-α. Indeed, we have demonstrated that synergistic induction of TNF-α could be observed when NFκB knock down oral tumors were cultured with either PBMCs or NK cells [192].

Since tumorigenic and non-tumorigenic human oral keratinocytes acquire sensitivity to NK cell mediated lysis when NFκB is inhibited, it is likely that this phenomenon is not specific to cancer or oral keratinocytes, and it may occur in other healthy non-transformed cell types. Indeed, when human primary monocytes were differentiated to dendritic cells they too became more resistant to NK cell mediated cytotoxicity [412]. Moreover, knock down of COX2 in primary mouse monocytes [412], or in mouse embryonic fibroblasts (manuscript in prep), resulted in the reversion or de-differentiation of the monocytes and fibroblasts respectively, and the activation of NK cell cytotoxicity. Indeed, it is likely that any disturbance in cellular differentiation may

predispose the cells to NK cell mediated cytotoxicity. Since STAT3 is an important factor increased during differentiation, blocking STAT3 is also critical in the activation of immune effectors [211]. In support of a critical role of STAT3 in immune evasion of tumor cells in humans, we and others have recently shown that glioblastoma multiforme (GBM) tumors display constitutive activation of STAT3 (Cacalano and Jewett, unpublished observation) [160], and poorly induce activating cytokines and tumor-specific cytotoxicity in human peripheral blood mononuclear cells (PBMCs) and NK cells. Ectopic expression of dominant-negative STAT3 in the GBM cells increased lysis of the tumor cells by the immune effectors and induced production of IFN-γ by the interacting immune effectors (manuscript in prep).

Since NFκB is shown to regulate IL-6 secretion in OSCCs, HOK-16B and HEp2 cells and secreted IL-6 in tumors is known to activate STAT3 expression and function, increase in NFκB nuclear function could in turn induce STAT3 activation and result in a significant resistance of tumors to NK cell mediated cytotoxicity. Therefore, targeted knock down of STAT3 or signaling pathways upstream of STAT3, such as NFκB, may de-differentiate the cells and predispose the cells to NK cell mediated cytotoxicity.

Induction of split anergy in NK cells after their co-culture with sensitive but not resistant tumors and after the triggering of CD16 on IL-2 treated NK cells

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity and induces cell death in a small subset of NK cells [136, 137]. On the

other hand, NK resistant tumors such as RAJI cells induce much less anergy or loss of NK cell cytotoxicity [136, 137]. Furthermore, following NK cell cultures with sensitive tumor-target cells but not resistant tumors, the target binding NK cells undergo phenotypic and functional changes. Target cell inactivated NK cells express CD16- CD56dim/- CD69+ phenotype [136, 137]. This phenotype has also been observed in several disease manifestations including HIV infection [468]. Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in several cancer patients including those of the oral and ovarian cancer patients [138, 139]. In addition, down-regulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [140]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [140]. These observations suggested that CD16 receptors may play an important role in target cell induced loss of NK cell cytotoxicity. Indeed, CD16:Ig fusion proteins are shown to bind to a variety of tumor-target cells indicating the existence of specific ligands for CD16 receptors on tumor cells [469]. Furthermore, we have previously shown that the triggering of CD16 on untreated or IL-2 treated NK cells was found to result in down-modulation of CD16 receptors and in a great loss of cytotoxicity in NK cells. In addition, a subset of NK cells was programmed to undergo apoptosis [136, 137, 141, 142]. Cell death of NK cells was shown to be regulated, in part, by endogenously secreted TNF-α from the NK cells [142]. Previous studies by other groups have also shown that IL-2 activated NK cells undergo cell death following cross-linking of the CD16 receptor [470, 471]. Thus, we have coined the term "split anergy" for the responses observed by NK cells after their interaction with

sensitive target cells or after the triggering of CD16 receptors by the antibody in combination with IL-2 treatment [136, 137, 142, 144, 145]. Indeed, three subpopulations of NK cells; namely Free, Binder and Killer NK cells with varying degrees of loss of cytotoxicity were identified after the formation of conjugates with K562 targets [141, 201, 472-474]. Free cells which did not bind or form conjugates with target cells were inactivated less, or exhibited the most cytotoxicity, whereas both Binder and Killer subsets exhibited significant loss of cytotoxicity. In contrast, Binder and Killer subsets but not Free NK subset secreted significant levels of cytokines and exhibited CD16-CD56dim/-CD69+ phenotype [141, 201, 472-474]. Treatment of NK cells with IL-2 and anti-CD16mAb also induced split anergy by significantly decreasing the NK cell cytotoxicity while increasing the cytokine secretion capabilities of NK cells. Furthermore, NK cells exhibited CD16-CD56dim/-CD69+ phenotype after treatment with the combination of IL-2 and anti-CD16mAb [142-145]. Loss of cytotoxicity in NK cells was significantly exacerbated when NK cells were either treated with F(ab)'$_2$ fragment of anti-CD16 mAb or treated with a combination of MHC-Class I and anti-CD16 mAb while the same treatments resulted in an increased secretion of cytokines [143, 145]. These results suggested that receptor signaling in NK cells in the presence of IL-2 is likely to result in a decrease in NK cell cytotoxicity while increasing secretion of cytokines by the NK cells. Therefore, three distinct functional outcomes could be observed in NK cells which have either interacted with sensitive tumor-target cells or were treated with anti-CD16 mAb in the presence of IL-2 treatment to induce split anergy, namely; 1-Loss of cytotoxicity, 2-gain in the ability to secrete cytokines and 3-death in a small subset of NK cells.

Split anergy in NK cells is induced by monocytes and those depleted of CD16+ subsets of monocytes

When hMSCs or hDPSCs were cultured with either viable or irradiated monocytes before they were exposed to IL-2 treated NK cells a significant decrease in NK cell mediated cytotoxicity could be observed against hMSCs or hDPSCs. Interestingly, significant lysis of hMSCs and hDPSCs by untreated NK cells was also significantly and reproducibly blocked by the addition of monocytes [205]. To determine whether CD16- subset of monocytes were also able to inhibit the cytotoxic function of NK cells in a 3 way interaction with the stem cells we used both the unsorted, and those sorted to remove CD16+ subsets in a 3 way killing assay with the NK cells. Both the total populations of monocytes and CD16- subsets were capable of inducing inhibition of NK cell cytotoxicity against stem cells [205]. We then determined whether decreased lysis of stem cells by NK cells was due to a competitive lysis of monocytes by the NK cells. We confirmed that monocytes were also lysed by the NK cells significantly. Furthermore, when we co-cultured stem cells with monocytes and sorted to remove the monocytes from the stem cells before assessing the killing function of NK cells, we could still observe significant inhibition of NK cell mediated lysis, arguing against the protection of stem cell lysis by NK cells being solely on the bases of competitive lysis of monocytes [205]. Therefore, even though lysis of monocytes by the NK cells may in part contribute to the prevention of NK cell lysis of stem cells, interaction of monocytes with stem cells can also provide resistance of stem cells against NK cell cytotoxicity. Decrease in NK cell lysis of hMSCs and hDPSCs was paralleled with a significant induction of IFN-γ.

Indeed, when hMSCs or hDPSCs were cultured with IL-2 treated NK cells alone we could observe significant induction of IFN-γ secretion. However, the highest increase was seen when IL-2 treated NK cells were cultured with hMSCs or hDPSCs in the presence of monocytes. Therefore, although decreased killing of stem cells by the NK cells could be observed in the presence of monocytes, synergistic secretion of IFN-γ by the NK cells in the presence of monocytes and stem cells could be observed, indicating an inverse relationship between cytotoxicity and IFN-γ secretion (split anergy). This was similar to the profiles which we had seen when NK cells were treated with IL-2 and anti-CD16 antibody in which significant decrease in cytotoxicity of NK cells could be observed in parallel with increased secretion of IFN-γ (split anergy) [142].

Tumor microenvironment may shape the function and phenotype of the NK cells

The above observations prompted us to speculate regarding the significance of interaction of monocytes with NK cells and stem cells. It is plausible that monocytes may serve as shields against NK cell lysis of stem cells. Similar to anti-CD16 antibody mediated effect on IL-2 treated NK cells, monocytes too can shield stem cells from killing by the NK cells by increasing the total IFN-γ release by the NK cells while decreasing the cytotoxic function of NK cells (split anergy), resulting in an increased protection and differentiation of stem cells. Indeed, monocytes also increased TNF-α, IL-6 and VEGF secretion in the co-cultures of stem cells with NK cells which could augment NFκB and increase differentiation of stem cells. The shielding effect of monocytes could be a more generalized function of other effectors since NK cells can also target fibroblasts and to a much lesser extent the T and B cells [205]. Whether

MDSCs or PMNs could also be targeted by the NK cells awaits future investigation. This may have significant implications regarding the role of NK cells in not only limiting inflammation, but also the significance of other immune effectors in shielding and limiting the cytotoxic function of NK cells against cancer or healthy stem cells in order to raise maximally the secretion of key cytokines for speedy and optimal differentiation of stem cells during inflammation. This is precisely what is observed in cancer patients in whom global decrease in NK, cytotoxic T cells and monocytes have all been reported [180].

Potential role of anergized NK cells in differentiation and regeneration of tissues

Even though conditioning of NK cells to support differentiation of cells is discussed in the context of tumors, induction of split anergy in NK cells, we believe, is an important conditioning step responsible for the repair of tissues during pathological processes irrespective of the type of pathology. In tumors since the generation and maintenance of cancer stem cells is higher, the majority, if not all of the NK cells, may be conditioned to support differentiation and repair of the tissues and as such the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble that of the anergic NK cells, i.e., decreased NK cell cytotoxicity, acquisition of CD16-/dimCD56-/dimCD69+ phenotype and augmented ability to secrete inflammatory cytokines (Fig. 1). Of course, the degree of the loss of NK cell cytotoxicity may be directly proportional to the load of cancer stem cells. Therefore, our results suggest two very important functions for the NK cells. One function is to limit the number of stem cells by selecting those with a greater potential for differentiation for the repair of the

tissues and second to support differentiation of the stem cells and subsequent regeneration of the tissues. To achieve these tasks NK cells have to acquire two different phenotypes and be conditioned to carry out both functions successfully. CD16+CD56+/dimCD69- subsets of NK cells are cytotoxic and will mediate cytotoxicity depending on which sensitive targets they encounter first. In respect to the oral squamous cell carcinomas since the majority of immune effectors can be found at the connective tissue area the chances are that they may first encounter and interact with either the other immune effectors or the effectors of connective tissue such as fibroblasts. However, there is also the possibility that NK cells may first encounter the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they too can become anergized (Fig. 1). Surprisingly, allogeneic CTLs were also found to target Glioblastoma stem-like cells and not their differentiated counterparts (Veronique Quillien, personal communication). By eliminating a subset of stem cells or after their interaction with other immune inflammatory cells or effectors of connective tissue NK cells could then be in a position to support differentiation of selected population of stem cells since they will be conditioned to lose cytotoxicity, induce cytokine and growth factor secretion and gain the CD16-/dimCD56dimCD69+ phenotype (Fig. 1). It is interesting to note that all of the immune effectors isolated from oral gingival tissues of healthy as well as diseased gingiva have CD69+ phenotype, with the exception that the numbers of immune effectors are much less in the healthy oral gingival tissues when compared to diseased tissues (manuscript in prep). Therefore, our results suggest two very important functions for the NK cells. One function is to kill and the other function is to be conditioned to support differentiation for the repair and regeneration of the tissues.

In vivo physiological relevance of above-mentioned observations could be seen in a subpopulation of NK cells in peripheral blood, uterine and liver NK cells which express low or no CD16 receptors, have decreased capacity to mediate cytotoxicity and is capable of secreting significant amounts of cytokines [213, 214]. In addition, 70% of NK cells become CD16 dim or negative immediately after allogeneic or autologous bone marrow transplantation [213]. Since NK cells lose their cytotoxic function and gain in cytokine secretion phenotype and down modulate CD16 receptors after their interaction with tumor cells or the stem cells [137, 142], it is tempting to speculate that in vivo identified CD16- NK cells and in vitro tumor induced CD16- NK cells have similar developmental pathways since they have similar if not identical functional properties.

The ultimate proof of concept in support of this model was recently obtained in our laboratory. We observed that anergized NK cells were directly responsible for the increased differentiation and resistance of a number of different stem cells including cancer stem cells against cytotoxic effectors (manuscript submitted). In addition, we now have evidence which supports the notion that the induction of anergy in NK cells is an active process which is induced by the triggering of CD16 receptor on the NK cells and is not due to degranulation and exhaustion of cytotoxic granules (manuscript in prep).

Our work collectively suggests that anergized NK cells are as important as the non-anergized NK cells in their effector functions. NK cells are not only important for the removal and shaping of the size of the stem cells but also their differentiation, and the

ultimate regeneration of the new tissues. The task of NK cells in this regard goes above and beyond their most appreciated function of being the effectors of first line defense against viral infection and malignancies. They too can be effectors of differentiation and tissue regeneration.

Conclusion

Recent advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between the immune effectors and the tumor cells. Effectors of the immune system are known to shape the tumor cells and to select for cancers with reduced immunogenicity and enhanced capacity to actively induce immunosuppression. However, the same effector mechanisms are likely responsible for the selection of healthy stem cells with enhanced capacity to induce immunosuppression for the ultimate goal of the regeneration of damaged or disturbed tissues and the resolution of inflammation. Much work has been done to identify strategies by which tumor cells evade the function of immune system. Altered expression of MHC molecules which block recognition and activation of T and NK cells are examples of mechanisms by which tumor cells evade the function of immune system. In addition, tumor cells by releasing immunosuppressive factors such as Fas, VEGF, IL-6, IL-10, TNF-α, GM-CSF and IL-1β, induce T and NK cell apoptosis, block lymphocyte homing and activation, and dampen macrophage and dendritic cell function. However, the same effector functions are also important in tissue repair. Furthermore, progress has been made in identification of the upstream mechanisms which control the expression of immunosuppressive factors in tumor cells. Two key control elements, NFκB and STAT3 were identified and shown to coordinately regulate the production of multiple tumor-derived immunosuppressive molecules and play a pivotal role in tumor cell immune suppression. The potential for these two signaling modules to repress immune responses is underscored by the finding that the pathways interact and may even amplify each other.

Based on the accumulated work presented in this review we suggest that NK cells may have two significant functions; one that relates to the removal of stem cells that are either defective or disturbed or in general more in numbers than are needed for the regeneration of damaged tissue. Therefore, they may select stem cells that are competent and are able to achieve the highest ability to regenerate tissues. In addition, NK cells may lyse other effectors in the connective tissue area in order to not only decrease inflammation but also to be conditioned to promote tissue regeneration. The second important task for NK cells is therefore, to support differentiation and promote tissue regeneration after altering their phenotype to cytokine secreting cells (Fig. 1). This process will not only remove cells that are damaged and have flaws in the differentiation process, but also it will ensure the regeneration of tissues and the resolution of inflammation. Thus, any disturbance in the NK cell function or in the process of differentiation of stem cells may result in chronic inflammation, causing continual tissue damage and recruitment of immune effectors to aid in tissue regeneration.

The inability of patient NK cells to contain cancer stem cells due to the flooding of NK cells by proliferating cancer stem cells and conversion of NK cells to cytokine secreting cells may likely be one mechanism by which cancer may progress and metastasize. Therefore, there should be two distinct strategies by the NK cells to eliminate tumors, one which targets stem cells and the other which targets differentiated cells. Since cancer stem cells were found to be more resistant to certain chemotherapeutic drugs but sensitive to NK cell mediated killing while differentiated oral tumors were more resistant to NK cell mediated killing but relatively more sensitive to

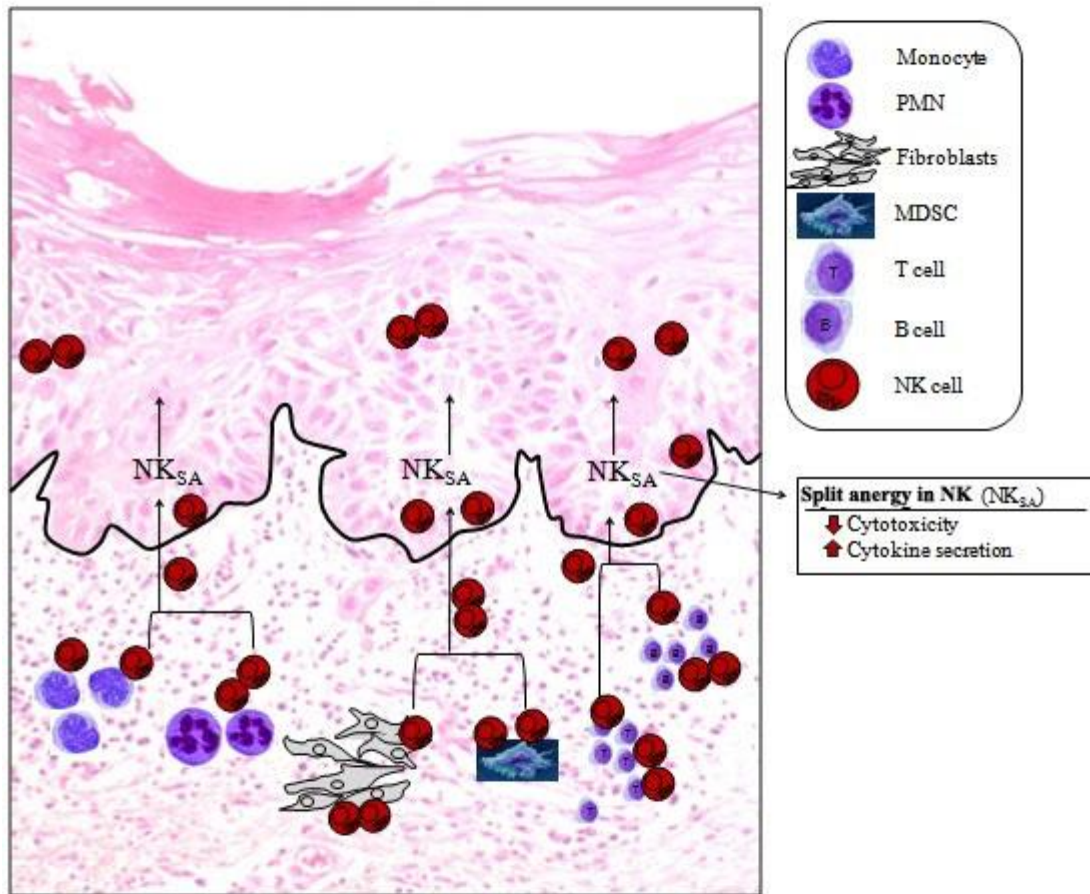
chemotherapeutic drugs, combination therapy should be considered for the elimination of both undifferentiated and differentiated tumors. In addition, since a great majority of patient NK cells have modified their phenotype to support differentiation of the cells, they may not be effective in eliminating the cancer stem cells. Therefore, these patients may benefit from repeated allogeneic NK cell transplantation at the site of the tumor for elimination of cancer stem cells. In this regard depletion of immunosuppressive effectors in the tumor microenvironment, which condition NK cells to lose cytotoxicity, via radiation or chemotherapeutic drugs should in theory provide a better strategy for successful targeting of tumors by the NK cells.

Figure Legend

Fig. 1 Hypothetical model of conditioning of NK cells by immune inflammatory cells and the effectors of connective tissue to modify their phenotypic and functional properties in order to support differentiation of the cells.

Hypothetical model of NK cell conditioning in the tumor microenvironment is over imposed on the H&E stained OSCC. Significant infiltration of immune effectors right beneath the epithelial layer can be seen in the connective tissue area where the immune inflammatory cells are likely to condition NK cells to lose cytotoxicity and to support differentiation of epithelial cells. Two hypothetical mechanisms for conditioning of NK cells to support differentiation of stem cells are shown. In one scenario, NK cells, may first encounter and interact with either the other immune effectors as listed in the figure or the effectors of connective tissue such as fibroblasts to undergo split anergy (NK_{SA}). In a second scenario, NK cells may also directly encounter the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they too can become anergized to support differentiation of other stem cells. Both mechanisms may be operational in the tumor microenvironment.

Figure 1



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Tumor induced inactivation of Natural Killer cell cytotoxic function; implication in growth, expansion and differentiation of Cancer stem cells

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Abstract

Accumulated evidence indicates that cytotoxic function of immune effectors is largely suppressed in the tumor microenvironment by a number of distinct effectors and their secreted factors. The aims of this review are to provide a rationale and a potential mechanism for immunosuppression in cancer and to demonstrate the significance of such immunosuppression in cellular differentiation and progression of cancer. To that end, we have recently shown that NK cells mediate significant cytotoxicity against primary oral squamous carcinoma stem cells (OSCSCs) as compared to their more differentiated oral squamous carcinoma cells (OSCCs). In addition, human embryonic stem cells (hESCs), Mesenchymal Stem Cells (hMSCs), dental pulp stem cells (hDPSCs) and induced pluripotent stem cells (hiPSCs) were all significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts or parental cells from which they were derived. We have also reported that inhibition of differentiation or reversion of cells to a less-differentiated phenotype by blocking NFκB or targeted knock down of COX2 in primary monocytes in vivo significantly augmented NK cell function. Total population of monocytes and those depleted of CD16(+) subsets were able to substantially prevent NK cell mediated lysis of OSCSCs, hMSCs and hDPSCs. Taken together, our results suggest that stem cells are significant targets of the NK cell cytotoxicity. The concept of split anergy in NK cells and its contribution to tissue repair and regeneration and in tumor resistance and progression will be discussed in this review.

Introduction

Effective immunosurveillance is important for the prevention of initiation and progression of cancer. Rapid progression of oral leukoplakia to carcinoma was previously observed after immunosuppression [405]. Furthermore, neoplasias of tongue and lip have been widely described in renal transplant patients [406-409], and finally induction of oral cavity cancers was second to liver cancer in patients after bone marrow transplantation [410]. In addition, there is substantial evidence which indicates that immune responses are inhibited by oral tumors, and this may largely be responsible for their induction and progression. This review will focus on the emerging new roles of NK cells in regulation of numbers, resistance and differentiation of cancer stem cells as well as healthy untransformed stem cells. In addition, the significance and role of anergic NK cells will be discussed in induction of tumor resistance, as well as in shaping the size and differentiation of healthy stem cells.

Immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, however, neither underlying physiological significance nor the exact mechanisms by which immunosuppression occurs are well understood. A number of factors responsible for the suppression of NK cell cytotoxicity in humans have been identified previously [175-180]. It is shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [181-184]. In addition, NK cell

cytotoxicity is suppressed after their interaction with stem cells [185-187]. In contrast, interaction of NK cells with the resistant tumors leads to a much lower suppression of NK cell cytotoxicity when compared to those dissociated from the NK sensitive target cells [136, 137]. Many mechanisms have been proposed for the functional inactivation of tumor associated NK cells including the over-expression of Fas ligand, the loss of mRNA for granzyme B [176] and decreased CD16 and its associated zeta chain [188] (Table I).

Many metastatic tumor cells exhibit constitutively elevated NFκB activity [189]. We have previously shown that NK resistant primary oral epithelial tumors demonstrate higher nuclear NFκB activity and secrete significant levels of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF), Interleukin(IL)-1β, IL-6 and IL-8 [190, 412]. Moreover, the addition of Non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit NFκB have the ability to reverse immunosuppression induced by a tobacco-specific carcinogen [413] in addition to their well established ability to decrease oral dysplasia as well as induction of overt oral cancer in transgenic animals [191].. The significance and exact mechanism by which NFκB nuclear function in oral tumors modulate and shape the function of key interacting immune effectors is starting to unravel. We have previously shown that inhibition of NFκB in primary OSCCs, or in non-tumorigenic oral keratinocytes (HOK-16B) leads to a significant increase in cytotoxicity and secretion of IFN-γ by the human NK cells [192, 193]. However, it is only now that the underlying significance and the physiological relevance of NFκB modulation in tumors or in primary cells responsible for the alteration of NK cell cytotoxic function is being clarified. Indeed, targeted inhibition of NFκB function in both the intestinal epithelial cells and the

myeloid cells was previously shown to result in a significant decrease in the size and the numbers of the tumor cells [194].

In this report we review the previous studies from our laboratory and those of the others regarding the factors and mechanisms involved in immunosuppression observed in cancer, and furthermore we discuss the emerging view from our laboratory which indicates that the stage of maturation and differentiation of healthy untransformed stem cells as well as transformed tumorigenic cancer stem cells is predictive of their sensitivity to NK cell lysis. In this regard we have previously demonstrated that OSCSCs, which are stem-like oral tumors, are significantly more susceptible to NK cell mediated cytotoxicity; whereas, their differentiated counterpart OSCCs are significantly more resistant [412]. In addition, hESCs and iPSCs as well as a number of other healthy normal stem cells such as hMSCs and hDPSCs were found to be significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts [412]. Based on these results, we propose that NK cells play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact. To be conditioned to drive differentiation, however, NK cells will have to first receive signals either from stem cells or those which have disturbed or defective capabilities to differentiate. Alternatively, NK cells by targeting other immune inflammatory cells or fibroblasts in tumor microenvironment may become conditioned to lose cytotoxicity and gain cytokine producing phenotype before they can aid in the differentiation of oral squamous cancer stem cells. These alterations in NK cell effector function will ultimately aid in driving differentiation of a population of surviving healthy

as well as transformed stem cells. Since the majority of NK cells in cancer patients have lost cytotoxic activity, they may eventually contribute rather than halt the progression of cancer by not only driving the differentiation of tumor cells but more importantly, by allowing the growth and expansion of the pool of cancer stem cells.

Immunosurveillance in the Prevention of Cancer

The theory of immunosurveillance was initially set forth by Burnet [414] to indicate that the key thymus dependent effectors were responsible for clearance of tumors [415, 416]. However, since then the concept of immunosurveillance has been expanded to include immunoediting as an important mechanism for the development of cancer [421, 422]. It was suggested that cancer immunoediting comprises of three phases: elimination, equilibrium and escape [422]. Elimination represents the classical concept of immunosurveillance. However, during equilibrium and escape the interaction and cross signaling between the immune effectors and the tumor cells generates tumors which are capable of gradual inactivation and death of the immune effector cells. The final stages of cancer development may result in the induction of less immunogenic tumors in the presence of fewer immune effectors capable of lysing the tumors. Thus, pressures exerted by tumor cells and immune effectors may eventually shape the microenvironment for the invading tumors. Similarly, elements of such interactions are also observed during the interaction of NK cells with healthy non-transformed human stem cells in which case the three phases of interaction may include elimination which marks the decrease in the numbers of stem cells due to the selection of stem cells by the NK cells, induction of

tolerance or anergy which denotes the conditioning of NK cells to lose cytotoxicity and support maturation and differentiation of stem cells, and finally the resolution phase which denotes elimination of anergized NK cells and differentiation of selected stem cells. Similarities and differences between these phases in cancer and during stem cell maturation will be discussed below.

Immune Responses in Cancer

Although limited success in the immunotherapy with cytokines or adaptively transferred effector cells in metastatic melanomas or renal cell carcinomas have previously been observed. Immunotherapy was found to be ineffective in the treatment of many other cancers [427-430]. The reasons for the failure of known immunotherapeutic modalities in many cancers including head and neck cancers are poorly understood. It has been hypothesized that a widespread paralysis of cytotoxic effectors residing inside the inflammatory infiltrate of advanced cancer patients is the main reason for poor prognosis [431, 432]. Furthermore, freshly isolated tumor infiltrating lymphocytes are not cytotoxic to autologous tumor cells and show a significantly reduced clonogenicity [182-184, 433]. Functional paralysis of cytotoxic cells have also been reported in a variety of cancers, notably breast [177, 178, 434, 435], renal [175], and colon [176]. More importantly, depletion of cytotoxic effectors in the tumor milieu has an unfavorable outcome for survival in cancer patients [434, 436-438]. Indeed, a significantly shorter survival rate is reported for colorectal carcinoma patients with little or moderate NK infiltration as compared to those with extensive infiltration [439]. A five year survival advantage was also seen with higher CD3 positive tumor infiltrating T cells than with lower T cell count

in the carcinoma of uterine cervix [438]. Regressing tumor grafts of oral origin contain significantly larger numbers of functional NK and T cells than those associated with the primary tumors [424], while patients with metastasis of head and neck cancers have low NK and T cell activity [425].

Defects in NK, T and DCs have been reported in oral cancer patients. Signaling abnormalities, spontaneous apoptosis and reduced proliferation of circulating T cells, DCs and TILs have been reported in patients with oral cancers [426, 440].

Mechanisms of Immunosuppression by Tumor Cells

Many mechanisms have been proposed for the functional inactivation of tumor associated lymphocytes [175-179] (Table I). Soluble products derived from renal cell carcinoma inhibit proliferative capacity of T cells infiltrating human tumors due to a downregulation of Janus kinase 3 (Jak 3), p56 (lck), p59 (fyn) and zap 70 [175]. Expression of Fas ligand by many human tumor cells including oral tumors has been hypothesized to be a major cause of lymphocyte depletion in the tumor microenvironment [179]. In mice, tumor induced immunosuppression has been associated with a decreased expression of the zeta-chain of the T cell receptor and the loss of mRNA for granzyme B [176]. Indeed, as observed in mice, the frequency of TCR-zeta positive and granzyme positive T cells are decreased in advanced stage head and neck carcinomas in humans, and the restoration of expression during in vitro stimulation suggests the presence of tumor derived suppressive factors [176]. However, caution should be exercised in extending the results from mice cancer model to human

disease model in terms of NK cell function due to an array of species specific differences. We have found that freshly isolated NK cells from humans have significant cytotoxic activity against sensitive tumors or healthy stem cells immediately after purification [145], however, freshly isolated NK cells from mice not only lacked any significant cytotoxicity against sensitive tumors such as YAC-1, immediately after purification or after an extended period of incubation, but also they required a much longer period of activation with IL-2 for the acquisition of significant cytotoxicity [475]. In addition, we have found that a greater amount of IL-2 was required for the activation of NK cells from mice to mediate cytotoxicity whereas in humans not only much lower amounts of IL-2 could trigger activation of cytotoxicity but also IL-2 treatment in humans required a very short period of time (hours and not days) for significant increase in cytotoxicity [145, 475]. These differences could be due to the strain of mice used or the source from which purified NK cells were isolated in mice and humans; i.e. spleen vs. peripheral blood or could be due to the inherent differences between human and mice NK cell function. The latter may be likely since species specific differences for the use of key surface receptors for the NK cell function have previously been reported by others [479]. Indeed, it was stated in a previous report that "The extraordinary and unanticipated divergence of human NK cell receptors and MHC class I ligands from their mouse counterparts can in part explain the difficulties experienced in finding informative mouse models of human diseases." Thus, mouse model "has been unimpressive in producing informative and translatable models of human diseases" [479]. It is, however, a very convenient model which can be genetically manipulated, and as such can give a wealth of knowledge if used in parallel with the human disease model confirming the observations obtained in

human disease and not vice versa. It is outside the scope of this review to provide a complete description of species specific differences of NK cell receptors. Interestingly, most studies reported for tumor mediated NK cell immunosuppression was conducted using human NK cells. Therefore, decreased CD16 and its associated zeta chains were observed in tumor infiltrating NK cells of patients with cancer [188] (Table I), thus, correlating in phenotype with our in vitro inactivated NK cells by sensitive tumors [137, 142]. Overall both secreted factors and direct cell-cell contact during the interaction of immune effectors with the tumor cells in patients were shown to be responsible for the suppression of immune effector function.

Immunosuppression mediated by secreted factors

Many secreted factors such as PGE2, Interleukin (IL)-6, Vascular Endothelial Growth Factor (VEGF) and Granulocyte Monocyte Colony Stimulating Factor (GM-CSF) are known to induce Immunosuppression in the tumor microenvironment. Immunosuppression linked to enhanced PGE2 synthesis has been documented in many human cancers [441]. Freshly excised human head and neck cancers demonstrated elevated levels of PGE2, transforming growth factor- β1 (TGFβ1) and interleukin-10 (IL-10) secretion which are known to upregulate the expression of Killer Immunoglobulin-like Receptors (KIRs) on the surface of NK, T and DCs and block immune effector function [442]. Furthermore, metastatic head and neck cancers released higher levels of above-mentioned inhibitory factors and lower levels of immune activating factors IFN-γ and IL-2 than did their corresponding primary tumors [442]. PGE2 overproduction in

tumor microenvironment was also shown to lead to dendritic cell (DC) abnormalities [443].

IL-6, another immunosuppressive factor is secreted constitutively by oral squamous carcinomas [209], and is found to be elevated in oral cancer patients [444]. IL-6 is known to interfere with IFN-γ signaling by the induction of Th2 differentiation [210]. In support of a role for IL-6 in mediating immune evasion of tumor cells, Menetrier-Caux et al showed that conditioned medium from human renal cell carcinoma cell lines blocked the differentiation of CD34+ bone marrow cells into immature DCs, and this inhibitory effect could be blocked with antibodies against IL-6 [445].

Other factors which have been implicated in immunosuppression in cancers are angiogenic factors such as VEGF [446] and cytokines such as G-CSF and GM-CSF. The finding that neutralizing antibodies to VEGF or GM-CSF could partially reverse the inhibitory effects of tumor cell supernatant on DC maturation demonstrated that these factors could interfere with DC differentiation and function [446]. Increased numbers of immature DCs were found in the peripheral blood of cancer patients with elevated levels of circulating VEGF [446].

Immunosuppression Mediated by Direct Cell-Cell Contact.

Contact-dependent immune suppression can occur by engagement of MHC class I molecules on CD8+CD28- suppressor cells with immunoglobulin-like transcript (ILT2 and ILT4) inhibitory receptors on DCs. Blocking of both MHC Class I and ILTs by

specific antibodies can reverse immunosuppression [447]. Similarly binding of c-type lectin receptors or Killer Immunoglobulin-like Receptors (KIRs) to MHC Class I ligands inhibit NK cell function [449, 480-483] In addition, the expression of co-stimulatory molecules, such as B7H1 on tumor cells and inhibitory DCs and T cells can inhibit T cell activation and proliferation [484].

Immunosuppressive effectors in tumor microenvironment

The tumor microenvironment consists of a number of heterogeneous cell populations with ability to suppress and limit the function and survival of cytotoxic immune effectors. Patients with cancer often have higher numbers of immature CD14+HLADR- monocytes [227, 453]. Tumor Associated Macrophages (TAMs) were previously shown to significantly influence and limit immune activation in the tumor microenvironment [454, 455]. In addition, Myeloid Derived Suppressor Cells (MDSCs) which are comprised of a number of distinct cell populations of myeloid origin and whose roles in immunosuppression have received significant attention in recent years are major cells capable of suppressing the cytotoxic function of T and possibly NK cells [453]. T cell dysfunction is shown to be induced by MDSCs by the secretion of IL-10, TGF-β, reactive oxygen species (ROS), arginase and Nitric Oxide synthase (NOS). T regulatory (Treg) and DC regulatory (DCreg) cells were also recently shown to have significant immunosuppressive roles in the tumor microenvironment [453]. Perhaps one of the most interesting observations regarding the immunosuppressive effectors is the identification of Cancer Associated Fibroblasts (CAFs) and Mesenchymal Stem Cells (MSCs) as two potential tumor promoters. Fibroblasts from tumor tissues demonstrate an

activated phenotype and have the ability to secrete many immunosuppressive factors such as TGF-β and VEGF [456]. We have also found that fibroblasts, as well as MSCs and CD14+HLA-DR- monocytes irrespective of their surface expression of CD16 are significantly more susceptible to NK cell mediated cytotoxicity [205], therefore, these cells may condition NK cells to become anergic (please see below). Indeed, in OSCCs the majority of recruited immune effectors are usually found in the connective tissue area where through cell-cell interaction with the immunosuppressive cells such as fibroblasts, monocytes-macrophages and to a lesser degree T and B cells [205] can condition NK cells by inhibiting the cytotoxic function of NK cells, while increasing the cytokine secretion capability of the NK cells resulting in differentiation and resistance of oral epithelial tumors (please see below).

Role of NFκB in tumor resistance

Although each one of the secreted factors indicated above individually can be responsible in part for the resistance of tumors, previous data obtained from different laboratories [193, 211] indicated that targeting transcription factors may be more effective in decreasing resistance of tumors to immune mediated cytotoxicity. Many tumor cells exhibit constitutively elevated NFκB activity [189]. Human leukemias and lymphomas as well as human solid tumors exhibit constitutively activated NFκB in the nucleus [189].

We have shown previously that NFκB nuclear function in a primary Oral tumor OSCCs and in a non-tumorigenic oral cells (HOK-16B) as well as in an established tumor

line, HEp-2 cells known to be Hela contaminant [193, 457-459], modulates and shapes the function of interacting immune effectors [192, 193]. It is believed that HEp-2 cells are Hela contaminants since cells of these lines are shown to contain Hela marker chromosomes (ATCC). Since knock down of NFκB was shown to increase the function of immune inflammatory cells in diverse cell types (please see below) it is not surprising to find similar patterns of immune activation in both oral and non-oral derived cell lines, even in those which have been derived from contaminants such as Hela cells. Since in addition, the majority if not all cells increase NFκB during their activation and differentiation, a knock down of NFκB is likely to revert the cells, irrespective of their cellular origin, to their less differentiated phenotypes resulting in the potential activation of the immune effectors in order to aid in their differentiation [412]. Therefore, inhibition of NFκB in oral tumors resulted in a significant activation of human NK cell cytotoxic function and increased IFN-γ secretion [412]. Similarly, inhibition of NFκB by Sulindac increased the functional activation of NK and enhanced anti-tumor cytotoxic activity [192, 193]. Inverse modulation of IFN-γ and IL-6 cytokine secretion was seen in co-cultures of NK cells with NFκB knock down OSCCs and HEp2 cells indicating that blocking NFκB in these cells serves to switch the balance from Th2 type responses to more of a Th1 type response [192, 412].

NFκB inhibition in mice and humans activate immune inflammatory functions

Targeted deletion of IKK-β in epidermis of mice has previously been shown in one study to lead to inflammatory skin manifestations similar to that seen in patients with Incontinentia Pigmenti (IP) [153]. Elevated levels of cytokines and chemokines have also

been demonstrated in the epidermis of patients and animals with Iκκγ and Iκκβ deletions [153, 460]. Blocking of NFκB function by deleting Iκκ-β in intestinal epithelial cells also dramatically decreased the rate of tumor formation without affecting the size of the tumors in colitis-associated cancer model [461, 462]. Moreover, deleting Iκκ-β in myeloid cells in the same model system resulted in the decrease in tumor size. These studies underscore the significance of the cross talk between different subsets of immune effectors with the epithelial cells in induction and progression of the intestinal tumors.

Mice with a keratinocyte-specific deletion of Iκκ–β demonstrated decreased proliferation of epidermal cells and developed TNF-α dependent inflammatory skin disease [153]. In contrast, in other studies in which NFκB function was blocked in dermal keratinocytes by a mutant IκB-α an increased proliferation and hyperplasia [463] and eventual development of cutaneous squamous cell carcinomas of skin were seen if mice were allowed to survive and reach adulthood [464, 465]. It is of interest to note that in these studies with diverse functional outcomes in keratinocytes, blocking TNF-α function resulted in the prevention of both the neoplastic transformation and the inflammatory skin disease [153, 465] . Thus, it appears that TNF-α is the primary factor mediating the pathological processes in both of these studies. Elevated numbers of immune inflammatory cells recruited to the site of epidemis are likely responsible for the increased secretion of TNF-α. Indeed, we have demonstrated that synergistic induction of TNF-α could be observed when NFκB knock down oral tumors were cultured with either PBMCs or NK cells [192].

NK cells lyse cancer stem cells, as well as hESCs, hiPSCs, hMSCs and hDPSCs but not their differentiated counterparts

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed when NK cells were co-incubated with OSCSCs which released significantly lower levels of GM-CSF, IL-6 and IL-8 and demonstrated decreased expression of phospho-Stat3, B7H1 and EGFR, and much lower constitutive NFκB activity when compared to differentiated OSCCs [412]. More importantly, OSCSCs expressed CD133 and $CD44^{bright}$ oral stem cell markers [412]. Increase in IFN-γ secretion was correlated with a decrease in secretion of IL-6 in co-cultures of NK cells with OSCSCs as compared to those co-cultured with OSCCs. Therefore, from these results a specific profile for differentiated NK resistant oral tumors emerged which demonstrated increased GM-CSF, IL-6 and IL-8 secretion in the context of decreased IFN-γ secretion during their interaction with the NK cells. In contrast, co-cultures of cancer stem cells with NK cells demonstrated increased IFN-γ in the context of lower GM-CSF, IL-6 and IL-8 secretion [205, 412]. In addition, three brain tumor stem cells which were previously characterized [259, 466, 467] were found to be significantly more susceptible to NK cell mediated cytotoxicity when compared to their differentiated counterparts which were significantly more resistant (manuscript submitted). Since OSCSCs and brain stem cells were significantly more susceptible to NK cell mediated cytotoxicity we reasoned that healthy, non-transformed primary stem cells may also be susceptible to NK cell mediated cytotoxicity. We demonstrated previously that NK cells lysed hMSCs, hDPSCs and hESCs significantly. All different types of stem cells became resistant to NK cell mediated cytotoxicity once they were differentiated [412]. In addition, higher sensitivity of hiPSCs to NK cell

mediated lysis was also observed when compared to parental line from which they were derived. Increased lysis of cancer stem cells or non-transformed healthy stem cells can be attributed to the use of allogeneic NK cells since obtaining matching autologous NK cells in most cases is often a significant challenge. However, our previous work using autologous NK cells exhibited similar cytotoxicity against hDPSCs when compared to lysis by allogeneic NK cells [205]. Taken together these results indicated that undifferentiated cells were targets of both allogeneic and autologous NK cells. Thus, the stage of differentiation of the cells is predictive of their susceptibility to NK cell mediated cytotoxicity.

Blocking NFκB revert the cells to more of an undifferentiated phenotype and increases NK cell mediated cytotoxicity against Oral tumors.

Since the degree of differentiation in the cells is predictive of their sensitivity to NK cell mediated cytotoxicity, we reasoned that blocking NFκB in the cells may de-differentiate and consequently revert the cells to more of undifferentiated phenotype, resulting in their increased susceptibility to NK cell mediated cytotoxicity. Indeed, blocking NFκB in oral tumors was also found to increase CD44 surface receptor expression, which is one of the hallmarks of stem cells (manuscript in prep). In addition, the profiles of cytokines secreted in the co-cultures of NK cells or PBMCs with NFκB knock down tumors resembled those secreted in the co-cultures of NK cells with the stem cells [192, 205, 412]. Since tumorigenic and non-tumorigenic human oral keratinocytes acquire sensitivity to NK cell mediated lysis when NFκB is inhibited, it is likely that this phenomenon is not specific to cancer, and it may occur during other pathological

conditions. Indeed, when human primary monocytes are differentiated to dendritic cells they too became more resistant to NK cell mediated cytotoxicity [412]. Moreover, knock down of COX2 in primary mouse monocytes [412], or in mouse embryonic fibroblasts (manuscript in prep), resulted in the reversion or de-differentiation of the monocytes and fibroblasts respectively, and the activation of NK cell cytotoxicity. Indeed, it is likely that any disturbance in cellular differentiation may predispose the cells to NK cell mediated cytotoxicity. Since STAT3 is an important factor increased during differentiation, blocking STAT3 is also critical in the activation of immune effectors [211]. In support of a critical role of STAT3 in immune evasion of tumor cells in humans, we and others have recently shown that glioblastoma multiforme (GBM) tumors display constitutive activation of STAT3 (Cacalano and Jewett, unpublished observation) [160], and poorly induce activating cytokines and tumor-specific cytotoxicity in human peripheral blood mononuclear cells (PBMCs) and NK cells. Ectopic expression of dominant-negative STAT3 in the GBM cells increased lysis of the tumor cells by the immune effectors and induced production of IFN-γ by the interacting immune effectors (manuscript in prep).

Since NFκB is shown to regulate IL-6 secretion in OSCCs, HOK-16B and HEp2 cells and secreted IL-6 in tumors is known to activate STAT3 expression and function, increase in NFκB nuclear function could in turn induce STAT3 activation and result in a significant resistance of tumors to NK cell mediated cytotoxicity. Therefore, targeted knock down of STAT3 or signaling pathways upstream of STAT3, such as NFκB, may de-differentiate the cells and predispose the cells to NK cell mediated cytotoxicity.

Induction of split anergy in NK cells after their co-culture with sensitive but not resistant tumors and after the triggering of CD16 on IL-2 treated NK cells

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity and induces cell death in a small subset of NK cells [136, 137]. On the other hand NK resistant tumors such as RAJI cells induce much less anergy or loss of NK cell cytotoxicity [136, 137]. Furthermore, following NK cell cultures with sensitive tumor-target cells but not resistant tumors, the target binding NK cells undergo phenotypic and functional changes. Target cell inactivated NK cells express CD16- CD56dim/- CD69+ phenotype [136, 137]. This phenotype has also been observed in several disease manifestations including HIV infection [468]. Significant downmodulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in several cancer patients including those of the oral and ovarian cancer patients [138, 139]. In addition, down-regulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [140]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [140]. These observations suggested that CD16 receptors may play an important role in target cell induced loss of NK cell cytotoxicity. Indeed, CD16:Ig fusion proteins are shown to bind to a variety of tumor-target cells indicating the existence of specific ligands for CD16 receptors on tumor cells [469]. Furthermore, we have previously shown that the triggering of CD16 on untreated or IL-2 treated NK cells was found to result in down-modulation of CD16 receptors and in a great loss of cytotoxicity in NK cells. In addition, a subset of NK cells

was programmed to undergo apoptosis [136, 137, 141, 142]. Cell death of NK cells was shown to be regulated, in part, by endogenously secreted TNF-α from the NK cells [142]. Previous studies by other groups have also shown that IL-2 activated NK cells undergo cell death following cross-linking of the CD16 receptor [470, 471]. Thus, we have coined the term "split anergy" for the responses observed by NK cells after their interaction with sensitive target cells or after the triggering of CD16 receptors by the antibody in combination with IL-2 treatment [136, 137, 142, 144, 145]. Indeed, three subpopulations of NK cells; namely Free, Binder and Killer NK cells with varying degrees of loss of cytotoxicity were identified after the formation of conjugates with K562 targets [141, 201, 472-474]. Free cells which did not bind or form conjugates with target cells were not inactivated, or exhibited the most cytotoxicity, whereas both Binder and Killer subsets exhibited significant loss of cytotoxicity. In contrast, Binder and Killer subsets but not Free NK subset secreted significant levels of cytokines and exhibited CD16-CD56dim/-CD69+ phenotype [141, 201, 472-474]. Treatment of NK cells with IL-2 and anti-CD16mAb also induced split anergy by significantly decreasing the NK cell cytotoxicity while increasing the cytokine secretion capabilities of NK cells. Furthermore, NK cells exhibited CD16-CD56dim/-CD69+ phenotype after treatment with the combination of IL-2 and anti-CD16mAb [142-145]. Loss of cytotoxicity in NK cells was significantly exacerbated when NK cells were either treated with F(ab)'$_2$ fragment of anti-CD16 mAb or treated with a combination of MHC-Class I and anti-CD16 mAb while the same treatments resulted in an increased secretion of cytokines [143, 145]. These results suggested that receptor signaling in NK cells in the presence of IL-2 is likely to result in a decrease in NK cell cytotoxicity while increasing secretion of cytokines by the NK cells.

Therefore, three distinct functional outcomes could be observed in NK cells which have either interacted with sensitive tumor-target cells or treated with anti-CD16 mAb in the presence of IL-2 treatment to induce split anergy, namely; 1-Loss of cytotoxicity, 2-gain in the ability to secrete cytokines and 3- death in a small subset of NK cells.

Split anergy in NK cells is induced by total monocytes and those depleted of CD16+ subsets of monocytes

When hMSCs or hDPSCs were cultured with either viable or irradiated monocytes before they were exposed to IL-2 treated NK cells a significant decrease in NK cell mediated cytotoxicity could be observed against hMSCs or hDPSCs. Interestingly, significant lysis of hMSCs and hDPSCs by untreated NK cells was also significantly and reproducibly blocked by the addition of monocytes [205]. To determine whether CD16- subset of monocytes were also able to inhibit the cytotoxic function of NK cells in a 3 way interaction with the stem cells we used both the unsorted, and those sorted to remove CD16+ subsets (CD16-) in a 3 way killing assay with the NK cells. Both the total populations of monocytes and CD16- subsets were capable of inducing inhibition of NK cell cytotoxicity against stem cells [205]. We then determined whether decreased lysis of stem cells by NK cells was due to a competitive lysis of monocytes by the NK cells. We confirmed that monocytes were also lysed by the NK cells significantly. Furthermore, when we co-cultured stem cells with monocytes and sorted to remove the monocytes from the stem cells before assessing the killing function of NK cells, we could still observe significant inhibition of NK cell mediated lysis, arguing against the protection of stem cell lysis by NK cells being solely on the bases of competitive lysis of

monocytes [205]. Therefore, even though lysis of monocytes by the NK cells may in part contribute to the prevention of NK cell lysis of stem cells, interaction of monocytes with stem cells can also provide resistance of stem cells against NK cell cytotoxicity. Decrease in NK cell lysis of hMSCs and hDPSCs was paralleled with a significant induction of IFN-γ. Indeed, when hMSCs or hDPSCs were cultured with IL-2 treated NK cells alone we could observe significant induction of IFN-γ secretion. However, the highest increase was seen when NK cells were cultured with hMSCs or hDPSCs in the presence of monocytes. Therefore, although decreased killing of stem cells by the NK cells could be observed in the presence of monocytes, synergistic secretion of IFN-γ by the NK cells in the presence of monocytes and stem cells could be observed, indicating an inverse relationship between cytotoxicity and IFN-γ secretion (split anergy). This was similar to the profiles which we had seen when NK cells were treated with IL-2 and anti-CD16 antibody in which significant decrease in cytotoxicity of NK cells could be observed in parallel with increased secretion of IFN-γ (split anergy) [142].

Tumor microenvironment may shape the function and phenotype of the NK cells

The above observations prompted us to speculate regarding the significance of interaction of monocytes with NK cells and stem cells. It is plausible that monocytes may serve as shields against NK cell lysis of stem cells. Similar to anti-CD16 antibody mediated effect on IL-2 treated NK cells, monocytes too can shield stem cells from killing by the NK cells by increasing the total IFN-γ release by the NK cells while decreasing the cytotoxic function of NK cells (split anergy), resulting in an increased protection and differentiation of stem cells. Indeed, monocytes also increased TNF-α, IL-

6 and VEGF secretion in the co-cultures of stem cells with NK cells which could augment NFκB and increase differentiation of stem cells. The shielding effect of monocytes could be a more generalized function of other effectors since NK cells can also target fibroblasts and to a much lesser extent the T and B cells [205]. Whether MDSCs or PMNs could also be targeted by the NK cells awaits future investigation. This may have significant implications regarding the role of NK cells in not only limiting inflammation, but also the significance of other immune effectors in shielding and limiting the cytotoxic function of NK cells against cancer or healthy stem cells in order to raise maximally the secretion of key cytokines for speedy and optimal differentiation of stem cells during inflammation. This is precisely what is observed in cancer patients in whom global decrease in NK, cytotoxic T cells and monocytes have all been reported [180].

Potential role of anergized NK cells in differentiation and regeneration of tissues

Even though conditioning of NK cells to support differentiation of cells is discussed in the context of tumors, induction of split anergy in NK cells, we believe, is an important conditioning step responsible for the repair of tissues during pathological processes irrespective of the type of pathology. In tumors since the generation and maintenance of cancer stem cells is higher, the majority if not all of the NK cells may be conditioned to support differentiation and repair of the tissues and as such the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble that of the anergic NK cells, i.e., decreased NK cell cytotoxicity, acquisition of CD16-/dimCD56-/dimCD69+ phenotype and augmented ability to secrete inflammatory

cytokines (Fig. 2). Of course, the degree of the loss of NK cell cytotoxicity may be directly proportional to the load of cancer stem cells. Therefore, our results suggest two very important functions for the NK cells. One function is to limit the number of stem cells by selecting those with a greater potential for differentiation for the repair of the tissues and second to support differentiation of the stem cells and subsequent regeneration of the tissues. To achieve these tasks NK cells have to acquire two different phenotypes and be conditioned to carry out both functions successfully.

CD16+CD56+/dimCD69- subsets of NK cells are cytotoxic and will mediate cytotoxicity depending on which sensitive targets they encounter first. In respect to the oral squamous cell carcinomas since the majority of immune effectors can be found at the connective tissue area the chances are that they may first encounter and interact with either the other immune effectors or the effectors of connective tissue such as fibroblasts. However, there is also the possibility that NK cells may first encounter the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they too can become anergized (Fig. 1). Surprisingly, allogeneic CTLs were also found to target Glioblastoma stem-like cells and not their differentiated counterparts (Veronique Quillien, personal communication) (Fig. 2). By eliminating a subset of stem cells or after their interaction with other immune inflammatory cells or effectors of connective tissue NK cells could then be in a position to support differentiation of selected population of stem cells since they will be conditioned to lose cytotoxicity, induce cytokine and growth factor secretion and gain the CD16-/dimCD56dimCD69+ phenotype. It is interesting to note that all of the immune effectors isolated from oral gingival tissues of healthy as well

as diseased gingivae have CD69+ phenotype, with the exception that the numbers of immune effectors are much less in the healthy oral gingival tissues when compared to diseased tissues (manuscript in prep). Therefore, our results suggest two very important functions for the NK cells. One function is to kill and the other function is to be conditioned to support differentiation for the repair and regeneration of the tissues.

In vivo physiological relevance of above-mentioned observations could be seen in a subpopulation of NK cells in peripheral blood, uterine and liver NK cells which express low or no CD16 receptors, have decreased capacity to mediate cytotoxicity and is capable of secreting significant amounts of cytokines [213, 214]. In addition, 70% of NK cells become CD16 dim or negative immediately after allogeneic or autologous bone marrow transplantation [213]. Since NK cells lose their cytotoxic function and gain in cytokine secretion phenotype and down modulate CD16 receptors after their interaction with tumor cells or the stem cells [137, 142], it is tempting to speculate that in vivo identified CD16- NK cells and in vitro tumor induced CD16- NK cells have similar developmental pathways since they have similar if not identical functional properties.

The ultimate proof of concept in support of this model was recently obtained in our laboratory. We observed that anergized NK cells directly were responsible for the increased differentiation and resistance of a number of different stem cells including cancer stem cells against cytotoxic effectors (manuscript submitted). In addition, we now have evidence which supports the notion that the induction of anergy in NK cells is an

active process which is induced by the triggering of CD16 receptor on the NK cells and is not due to degranulation and exhaustion of cytotoxic granules (manuscript in prep).

Our work collectively suggests that anergized NK cells are as important as the non-anergized NK cells in their effector functions. NK cells are not only important for the removal and shaping of the size of the stem cells but also their differentiation, and the ultimate regeneration of the new tissues. The task of NK cells in this regard goes above and beyond their most appreciated function of being the effectors of first line defense against viral infection and malignancies. They too can be effectors of differentiation and tissue regeneration.

Conclusion

Recent advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between the immune effectors and the tumor cells. Effectors of the immune system are known to shape the tumor cells (immunoediting) and to select for cancers with reduced immunogenicity and enhanced capacity to actively induce immunosuppression. However, the same effector mechanisms are likely responsible for the selection of healthy stem cells with enhanced capacity to induce immunosuppression for the ultimate goal of the regeneration of damaged or disturbed tissues and the resolution of inflammation. Much work has been done to identify strategies by which tumor cells evade the function of immune system. Altered expression of MHC molecules which block recognition and activation of T and NK cells are examples of mechanisms by which tumor cells evade the function of immune system. In addition, tumor cells by releasing immunosuppressive factors such as Fas, VEGF, IL-6, IL-10, TNF-α, GM-CSF and IL-1β, induce T and NK cell apoptosis, block lymphocyte homing and activation, and dampen macrophage and dendritic cell function. However, the same effector functions are also important in tissue repair. Furthermore, progress has been made in identification of the upstream mechanisms which control the expression of immunosuppressive factors in tumor cells. Two key control elements, NFκB and STAT3 were identified and shown to coordinately regulate the production of multiple tumor-derived immunosuppressive molecules and play a pivotal role in tumor cell immune suppression. The potential for these two signaling modules to repress immune responses is underscored by the finding that the pathways interact and may even amplify each other.

One model for NFκB-STAT3-mediated immunosuppression suggests that NFκB-induced IL-6 expression activates STAT3 in tumor cells and modulates the production of inhibitory cytokines and chemokines resulting in decreased T cell infiltration and activation. However, the same mechanisms are likely to be important for normal tissue regeneration and induction of resistance to NK and T cell mediated cytotoxicity.

Based on the accumulated work presented in this review we suggest that NK cells may have two significant functions; one that relates to the removal of stem cells that are either defective or disturbed or in general more in numbers than are needed for the regeneration of damaged tissue. Therefore, the first task is to select stem cells that are competent and are able to achieve the highest ability to regenerate tissues. The second important task for NK cells is to support differentiation of the selected cells after altering their phenotype to cytokine secreting cells (Fig. 2). This process will not only remove cells that are damaged and have flaws in the differentiation process, but also it will ensure the regeneration of tissues. Therefore, when suboptimal differentiation or defective cellular regeneration occurs, a chronic inflammatory process may be established causing continual tissue damage and recruitment of NK cells to aid in tissue regeneration.

The inability of patient NK cells to contain cancer stem cells due to flooding of NK cells by proliferating cancer stem cells and conversion of NK cells to cytokine secreting cells may likely be one mechanism by which cancer may progress and metastasize. Therefore, there should be two distinct strategies by the NK cells to eliminate tumors, one which targets stem cells and the other which targets differentiated

cells. Since cancer stem cells were found to be more resistant to certain chemotherapeutic drugs but sensitive to NK cell mediated killing while differentiated tumors are more resistant to NK cell mediated killing but relatively more sensitive to chemotherapeutic drugs, combination therapy should be considered for the elimination of both undifferentiated and differentiated tumors. In addition, since a great majority of patient NK cells have modified their phenotype to support differentiation of the cells, they may not be effective in eliminating the cancer stem cells. Therefore, these patients may benefit from repeated allogeneic NK cell transplantation at the site of the tumor for elimination of cancer stem cells. In this regard depletion of immunosuppressive effectors in the tumor microenvironment, which condition NK cells to lose cytotoxicity, via radiation or chemotherapeutic drugs should in theory provide a better strategy for successful targeting of tumors by the NK cells.

Table I. Mechanisms of immune evasion by tumor cells

Defect	Mechanism	References
Loss of T cell Recognition	Decreased MHC class I expression/shedding of class I; decreased TCR zeta chain expression, expression of co-stimulatory molecules (B7H1) on tumor cells	[176, 193, 452, 485]
Altered NK cell recognition	Decreased expression of NK receptor (NKG2D) ligands, increased MHC class I expression, decreased expression of adhesion molecules, CD16 and zeta chain	[188, 193, 486, 487]
Loss of NK cell function	Induction of anergy in NK cells. Activation of upstream transcription factors (NFκB) in differentiated tumors and tumor cell production of inhibitory factors (IL-10, IL-6, IL-1β, PGE2, GM-CSF, IL-8). Decreased IFN-g secretion by the NK cells when co-cultured with increased NFkB function in tumors	[136, 137, 141, 193, 201, 209, 413, 441, 442, 444, 472-474, 488-491]
Enhanced tumor cell survival/resistance to killing	Expression of anti-apoptotic molecules by tumor cells via activation of upstream transcription factors (c-Myc, AP-1, NFκB, STAT3)	[492-497]
Increased NK and T cell apoptosis	TNF-α induced apoptosis, Fas ligand expression by tumor cells and membranous vesicles; Fas-mediated apoptosis of responding T cells, expression of DF3 and Muc1 in tumor cells	[142, 178, 498, 499]
Inhibition of macrophage/DC maturation and function	Tumor cell production of inhibitory cytokines (VEGF, IL-6, GM-CSF) and activation of STAT3-mediated transcription in DCs.	[210, 211, 442, 445, 446, 488, 500-507]
Inhibition of T cell chemotaxis to tumor microenvironment	Constitutive STAT3 activation in tumor cells, decreased expression of T cell chemotactic factors (RANTES, IP-10)	[211]
Increased recruitment or function of CD14+HLADR- monocytes,Tumor-associated Macrophages, MDSCs, Cancer Associated Fibroblasts, MSCs, Tregs and DCregs	Suppression of cytotoxic immune effectors	[227, 453]; [454, 455]; [456]

Figure Legend

Fig. 1 Schematic representation of simplified hypothetical model of oral cancer stem cell differentiation by NK cells

Based on our previous results [205, 412], OSCSCs initially express the phenotype listed in the figure (left panel). Upon conditioning of NK cells to lose cytotoxicity and gain in cytokine secretion and expressing CD16-CD56dim/-CD69+ surface phenotype (middle panel), either by immune inflammatory cells such as monocytes (for more detail see figure 2) or stem cells, the phenotype of stem cells is modified as shown in the right panel, and they become differentiated to support tissue regeneration.

Fig. 2 Immune inflammatory cells are mainly concentrated in the connective tissue area right beneath the epithelial layer of OSCC.

The slides from OSCC were prepared and stained with H&E (left panel). At the right panel the hypothetical model of NK cell conditioning in the tumor microenvironment is over imposed on the actual slide shown on the left panel. On the left panel significant infiltration of immune effectors right beneath the epithelial layer can be seen in the connective tissue area where the immune inflammatory cells are likely to condition NK cells to lose cytotoxicity and to support differentiation of epithelial cells. At the right panel two hypothetical mechanisms for conditioning of NK cells to support differentiation of stem cells are shown. In one scenario, NK cells, may first encounter and interact with either the other immune effectors as listed in the figure or the effectors of connective tissue such as fibroblasts to undergo split anergy (NK_{SA}). In a second

scenario, NK cells may also directly encounter the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they too can become anergized to support differentiation of other stem cells. Both mechanisms may be operational in the tumor microenvironment.

Figure 1

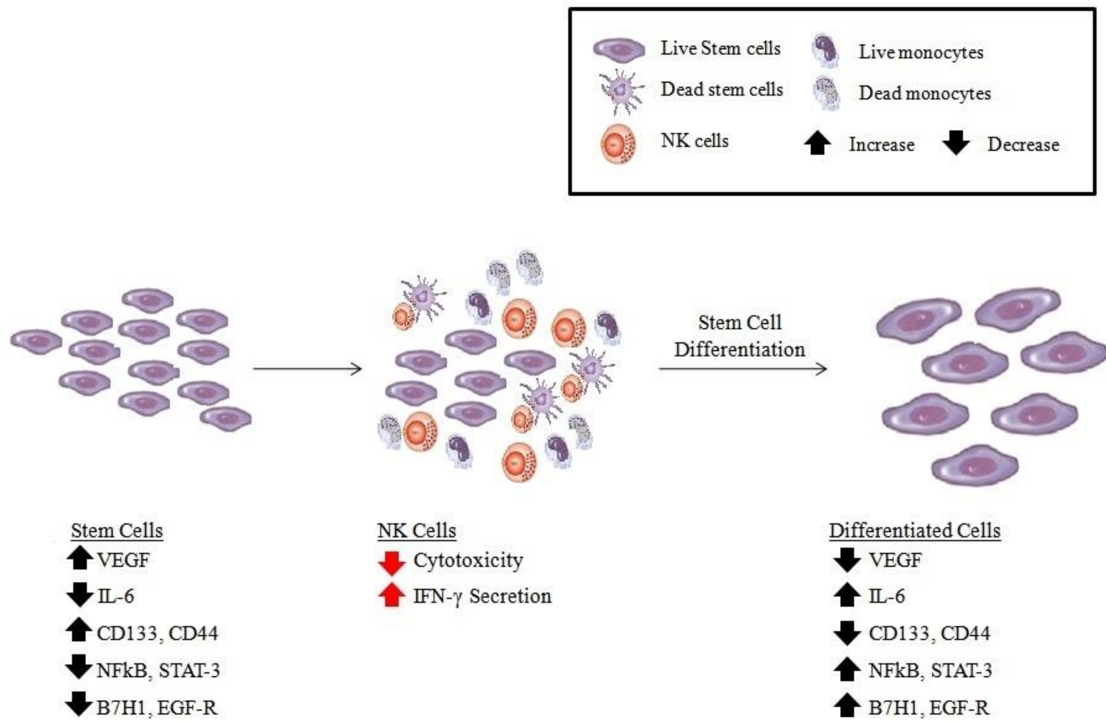
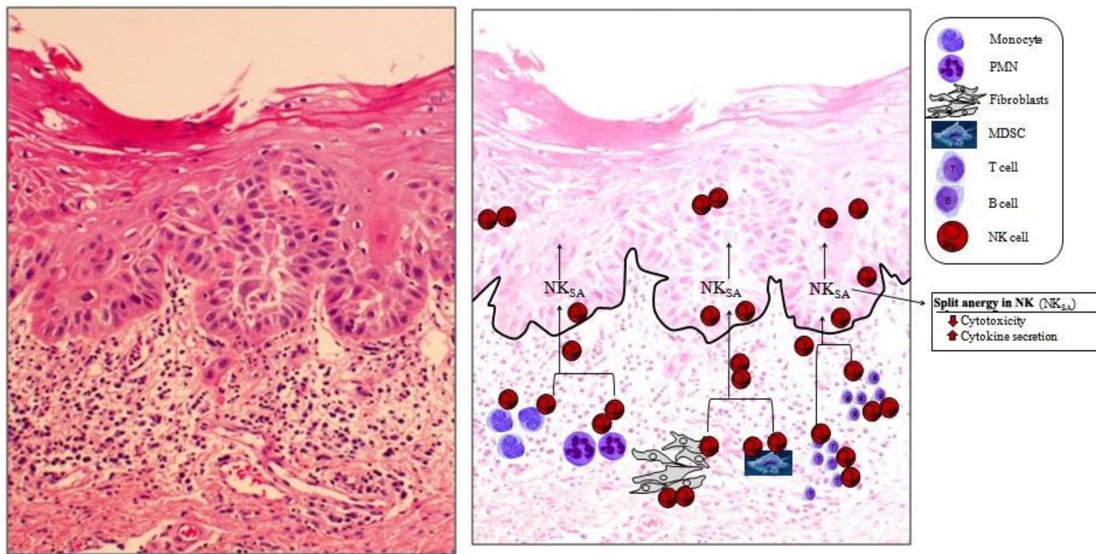


Figure 2



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Dual functions of Natural Killer cells in selection and differentiation of stem cells; Role in regulation of inflammation and regeneration of tissues

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Abstract

Accumulated evidence from our laboratory indicates that conditioned or anergized NK cells have the ability to induce resistance of healthy stem cells and transformed cancer stem cells through both secreted factors and direct cell-cell contact by inducing differentiation. Cytotoxic function of NK cells is suppressed in the tumor microenvironment by a number of distinct effectors and their secreted factors. Furthermore, decreased peripheral blood NK cell function has been documented in many cancer patients. We have previously shown that NK cells mediate significant cytotoxicity against primary oral squamous carcinoma stem cells (OSCSCs) as compared to their more differentiated oral squamous carcinoma cells (OSCCs). In addition, human embryonic stem cells (hESCs), human mesenchymal stem cells (hMSCs), human dental pulp stem cells (hDPSCs) and induced human pluripotent stem cells (hiPSCs) were all significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts or parental cells from which they were derived. We have also reported that inhibition of differentiation or reversion of cells to a less-differentiated phenotype by blocking NFκB or gene deletion of COX2 significantly augmented NK cell function. Furthermore, the induction of resistance of the stem cells to NK cell mediated cytotoxicity and their subsequent differentiation is amplified when either the stem cells or the NK cells were cultured in the presence of monocytes. Therefore, we propose that the two stages of NK cell maturation namely CD16+CD56dimCD69- NK cells are important for the lysis of stem cells or poorly differentiated cells whereas the CD16dim/-CD56dim/+CD69+NK cells are important for differentiation and eventual regeneration of

the tissues and the resolution of inflammation, thus potentially serving as regulatory NK cells (NK_{reg}). CD16 receptor on the NK cells were found to be the receptor with significant potential to induce NK cell anergy, however, our recent data indicated that NKp46 but not NKp30 or NKp44 were also able to induce significant anergy in NK cells, although the levels were less when compared to CD16 receptor triggering. The concept of split anergy in NK cells and generation of NK_{reg} and its contribution to cell differentiation, tissue repair and regeneration and in tumor resistance will be discussed in this review.

Introduction

NK cells are known to mediate direct cytotoxicity as well as antibody-dependent cellular cytotoxicity (ADCC) against a variety of tumor cells. By producing key cytokines and chemokines, NK cells are also known to regulate the functions of other cells [508, 509]. Human NK cells are identified by the cell surface expression of CD16 and CD56. NK cells mediate their function through a number of important activating and inhibitory cell receptors listed in Table 1 [510]. It is thought that the balance between activating and inhibitory signals which NK cells receive from their surface receptors determines their functional fate. Many of the receptors listed in table 1 including CD16, killer immunoglobulin like receptors (KIR), NKG2 family of receptors which form a heterodimer with CD94, NKG2D and natural cytotoxicity receptors (NCR) have all been the subject of many studies. Likewise, several key cytokines, chemokines and adhesion molecules are found to have significant roles in maturation, differentiation, and effector function of NK cells. Much less is known about the function of Toll Like Receptors (TLRs), NOD-Like Receptors (NLRs) and RIG like Receptors (RLRs) in NK cell effector function.

Recent advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between the immune effectors and the tumor cells. Effectors of the immune system are known to shape the maturation of tumor cells, and to select for cancers with reduced immunogenicity. However, recent data from our laboratory indicted that the same effector mechanisms are likely responsible for

shaping the maturation of healthy stem cells for the ultimate goal of the regeneration of damaged or disturbed tissues and the resolution of inflammation. Although, immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, neither their underlying physiological significance nor the exact mechanisms by which immunosuppression occurs are completely understood.

A number of factors responsible for the suppression of NK cell cytotoxicity in humans have been identified previously [175-180, 476 2010]. It is shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [181-184]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [185-187]. In contrast, interaction of NK cells with the resistant tumors does not lead to a significant suppression of NK cell cytotoxicity when compared to those dissociated from the NK sensitive target cells [136, 137]. Traditionally, the suppression of NK cell cytotoxic function after their interaction with the sensitive cells was sometimes perceived to be due to the exhaustion of cytotoxic granules from the NK cells, however, our recent data indicates that such suppression is physiological and it is an important step in maturation of NK cells to support differentiation of other cells and in resolution of inflammation.

Many mechanisms have been proposed for the functional inactivation of tumor associated NK cells including the over-expression of Fas ligand, the loss of mRNA for

granzyme B [176] and decreased CD16 and its associated zeta chain [188], some of which are also observed in NK cells which have been conditioned to support differentiation of healthy stem cells (data not shown).

In this report we review the previous studies from our laboratory regarding the factors and mechanisms involved in NK cell immunosuppression observed in cancer, and after interaction with healthy stem cells, and furthermore we discuss the emerging view from our laboratory which indicates that NK cells are the effectors of selection, differentiation and resistance of undifferentiated or stem like cells. In this regard we have reported previously that the stage of maturation and differentiation of healthy untransformed stem cells such as hESCs and hiPSCs, hMSCs and hDPSCs as well as transformed tumorigenic cancer stem cells is predictive of their sensitivity to NK cell lysis [412 2010]. Based on our results, we propose that NK cells play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact. To be conditioned to drive differentiation, NK cells may have to first receive signals through their key surface receptors either from healthy stem cells or those which have disturbed or defective capabilities to differentiate. In addition, NK cells by targeting other immune inflammatory cells or fibroblasts in the tumor microenvironment may become conditioned to lose cytotoxicity and gain cytokine producing phenotype before they can aid in differentiation of stem cells. These alterations in NK cell effector function will ultimately aid in driving differentiation of a population of surviving healthy as well as transformed stem cells. In cancer patients since the majority of NK cells have lost cytotoxic activity, they may eventually contribute rather

than halt the progression of cancer by allowing the growth and expansion of the pool of cancer stem cells. Below we will describe the evidence regarding the conditioning of the NK cells through key surface receptors and cytokines, differential targeting of stem cells and differentiated cells by the NK cells, and finally the role of conditioned NK cells in driving differentiation and resistance of undifferentiated tumors and healthy stem cells. In this regard conditioned NK cells function similarly to T regulatory cells in providing anti-inflammatory signals resulting in the resolution of inflammation and regeneration of the tissues.

Induction of split anergy in NK cells after their co-culture with sensitive but not resistant tumors and after the triggering of CD16 and NKp46 but not NKp30 or NKp44 receptors

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity and induces cell death in a small subset of NK cells [136, 137]. On the other hand, NK resistant tumors such as RAJI cells induce much less anergy or loss of NK cell cytotoxicity [136, 137]. Furthermore, following NK cell cultures with sensitive tumor-target cells but not resistant tumors, the target binding NK cells undergo phenotypic and functional changes. Target cell inactivated NK cells express CD16- CD56dim/- CD69+ phenotype [136, 137]. This phenotype has also been observed in several disease manifestations including HIV infection [468]. Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in several cancer patients including those of the oral and ovarian cancer patients

[138, 139]. In addition, down-regulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [140]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [140]. These observations suggested that CD16 receptors could likely play an important role in target cell induced loss of NK cell cytotoxicity. Indeed, CD16:Ig fusion proteins were shown to bind to a variety of tumor-target cells indicating the existence of specific ligands for CD16 receptors on tumor cells [469]. Furthermore, we have previously shown that the triggering of CD16 on untreated or IL-2 treated NK cells was found to result in down-modulation of CD16 receptors and in a great loss of cytotoxicity in NK cells. In addition, a subset of NK cells was programmed to undergo apoptosis [136, 137, 141, 142]. Cell death of NK cells was shown to be regulated, in part, by endogenously secreted TNF-α from the NK cells [142]. Previous studies by other groups have also shown that IL-2 activated NK cells undergo cell death following cross-linking of the CD16 receptor [470, 471]. Thus, we have coined the term “split anergy” for the responses observed by NK cells after their interaction with sensitive target cells or after the triggering of CD16 receptors by the antibody in combination with IL-2 treatment [136, 137, 142, 144, 145]. Indeed, three subpopulations of NK cells; namely Free, Binder and Killer NK cells with varying degrees of loss of cytotoxicity were identified after the formation of conjugates with K562 targets [141, 201, 472-474]. Free cells which did not bind or form conjugates with target cells were inactivated less, or exhibited the most cytotoxicity, whereas both Binder and Killer subsets exhibited significant loss of cytotoxicity. In contrast, Binder and Killer subsets but not Free NK subset secreted significant levels of cytokines and exhibited CD16-

CD56dim/-CD69+ phenotype [141, 201, 472-474]. Treatment of NK cells with IL-2 and anti-CD16mAb also induced split anergy by significantly decreasing the NK cell cytotoxicity while increasing the cytokine secretion capabilities of NK cells. Furthermore, NK cells exhibited CD16-CD56dim/-CD69+ phenotype after treatment with the combination of IL-2 and anti-CD16mAb [142-145]. Loss of cytotoxicity in NK cells was significantly exacerbated when NK cells were either treated with F(ab)'$_2$ fragment of anti-CD16 mAb or treated with a combination of MHC-Class I and anti-CD16 mAbs while the same treatments resulted in an increased secretion of cytokines [143, 145]. Based on our recent results NKp46 mAb but not those of NKp30 or NKp44 were also able to induce significant NK cell anergy in the presence and absence of IL-2 correlating with their increased expression on untreated and IL-2 treated NK cells when compared to NKp30 and NKp44 expression (manuscript submitted). Moreover, addition of bacteria or their extracts conditioned NK cells to support differentiation of OSCSCs (unpublished results). These results suggested that receptor signaling in NK cells in the presence of IL-2 is likely to result in a decrease in NK cell cytotoxicity while increasing secretion of cytokines by the NK cells. Therefore, three distinct functional outcomes could be observed in NK cells which have either interacted with sensitive tumor-target cells or were treated with anti-CD16 or anti-NKp46 mAbs in the presence of IL-2 treatment to induce split anergy, namely; 1-loss of cytotoxicity, 2-gain in the ability to secrete cytokines and 3- death in a small subset of NK cells.

Split anergy in NK cells is induced by total populations of monocytes and those depleted of CD16+ subset

When hMSCs or hDPSCs were cultured with either viable or irradiated monocytes before they were exposed to IL2-treated NK cells a significant decrease in NK cell mediated cytotoxicity could be observed against hMSCs or hDPSCs. Interestingly, lysis of hMSCs and hDPSCs by untreated NK cells was also reproducibly blocked by the addition of monocytes [205 2010]. To determine whether depletion of CD16+ subset of Monocytes from the total population of Monocytes change the cytotoxic function of NK cells in a 3 way interaction with the stem cells we used both the unsorted, and those sorted to remove CD16+ subsets in a 3 way killing assay with the NK cells. Both the total populations of monocytes and CD16- subsets were capable of inducing inhibition of NK cell cytotoxicity against stem cells [205 2010]. We then determined whether decreased lysis of stem cells by NK cells was due to a competitive lysis of monocytes by the NK cells. We confirmed that monocytes were also lysed by the NK cells significantly. Furthermore, when we co-cultured stem cells with monocytes and sorted to remove the monocytes from the stem cells before assessing the killing function of NK cells, we could still observe significant inhibition of NK cell mediated lysis, arguing against the protection of stem cell lysis by NK cells being solely on the bases of competitive lysis of monocytes [205 2010]. Therefore, even though lysis of monocytes by the NK cells may in part contribute to the prevention of NK cell lysis of stem cells, interaction of monocytes with stem cells can also provide resistance of stem cells against NK cell cytotoxicity. Decrease in NK cell lysis of hMSCs and hDPSCs was paralleled with a significant induction of IFN-γ. Indeed, when hMSCs or hDPSCs were cultured with IL-2 treated NK cells alone we could observe significant induction of IFN-γ secretion.

However, the highest increase was seen when IL2-treated NK cells were cultured with hMSCs or hDPSCs in the presence of monocytes. Therefore, although decreased killing of stem cells by the NK cells could be observed in the presence of monocytes, synergistic secretion of IFN-γ by the NK cells in the presence of monocytes and stem cells could be observed, indicating an inverse relationship between cytotoxicity and IFN-γ secretion (split anergy). This was similar to the profiles which we had seen when NK cells were treated with IL-2 and anti-CD16 mAb in which significant decrease in cytotoxicity of NK cells could be observed in parallel with increased secretion of IFN-γ [142].

Immunosuppressive effectors in tumor and non-transformed inflammatory microenvironment

Both the tumor microenvironment as well as non-transformed inflammatory microenvironment consists of a number of heterogeneous cell populations with ability to suppress and limit the function of cytotoxic immune effectors. Patients with cancer often have higher numbers of immature monocytes serving as Myeloid Derived Suppressor Cells (MDSCs) expressing CD14+HLADR- phenotype [227 2010, 453 2010]. Tumor associated Macrophages (TAMs) were previously shown to significantly influence and limit immune activation in the tumor microenvironment [454, 455 2010]. In addition, MDSCs which are comprised of a number of distinct cell populations of myeloid origin and whose roles in immunosuppression have received significant attention in recent years are major cells capable of suppressing the cytotoxic function of T and NK cells [453 2011]. T cell dysfunction is shown to be induced by MDSCs by the increased secretion of IL-10, TGF-β, induction of reactive oxygen species (ROS), and increased expression of

arginase-1 and inducible nitric oxide synthase (iNOS). T regulatory (Treg) and DC regulatory (DCreg) cells were also recently shown to have significant immunosuppressive roles in the tumor microenvironment [453 2010]. Perhaps one of the most intriguing observations regarding the immunosuppressive effectors is the identification of Cancer Associated Fibroblasts (CAFs) and Mesenchymal Stem Cells (MSCs) as two potential tumor promoters. Fibroblasts from tumor tissues demonstrate an activated phenotype and have the ability to secrete many immunosuppressive factors such as TGF-β and VEGF [456 2011]. We have also found that undifferentiated fibroblasts, as well as MSCs and CD14+HLA-DR- monocytes are significantly more susceptible to NK cell mediated cytotoxicity [205 2010], therefore, these cells may condition NK cells to undergo split anergy (please see below). Indeed, in oral epithelial tumors the majority of recruited immune effectors are usually found in the connective tissue area where through cell-cell interaction with the immunosuppressive cells such as fibroblasts, monocytes-macrophages and to a lesser extent T and B cells [205 2010] can condition NK cells to lose cytotoxicity and gain in cytokine secretion capabilities, resulting in differentiation and resistance of oral epithelial tumors (please see below).

Differentiated cells are lysed significantly less by the NK cells and do not trigger secretion of cytokines by the NK cells when compared to their undifferentiated counterparts

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed when NK cells were co-incubated with OSCSCs which released significantly lower levels of GM-CSF, IL-6 and IL-8 and demonstrated decreased expression of phospho-Stat3,

B7H1 and EGFR, and much lower constitutive NFκB activity when compared to differentiated OSCCs [412 2010]. More importantly, OSCSCs expressed CD133 and CD44^{bright} oral tumor stem cell markers [412 2010]. Increase in IFN-γ secretion was correlated with a decrease in secretion of IL-6 in co-cultures of NK cells with OSCSCs as compared to those co-cultured with OSCCs. Therefore, from these results a specific profile for differentiated NK resistant oral tumors emerged which demonstrated increased GM-CSF, IL-6 and IL-8 secretion in the context of decreased IFN-γ secretion during their interaction with the NK cells. In contrast, co-cultures of cancer stem cells with NK cells demonstrated increased IFN-γ in the context of lower GM-CSF, IL-6 and IL-8 secretion [205 2010, 412 2010]. In addition, three brain tumor stem cells which were previously characterized [259, 466, 467] were found to be significantly more susceptible to NK cell mediated cytotoxicity when compared to their differentiated counterparts which were significantly more resistant (manuscript submitted). Since OSCSCs and brain cancer stem cells were significantly more susceptible to NK cell mediated cytotoxicity we reasoned that healthy, non-transformed primary stem cells may also be susceptible to NK cell mediated cytotoxicity. We demonstrated previously that NK cells lysed hMSCs, hDPSCs and hESCs significantly. All different types of stem cells became resistant to NK cell mediated cytotoxicity once they were differentiated [412 2010]. In addition, higher sensitivity of hiPSCs to NK cell mediated lysis was also observed when compared to parental line from which they were derived. Increased lysis of cancer stem cells or non-transformed healthy stem cells may be attributed to the use of allogeneic NK cells, however, our previous work using autologous NK cells exhibited similar levels of cytotoxicity against hDPSCs when compared to lysis by allogeneic NK cells [205 2010].

Taken together these results indicated that undifferentiated cells were targets of both allogeneic and autologous NK cells. Thus, the stage of differentiation of the cells is predictive of their susceptibility to NK cell mediated cytotoxicity.

Blocking NFκB reverts the oral tumors to more of an undifferentiated phenotype resulting in an increase in NK cell mediated cytotoxicity.

Since the degree of differentiation in the cells was predictive of their sensitivity to NK cell mediated cytotoxicity, we reasoned that blocking NFκB in the cells may de-differentiate and subsequently revert the cells to more of undifferentiated phenotype, resulting in their increased susceptibility to NK cell mediated cytotoxicity. Indeed, blocking NFκB in oral tumors was found to increase CD44 surface receptor expression, which is one of the hallmarks of stem cells (unpublished results). In addition, we have shown previously that NFκB nuclear function in a primary Oral tumor OSCCs and in a non-tumorigenic oral cells (HOK-16B) as well as in an established tumor line, HEp-2 cells known to be Hela contaminant [193, 457-459], modulates and shapes the function of interacting NK cells [192, 193]. Since knock down of NFκB was shown to increase the function of immune inflammatory cells in diverse cell types (please see below) it is not surprising to find similar patterns of immune activation in both oral and non-oral derived tumor cells. In addition, since the majority, if not all cells, increase NFκB during their activation and differentiation, a knock down of NFκB is likely to revert the cells, irrespective of their cellular origin, to a less differentiated phenotype resulting in the potential activation of the immune effectors in order to aid in their differentiation [412 2010]. Similarly, inhibition of NFκB by Sulindac increased the functional activation of

NK and enhanced anti-tumor cytotoxic activity [192, 193]. Inverse modulation of IFN-γ and IL-6 cytokine secretion was seen in co-cultures of NK cells with NFκB knock down OSCCs, HOK-16B and HEp2 cells indicating that blocking NFκB in these cells serves to switch the balance from Th2 type responses to more of a Th1 type response [192, 412 2010].

In agreement with our studies, targeted deletion of IKK-β in epidermis of mice has previously been shown in one study to lead to inflammatory skin manifestations [153]. Elevated levels of cytokines and chemokines have also been demonstrated in the epidermis of patients and animals with Iκκγ and Iκκβ deletions [153, 460]. Mice with a keratinocyte-specific deletion of Iκκ−β demonstrated decreased proliferation of epidermal cells and developed TNF-α dependent inflammatory skin disease [153]. In contrast, in other studies in which NFκB function was blocked in dermal keratinocytes by a mutant IκB-α an increased proliferation and hyperplasia [463] and eventual development of cutaneous squamous cell carcinomas of skin were seen if mice were allowed to survive and reach adulthood [464, 465]. It is of interest to note that in these studies with diverse functional outcomes in keratinocytes, blocking TNF-α function resulted in the prevention of both the neoplastic transformation and the inflammatory skin disease [153, 465]. Elevated numbers of immune inflammatory cells recruited to the site of epidermis are likely responsible for the increased secretion of TNF-α. Indeed, we have demonstrated that synergistic induction of TNF-α could be observed when NFκB knock down oral tumors were cultured with either PBMCs or NK cells [192].

Since tumorigenic and non-tumorigenic human oral keratinocytes acquire sensitivity to NK cell mediated lysis when NFκB is inhibited, it is likely that this phenomenon is not specific to cancer or oral keratinocytes, and it may occur in other healthy non-transformed cell types. Indeed, when human primary monocytes were differentiated to dendritic cells they too became more resistant to NK cell mediated cytotoxicity [412 2010]. Moreover, knock down of COX2 in primary mouse monocytes [412 2010], or in mouse embryonic fibroblasts (unpublished observations), resulted in the reversion or de-differentiation of the monocytes and fibroblasts respectively, and the activation of NK cell cytotoxicity. Indeed, it is likely that any disturbance in cellular differentiation may predispose the cells to NK cell mediated cytotoxicity. Since STAT3 is an important factor increased during differentiation, blocking STAT3 is also critical in the activation of immune effectors [211]. In support of a critical role of STAT3 in immune evasion of tumor cells in humans, we and others have recently shown that glioblastoma multiforme (GBM) tumors display constitutive activation of STAT3 (Cacalano and Jewett, unpublished observation) [160], and poorly induce activating cytokines and tumor-specific cytotoxicity in human peripheral blood mononuclear cells (PBMCs) and NK cells. Ectopic expression of dominant-negative STAT3 in the GBM cells increased lysis of the tumor cells by the immune effectors and induced production of IFN-γ by the interacting immune effectors (unpublished publications).

Since NFκB is shown to regulate IL-6 secretion in OSCCs, HOK-16B and HEp2 cells and secreted IL-6 in tumors is known to activate STAT3 expression and function, increase in NFκB nuclear function could in turn induce STAT3 activation and result in a

significant resistance of tumors to NK cell mediated cytotoxicity. Therefore, targeted knock down of STAT3 or signaling pathways upstream of STAT3, such as NFκB, should de-differentiate the cells and predispose the cells to NK cell mediated cytotoxicity.

NK cells are conditioned in the tumor or in non-transformed inflammatory microenvironment to drive differentiation

The theory of immunosurveillance was postulated by Burnet [414] to indicate that the key thymus dependent effectors were responsible for clearance of tumors [415, 416]. However, since then the concept of immunosurveillance has been expanded to include immunoediting as an important mechanism for the development of cancer [421, 422]. It was suggested that cancer immunoediting comprises of three phases: elimination, equilibrium and escape [422]. Elimination represents the classical concept of immunosurveillance. However, during equilibrium and escape the interaction and cross signaling between the immune effectors, the tumor cells, and the effectors of the connective tissue in the tumor microenvironment is likely to result in the generation of differentiated tumors which are resistant to cytotoxic function of NK and CTLs and thus are capable of ceasing or lowering the inflammatory responses. Therefore, the final stages of cancer progression may result in the induction of less immunogenic tumors in the presence of fewer immune effectors capable of lysing them. Thus, pressures exerted by the tumor cells and immune effectors may eventually shape the microenvironment for the growth, expansion and invasion of tumors. Similarly, a variation of such interactions may also be observed during the interaction of NK cells with healthy non-transformed human

stem cells in which case the three phases of interaction may include elimination which marks the decrease in the numbers of stem cells or other immune effectors in the inflammatory microenvironment, potentially resulting in the selection of stem cells by the NK cells, induction of tolerance or anergy which denotes the conditioning of NK cells by the stem cells and/or by the other effectors of microenvironment to lose cytotoxicity and support maturation and differentiation of remaining stem cells, and finally the resolution phase which denotes the elimination of anergized NK cells and generation of less immunogenic differentiated cells. Similarities and differences between these phases in cancer and during stem cell maturation will be discussed below.

Potential role of anergized NK cells in differentiation and regeneration of tissues

Induction of split anergy in NK cells could be an important conditioning step responsible for the repair of tissues during pathological processes irrespective of the type of pathology. In tumors since the generation and maintenance of cancer stem cells is higher, the majority, if not all of the NK cells, may be conditioned to support differentiation and repair of the tissues and as such the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble that of the anergic NK cells, i.e., decreased NK cell cytotoxicity, acquisition of CD16-/dimCD56dim/+CD69+ phenotype and augmented ability to secrete inflammatory cytokines (Fig. 1). Of course, the degree of the loss of NK cell cytotoxicity may be directly proportional to the load of cancer stem cells. Therefore, our results suggest two very important functions for the NK cells. One function is to limit the number of stem cells and immune inflammatory cells by selecting those with a greater potential for differentiation for the repair of the tissues and

second to support differentiation of the stem cells and subsequent regeneration of the tissues. To achieve these tasks NK cells have to acquire two different phenotypes and be conditioned to carry out both functions successfully. CD16+CD56dimCD69- subsets of NK cells are cytotoxic and will mediate cytotoxicity depending on which cells they encounter first. In respect to the oral squamous cell carcinomas since the majority of immune effectors can be found at the connective tissue area the chances are that they may first encounter and interact with either the other immune inflammatory cells or the effectors of connective tissue such as fibroblasts. However, there is also the possibility that NK cells may first encounter the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they too can become anergized (Fig. 1). Surprisingly, allogeneic CTLs were also found to target Glioblastoma stem-like cells and not their differentiated counterparts (unpublished observation). By eliminating a subset of stem cells or after their interaction with other immune inflammatory cells or effectors of connective tissue NK cells could then be in a position to support differentiation of selected population of stem cells since they will be conditioned to lose cytotoxicity, induce cytokine and growth factor secretion and gain the CD16-/dimCD56dim/+CD69+ phenotype (Fig. 1). It is interesting to note that all of the immune effectors isolated from the oral gingival tissues of healthy as well as diseased gingiva have CD69+ phenotype, with the exception that the numbers of immune effectors are much less in the healthy oral gingival tissues when compared to diseased tissues (unpublished observation). Therefore, our results suggest two very important functions for the NK cells. One function is to kill and the other function is to be conditioned to support differentiation for the repair and regeneration of the tissues.

In vivo physiological relevance of above-mentioned observations could be seen in a subpopulation of NK cells in peripheral blood, uterine and liver NK cells which express low or no CD16 receptors, have decreased capacity to mediate cytotoxicity and is capable of secreting significant amounts of cytokines [213, 214]. In addition, 70% of NK cells become CD16 dim or negative immediately after allogeneic or autologous bone marrow transplantation [213]. Since NK cells lose their cytotoxic function and gain in cytokine secretion phenotype and down modulate CD16 receptors after their interaction with tumor cells or the stem cells [137, 142], it is tempting to speculate that in vivo identified CD16- NK cells and in vitro tumor induced CD16- NK cells have similar maturational pathways since they have similar if not identical functional properties.

The proof of concept in support of this model was recently obtained in our laboratory. We observed that anergized NK cells were directly responsible for the increased differentiation and resistance of a number of different stem cells including cancer stem cells and dental pulp stem cells against cytotoxic effectors (manuscript submitted). In addition, we now have evidence which supports the notion that the induction of anergy in NK cells is an active process which is induced by the triggering of CD16 receptor on the NK cells and is not due to degranulation and exhaustion of cytotoxic granules (manuscript in prep).

Our work collectively suggests that anergized NK cells are as important as the non-anergized NK cells in their effector functions. NK cells are not only important for

the removal and shaping of the size of the stem cells but also their differentiation, and the eventual regeneration of the new tissues. The task of NK cells in this regard goes above and beyond their most appreciated function of being the effectors of first line defense against viral infection and malignancies. They too are the effectors of differentiation and tissue regeneration.

Tumor microenvironment may shape the function and phenotype of the NK cells

The above observations prompted us to speculate regarding the significance of interaction of monocytes with NK cells and stem cells. It is plausible that monocytes may serve as shields against NK cell lysis of stem cells. Similar to anti-CD16 mAb mediated effect on IL2-treated NK cells, monocytes too can shield stem cells from killing by the NK cells by increasing the total IFN-γ release by the NK cells while decreasing the cytotoxic function of NK cells (split anergy), resulting in an increased protection and differentiation of stem cells. Indeed, monocytes also increased TNF-α, IL-6 and VEGF secretion in the co-cultures of stem cells with NK cells which could augment NFκB and increase differentiation of stem cells. The shielding effect of monocytes could be a more generalized function of other effectors since NK cells can also target fibroblasts and to a much lesser extent the T and B cells [205 2010]. Whether other MDSCs such as PMNs, mast cells, etc could also be targeted by the NK cells awaits future investigation. This may have significant implications for the role of NK cells in not only limiting inflammation, but also the significance of other immune effectors in shielding and limiting the cytotoxic function of NK cells against cancer or healthy stem cells in order to raise maximally the secretion of key cytokines for speedy and optimal differentiation of

stem cells during inflammation. This is precisely what is observed in cancer patients in whom global decrease in NK, cytotoxic T cells and monocytes have all been reported [180].

Potential homeostatic regulation of differentiation of stem cells in the tissue microenvironment by conditioned NK cells

Recent data from our laboratory indicates that NK cells may be capable of regulating homeostatic differentiation of the stem cells. In this regard, the two stages of NK cells to mediate cytotoxicity and secretion of cytokines may be important for homeostatic regulation of cell differentiation since cytotoxic NK cells if they first encounter cancer stem cells or healthy stem cells they will likely eliminate them, however, if they encounter them after they are conditioned by the effectors of stroma, they may drive stem cell differentiation. Some of the terminally differentiated tumor cells or healthy cells may also be eliminated by cytokines secreted from the conditioned NK cells. Therefore, the potential outcome of such regulation by the NK cells may be the generation and maintenance of moderately to highly differentiated cells. These two stages of NK cell development may ensure that the number of stem cells as well as terminally differentiated cells remain very low in the stroma favoring the generation of moderately to highly differentiated cells. These hypotheses are currently under investigation in our laboratory. Indeed, all of the immune effectors dissociated from either healthy gingival tissues or periodontal gingiva are found to have an activated phenotype when compared to peripheral blood since they have elevated CD69 expression, and the magnitude of CD69 expression is similar between those recovered from healthy and periodontal

gingivae, with the periodontal gingiva having higher numbers of immune effectors when compared to healthy gingiva (Fig. 2). Similarly, all T cells in normal breast tissue as well as breast tumor microenvironment were found to have high CD69 expression, with the breast tumor tissue having higher numbers of immune effectors when compared to normal breast tissue when compared to the peripheral blood [68]. The finding of CD69+ immune cells in healthy as well as tumor microenvironments suggest the general role of the activated immune effectors in repair and maintenance of tissue homeostasis, however, since potentially more cancer stem cell is generated by the tumor cells, immune cells may become overwhelmed and are unable to cope with the proliferating tumor cells.

Common functional outcomes between conditioned/anergized NK cells and T regulatory cells

Because of the ability to drive differentiation, conditioned NK cells may have the ability to halt inflammation since differentiated cells are no longer targeted by the NK cells and they do not induce cytokine secretion by the NK cells [18, 19]. This function of NK cells may be similar to T regulatory cells since they are inhibitory and are capable of decreasing the magnitude of inflammation in a number of previous studies. Thus in this respect anergized or conditioned NK cells may have regulatory or tolerizing function (NK_{reg}). Therefore, although immunosuppression in the tumor microenvironment is not advantageous for the cancer patients, it is indeed, an important function which may not only stimulate differentiation, but it may also halt inflammation.

All of the immune effectors in the mucosal immune system including in the oral cavity are of activated phenotype, ie, they express CD69 early activation antigen. These cells, including NK cells are likely conditioned in the mucosa to support differentiation and resistance of the epithelial cells. Such environment is anti-inflammatory since the majority of immune cells is tolerant of ingested food particles since it contains many regulatory cells including T cells, Dendritic cells and conditioned NK cells. However, once the threshold which keeps the inflammation at bay is decreased in the mucosa due likely to disturbance in homeostatic differentiation of the cells, immune inflammatory effectors are recruited and activated which may result in tissue damage and establishment of chronic inflammation. Indeed, our preliminary *in vitro* and *in vivo* observations in humans indicated that the consumption of a newly developed food supplement with potent ability to condition NK cells to support differentiation of OSCSCs and hDPSCs (manuscript in prep.) was able to relieve chronic inflammation and pain, and resulted in the resolution of inflammatory mouth ulcers, decrease in the periodontal pocket depth and decrease swelling and edema in the oral mucosa. Furthermore, the number of neutrophils in the blood of a donor who had chronically decreased levels of neutrophils for over ten years rose to the normal levels after the consumption of the food supplement. Both the number and function of NK cells in the blood was substantially higher after the consumption of supplement.

Autologous and allogeneic NK cells drive differentiation of stem cells to a similar extent and result in the resistance of differentiated cells to NK cell mediated cytotoxicity

To determine whether there are differences between conditioned/anergized autologous and allogeneic NK cells in driving differentiation of dental pulp stem cells we obtained dental pulp stem cells after extraction of molars and highly purified NK cells from the same individuals and compared their ability to differentiate hDPSCs to conditioned/anergized NK cells obtained from different donors. Both formaldehyde fixed conditioned NK cells and soluble factors secreted from conditioned autologous and allogeneic NK cells were able to drive differentiation of hDPSCs as determined by the surface expression of CD44, and resulted in the resistance of differentiated hDPSCs against cytotoxicity mediated by freshly isolated untreated and IL-2 treated NK cells. Moreover, autologous and allogeneic NK cells did not secrete cytokines when cultured with differentiated hDPSCs (manuscript submitted). The ability of NK cells to differentiate hDPSCs and cause resistance of the differentiated hDPSCs to NK cell-mediated cytotoxicity was blocked when the combination of antibodies and not a single antibody to the key cytokines secreted by the NK cells was added, indicating the significance of synergistic interaction of the cytokines in driving stem cell differentiation (manuscript submitted). Several targets of synergistic modulation of stem cell differentiation by the NK cells are identified and their role in driving differentiation of the stem cells is currently under investigation in our laboratory.

Conclusion

Recent advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between the immune effectors and the tumor cells in the tumor microenvironment. Effectors of the immune system are known to shape the tumor cells and to select for cancers with reduced immunogenicity. However, the same effector mechanisms are likely responsible for shaping the maturation of healthy stem cells for the ultimate goal of the repair and the regeneration of damaged or disturbed tissues and the resolution of inflammation. Much work has been done to identify strategies by which tumor cells evade the function of immune system. Altered expression of MHC molecules which block recognition and activation of T and NK cells are examples of mechanisms by which tumor cells evade the function of immune system. In addition, tumor cells by releasing immunosuppressive factors such as Fas, VEGF, IL-6, IL-10, TNF-α, GM-CSF and IL-1β, induce T and NK cell apoptosis, block lymphocyte homing and activation, and dampen macrophage and dendritic cell function. However, the same effector functions are also important in tissue repair. Furthermore, progress has been made in identification of the upstream mechanisms which control the expression of immunosuppressive factors in tumor cells. Two key control elements, NFκB and STAT3 were identified and shown to coordinately regulate the production of multiple tumor-derived immunosuppressive molecules and play a pivotal role in tumor cell immune suppression. The potential for these two signaling modules to repress immune responses is underscored by the finding that the pathways interact and may even amplify each other.

Based on the accumulated work presented in this review, we suggest that NK cells may have two significant functions; one that relates to the removal and/or selection of stem cells or undifferentiated cells. Additionally, NK cells may lyse other effectors in the connective tissue area including immune effectors such as monocytes or other MDSCs as well as fibroblasts in order to not only decrease inflammation but also become conditioned to promote differentiation of stem cells and eventual regeneration of the tissues. The second important task for NK cells is therefore, to support differentiation and promote tissue regeneration after altering their phenotype to cytokine secreting cells (Fig. 1). This process will not only remove cells that are damaged and have flaws in the differentiation process, but also it will ensure the regeneration of tissues and the resolution of inflammation. Thus, any disturbance in the NK cell function or in the process of differentiation of stem cells may result in chronic inflammation, causing continual tissue damage and recruitment of immune effectors to aid in tissue regeneration.

The inability of patient NK cells to contain cancer stem cells potentially due to the flooding of NK cells by proliferating cancer stem cells and conversion of NK cells to cytokine secreting cells may likely be one mechanism by which cancer may progress and metastasize. Therefore, there should be two distinct strategies by the NK cells to eliminate tumors, one which targets stem cells and the other which targets differentiated cells. Since cancer stem cells were found to be more resistant to certain chemotherapeutic drugs but sensitive to NK cell mediated killing while differentiated oral tumors were more resistant to NK cell mediated killing but relatively more sensitive to

chemotherapeutic drugs, combination therapy should in theory be effective in the elimination of both undifferentiated and differentiated tumors. In addition, since a great majority of patient NK cells have modified their phenotype to support differentiation of the cells, they may not be effective in eliminating the cancer stem cells. Therefore, these patients may benefit from repeated allogeneic NK cell transplantation for elimination of cancer stem cells. In this regard, depletion of immunosuppressive effectors in the tumor microenvironment, which condition NK cells to lose cytotoxicity, via radiation or chemotherapeutic drugs should in theory provide a better strategy for successful targeting of tumors by the NK cells.

Table 1- List of NK cell Activating and Inhibitory surface receptors and their ligands

Receptors	Ligands
Activating/inhibitory Receptors	
FcγRIII (CD16)	Fc of antibodies
CD2	CD58 (LFA-3)
LFA-1	ICAM-1
2B4	CD48
CD69	Unknown
DNAM-1 (CD226)	CD112, CD155
NKp80	AICL
Tactile (CD96)	CD155, CD111
TIGIT	CD112,CD113,CD155
CRTAM	TSLC1
C-type Lectin receptors –Activating/Inhibitory	
CD94/NKG2A/B	HLA-E
NKG2D	MICA, MICB, ULBP-1, ULBP -2, ULBP -3, ULBP -4, ULBP -5, ULBP -6
CD94/NKG2C	HLA-E
CD94/NKG2E/H	HLA-E, Qa-1b
Natural cytotoxicity receptors (NCR)	
NKp46 (NCR1)	Viral Hemagglutinin
NKp44 (NCR2)	Viral Hemagglutinin
NKp30 (NCR3)	B7h6, HCMV-pp65
Killer IG-like (KIR) – Activating/Inhibitory	
KIR2DLs, KIR3DLs, KIR2DS	HLA-C, HLA-B, HLA-A, HLA-G
Cytokines, growth factors and chemokines	Cytokines, growth factors and chemokine ligands
Toll-like receptors (TLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR)	Bacterial DNA, LPS, peptidoglycan, teichoic acids, flagellin, pilin, viral dsRNA and fungi zymosan

Figure Legend

Fig. 1 Hypothetical model of conditioning of NK cells by immune inflammatory cells and the effectors of connective tissue to modify NK cell phenotypic and functional properties in order to support differentiation of the cells and the resolution of inflammation.

Hypothetical model of NK cell conditioning in the tumor microenvironment as well as in non-transformed immune inflammatory microenvironment is shown in this figure. Significant infiltration of immune effectors right beneath the epithelial layer can be seen in the connective tissue area where the immune inflammatory cells are likely to condition NK cells to lose cytotoxicity and gain the ability to secrete cytokines, a term which we have previously coined as split anergy in NK cells, and to support differentiation of the basal epithelial layer containing stem cells. NK cells are likely to encounter and interact with the other immune effectors such as monocytes or other myeloid-derived suppressor cells (MDSCs), and in tumor microenvironment with the tumor-associated macrophages (TAMs), or with connective tissue-associated fibroblasts (CAF) in order to be conditioned to form regulatory NK cells (NK_{reg}). NK cells may also directly interact with the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they can become conditioned to support differentiation of other stem cells. In addition, bacteria through the binding to Toll like receptors can further aid in the generation of $NK_{reg.}$ All the above mentioned mechanisms may be operational during inflammatory processes in the tumor microenvironment or in healthy non-transformed inflammatory microenvironment. NK cell-differentiated

epithelial cells will no longer be killed or induce cytokine secretion by the NK cells, therefore, resulting in the resolution of inflammation.

Fig. 2. The majority of the immune effectors in healthy and periodontal gingiva are of activated phenotype.

Immune infiltrates from healthy and periodontal patient gingiva were dissociated, and the levels of CD69 expression on CD45+ immune effectors were determined using flow cytometry.

Figure 1

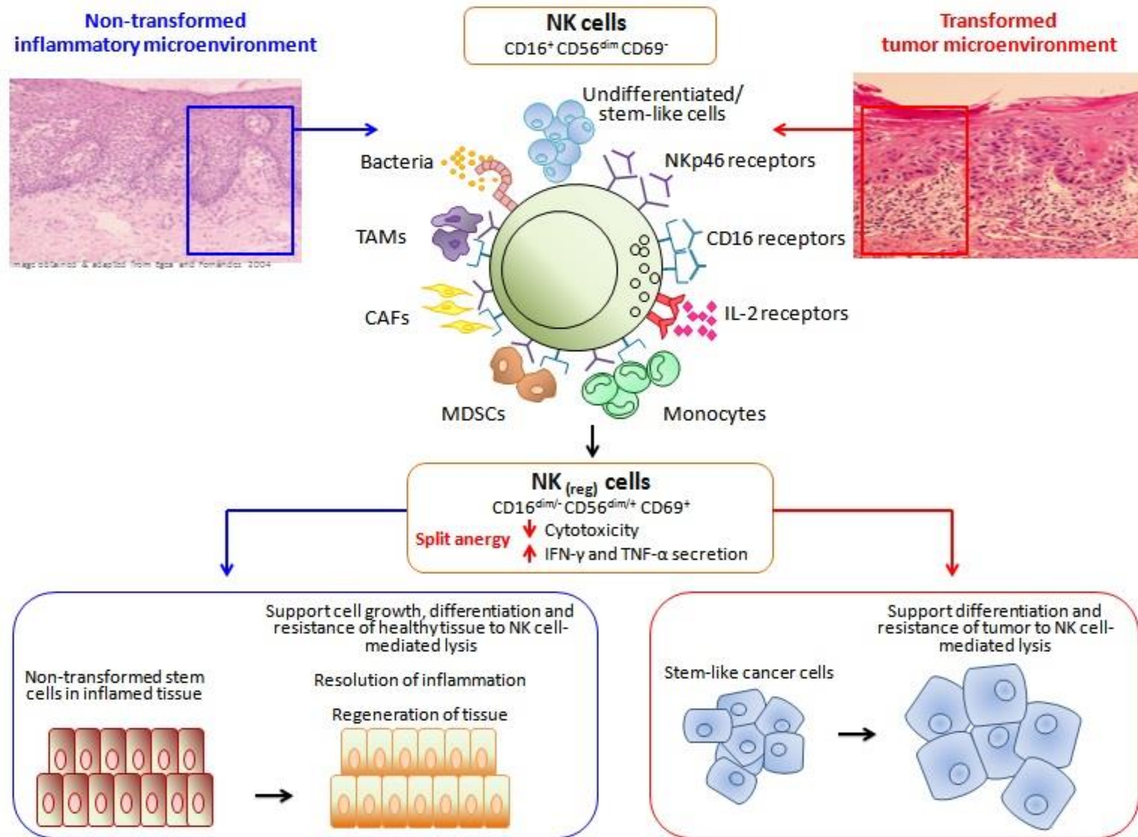
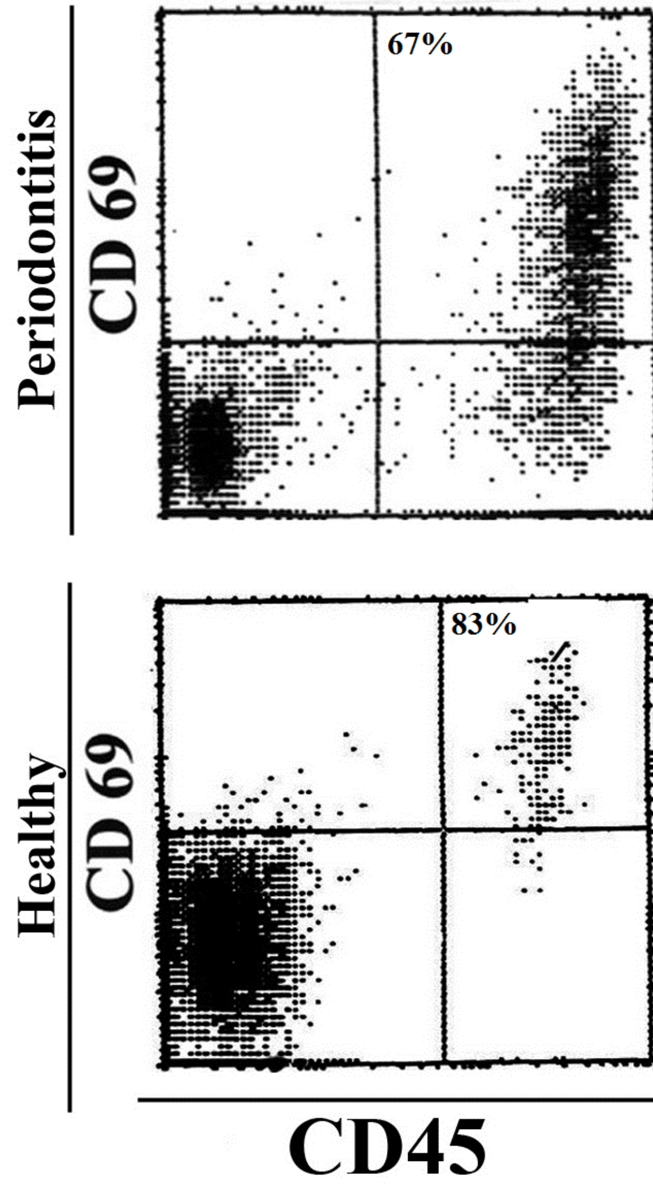


Figure 2



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Natural Killer cells as the effectors of selection and differentiation of stem cells; Role in resolution of inflammation

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Abstract

We have previously demonstrated evidence for the role of NK cells in specific elimination of healthy stem cells (hMSCs, hDPSCs, hESC and hiPSCs) as well as cancer stem cells and not their differentiated counterparts. There is also a stage wise susceptibility to NK cell mediated cytotoxicity in tumors in which case the poorly differentiated tumors are lysed much more than their moderately differentiated tumors. The well differentiated tumors were lysed the least when compared to either the moderately differentiated tumors or to poorly differentiated tumors. We have also reported that inhibition of differentiation or reversion of cells to a less-differentiated stage by blocking NFκB or gene deletion of COX2 significantly augmented NK cell cytotoxicity against both transformed and healthy cells. Additionally, the cytotoxic function of NK cells is severely inhibited against stem cells when they are cultured in the presence of monocytes. Therefore, we propose that the two stages of NK cell maturation namely CD16+CD56dimCD69- NK cells are important for the selection of stem cells whereas the CD16dim/-CD56dim/+CD69+NK cells are important for differentiation and eventual regeneration of the tissues and the resolution of inflammation, thus serving as regulatory NK cells (NK_{reg}). The concept of split anergy in NK cells and generation of NK_{reg} and its contribution to cell differentiation, tissue repair and regeneration and in tumor resistance will be discussed in this review.

Introduction

Effectors of the immune system are thought to shape the survival and maturation of tumor cells and to select for cancers with reduced immunogenicity. However, recent data from our laboratory indicted that the same effector mechanisms are likely responsible for shaping the survival and maturation of healthy stem cells for the ultimate goal of the regeneration of damaged tissues and the resolution of inflammation. Although, immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, neither their underlying physiological significance nor the exact mechanisms by which immunosuppression occurs are completely understood.

NK cells arise from the bone marrow and constitute 5-15% of total lymphocytes in the peripheral blood. They are known to mediate direct natural cytotoxicity as well as antibody-dependent cellular cytotoxicity (ADCC). By producing key cytokines and chemokines NK cells are known to regulate the functions of other immune cells [508, 509]. Conventional human NK cells are identified by the expression of CD16 and CD56, and by the lack of surface CD3 expression. NK cells mediate their function through a number of important activating and inhibitory cell receptors listed in Table 1 [511] . It is thought that the balance between activating and inhibitory signals which NK cells receive from their surface receptors determines their functional fate [511] . Many of the receptors listed in table 1 including CD16, killer immunoglobulin like receptors (KIR), NKG2 family of receptors which form a heterodimer with CD94, NKG2D and natural

cytotoxicity receptors (NCR) have all been the subject of many studies. Likewise, several key cytokines, chemokines and adhesion molecules are found to have significant roles in maturation, differentiation, and effector function of NK cells. However, little is known regarding the function of Toll Like Receptors (TLRs), NOD-Like Receptors (NLRs) and RIG like Receptors (RLRs) in NK cell effector function.

Much less is known regarding the interaction of NK cells with other immune effectors or the effectors of the connective tissue in the tumor microenvironment. Considering that NK cells may reside primarily in the immune rich compartments, it is likely that other immune effectors may shape the phenotype and function of NK cells. In this regard, very little is known regarding the interaction of NK cells with Myeloid Derived Suppressor Cells (MDSCs) which is known to induce suppression in T cells [512, 513].

The association of distinct effector functions with certain NK cell subsets is thought to be developmentally regulated [514, 515] . In this regard, previous studies have identified two distinct subsets of NK cells namely $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$ subpopulations based on their phenotypic and functional analysis [509]. The $CD56^{dim}CD16^{bright}$ NK subset is the major subset in the peripheral blood which mediates cytotoxicity whereas the $CD56^{bright}CD16^{dim}$ subset constitutes a minor subpopulation of NK cells in the peripheral blood and its role is in secretion of cytokines [509]. $CD56^{bright}CD16^{dim}$ subset does not mediate cytotoxicity. The $CD56^{bright}CD16^{dim}$ NK cells are thought to be precursors to the $CD56^{dim}CD16^{bright}$ NK subset [516].

Although a lot is known about the inhibitory and activating receptors that modulate the function of NK cells, and many previous studies have indicated that NK cells may recognize and become activated by irradiated or stressed cells [509, 511], no previous studies have shown the role of NK cells in recognition, selection and differentiation of stem cells and their potential role in the resolution of inflammation. In this review we provide a summary of factors and mechanisms involved in shaping the function of NK cells in cancer, and after interaction with healthy stem cells, and furthermore we discuss the emerging view from our laboratory which indicates that the NK cells may behave as the effectors of selection and differentiation of stem cells, and their subsequent resistance to NK cell mediated cytotoxicity.

Split anergy in NK cells is induced after their binding to sensitive but not resistant tumors and after the triggering of CD16 receptors

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity while it triggers significant induction of TNF-α and IFN-γ from the NK cells [136, 137]. In contrast, NK resistant tumors such as RAJI cells induce very little loss of NK cell cytotoxicity or secretion of cytokines [136, 137]. Indeed, there is an inverse relationship between target cell susceptibility to NK cell lysis and its ability to inactivate the cytotoxic function of NK cells (Table 2). Moreover, following NK cell cultures with sensitive tumor-target cells but not resistant tumors, the target binding NK cells undergo phenotypic and functional changes. Target cell inactivated NK cells express CD16- CD56dim/- CD69+ phenotype [136, 137]. This phenotype has also been observed in

several disease manifestations including HIV infection [468]. Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in several cancer patients including those of the oral and ovarian cancer patients [138, 139]. In addition, down-regulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [140]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [140]. These observations suggested that CD16 receptors could likely play an important role in target cell induced loss of NK cell cytotoxicity. Indeed, CD16:Ig fusion proteins were shown to bind to a variety of tumor-target cells indicating the existence of specific ligands for CD16 receptors on tumor cells [469]. Furthermore, we have previously shown that the triggering of CD16 on untreated or IL-2 treated NK cells was found to result in down-modulation of CD16 receptors and in a great loss of cytotoxicity in NK cells. In addition, a small subset of NK cells was programmed to undergo apoptosis [136, 137, 141, 142]. Cell death of NK cells was shown to be regulated, in part, by endogenously secreted TNF-α from the NK cells [142]. Previous studies by other groups have also shown that a subset of IL-2 activated NK cells undergo cell death following cross-linking of the CD16 receptor [470, 471]. Addition of antibodies to CD56 or LFA-1 did not cause any decrease in NK cell cytotoxicity demonstrating the specificity of CD16 mAb signaling in mediating inhibition of NK cell cytotoxicity [143]. Thus, we had coined the term “split anergy” for the responses observed by NK cells after their interaction with sensitive target cells or after the triggering of CD16 receptors by the antibody in combination with IL-2 treatment [136, 137, 142, 144, 145]. Indeed, three subpopulations of NK cells; namely Free, Binder

and Killer NK cells with varying degrees of loss of cytotoxicity were identified after the formation of conjugates with K562 targets. Free cells which did not bind or form conjugates with target cells were inactivated less, or exhibited the most cytotoxicity, whereas both Binder, those that bound but did not kill their bound tumors, and Killer subsets, which bound and killed their bound tumors exhibited significant loss of cytotoxicity. In contrast, Binder and Killer subsets but not Free NK subset secreted significant levels of cytokines and exhibited CD16-CD56dim/-CD69+ phenotype [141, 201, 472, 474]. Treatment of NK cells with IL-2 and anti-CD16mAb also induced split anergy by significantly decreasing the NK cell cytotoxicity while increasing the cytokine secretion capabilities of NK cells. Furthermore, NK cells exhibited CD16-CD56dim/-CD69+ phenotype after treatment with the combination of IL-2 and anti-CD16mAb [142-145]. Loss of cytotoxicity in NK cells was significantly exacerbated when NK cells were either treated with F(ab)'$_2$ fragment of anti-CD16 mAb with IL-2 or treated with a combination of MHC-Class I and anti-CD16 mAbs in combination with IL-2 while the same treatments resulted in an increased secretion of cytokines [143, 145]. Based on our recent results, NKp46 mAb were also able to induce significant NK cell anergy in the presence and absence of IL-2 correlating with their increased expression on untreated and IL-2 treated NK cells (manuscript submitted). Moreover, addition of bacteria or their extracts in the presence of CD16 receptor signaling and IL-2 was able to induce synergistic decrease in NK cell cytotoxicity while increasing the induction of cytokine release substantially (manuscript in prep). Because anergy in NK cells is an active process and is induced via signaling receptors such as CD16 and NKp46 and not through LFA1 or LFA3 or CD56 it is likely that binding of agonistic antibodies or their ligands

signal the NK cells to become tolerant in a manner similar to that obtained when anti-CD3 antibody is administered to T cells [517]. In addition, the magnitude of signaling through the receptors on NK cells may determine the extent and levels of anergy induced in NK cells. Therefore, these results suggested that receptor signaling in NK cells via key surface receptors in the presence of IL-2 is likely to result in a rapid loss of NK cell cytotoxicity while continuing to increase secretion of cytokines by the NK cells.

Localization of NK cells within immune rich compartments away from the epithelial tumor nests may provide clues to the significance of split anergy in NK cells induced by other immune effectors

Most NK cells are found within immune rich compartments away from the tumor nests in several tumor types, including oral (Fig. 1A , 1B and 1C), colon (Fig. 1D) , and breast tumors (Fig. 1E)[518-520] . Although some NK cells may be found within the tumor nests, it appears that most cells are seen within the immune compartment associated with other immune effectors such as Mast cells (Fig. 1). This indicates that perhaps NK cells may first encounter and bind to other immune effectors such as Mast cells (Fig. 1) or monocytes, known as Myeloid Derived Suppressor Cells (MDSCs). The interaction of NK cells with MDSCs may in turn induce split anergy in NK cells and condition these cells to become cells that can support differentiation of epithelial tumors, as well as the surrounding immune effectors. Indeed, in normal colon tissue the NK cells are found under the myoepithelial layer bound to Mast cells (Fig. 1D) or in oral mucosa they are found under the basal epithelium where undifferentiated proliferating cells reside (Fig. 1A, 1B and 1C). Likewise, stem cells reside in the basal layer of oral mucosa [521].

Therefore, it is likely that NK cells may be conditioned or anergized by other immune effectors or the effectors of connective tissue to support differentiation of epithelial layer. In cancer, NK cells may obtain entry to the tumor site after the myoepithelial layer forming the tumor capsule is disrupted [518-520], therefore, any NK cells which have escaped from the binding and anergizing effects of MDSCs may be able to lyse some of the tumor stem cells, in addition to inducing differentiation in a fraction of the tumor cells (Fig. 1C, 1D and 1E).

Potential roles of Monocytes and other Myeloid Derived Suppressor Cells in induction of split anergy in NK cells

When either the tumor cells or healthy non-transformed stem cells were cultured with viable or irradiated monocytes before they were exposed to IL2-treated NK cells, a significant decrease in NK cell mediated cytotoxicity could be observed against tumors and healthy stem cells [19]. Interestingly, significant lysis of tumors by untreated NK cells was also reproducibly blocked by the addition of monocytes [19] . We then determined whether decreased lysis of tumors or stem cells by NK cells was due to a competitive lysis of monocytes by the NK cells. We confirmed that monocytes were also lysed by the NK cells significantly. Furthermore, when we co-cultured tumors or healthy stem cells with monocytes and sorted to remove the monocytes from the tumors or stem cells before assessing the killing function of NK cells, we could still observe some inhibition of NK cell mediated lysis, indicating the ability of monocytes to induce protection of tumors or healthy stem cells from NK cell mediated lysis [19]. Therefore, even though lysis of monocytes by the NK cells may in part contribute to the prevention

of NK cell lysis of stem cells, interaction of monocytes with stem cells can also provide resistance of stem cells against NK cell cytotoxicity. Decrease in NK cell lysis of tumors or healthy stem cells was paralleled with a significant induction of IFN-γ. Indeed, when tumors or healthy stem cells were cultured with IL-2 treated NK cells alone we could observe significant induction of IFN-γ secretion. However, synergistic increase was seen when IL2-treated NK cells were cultured with tumors or healthy stem cells in the presence of monocytes. Therefore, although decreased killing of tumors or healthy stem cells by the NK cells could be observed in the presence of monocytes, synergistic secretion of IFN-γ by the NK cells in the presence of monocytes and stem cells could be observed, indicating an inverse relationship between cytotoxicity and IFN-γ secretion (split anergy). This was similar to the profiles which we had seen when NK cells were treated with IL-2 and anti-CD16 mAb in which significant decrease in cytotoxicity of NK cells could be observed in parallel with increased secretion of IFN-γ [142]. Whether Mast cells or other types of MDSCs are able to induce split anergy in NK cells requires further investigation.

The degree of cellular differentiation determines sensitivity to NK cell lysis

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed when NK cells were co-incubated with stem-like OSCSCs which released significantly lower levels of GM-CSF, IL-6 and IL-8 and demonstrated decreased expression of phospho-Stat3, B7H1 and EGFR, and much lower constitutive NFκB activity when compared to differentiated OSCCs. More importantly, OSCSCs expressed CD133 and $CD44^{bright}$ oral stem cell markers [18, 19], whereas differentiated OSCCs express lower

CD44 surface receptors. To assess whether the stage of differentiation of other tumor types also correlated with their sensitivity to NK cell mediated lysis we selected five pancreatic lines at different stages of differentiation based on a number of criteria including sphere formation and immunohistochemical analysis (Sipos, 2003; Deer 2010). Panc-1 and MP-2, two poorly differentiated, BXPC3 and HPAF, two moderately differentiated and CAPAN-1, a well differentiated pancreatic tumors were co-cultured with the NK cells and NK cell mediated cytotoxicity were determined in a 4 hour ^{51}Cr release assay. There was a significant correlation between the stage of differentiation of the tumors and the level of NK cell mediated lysis [236]. The highest NK cell cytotoxicity was obtained against poorly differentiated tumors Panc-1 and MP-2, intermediate lysis against moderately differentiated BXPC3 and HPAF and the lowest lysis was obtained against well differentiated CAPAN-1 cells. In addition, two Glioblastoma Multiforme (GBM) stem-like tumors XO1-NS and XO2-NS which were previously isolated and characterized [259, 466, 467] were found to be significantly more susceptible to NK cell mediated cytotoxicity when compared to differentiated U87 GBM tumors [236]. Since most stem-like tumors or poorly differentiated cells were significantly more susceptible to NK cell mediated cytotoxicity we reasoned that healthy, non-transformed primary stem cells may also be susceptible to NK cell mediated cytotoxicity. We demonstrated previously that NK cells lysed hMSCs, hDPSCs and hESCs significantly (Table 3). All different types of stem cells became resistant to NK cell mediated cytotoxicity once they were differentiated (Table 3). In addition, higher sensitivity of hiPSCs to NK cell mediated lysis was also observed when compared to parental line from which they were derived [18, 19] (Table 3). Differentiation of XO1-

NS and XO2-NS also rendered them more resistant to NK cell mediated cytotoxicity (Table 3, manuscript submitted). Increased lysis of cancer stem cells or non-transformed healthy stem cells may be attributed to the use of allogeneic NK cells, however, our previous work using autologous NK cells exhibited similar levels of cytotoxicity against hDPSCs when compared to lysis by allogeneic NK cells [19]. Taken together these results indicated that undifferentiated cells are targets of both allogeneic and autologous NK cells, and that the degree of cellular differentiation correlates inversely with the level and extent of their susceptibility to NK cell mediated cytotoxicity (Fig. 2).

De-differentiation of the epithelial cells renders them susceptible to NK cell mediated cytotoxicity

Since the degree of differentiation in the cells is predictive of their sensitivity to NK cell mediated cytotoxicity, we reasoned that blocking NFκB in the cells may de-differentiate and subsequently increase their susceptibility to NK cell mediated cytotoxicity. Indeed, blocking NFκB in oral tumors was found to increase CD44 surface receptor expression, which is one of the hallmarks of stem cells (unpublished results). In addition, blocking of NFκB nuclear function in a primary Oral tumor OSCCs and in a non-tumorigenic oral cells (HOK-16B) as well as in an established tumor line, HEp-2 cells known to be Hela contaminant [193, 457-459], augmented cytotoxicity and the release of key cytokines such as IFN-γ from the NK cells [192, 193]. Similarly, inhibition of NFκB by Sulindac increased the functional activation of NK and enhanced anti-tumor cytotoxic activity [192, 193].

In agreement with our studies, targeted deletion of Iκκ−β in epidermis of mice has previously been shown in one study to lead to inflammatory skin manifestations [153]. Elevated levels of cytokines and chemokines have also been demonstrated in the epidermis of patients and animals with Iκκγ and Iκκβ deletions [153, 460]. Mice with a keratinocyte-specific deletion of Iκκ−β demonstrated decreased proliferation of epidermal cells, and developed TNF-α dependent inflammatory skin disease [153]. In contrast, in other studies in which NFκB function was blocked in dermal keratinocytes by a mutant IκB-α an increased proliferation and hyperplasia [463] and eventual development of cutaneous squamous cell carcinomas of skin were seen if mice were allowed to survive and reach adulthood [464, 465].

Since tumorigenic and non-tumorigenic human oral keratinocytes acquire sensitivity to NK cell mediated lysis when NFκB is inhibited, it is likely that this phenomenon is not specific to cancer or oral keratinocytes, and it may occur in other healthy non-transformed cell types. Indeed, when human primary monocytes were differentiated to dendritic cells they too became more resistant to NK cell mediated cytotoxicity [18] . Moreover, knock down of COX2 in primary mouse monocytes, or in mouse embryonic fibroblasts (unpublished observations), resulted in the reversion or de-differentiation of the monocytes and fibroblasts respectively, and the activation of NK cell cytotoxicity. Indeed, it is likely that any disturbance in cellular differentiation may predispose the cells to NK cell mediated cytotoxicity. Since STAT3 is an important factor increased during differentiation, blocking STAT3 is also critical in the activation of immune effectors [211]. In support of a critical role of STAT3 in immune evasion of

tumor cells in humans, we and others have recently shown that GBM tumors display constitutive activation of STAT3 (Cacalano and Jewett, unpublished observation) [160], and poorly induce activating cytokines and tumor-specific cytotoxicity in human peripheral blood mononuclear cells (PBMCs) and NK cells. Ectopic expression of dominant-negative STAT3 in the GBM tumors increased lysis of the tumor cells by the immune effectors and induced production of IFN-γ by the interacting immune effectors (unpublished observations).

Since NFκB is shown to regulate IL-6 secretion in OSCCs, HOK-16B and HEp2 cells and secreted IL-6 in tumors is known to activate STAT3 expression and function, increase in NFκB nuclear function could in turn induce STAT3 activation and result in a significant resistance of tumors to NK cell mediated cytotoxicity. Indeed, inhibition of NFκB in oral tumors resulted in a significant decrease in IL-6 secretion by the tumor cells and the induction of IFN-γ secretion by the NK cells [19, 192] . Therefore, targeted knock down of STAT3 or signaling pathways upstream of STAT3, such as NFκB, may de-differentiate the cells and predispose the cells to NK cell mediated cytotoxicity.

Differentiation of the cells as a potential mechanism for the immuno-selection

The three stages of selection, equilibrium and escape has been hypothesized to govern the interaction of immune cells with their respective tumors [421, 422]. Specific interaction of healthy stem cells with the immune cells has potential basis on selection, differentiation and resolution of inflammation. Our findings demonstrate that there are two distinct mechanisms for the selection of stem cells; 1-mediated by the

perforin/granzme lysis and 2-mediated by the synergistic action of cytokines induced by the NK cells which eliminate poorly differentiating and highly differentiating target cells respectively leaving those that perhaps have intermediate phenotype (manuscript in prep). In this regard at the initial stages of differentiation, some of the stem cells may be selected by the granule exocytosis. Those which survive the process of initial elimination by the NK cells will differentiate by the aid of cytokines produced by anergized NK cells. The second mechanism of selection will take place through the synergistic action of cytokines in which case either those that have decreased capacity to differentiate are eliminated or those which are highly differentiated are subject to activation induced cell death. At present, we are unable to differentiate between the two modes, although we believe that the activation induced cell death may likely be the mechanism by which highly differentiated cells are eliminated. Thus, it appears that selection by the immune cells favor those that may not only have defects in differentiation but also those that perhaps have performed their tasks and are no longer needed to remain in the environment.

Not all types of differentiated cells undergo selection through the function of cytokines. Our data demonstrated that oral tumors were clearly more sensitive to increased induction of death by the synergistic action of cytokines since the addition of the combination of cytokines induced cell death in a fraction of tumor cells. In contrast, lung tumors were induced to differentiate but did not undergo cell death by the addition of cytokines (manuscript in prep). Indeed, both oral and lung tumor stem cells were susceptible to NK cell mediated lysis and both differentiated when either supernatants or

fixed NK cells were added, however, only oral tumors were induced to undergo cell death by the action of cytokines.

Split anergy in NK cells is a potential mechanism for the induction of target cell differentiation whereby cells become resistant to the function of NK cells; switch of NK cell function from effector to regulatory cells

Induction of split anergy in NK cells could be an important conditioning step responsible for the repair of tissues during pathological processes irrespective of the type of pathology. In tumors since the generation and maintenance of cancer stem cells is higher, the majority, if not all of the NK cells, may be conditioned to support differentiation and as such the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble that of the anergic NK cells, i.e., decreased NK cell cytotoxicity, acquisition of CD16-/dimCD56dim/+CD69+ phenotype and augmented ability to secrete inflammatory cytokines. Of course, the degree of the loss of NK cell cytotoxicity may be directly proportional to the load of cancer stem cells, stage of the disease and their metastatic potential. Therefore, our results suggest two very important functions for the NK cells. One function is to limit the numbers of proliferating stem cells and immune inflammatory cells by selecting those with a greater potential to differentiate and second to support differentiation of the stem cells and subsequent regeneration of the tissues and the resolution of inflammation. To achieve these tasks NK cells have to acquire two different phenotypes, and be conditioned to carry out both functions successfully. CD16+CD56dimCD69- subsets of NK cells are cytotoxic and will mediate cytotoxicity depending on which cells they encounter first. As mentioned above, in

respect to the oral squamous cell carcinomas since the majority of immune effectors are found at the connective tissue area the chances are that they may first encounter and interact with either the other immune effectors or the effectors of connective tissue such as fibroblasts. However, there is also the possibility that NK cells may first encounter the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they too can become anergized. Surprisingly, allogeneic CTLs were also found to target Glioblastoma stem-like cells and not their differentiated counterparts (unpublished observation). By eliminating a subset of stem cells or after their interaction with other immune inflammatory cells or effectors of connective tissue NK cells could then be in a position to support differentiation of selected population of stem cells since they will be conditioned to lose cytotoxicity, induce cytokine and growth factor secretion and gain the CD16-/dimCD56dim/+CD69+ phenotype. It is interesting to note that all of the immune effectors isolated from oral gingival tissues of healthy as well as diseased gingiva have CD69+ phenotype, with the exception that the numbers of immune effectors are much less in the healthy oral gingival tissues when compared to diseased tissues [237]. Therefore, our results suggest two very important functions for the NK cells. One function is to kill and the other function is to support differentiation for the repair and regeneration of the tissues.

In vivo physiological relevance of above-mentioned observations could be seen in a subpopulation of NK cells in peripheral blood, uterine and liver NK cells which express low or no CD16 receptors, have decreased capacity to mediate cytotoxicity and is capable of secreting significant amounts of cytokines [213, 214]. In addition, 70% of NK cells

become CD16 dim or negative immediately after allogeneic or autologous bone marrow transplantation [213]. Since NK cells lose their cytotoxic function and gain in cytokine secretion phenotype and down modulate CD16 receptors after their interaction with tumor cells or the stem cells [137, 142], it is tempting to speculate that in vivo identified CD16- NK cells and in vitro tumor induced CD16- NK cells have similar developmental pathways since they have similar if not identical functional properties.

This hypothesis was supported by the observation that anergized NK cells were directly responsible for the increased differentiation and resistance to NK cell mediated cytotoxicity of a number of different stem cells including cancer stem cells and dental pulp stem cells (manuscript submitted). As presented in Fig. 3 when OSCSCs were cultured with supernatants or paraformaldehyde fixed NK cells (manuscript submitted) treated with IL-2 and anti-CD16 mAb, they became resistant to cytotoxicity mediated by freshly isolated untreated or IL-2 treated NK cells. IL-2 treated NK cell supernatants or paraformaldehyde fixed IL-2 treated NK cells were also able to impart some resistance to OSCSCs but the highest levels of resistance were achieved when NK cells were treated with IL-2 and anti-CD16mAb (Fig. 3). No significant differences in resistance of OSCSCs to NK cell mediated lysis can be achieved by either culturing the OSCSCs with the supernatants or cells from untreated or anti-CD16mAb treated NK cells (Fig. 3). The resistance in OSCSCs induced by anergized NK cells correlated with a decrease in CD44 receptor expression and in an increase in B7H1 expression (manuscript submitted), two surface receptors which were inversely expressed in differentiated OSCCs and in OSCSCs [18]. In addition, we now have evidence which supports the notion that the

induction of anergy in NK cells is an active process which is induced by the triggering of CD16 receptor on the NK cells, and is regulated by the functions of cystatins and cathepsins, and is not due to degranulation and exhaustion of cytotoxic granules (unpublished results).

Our work collectively suggests that anergized NK cells are as important as the non-anergized NK cells in their effector functions. NK cells are not only important for the removal and shaping of the size of the stem cells which is mediated by the effector NK cells but also their differentiation, and the eventual regeneration of the new tissues which is mediated by their switch to anergized/regulatory NK cells. The task of NK cells in this regard goes above and beyond their most appreciated function of being the effectors of first line defense against viral infection and malignancies. They too can be effectors of differentiation and tissue regeneration.

Similarities in immune cell effector function in inflammatory tumor microenvironment and in non-transformed inflammatory microenvironment

The concept of tumor immunosurveillance has previously been expanded to include immunoediting as an important mechanism for the development of cancer [421, 422]. It was suggested that cancer immunoediting comprises of three phases: elimination, equilibrium and escape [422]. Elimination represents the classical concept of immunosurveillance. However, during equilibrium and escape the interaction and cross signaling between the immune effectors including NK cells, the tumor cells, and perhaps the effectors of the connective tissue in the tumor microenvironment may result in the

generation of tumors which are capable of gradual suppression of the NK cell cytotoxic function. The final stages of cancer development may result in the induction of resistant tumors in the presence of fewer immune effectors capable of lysing the tumors [422]. Thus, pressures exerted by the tumor cells and immune effectors may eventually shape the microenvironment for the growth, expansion and invasion of tumors. Similarly, a variation of such interactions may also be observed during the interaction of NK cells with healthy non-transformed human stem cells in non-transformed inflammatory microenvironment in which case the three phases of interaction may include elimination which marks the decrease in the numbers of proliferating stem cells or other immune effectors in the inflammatory microenvironment, potentially resulting in the selection of stem cells by the NK cells, induction of tolerance or anergy which denotes the conditioning of NK cells by the stem cells and/or by the other effectors of microenvironment to become regulatory cells and support maturation and differentiation of remaining stem cells, and finally the resolution phase which denotes the elimination of anergized NK cells and generation of less immunogenic differentiated cells.

Immunosuppressive effectors in tumor and in non-transformed inflammatory microenvironment

Both the tumor microenvironment as well as non-transformed inflammatory microenvironment consists of a number of heterogeneous cell populations with ability to suppress and limit the function of cytotoxic immune effectors. Patients with cancer often have higher numbers of immature monocytes serving as Myeloid Derived Suppressor Cells (MDSCs) expressing CD14+HLADR- phenotype [227, 522] . Tumor associated

Macrophages (TAMs) were previously shown to significantly influence and limit immune activation in the tumor microenvironment [454, 523]. In addition, MDSCs which are comprised of a number of distinct cell populations of myeloid origin and whose roles in immunosuppression have received significant attention in recent years are major cells capable of suppressing the cytotoxic function of T and NK cells [522] . As mentioned above, NK cells are found to be in close proximity and often bound to Mast cells, a subset of MDSCs in both healthy and transformed inflammatory microenvironments (Fig. 1). T cell dysfunction is shown to be induced by MDSCs by the increased secretion of IL-10, TGF-β, induction of reactive oxygen species (ROS), and increased expression of arginase-1 and inducible nitric oxide synthase (iNOS). T regulatory (Treg) and DC regulatory (DCreg) cells were also recently shown to have significant immunosuppressive roles in the tumor microenvironment [522] . Perhaps one of the most intriguing observations regarding the immunosuppressive effectors is the identification of Cancer Associated Fibroblasts (CAFs) and Mesenchymal Stem Cells (MSCs) as two potential tumor promoters. Fibroblasts from tumor tissues demonstrate an activated phenotype and have the ability to secrete many immunosuppressive factors such as TGF-β and VEGF [456 2011]. We have also found that undifferentiated fibroblasts, as well as MSCs and CD14+HLA-DR- monocytes are significantly more susceptible to NK cell mediated cytotoxicity [19] , therefore, these cells may condition NK cells to undergo split anergy and become regulatory NK cells. Indeed, as shown above in oral epithelial tumors the majority of recruited immune effectors are usually found in the connective tissue area where through cell-cell interaction with the immunosuppressive cells such as fibroblasts, monocytes-macrophages and to a lesser extent T and B cells [19] can

generate regulatory NK cells, resulting in differentiation and resistance of oral epithelial tumors.

Tumor microenvironment may shape the function and phenotype of the NK cells

Based on the work presented in this review, it is possible that the resident and recruited immune effectors in tumor microenvironment such as monocytes may serve as shields against NK cell lysis of cancer stem cells. Monocytes can shield cancer stem cells from killing by the NK cells by increasing the total IFN-γ release from the NK cells while decreasing the cytotoxic function of NK cells, resulting in an increased protection and differentiation of cancer stem cells. Indeed, monocytes also increase TNF-α, IL-6 and VEGF secretion in the co-cultures of cancer stem cells with NK cells which could augment NFκB and increase differentiation of cancer stem cells. The shielding effect of monocytes could be a more generalized function of other effectors since NK cells can also target fibroblasts [19]. Whether other MDSCs such as PMNs can also be targeted by the NK cells awaits future investigation. This may have significant implications for the role of NK cells in not only limiting inflammation, but also the significance of other immune effectors in shielding and limiting the cytotoxic function of NK cells against cancer or healthy stem cells in order to raise maximally the secretion of key cytokines for speedy and optimal differentiation of cancer stem cells during inflammation (Fig. 4). This is precisely what is observed in cancer patients in whom global decrease in NK, cytotoxic T cells and monocytes have all been reported [180].

Potential functional similarities between induced regulatory NK cells and T regulatory cells

Because of their ability to drive differentiation, anergized NK cells may have the ability to halt inflammation since differentiated cells are no longer targeted by the NK cells and T cells and they do not induce cytokine secretion by the NK and T cells (Fig. 4) [18, 19]. This function of NK cells is similar to T regulatory cells since they are inhibitory and are capable of decreasing the magnitude of inflammation in a number of previous studies. Therefore, although immunosuppression in the tumor microenvironment is not advantageous for the patient, it is indeed, an important function which may not only stimulate differentiation, but it may also halt inflammation.

The majority if not all of the effectors in the mucosal immune system including in the oral cavity are of activated phenotype, ie, they express CD69 early activation antigen. These cells, including NK cells are likely conditioned in the mucosa to support differentiation and resistance of the epithelial cells. Such environment is anti-inflammatory since the majority of immune cells is tolerant of ingested food particles and self-tissues and is known to contain many regulatory cells including T cells, dendritic cells and likely regulatory NK cells. However, once the threshold which keeps the inflammation at bay is decreased in the mucosa, immune effectors are activated and may cause tissue damage and establishment of chronic inflammation. Indeed, in this regard our preliminary *in vivo* observations in humans consuming a combination of proprietary probiotic bacterial strains with potent ability to condition NK cells to support differentiation of OSCSCs and hDPSCs (manuscript in prep) was able to relieve chronic

inflammation and pain, and resulted in the resolution of inflammatory mouth ulcers, and oral edema. Furthermore, the number of neutrophils in the blood of a donor who had chronically decreased levels of neutrophils rose to the normal levels and both the numbers and function of NK cells in the blood improved substantially after the consumption of probiotic bacterial strain.

Our results in the conditioning of NK cells to become regulatory NK cells with receptor signaling in the presence of LPS and IL-2 is in line with the numerous anti-inflammatory benefits which are achieved by the consumption of probiotic bacteria in the gut.

Conclusions

Much work has been done to identify strategies by which tumor cells evade the function of immune system. Altered expression of MHC molecules which block recognition and activation of T and NK cells are examples of mechanisms by which tumor cells evade the function of immune system. In addition, tumor cells by releasing immunosuppressive factors such as Fas, VEGF, IL-6, IL-10, TNF-α, GM-CSF and IL-1β, induce T and NK cell apoptosis, block lymphocyte homing and activation, and dampen macrophage and dendritic cell function. However, the same effector functions are also important in tissue repair.

Based on the accumulated work presented in this review, we suggest that NK cells may have two significant functions; one that relates to the removal of excess proliferating stem cells and their subsequent selection. In this regard, NK cells could also lyse other effectors in the connective tissue area in order to not only decrease inflammation but also to be conditioned to promote differentiation and resistance of selected stem cells and eventual regeneration of the tissues (Fig. 4). The second important task for NK cells is therefore, to support differentiation and promote tissue regeneration after altering their phenotype to cytokine secreting cells (Fig. 4). This process will not only remove cells that are perhaps damaged and have flaws in the differentiation process or in general are more than needed, but also it will ensure the proper regeneration of tissues and the resolution of inflammation. Thus, any disturbance in the NK cell function or in the process of selection and differentiation of stem cells may result in chronic inflammation,

causing continual tissue damage and recruitment of immune effectors to aid in tissue regeneration.

The inability of patient NK cells to contain cancer stem cells due to the flooding of NK cells by proliferating cancer stem cells and conversion of NK cells to regulatory NK cells may likely be one mechanism by which cancer may progress and metastasize. Therefore, there should be two distinct strategies by the NK cells to eliminate tumors, one which targets stem cells and the other which targets differentiated cells. Since cancer stem cells were found to be more resistant to certain chemotherapeutic drugs but sensitive to NK cell mediated killing while differentiated oral tumors were more resistant to NK cell mediated killing but relatively more sensitive to chemotherapeutic drugs, combination therapy should be considered for the elimination of both undifferentiated and differentiated tumors. In addition, since a great majority of patient NK cells may have switched to regulatory function to support differentiation of the proliferating cancer stem cells, they may not be effective in eliminating the cancer stem cells. Therefore, these patients may benefit from repeated allogeneic NK cell transplantation for elimination of cancer stem cells. In this regard, depletion of immunosuppressive effectors in the tumor microenvironment, which condition NK cells to become regulatory cells, via radiation or chemotherapeutic drugs should in theory provide a better strategy for successful targeting of tumors by the NK cells.

Table 1- List of NK cell Activating and Inhibitory surface receptors and their ligands

Receptors	Ligands
Activating/inhibitory Receptors	
FcγRIII (CD16)	Fc of antibodies
CD2	CD58 (LFA-3)
LFA-1	ICAM-1
2B4	CD48
CD69	Unknown
DNAM-1 (CD226)	CD112, CD155
NKp80	AICL
Tactile (CD96)	CD155, CD111
TIGIT	CD112,CD113,CD155
CRTAM	TSLC1
C-type Lectin receptors –Activating/Inhibitory	
CD94/NKG2A/B	HLA-E
NKG2D	MICA, MICB, ULBP-1, ULBP -2, ULBP -3, ULBP -4, ULBP -5, ULBP -6
CD94/NKG2C	HLA-E
CD94/NKG2E/H	HLA-E, Qa-1b
Natural cytotoxicity receptors (NCR)	
NKp46 (NCR1)	Viral Hemagglutinin
NKp44 (NCR2)	Viral Hemagglutinin
NKp30 (NCR3)	B7h6, HCMV-pp65
Killer IG-like (KIR) – Activating/Inhibitory	
KIR2DLs, KIR3DLs, KIR2DS	HLA-C, HLA-B, HLA-A, HLA-G
Cytokines, growth factors and chemokines	Cytokines, growth factors and chemokine ligands
Toll-like receptors (TLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR)	Bacterial DNA, LPS, peptidoglycan, teichoic acids, flagellin, pilin, viral dsRNA and fungi zymosan

Table 2- Susceptibility of a number of different stem cells but not their differentiated counterparts to NK cell mediated cytotoxicity.

Type of cells	Stem cells	Differentiated	Parental cells
hESCs	++++++	+	
hMSCs	++++++	+	
hDPSCs	++++++	+	
hiPSCs	++++++		+
GBMs	++++++	+	

The susceptibility of a number of stem cells and their differentiated counterparts and the parental cells from which hiPSCs were derived to NK cell mediated cytotoxicity was determined using standard 4 hour 51Cr release assay [18]. The higher the number of + signs the more susceptibility to NK cell mediated cytotoxicity.

Table 3. Inverse association of target cell susceptibility to NK cell lysis and its ability to inactivate the cytotoxic function of NK cells

Targets	Sensitivity to NK cell lysis	NK cell cytotoxicity against K562 after exposure and dissociation from the targets (LU30/10^6 cells)
K562	+++++	1.7(+)
U937	+++++	5(+)
222	++++	13(++)
PA1	+++	28(+++)
Daudi	++	38(++++)
SKOV.3	++	44(++++)
Raji	+	62(+++++)
Media	-	131

Target cell sensitivity (K562, U937, 222, PA1, Daudi, SKOV3, and Raji) to NK cell lysis was determined in a 4-h ^{51}Cr release assay as shown in the middle column. The signs +++++ to + represents high to low sensitivity to lysis of target cells listed in left column by the NK cells. NK cells were then treated with IFN-α (1000 u/ml) and cultured with each of K562, U937, 222, PA1, Daudi, SKOV3, and Raji. After an overnight incubation, the NK cells were dissociated from the target cells and added to ^{51}Cr labeled K562 target cells and cytotoxicity assessed in a 4h ^{51}Cr release assay. One Lu30/10^6

represents the number of effector cells required to lyse 30% of target cells. The signs +++++ to + in the right column represents high to low sensitivity of K562 to lysis by the target cell inactivated NK cells.

Figure legends

Fig. 1. Immunohistochemical analysis of oral, colon, and breast tumor tissues

(Fig. 1A) Healthy inflammatory oral tissue was stained with antibodies to CD16 (Brown), Mast cells (Blue) and Smooth Muscle Actin (SMA) (red). Immunohistochemical analysis indicated that NK cells (thick arrows) and Mast cells (thin arrows) are located underneath the base of the normal oral epithelium (star), immediately below the basal layer where stem cells and undifferentiated epithelial cells reside. Please note that epithelial layer is devoid of the infiltrating immune cells. (Fig. 1B) The slides from OSCC were prepared and stained with H&E. Infiltration of immune effectors right beneath the epithelial layer can be seen in the connective tissue area where the immune inflammatory cells are likely to condition NK cells to lose cytotoxicity and to support differentiation of epithelial cells. (Fig. 1C), oral tumor tissue was stained with the antibodies against CD16, Mast cells and SMA as indicated above. Immunohistochemical analysis indicated that NK cells (thick arrows) and Mast cells (thin arrows) are located along the oral tumor capsules, with only few infiltrating immune cells seen within the epithelium (star). (Fig. 1C), Colorectal tumor tissue is stained as described above. Immunohistochemical analysis indicated that NK cells (thick arrows) and Mast cells (thin arrows) are located along the colorectal epithelial capsules, with only few infiltrating immune cells seen within the normal (black stars) and tumor (yellow star) epithelium. (Fig. 1D), breast tumor tissue is stained as described above. Immunohistochemical analysis indicated that NK cells (thick arrows) and Mast cells (thin arrows) are located

along the breast tumor capsules, with only few infiltrating immune cells seen within the epithelium (star).

Fig. 2. Inverse association between cellular differentiation and NK cell cytotoxicity

Based on the accumulated data from our laboratory inverse relationship between cellular differentiation and NK cell mediated cytotoxicity is directly proportional.

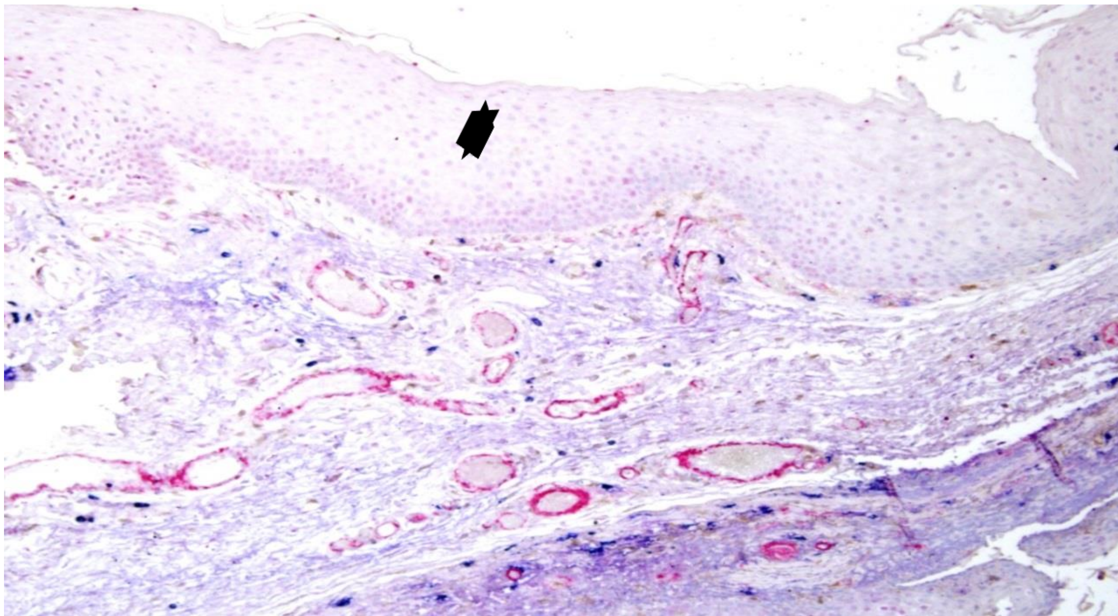
Fig. 3. Supernatants from anergized NK cells induce the highest resistance of OSCSCs against NK cell mediated cytotoxicity.

Highly purified NK cells at ($1X10^6$ cells/ml) were either left untreated or treated with IL-2 (1000u/ml), anti-CD16 mAb (3µg/ml) or a combination of IL-2 (1000u/ml) and anti-CD16 mAb (3µg/ml) for 24 hours before they were harvested and used to induce differentiation of OSCSCs. OSCSCs at $1X10^6$ cells were added to each plate in 10 ml of media and the cells were allowed to adhere before the NK cell supernatants were added to each plate. A total of 180 microliter of supernatants were added at day 1, 3 and 5 and the levels of NK cell cytotoxicity were determined using freshly isolated untreated (Fig. 2A) and IL-2 treated (1000u/ml) (Fig. 2B) NK cells in a 4 hour 51Cr release assay on the 6th day.

Fig. 4. Hypothetical model of induction of regulatory NK cells by immune inflammatory cells and by the effectors of connective tissue to support differentiation of the non-transformed stem cells and cancer stem cells.

Hypothetical model of NK cell conditioning in the tumor microenvironment as well as in non-transformed immune inflammatory microenvironment is shown in this figure. Significant infiltration of immune effectors right beneath the epithelial layer can be seen in the connective tissue area where the immune inflammatory cells are likely to condition NK cells to lose cytotoxicity and gain the ability to secrete cytokines, a term which we have previously coined as split anergy in NK cells, and to support differentiation of the basal epithelial layer containing stem cells. NK cells are likely to encounter and interact with the other immune effectors such as monocytes or other myeloid-derived suppressor cells (MDSCs), and in tumor microenvironment with the tumor-associated macrophages (TAMs), or with connective tissue-associated fibroblasts (CAF) in order to be conditioned to form regulatory NK cells (NK_{reg}). NK cells may also directly interact with the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they can become conditioned to support differentiation of other stem cells. In addition, bacteria through the binding to Toll like receptors can further aid in the generation of $NK_{reg.}$ All the above mentioned mechanisms may be operational during inflammatory processes in the tumor microenvironment or in healthy non-transformed inflammatory microenvironment. NK cell-differentiated epithelial cells will no longer be killed or induce cytokine secretion by the NK cells, therefore, resulting in the resolution of inflammation.

Fig. 1A



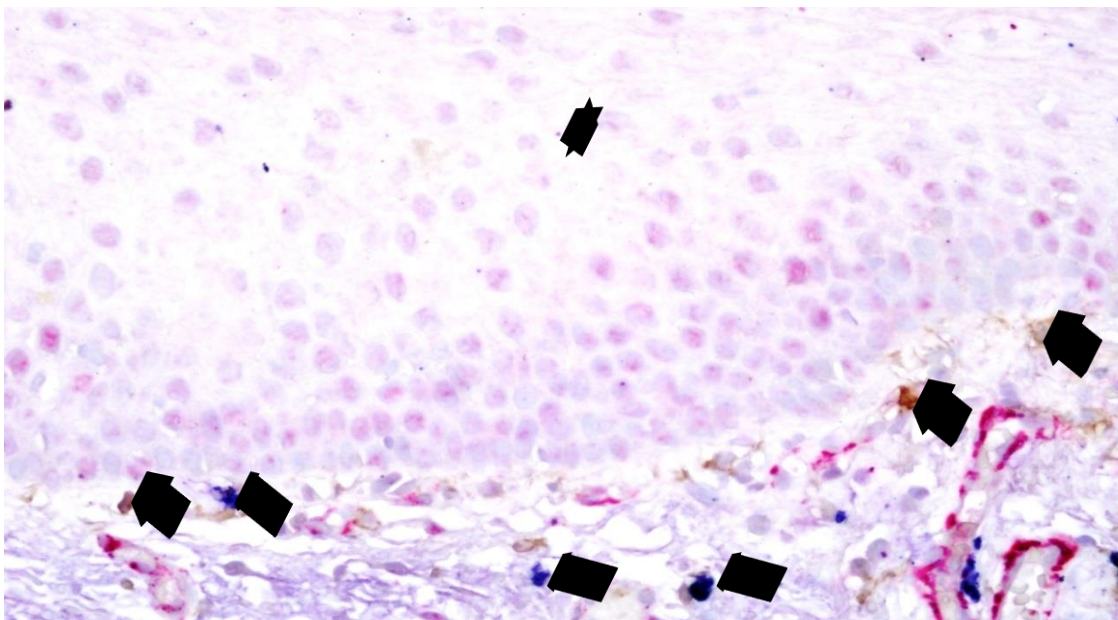


Fig. 1B

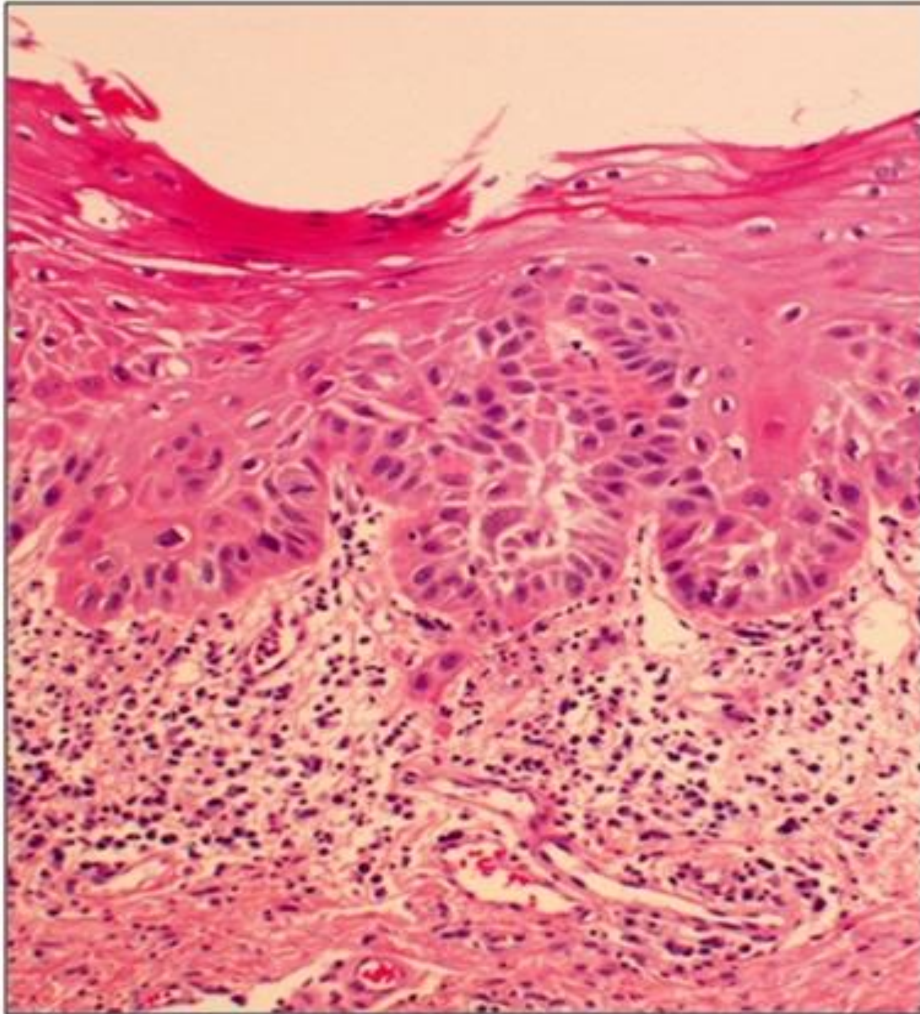
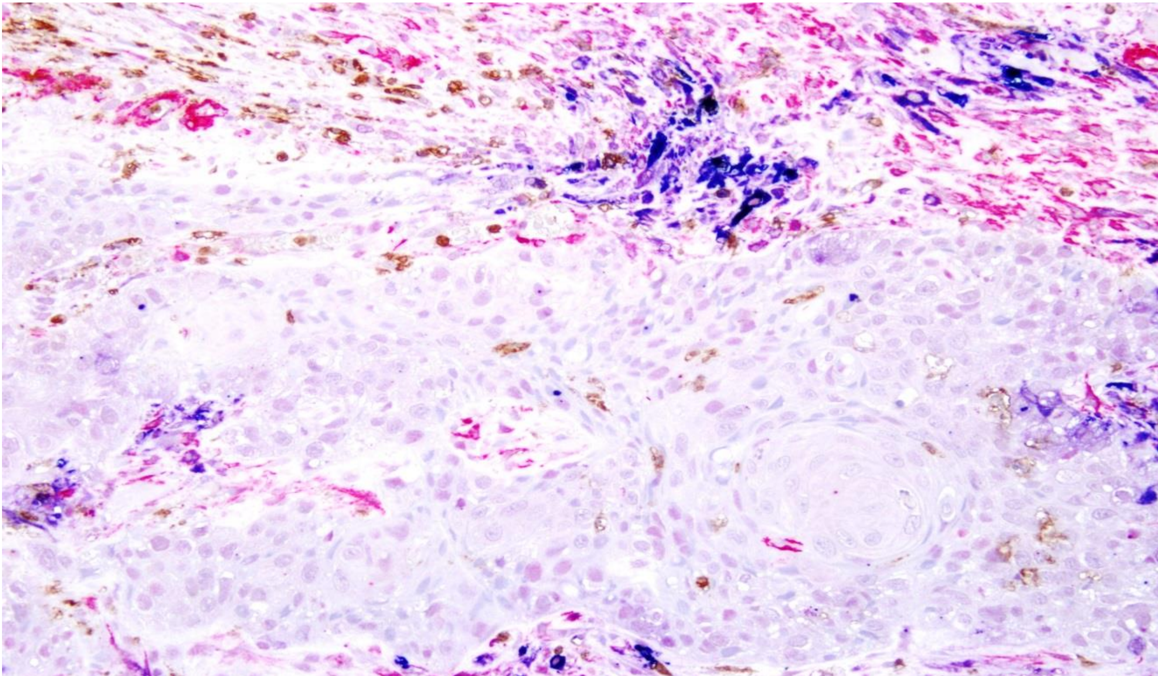


Fig. 1C



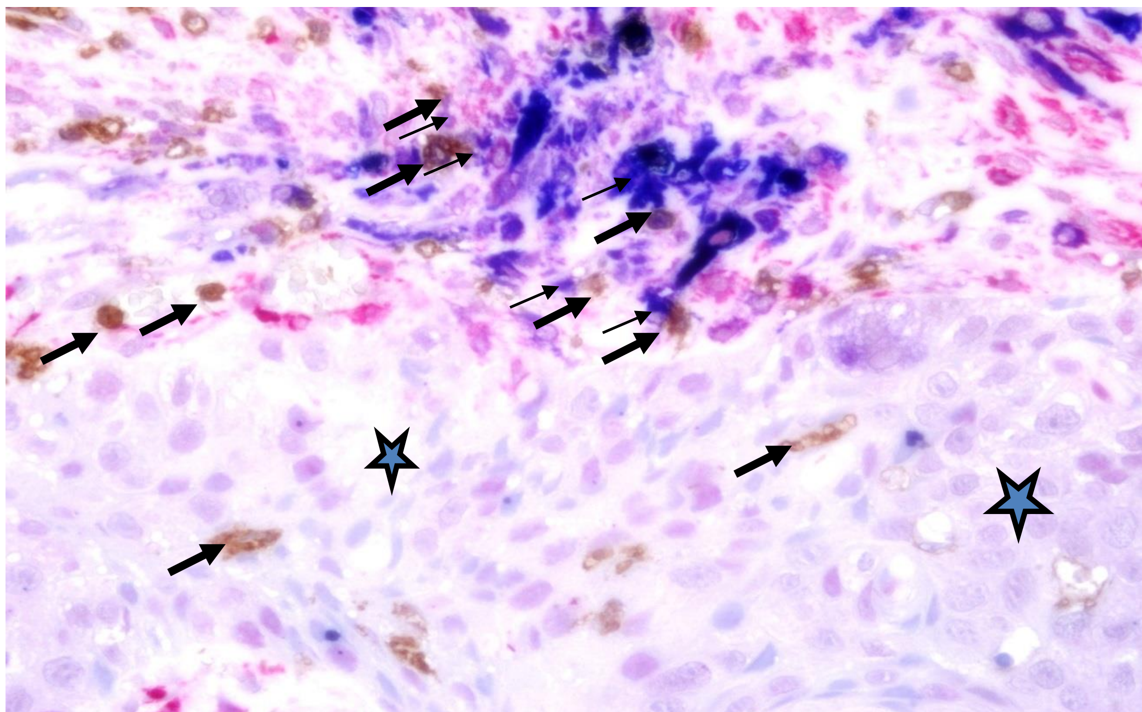
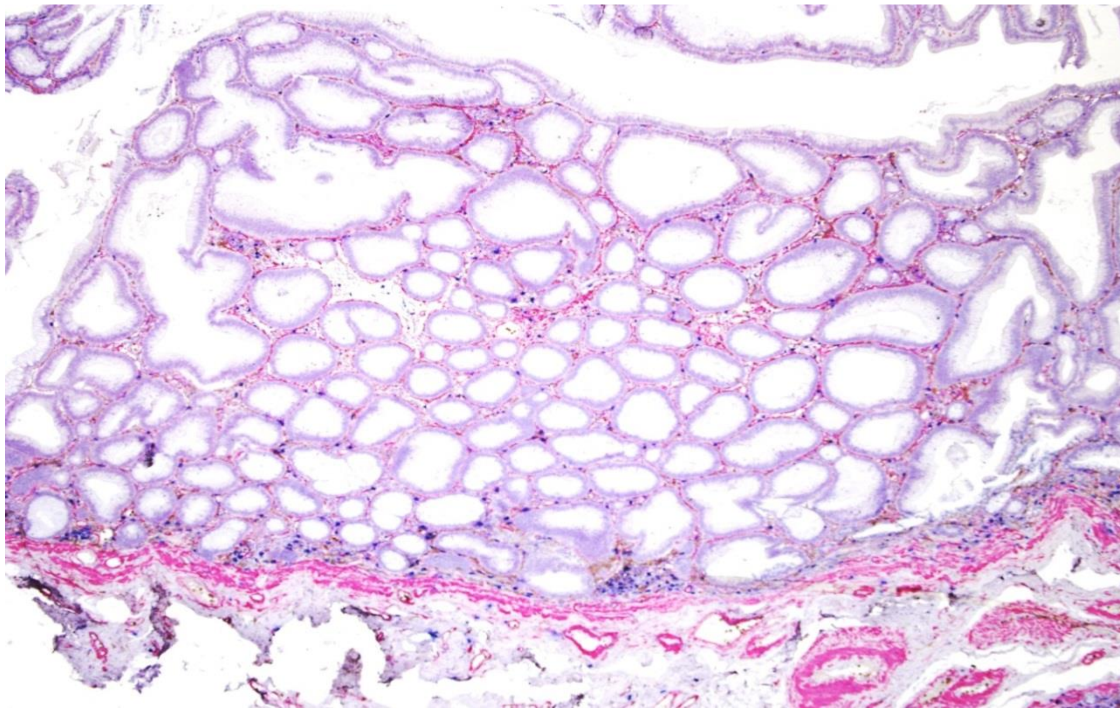


Fig. 1D



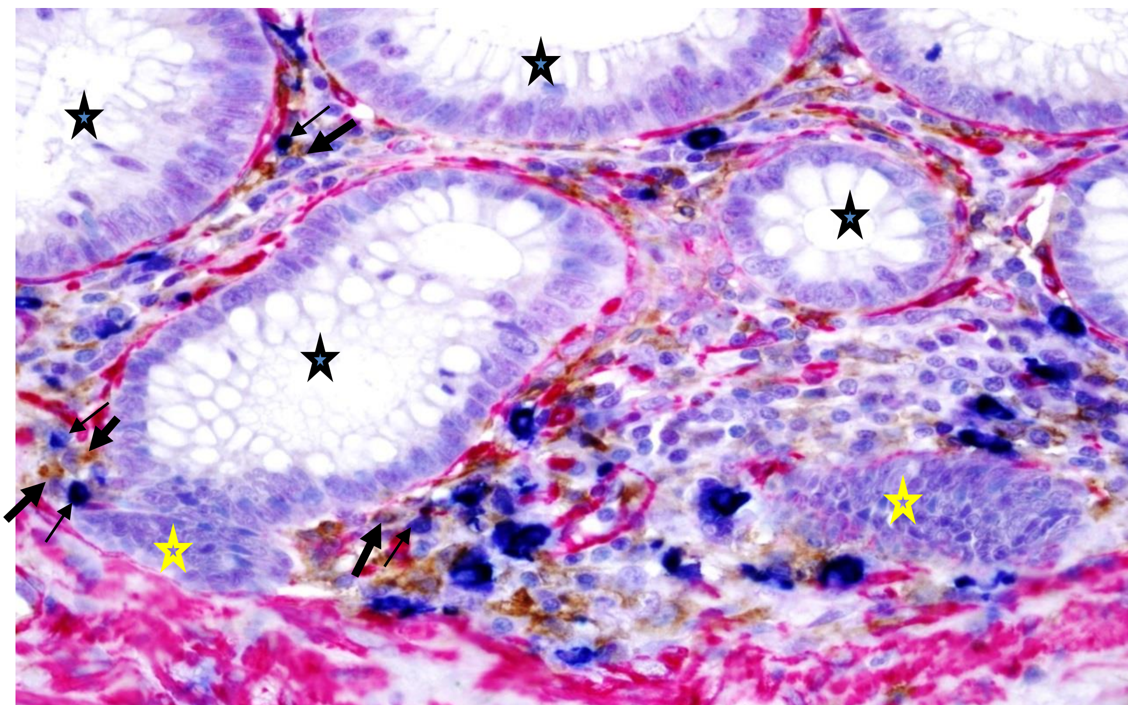


Fig. 1E

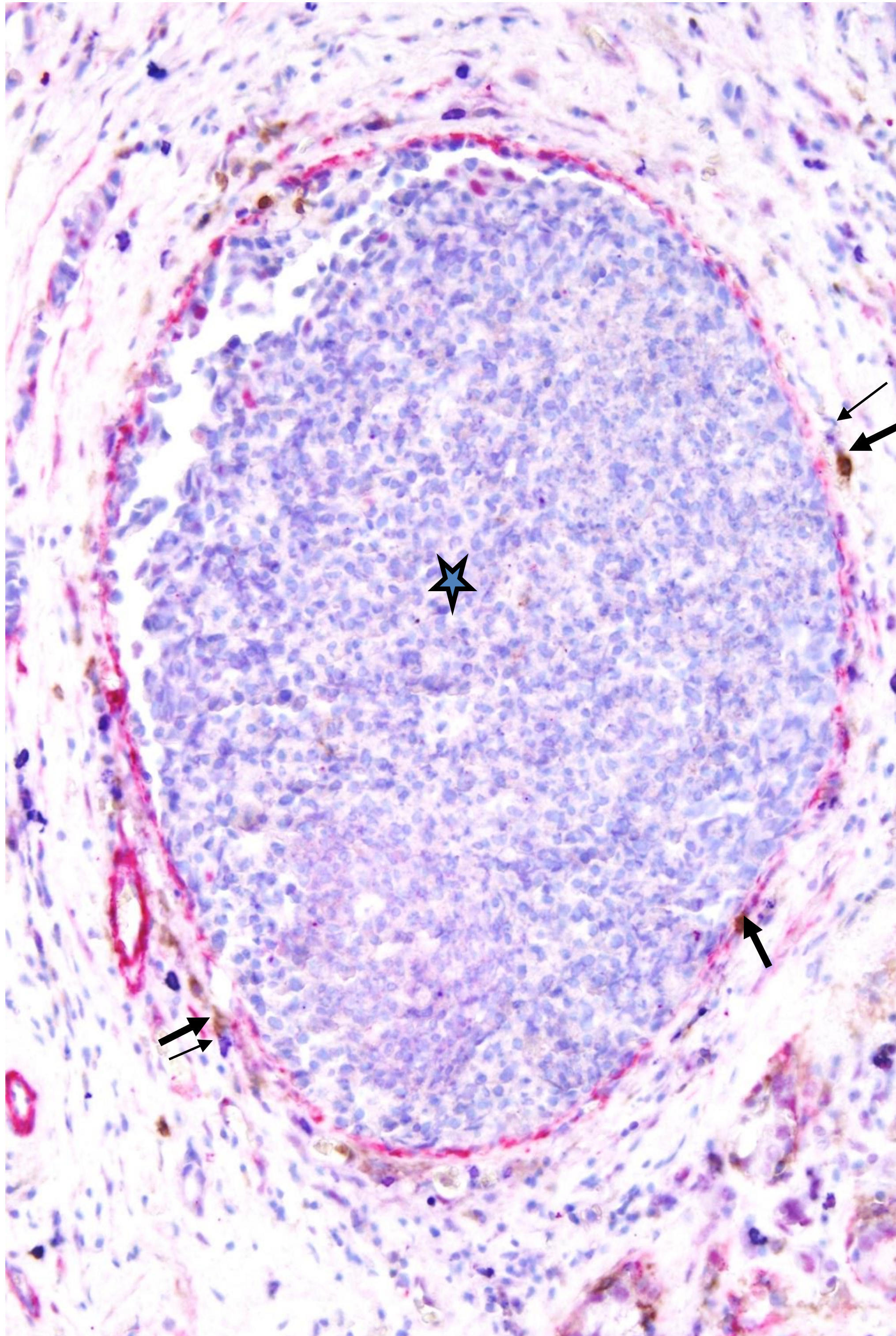


Fig. 2

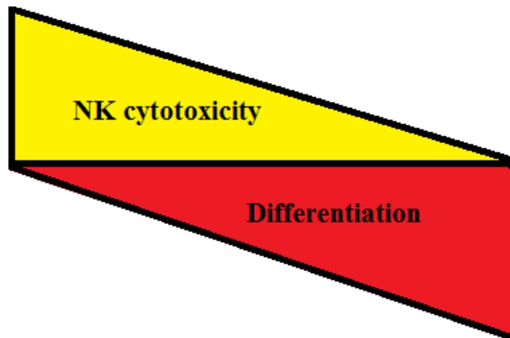


Fig. 3

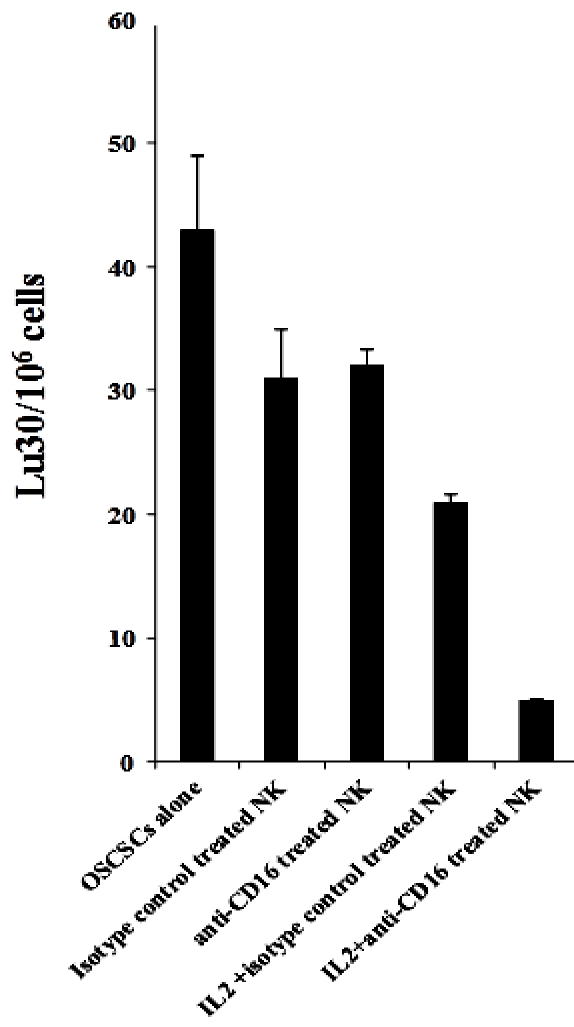
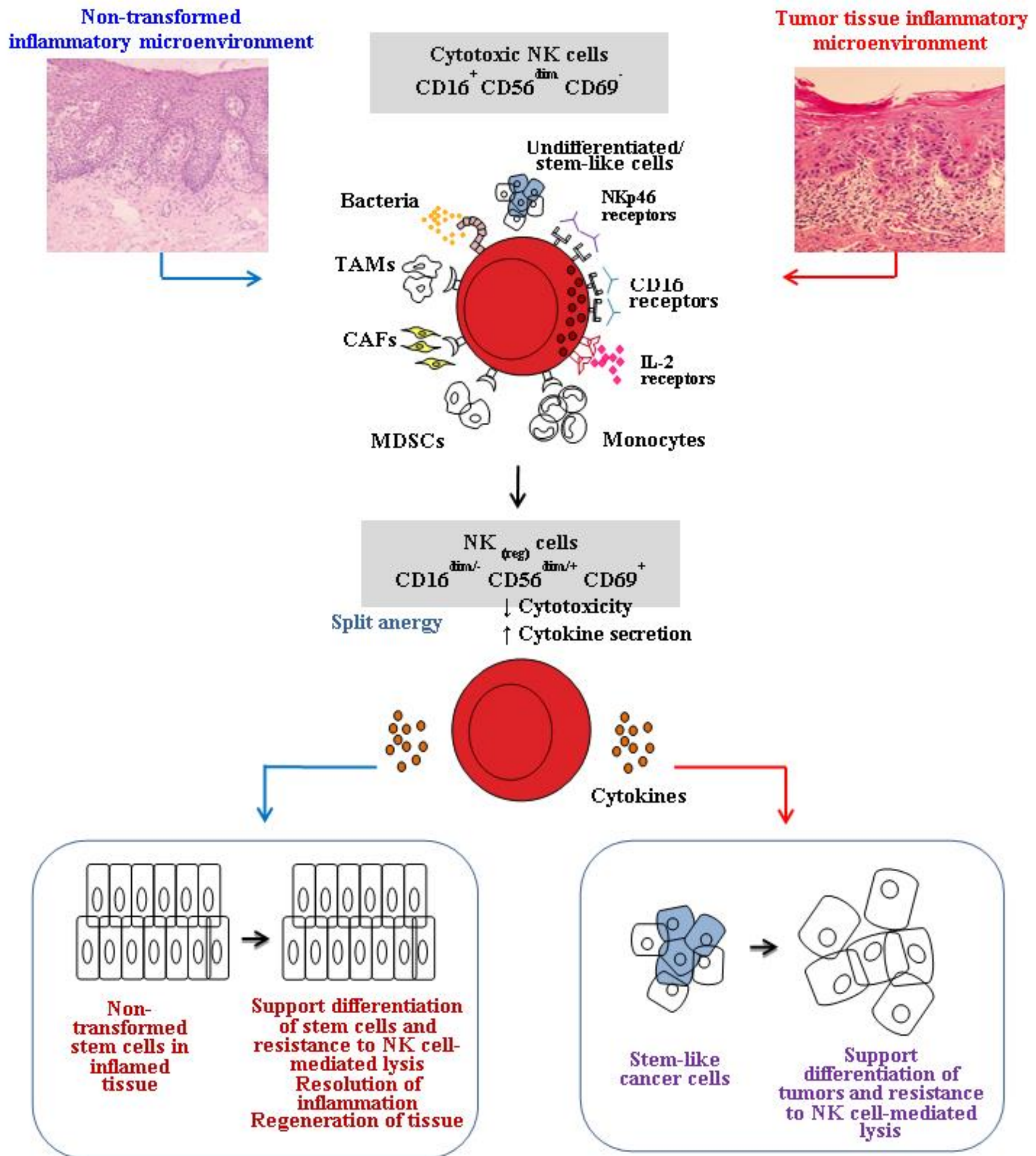


Fig. 4



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Tumor microenvironment may shape the function and phenotype of NK cells through the induction of split anergy and generation of regulatory NK cells

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Abstract

Cytotoxic function of NK cells is suppressed in the tumor microenvironment by a number of distinct effectors. Furthermore, decreased peripheral blood NK cell cytotoxicity has been documented in cancer patients. We have previously demonstrated evidence for the role of NK cells in specific elimination of stem cells and not their differentiated counterparts. In this regard, NK cells were found to mediate significant cytotoxicity against primary oral squamous carcinoma stem cells (OSCSCs) as compared to their more differentiated oral squamous carcinoma cells (OSCCs). In addition, human embryonic stem cells (hESCs), human mesenchymal stem cells (hMSCs), human dental pulp stem cells (hDPSCs) and induced human pluripotent stem cells (hiPSCs) were all significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts or parental cells from which they were derived. There is also a stage wise susceptibility to NK cell mediated cytotoxicity in pancreatic tumors in which case the poorly differentiated tumors are lysed much more than their moderately differentiated tumors. The well differentiated pancreatic tumors were lysed the least when compared to either the moderately differentiated tumors or to poorly differentiated tumors. We have also reported that inhibition of differentiation or reversion of cells to a less-differentiated stage by blocking NFκB or gene deletion of COX2 significantly augmented NK cell cytotoxicity against both transformed and healthy cells. Therefore, we propose that the two stages of NK cell maturation namely CD16+CD56dimCD69- NK cells are important for the selection of stem cells whereas the CD16dim/-CD56dim/+CD69+NK cells are important for differentiation and eventual regeneration of the tissues and the resolution of

inflammation, thus serving as regulatory NK cells (NK_{reg}). The concept of split anergy in NK cells and generation of NK_{reg} and its contribution to cell differentiation, tissue repair and regeneration and in tumor resistance will be discussed in this chapter.

Introduction

Advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between the immune effectors and the tumor cells. Effectors of the immune system are thought to shape the survival and maturation of tumor cells and to select for cancers with reduced immunogenicity. However, recent data from our laboratory indicted that the same effector mechanisms are likely responsible for shaping the survival and maturation of healthy stem cells for the ultimate goal of the regeneration of damaged tissues and the resolution of inflammation. Although, immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, neither their underlying physiological significance nor the exact mechanisms by which immunosuppression occurs are completely understood.

NK cells arise from the bone marrow and constitute 5-15% of total lymphocytes in the peripheral blood. They are known to mediate direct natural cytotoxicity as well as antibody-dependent cellular cytotoxicity (ADCC). By producing key cytokines and chemokines NK cells are known to regulate the functions of other immune cells [508, 509]. Conventional human NK cells are identified by the expression of CD16 and CD56, and by the lack of surface CD3 expression. NK cells mediate their function through a number of important activating and inhibitory cell receptors listed in Table 1 [510]. It is thought that the balance between activating and inhibitory signals which NK cells receive from their surface receptors determines their functional fate [510]. Many of the receptors

listed in table 1 including CD16, killer immunoglobulin like receptors (KIR), NKG2 family of receptors which form a heterodimer with CD94, NKG2D and natural cytotoxicity receptors (NCR) have all been the subject of many studies. Likewise, several key cytokines, chemokines and adhesion molecules are found to have significant roles in maturation, differentiation, and effector function of NK cells. Much less is known regarding the function of Toll Like Receptors (TLRs), NOD-Like Receptors (NLRs) and RIG like Receptors (RLRs) in NK cell effector function.

The extracellular domains of KIRs are homologous, irrespective of whether they are transmitting activating or inhibiting signals. However, the functional fate of these receptors are determined by their intracellular domains for which they either have an immunoreceptor tyrosine-based activation motif (ITAM) or immunoreceptor tyrosine-based inhibition motif (ITIM) responsible for delivering an activating or inhibitory signals respectively.

The association of distinct effector functions with certain NK cell subsets is thought to be developmentally regulated [524, 525]. In this regard, previous studies have identified two distinct subsets of NK cells namely $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$ subpopulations based on their phenotypic and functional analysis [509]. The $CD56^{dim}CD16^{bright}$ NK subset is the major subset in the peripheral blood which mediates cytotoxicity whereas the $CD56^{bright}CD16^{dim}$ subset constitutes a minor subpopulation of NK cells in the peripheral blood and its role is in secretion of cytokines

[509]. $CD56^{bright}CD16^{dim}$ subset does not mediate cytotoxicity. The $CD56^{bright}CD16^{dim}$ NK cells are thought to be precursors to the $CD56^{dim}CD16^{bright}$ NK subset [516].

Although a lot is known about the inhibitory and activating receptors that modulate the function of NK cells, and many previous studies have indicated that NK cells may recognize and become activated by irradiated or stressed cells [509, 510], no previous studies have shown the role of NK cells in recognition, selection and differentiation of stem cells and their potential role in the resolution of inflammation. In this chapter we provide data regarding the factors and mechanisms involved in shaping the function of NK cells in cancer, and after interaction with healthy stem cells, and furthermore we discuss the emerging view from our laboratory which indicates that the NK cells may behave as the effectors of selection, differentiation and resistance of undifferentiated or stem like cells. The concept of split anergy in NK cells and its role in the switch of NK cell function from effector to regulatory cell function and its contribution to cell differentiation, tissue repair and regeneration and in tumor resistance will be discussed in this chapter.

Cancer stem-like tumors or poorly differentiated tumors as well as non-transformed stem cells are lysed significantly more by the NK cells when compared to their differentiated counterparts

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed when NK cells were co-incubated with OSCSCs which released significantly lower levels of GM-CSF, IL-6 and IL-8 and demonstrated decreased expression of phospho-Stat3,

B7H1 and EGFR, and much lower constitutive NFκB activity when compared to differentiated OSCCs (Fig. 1A) and [412 2010]. More importantly, OSCSCs expressed CD133 and CD44^{bright} oral stem cell markers [412 2010], whereas differentiated OSCCs express lower CD44 surface receptors. To assess whether the stage of differentiation of other tumor types also correlated with their sensitivity to NK cell mediated lysis we selected five pancreatic lines at different stages of differentiation based on a number of criteria including sphere formation and immunohistochemical analysis (Sipos, 2003; Deer 2010). Panc-1 and MP-2, two poorly differentiated, BXPC3 and HPAF, two moderately differentiated and CAPAN-1, a well differentiated pancreatic tumors were co-cultured with the NK cells and NK cell mediated cytotoxicity were determined in a 4 hour ^{51}Cr release assay. There was a significant correlation between the stage of differentiation of the tumors and the level of NK cell mediated lysis (Fig. 1B). The highest NK cell cytotoxicity was obtained against poorly differentiated tumors Panc-1 and MP-2, intermediate lysis against moderately differentiated BXPC3 and HPAF and the lowest lysis was obtained against well differentiated CAPAN-1 cells (Fig. 1B). Both untreated and IL-2 treated NK cells lysed poorly differentiated tumors much more than the differentiated tumors. Anti-CD16mAb treatment of NK cells abolished NK cell mediated cytotoxicity against all tumors and the combination of IL-2 and anti-CD16mAb significantly reduced IL-2 mediated lysis as expected (Fig. 1B). In addition, two Glioblastoma Multiforme (GBM) stem-like tumors XO1-NS and XO2-NS which were previously isolated and characterized [259, 466, 467] were found to be significantly more susceptible to NK cell mediated cytotoxicity when compared to differentiated U87 GBM tumors (Fig. 1C). Since most stem-like tumors or poorly differentiated cells were

significantly more susceptible to NK cell mediated cytotoxicity we reasoned that healthy, non-transformed primary stem cells may also be susceptible to NK cell mediated cytotoxicity. We demonstrated previously that NK cells lysed hMSCs, hDPSCs and hESCs significantly (Table 2). All different types of stem cells became resistant to NK cell mediated cytotoxicity once they were differentiated (Table 2) [412 2010]. In addition, higher sensitivity of hiPSCs to NK cell mediated lysis was also observed when compared to parental line from which they were derived (Table 2). Differentiation of XO1-NS and XO2-NS also rendered them more resistant to NK cell mediated cytotoxicity (Table 2, manuscript submitted). Increased lysis of cancer stem cells or non-transformed healthy stem cells may be attributed to the use of allogeneic NK cells, however, our previous work using autologous NK cells exhibited similar levels of cytotoxicity against hDPSCs when compared to lysis by allogeneic NK cells [205 2010]. Taken together these results indicated that undifferentiated cells are targets of both allogeneic and autologous NK cells. Thus, the stage of differentiation of the cells is predictive of their susceptibility to NK cell mediated cytotoxicity.

De-differentiation of the epithelial cells activates NK cell function

Since the degree of differentiation in the cells is predictive of their sensitivity to NK cell mediated cytotoxicity, we reasoned that blocking NFκB in the cells may de-differentiate and subsequently revert the cells to more of undifferentiated phenotype, resulting in their increased susceptibility to NK cell mediated cytotoxicity. Indeed, blocking NFκB in oral tumors was found to increase CD44 surface receptor expression, which is one of the hallmarks of stem cells (unpublished results). In addition, blocking of

NFκB nuclear function in a primary Oral tumor OSCCs and in a non-tumorigenic oral cells (HOK-16B) as well as in an established tumor line, HEp-2 cells known to be Hela contaminant [193, 457-459], augmented cytotoxicity and the release of key cytokines such as IFN-γ from the NK cells [192, 193]. Similarly, inhibition of NFκB by Sulindac increased the functional activation of NK and enhanced anti-tumor cytotoxic activity [192, 193].

In agreement with our studies, targeted deletion of IKK-β in epidermis of mice has previously been shown in one study to lead to inflammatory skin manifestations [153]. Elevated levels of cytokines and chemokines have also been demonstrated in the epidermis of patients and animals with Iκκγ and Iκκβ deletions [153, 460]. Mice with a keratinocyte-specific deletion of Iκκ−β demonstrated decreased proliferation of epidermal cells, and developed TNF-α dependent inflammatory skin disease [153]. In contrast, in other studies in which NFκB function was blocked in dermal keratinocytes by a mutant IκB-α an increased proliferation and hyperplasia [463] and eventual development of cutaneous squamous cell carcinomas of skin were seen if mice were allowed to survive and reach adulthood [464, 465]. It is of interest to note that in these studies with diverse functional outcomes in keratinocytes, blocking TNF-α function resulted in the prevention of both the neoplastic transformation and the inflammatory skin disease [153, 465]. Elevated numbers of immune inflammatory cells recruited to the site of epidermis are likely responsible for the increased secretion of TNF-α. Indeed, we have demonstrated that synergistic induction of TNF-α could be observed when NFκB knock down oral tumors were cultured with either PBMCs or NK cells [192].

Since tumorigenic and non-tumorigenic human oral keratinocytes acquire sensitivity to NK cell mediated lysis when NFκB is inhibited, it is likely that this phenomenon is not specific to cancer or oral keratinocytes, and it may occur in other healthy non-transformed cell types. Indeed, when human primary monocytes were differentiated to dendritic cells they too became more resistant to NK cell mediated cytotoxicity [412 2010]. Moreover, knock down of COX2 in primary mouse monocytes [412 2010], or in mouse embryonic fibroblasts (unpublished observations), resulted in the reversion or de-differentiation of the monocytes and fibroblasts respectively, and the activation of NK cell cytotoxicity. Indeed, it is likely that any disturbance in cellular differentiation may predispose the cells to NK cell mediated cytotoxicity. Since STAT3 is an important factor increased during differentiation, blocking STAT3 is also critical in the activation of immune effectors [211]. In support of a critical role of STAT3 in immune evasion of tumor cells in humans, we and others have recently shown that GBM tumors display constitutive activation of STAT3 (Cacalano and Jewett, unpublished observation) [160], and poorly induce activating cytokines and tumor-specific cytotoxicity in human peripheral blood mononuclear cells (PBMCs) and NK cells. Ectopic expression of dominant-negative STAT3 in the GBM tumors increased lysis of the tumor cells by the immune effectors and induced production of IFN-γ by the interacting immune effectors (unpublished observations).

Since NFκB is shown to regulate IL-6 secretion in OSCCs, HOK-16B and HEp2 cells and secreted IL-6 in tumors is known to activate STAT3 expression and function, increase in NFκB nuclear function could in turn induce STAT3 activation and result in a significant resistance of tumors to NK cell mediated cytotoxicity. Indeed, inhibition of NFκB in oral tumors resulted in a significant decrease in IL-6 secretion by the tumor cells and the induction of IFN-γ secretion by the NK cells [192, 412]. Therefore, targeted knock down of STAT3 or signaling pathways upstream of STAT3, such as NFκB, may de-differentiate the cells and predispose the cells to NK cell mediated cytotoxicity.

Split anergy in IL-2 treated NK cells is induced after their binding to sensitive but not resistant tumors and after the triggering of CD16 receptors

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity while it triggers significant induction of TNF-α and IFN-γ from the NK cells [136, 137]. In contrast, NK resistant tumors such as RAJI cells induce very little loss of NK cell cytotoxicity or secretion of cytokines [136, 137]. Moreover, following NK cell cultures with sensitive tumor-target cells but not resistant tumors, the target binding NK cells undergo phenotypic and functional changes. Target cell inactivated NK cells express CD16-CD56dim/- CD69+ phenotype [136, 137]. This phenotype has also been observed in several disease manifestations including HIV infection [468]. Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in several cancer patients including those of the oral and ovarian cancer patients [138, 139]. In addition, down-regulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor

cells [140]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [140]. These observations suggested that CD16 receptors could likely play an important role in target cell induced loss of NK cell cytotoxicity. Indeed, CD16:Ig fusion proteins were shown to bind to a variety of tumor-target cells indicating the existence of specific ligands for CD16 receptors on tumor cells [469]. Furthermore, we have previously shown that the triggering of CD16 on untreated or IL-2 treated NK cells was found to result in down-modulation of CD16 receptors and in a great loss of cytotoxicity in NK cells. In addition, a small subset of NK cells was programmed to undergo apoptosis [136, 137, 141, 142]. Cell death of NK cells was shown to be regulated, in part, by endogenously secreted TNF-α from the NK cells [142]. Previous studies by other groups have also shown that a subset of IL-2 activated NK cells undergo cell death following cross-linking of the CD16 receptor [470, 471]. Addition of antibodies to CD56 or LFA-1 did not cause any decrease in NK cell cytotoxicity demonstrating the specificity of CD16 mAb signaling in mediating inhibition of NK cell cytotoxicity [143]. Thus, we had coined the term “split anergy” for the responses observed by NK cells after their interaction with sensitive target cells or after the triggering of CD16 receptors by the antibody in combination with IL-2 treatment [136, 137, 142, 144, 145]. Indeed, three subpopulations of NK cells; namely Free, Binder and Killer NK cells with varying degrees of loss of cytotoxicity were identified after the formation of conjugates with K562 targets [141, 201, 472-474]. Free cells which did not bind or form conjugates with target cells were inactivated less, or exhibited the most cytotoxicity, whereas both Binder, those that bound but did not kill their bound tumors, and Killer subsets, which bound and killed their bound tumors

exhibited significant loss of cytotoxicity. In contrast, Binder and Killer subsets but not Free NK subset secreted significant levels of cytokines and exhibited CD16-CD56dim/-CD69+ phenotype [141, 201, 472-474]. Treatment of NK cells with IL-2 and anti-CD16mAb also induced split anergy by significantly decreasing the NK cell cytotoxicity while increasing the cytokine secretion capabilities of NK cells. Furthermore, NK cells exhibited CD16-CD56dim/-CD69+ phenotype after treatment with the combination of IL-2 and anti-CD16mAb [142-145]. Loss of cytotoxicity in NK cells was significantly exacerbated when NK cells were either treated with F(ab)'$_2$ fragment of anti-CD16 mAb with IL-2 or treated with a combination of MHC-Class I and anti-CD16 mAbs in combination with IL-2 while the same treatments resulted in an increased secretion of cytokines [143, 145]. Based on our recent results NKp46 mAb were also able to induce significant NK cell anergy in the presence and absence of IL-2 correlating with their increased expression on untreated and IL-2 treated NK cells (manuscript submitted). Moreover, addition of bacteria or their extracts in the presence of CD16 receptor signaling and IL-2 was able to induce synergistic decrease in NK cell cytotoxicity while increasing the induction of cytokine release substantially (manuscript in prep). Because anergy in NK cells is an active process and it is induced via signaling receptors such as CD16 and NKp46 and not through LFA1 or LFA3 or CD56 it is likely that binding of agonistic antibodies or their ligands signal the NK cells to become tolerant in a manner similar to that obtained when anti-CD3 antibody is administered to T cells [517]. In addition, the magnitude of signaling through the receptors on NK cells may determine the extent and levels of anergy induced in NK cells. Therefore, these results suggested that receptor signaling in NK cells via key surface receptors in the presence of IL-2 is likely

to result in a rapid loss of NK cell cytotoxicity while continuing to increase secretion of cytokines by the NK cells.

Split anergy in NK cells is induced by monocytes

When hMSCs or hDPSCs were cultured with either viable or irradiated monocytes before they were exposed to IL2-treated NK cells a significant decrease in NK cell mediated cytotoxicity could be observed against hMSCs or hDPSCs. Interestingly, significant lysis of hMSCs and hDPSCs by untreated NK cells was also reproducibly blocked by the addition of monocytes [205 2010]. We then determined whether decreased lysis of stem cells by NK cells was due to a competitive lysis of monocytes by the NK cells. We confirmed that monocytes were also lysed by the NK cells significantly. Furthermore, when we co-cultured stem cells with monocytes and sorted to remove the monocytes from the stem cells before assessing the killing function of NK cells, we could still observe significant inhibition of NK cell mediated lysis, arguing against the protection of stem cell lysis by NK cells being solely on the bases of competitive lysis of monocytes [205 2010]. Therefore, even though lysis of monocytes by the NK cells may in part contribute to the prevention of NK cell lysis of stem cells, interaction of monocytes with stem cells can also provide resistance of stem cells against NK cell cytotoxicity. Decrease in NK cell lysis of hMSCs and hDPSCs was paralleled with a significant induction of IFN-γ. Indeed, when hMSCs or hDPSCs were cultured with IL-2 treated NK cells alone we could observe significant induction of IFN-γ secretion. However, the highest increase was seen when IL2-treated NK cells were cultured with hMSCs or hDPSCs in the presence of monocytes. Therefore, although decreased killing

of stem cells by the NK cells could be observed in the presence of monocytes, synergistic secretion of IFN-γ by the NK cells in the presence of monocytes and stem cells could be observed, indicating an inverse relationship between cytotoxicity and IFN-γ secretion (split anergy). This was similar to the profiles which we had seen when NK cells were treated with IL-2 and anti-CD16 mAb in which significant decrease in cytotoxicity of NK cells could be observed in parallel with increased secretion of IFN-γ [142].

Induction of split anergy in NK cells is a potential mechanism for the switch from effector to regulatory function

Induction of split anergy in NK cells could be an important conditioning step responsible for the repair of tissues during pathological processes irrespective of the type of pathology. In tumors since the generation and maintenance of cancer stem cells is higher, the majority, if not all of the NK cells, may be conditioned to support differentiation and repair of the tissues and as such the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble that of the anergic NK cells, i.e., decreased NK cell cytotoxicity, acquisition of CD16-/dimCD56dim/+CD69+ phenotype and augmented ability to secrete inflammatory cytokines. Of course, the degree of the loss of NK cell cytotoxicity may be directly proportional to the load of cancer stem cells. Therefore, our results suggest two very important functions for the NK cells. One function is to limit the numbers of proliferating stem cells and immune inflammatory cells by selecting those with a greater potential for differentiation for the repair of the tissues and second to support differentiation of the stem cells and subsequent regeneration of the tissues. To achieve these tasks NK cells have to acquire two different

phenotypes, and be conditioned to carry out both functions successfully. CD16+CD56dimCD69- subsets of NK cells are cytotoxic and will mediate cytotoxicity depending on which cells they encounter first. In respect to the oral squamous cell carcinomas since the majority of immune effectors can be found at the connective tissue area the chances are that they may first encounter and interact with either the other immune effectors or the effectors of connective tissue such as fibroblasts. However, there is also the possibility that NK cells may first encounter the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they too can become anergized. Surprisingly, allogeneic CTLs were also found to target Glioblastoma stem-like cells and not their differentiated counterparts (unpublished observation). By eliminating a subset of stem cells or after their interaction with other immune inflammatory cells or effectors of connective tissue NK cells could then be in a position to support differentiation of selected population of stem cells since they will be conditioned to lose cytotoxicity, induce cytokine and growth factor secretion and gain the CD16-/dimCD56dim/+CD69+ phenotype. It is interesting to note that all of the immune effectors isolated from oral gingival tissues of healthy as well as diseased gingiva have CD69+ phenotype, with the exception that the numbers of immune effectors are much less in the healthy oral gingival tissues when compared to diseased tissues (unpublished observation). Therefore, our results suggest two very important functions for the NK cells. One function is to kill and the other function is to support differentiation for the repair and regeneration of the tissues.

In vivo physiological relevance of above-mentioned observations could be seen in a subpopulation of NK cells in peripheral blood, uterine and liver NK cells which express low or no CD16 receptors, have decreased capacity to mediate cytotoxicity and is capable of secreting significant amounts of cytokines [213, 214]. In addition, 70% of NK cells become CD16 dim or negative immediately after allogeneic or autologous bone marrow transplantation [213]. Since NK cells lose their cytotoxic function and gain in cytokine secretion phenotype and down modulate CD16 receptors after their interaction with tumor cells or the stem cells [137, 142], it is tempting to speculate that in vivo identified CD16- NK cells and in vitro tumor induced CD16- NK cells have similar developmental pathways since they have similar if not identical functional properties.

The proof of concept in support of this model was recently obtained in our laboratory. We observed that anergized NK cells were directly responsible for the increased differentiation and resistance of a number of different stem cells including cancer stem cells and dental pulp stem cells against cytotoxic effectors (Fig. 2) (manuscript submitted). As presented, when OSCSCs were cultured with supernatants (Fig. 2) or paraformaldehyde fixed NK cells (manuscript submitted) treated with IL-2 and anti-CD16 mAb, they became resistant to cytotoxicity mediated by freshly isolated untreated or IL-2 treated NK cells. IL-2 treated NK cell supernatants or paraformaldehyde fixed NK cells were also able to impart some resistance to OSCSCs but the highest levels of resistance were achieved when NK cells were treated with IL-2 and anti-CD16mAb (Fig. 2). No significant differences in resistance of OSCSCs to NK cell mediated lysis can be achieved by either culturing the OSCSCs with the supernatants

or cells from untreated or anti-CD16mAb treated NK cells (Fig. 2). The resistance in OSCSCs induced by anergized NK cells correlated with a decrease in CD44 receptor expression and in an increase in B7H1 expression (data not shown), two surface receptors which were inversely expressed in differentiated OSCCs and in OSCSCs [412]. In addition, we now have evidence which supports the notion that the induction of anergy in NK cells is an active process which is induced by the triggering of CD16 receptor on the NK cells and is not due to degranulation and exhaustion of cytotoxic granules (unpublished results).

Our work collectively suggests that anergized NK cells are as important as the non-anergized NK cells in their effector functions. NK cells are not only important for the removal and shaping of the size of the stem cells which is mediated by the effector NK cells but also their differentiation, and the eventual regeneration of the new tissues which is mediated by their switch to regulatory NK cells. The task of NK cells in this regard goes above and beyond their most appreciated function of being the effectors of first line defense against viral infection and malignancies. They too can be effectors of differentiation and tissue regeneration.

Similarities in immune cell effector function in inflammatory tumor microenvironment and in non-transformed inflammatory microenvironment

The concept of tumor immunosurveillance has previously been expanded to include immunoediting as an important mechanism for the development of cancer [421, 422]. It was suggested that cancer immunoediting comprises of three phases: elimination,

equilibrium and escape [422]. Elimination represents the classical concept of immunosurveillance. However, during equilibrium and escape the interaction and cross signaling between the immune effectors including NK cells, the tumor cells, and perhaps the effectors of the connective tissue in the tumor microenvironment may result in the generation of tumors which are capable of gradual suppression of the NK cell cytotoxic function. The final stages of cancer development may result in the induction of resistant tumors in the presence of fewer immune effectors capable of lysing the tumors [422]. Thus, pressures exerted by the tumor cells and immune effectors may eventually shape the microenvironment for the growth, expansion and invasion of tumors. Similarly, a variation of such interactions may also be observed during the interaction of NK cells with healthy non-transformed human stem cells in non-transformed inflammatory microenvironment in which case the three phases of interaction may include elimination which marks the decrease in the numbers of proliferating stem cells or other immune effectors in the inflammatory microenvironment, potentially resulting in the selection of stem cells by the NK cells, induction of tolerance or anergy which denotes the conditioning of NK cells by the stem cells and/or by the other effectors of microenvironment to become regulatory cells and support maturation and differentiation of remaining stem cells, and finally the resolution phase which denotes the elimination of anergized NK cells and generation of less immunogenic differentiated cells.

Immunosuppressive effectors in tumor and in non-transformed inflammatory microenvironment

Both the tumor microenvironment as well as non-transformed inflammatory microenvironment consists of a number of heterogeneous cell populations with ability to suppress and limit the function of cytotoxic immune effectors. Patients with cancer often have higher numbers of immature monocytes serving as Myeloid Derived Suppressor Cells (MDSCs) expressing CD14+HLADR- phenotype [227 2010, 453 2010]. Tumor associated Macrophages (TAMs) were previously shown to significantly influence and limit immune activation in the tumor microenvironment [454, 455 2010]. In addition, MDSCs which are comprised of a number of distinct cell populations of myeloid origin and whose roles in immunosuppression have received significant attention in recent years are major cells capable of suppressing the cytotoxic function of T and NK cells [453 2011]. T cell dysfunction is shown to be induced by MDSCs by the increased secretion of IL-10, TGF-β, induction of reactive oxygen species (ROS), and increased expression of arginase-1 and inducible nitric oxide synthase (iNOS). T regulatory (Treg) and DC regulatory (DCreg) cells were also recently shown to have significant immunosuppressive roles in the tumor microenvironment [453 2010]. Perhaps one of the most intriguing observations regarding the immunosuppressive effectors is the identification of Cancer Associated Fibroblasts (CAFs) and Mesenchymal Stem Cells (MSCs) as two potential tumor promoters. Fibroblasts from tumor tissues demonstrate an activated phenotype and have the ability to secrete many immunosuppressive factors such as TGF-β and VEGF [456 2011]. We have also found that undifferentiated fibroblasts, as well as MSCs and CD14+HLA-DR- monocytes are significantly more susceptible to NK cell mediated cytotoxicity [205 2010], therefore, these cells may condition NK cells to undergo split anergy and become regulatory NK cells. Indeed, in oral epithelial tumors

the majority of recruited immune effectors are usually found in the connective tissue area where through cell-cell interaction with the immunosuppressive cells such as fibroblasts, monocytes-macrophages and to a lesser extent T and B cells [205 2010] can generate regulatory NK cells, resulting in differentiation and resistance of oral epithelial tumors.

Tumor microenvironment may shape the function and phenotype of the NK cells

Based on the work presented in this chapter, it is possible that the resident and recruited immune effectors in tumor microenvironment such as monocytes may serve as shields against NK cell lysis of stem cells (Fig. 3). Monocytes can shield stem cells from killing by the NK cells by increasing the total IFN-γ release from the NK cells while decreasing the cytotoxic function of NK cells, resulting in an increased protection and differentiation of stem cells. Indeed, monocytes also increase TNF-α, IL-6 and VEGF secretion in the co-cultures of stem cells with NK cells which could augment NFκB and increase differentiation of stem cells. The shielding effect of monocytes could be a more generalized function of other effectors since NK cells can also target fibroblasts (Fig. 3) [205 2010]. Whether other MDSCs such as PMNs can also be targeted by the NK cells awaits future investigation. This may have significant implications for the role of NK cells in not only limiting inflammation, but also the significance of other immune effectors in shielding and limiting the cytotoxic function of NK cells against cancer or healthy stem cells in order to raise maximally the secretion of key cytokines for speedy and optimal differentiation of stem cells during inflammation (Fig. 3). This is precisely what is observed in cancer patients in whom global decrease in NK, cytotoxic T cells and monocytes have all been reported [180].

Potential functional similarities between induced regulatory NK cells and T regulatory cells

Because of their ability to drive differentiation, anergized NK cells may have the ability to halt inflammation since differentiated cells are no longer targeted by the NK cells and T cells and they do not induce cytokine secretion by the NK and T cells (Fig. 3) [18, 19]. This function of NK cells is similar to T regulatory cells since they are inhibitory and are capable of decreasing the magnitude of inflammation in a number of previous studies. Therefore, although immunosuppression in the tumor microenvironment is not advantageous for the patient, it is indeed, an important function which may not only stimulate differentiation, but it may also halt inflammation.

The majority if not all of the effectors in the mucosal immune system including in the oral cavity are of activated phenotype, ie, they express CD69 early activation antigen. These cells, including NK cells are likely conditioned in the mucosa to support differentiation and resistance of the epithelial cells. Such environment is anti-inflammatory since the majority of immune cells is tolerant of ingested food particles and self-tissues and is known to contain many regulatory cells including T cells, Dendritic cells and likely regulatory NK cells. However, once the threshold which keeps the inflammation at bay is decreased in the mucosa, immune effectors are activated and may cause tissue damage and establishment of chronic inflammation. Indeed, in this regard our preliminary *in vivo* observations in humans consuming a combination of proprietary probiotic bacterial strains with potent ability to condition NK cells to support

differentiation of OSCSCs and hDPSCs (manuscript in prep) was able to relieve chronic inflammation and pain, and resulted in the resolution of inflammatory mouth ulcers, and oral edema. Furthermore, the number of neutrophils in the blood of a donor who had chronically decreased levels of neutrophils rose to the normal levels and both the numbers and function of NK cells in the blood improved substantially after the consumption of probiotic bacterial strain.

Our results in the conditioning of NK cells to become regulatory NK cells with receptor signaling in the presence of LPS and IL-2 is in line with the numerous anti-inflammatory benefits which are achieved by the consumption of probiotic bacteria in the gut.

Conclusions

Much work has been done to identify strategies by which tumor cells evade the function of immune system. Altered expression of MHC molecules which block recognition and activation of T and NK cells are examples of mechanisms by which tumor cells evade the function of immune system. In addition, tumor cells by releasing immunosuppressive factors such as Fas, VEGF, IL-6, IL-10, TNF-α, GM-CSF and IL-1β, induce T and NK cell apoptosis, block lymphocyte homing and activation, and dampen macrophage and dendritic cell function. However, the same effector functions are also important in tissue repair.

Based on the accumulated work presented in this chapter, we suggest that NK cells may have two significant functions; one that relates to the removal of excess proliferating stem cells and their selection. In this regard, NK cells could also lyse other effectors in the connective tissue area in order to not only decrease inflammation but also to be conditioned to promote differentiation and resistance of selected stem cells and eventual regeneration of the tissues (Fig. 3). The second important task for NK cells is therefore, to support differentiation and promote tissue regeneration after altering their phenotype to cytokine secreting cells (Fig. 3). This process will not only remove cells that are perhaps damaged and have flaws in the differentiation process or in general are more than needed, but also it will ensure the proper regeneration of tissues and the resolution of inflammation. Thus, any disturbance in the NK cell function or in the process of selection and differentiation of stem cells may result in chronic inflammation,

causing continual tissue damage and recruitment of immune effectors to aid in tissue regeneration.

The inability of patient NK cells to contain cancer stem cells due to the flooding of NK cells by proliferating cancer stem cells and conversion of NK cells to regulatory NK cells may likely be one mechanism by which cancer may progress and metastasize. Therefore, there should be two distinct strategies by the NK cells to eliminate tumors, one which targets stem cells and the other which targets differentiated cells. Since cancer stem cells were found to be more resistant to certain chemotherapeutic drugs but sensitive to NK cell mediated killing while differentiated oral tumors were more resistant to NK cell mediated killing but relatively more sensitive to chemotherapeutic drugs, combination therapy should be considered for the elimination of both undifferentiated and differentiated tumors. In addition, since a great majority of patient NK cells may have switched to regulatory function to support differentiation of the proliferating cancer stem cells, they may not be effective in eliminating the cancer stem cells. Therefore, these patients may benefit from repeated allogeneic NK cell transplantation for elimination of cancer stem cells. In this regard, depletion of immunosuppressive effectors in the tumor microenvironment, which condition NK cells to become regulatory cells, via radiation or chemotherapeutic drugs should in theory provide a better strategy for successful targeting of tumors by the NK cells.

Figure legends

Fig. 1-(A). Augmented NK cell cytotoxicity against OSCSCs as compared to differentiated OSCCs

NK cells were left untreated or treated with IL-2 (1000 u/ml) or anti-CD16 mAb (3µg/ml) or a combination of IL-2 (1000 u/ml) and anti-CD16 mAb (3µg/ml) for 12-24 hours before they were added to ^{51}Cr labeled primary oral tumors. NK cell cytotoxicity was determined using a standard ^{51}Cr release assay and the lytic units 30/10^6 cells were calculated using inverse number of effectors required to lyse 30% of the tumor cells X 100. Differences between untreated, anti-CD16 mAb treated or IL-2 and/or anti-CD16 mAb treated NK cell cytotoxicity between UCLA-OSCCs and UCLA-OSCSCs were significant at a p value of <0.05. One of four representative experiments is shown in this figure.

Fig. 1-(B). Correlation between the stage of differentiation of pancreatic tumors and susceptibility to NK cell mediated cytotoxicity.

Highly purified NK cells at (1X10^6 cells/ml) were either left untreated or treated with IL-2 (1000u/ml), anti-CD16 mAb (3µg/ml) or a combination of IL-2 (1000u/ml) and anti-CD16 mAb (3µg/ml) for 12-24 hours before they were washed and used in cytotoxicity assay against poorly differentiated Panc-1 and MP2, moderately differentiated BXPC3 and HPAF and well differentiated CAPAN-1 pancreatic tumors. NK cell cytotoxicity was determined using a standard ^{51}Cr release assay and the lytic units 30/10^6 were determined using inverse number of effectors required to lyse 30% of

the tumor cells X 100. Differences between untreated, IL-2 treated or IL-2 and anti-CD16mAb treated NK cell killing between poorly differentiated as compared to moderately differentiated and well differentiated tumors are significant at a p value of <0.05. One of five representative experiments is shown in this figure.

Fig. 1(C) XO1-NS and XO2-NS GBM stem-like tumors are lysed significantly more than differentiated U87 GBMs.

Highly purified NK cells at ($1X10^6$ cells/ml) were either left untreated or treated with IL-2 (1000u/ml), anti-CD16 mAb (3μg/ml) or a combination of IL-2 (1000u/ml) and anti-CD16 mAb (3μg/ml) for 12-24 hours before they were washed and used in cytotoxicity assay against XO1-NS, XO2-NS and U87 GBMs. NK cell cytotoxicity was determined using a standard ^{51}Cr release assay and the lytic units $30/10^6$ were determined using inverse number of effectors required to lyse 30% of the tumor cells X 100. Differences between untreated, anti-CD16mAb treated or IL-2 and/or anti-CD16mAb treated NK cell killing between XO1-NS or XO2-NS and U87 GBMs were significant at a p value of <0.05. One of four representative experiments is shown in this figure.

Fig. 2. Supernatants from anergized NK cells induce the highest resistance of OSCSCs against NK cell mediated cytotoxicity.

Highly purified NK cells at ($1X10^6$ cells/ml) were either left untreated or treated with IL-2 (1000u/ml), anti-CD16 mAb (3μg/ml) or a combination of IL-2 (1000u/ml) and anti-CD16 mAb (3μg/ml) for 24 hours before they were harvested and used to induce differentiation of OSCSCs. OSCSCs at $1X10^6$ cells were added to each plate in 10 ml of

media and the cells were allowed to adhere before the NK cell supernatants were added to each plate. A total of 180 microliter of supernatants were added at day 1, 3 and 5 and the levels of NK cell cytotoxicity were determined using freshly isolated untreated (Fig. 2A) and IL-2 treated (1000u/ml) (Fig. 2B) NK cells in a 4 hour 51Cr release assay on the 6th day.

Fig. 3 Hypothetical model of induction of regulatory NK cells by immune inflammatory cells and by the effectors of connective tissue to support differentiation of the non-transformed stem cells and cancer stem cells.

Hypothetical model of NK cell conditioning in the tumor microenvironment as well as in non-transformed immune inflammatory microenvironment is shown in this figure. Significant infiltration of immune effectors right beneath the epithelial layer can be seen in the connective tissue area where the immune inflammatory cells are likely to condition NK cells to lose cytotoxicity and gain the ability to secrete cytokines, a term which we have previously coined as split anergy in NK cells, and to support differentiation of the basal epithelial layer containing stem cells. NK cells are likely to encounter and interact with the other immune effectors such as monocytes or other myeloid-derived suppressor cells (MDSCs), and in tumor microenvironment with the tumor-associated macrophages (TAMs), or with connective tissue-associated fibroblasts (CAF) in order to be conditioned to form regulatory NK cells (NK_{reg}). NK cells may also directly interact with the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they can become conditioned to support differentiation of other stem cells. In addition, bacteria through the binding to Toll like

receptors can further aid in the generation of $NK_{reg.}$ All the above mentioned mechanisms may be operational during inflammatory processes in the tumor microenvironment or in healthy non-transformed inflammatory microenvironment. NK cell-differentiated epithelial cells will no longer be killed or induce cytokine secretion by the NK cells, therefore, resulting in the resolution of inflammation.

Table 1- List of NK cell Activating and Inhibitory surface receptors and their ligands

Receptors	Ligands
Activating/inhibitory Receptors	
FcγRIII (CD16)	Fc of antibodies
CD2	CD58 (LFA-3)
LFA-1	ICAM-1
2B4	CD48
CD69	Unknown
DNAM-1 (CD226)	CD112, CD155
NKp80	AICL
Tactile (CD96)	CD155, CD111
TIGIT	CD112,CD113,CD155
CRTAM	TSLC1
C-type Lectin receptors –Activating/Inhibitory	
CD94/NKG2A/B	HLA-E
NKG2D	MICA, MICB, ULBP-1, ULBP -2, ULBP -3, ULBP -4, ULBP -5, ULBP -6
CD94/NKG2C	HLA-E
CD94/NKG2E/H	HLA-E, Qa-1b
Natural cytotoxicity receptors (NCR)	
NKp46 (NCR1)	Viral Hemagglutinin
NKp44 (NCR2)	Viral Hemagglutinin
NKp30 (NCR3)	B7h6, HCMV-pp65
Killer IG-like (KIR) – Activating/Inhibitory	
KIR2DLs, KIR3DLs, KIR2DS	HLA-C, HLA-B, HLA-A, HLA-G
Cytokines, growth factors and chemokines	Cytokines, growth factors and chemokine ligands
Toll-like receptors (TLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR)	Bacterial DNA, LPS, peptidoglycan, teichoic acids, flagellin, pilin, viral dsRNA and fungi zymosan

Table 2- Susceptibility of a number of different stem cells but not their differentiated counterparts to NK cell mediated cytotoxicity.

Type of cells	Stem cells	Differentiated	Parental cells
hESCs	++++++	+	
hMSCs	++++++	+	
hDPSCs	++++++	+	
hiPSCs	++++++		+
GBMs	++++++	+	

The susceptibility of a number of stem cells and their differentiated counterparts and the parental cells from which hiPSCs were derived to NK cell mediated cytotoxicity was determined using standard 4 hour 51Cr release assay [412]. The higher the number of + signs the more susceptibility to NK cell mediated cytotoxicity

Fig. 1A

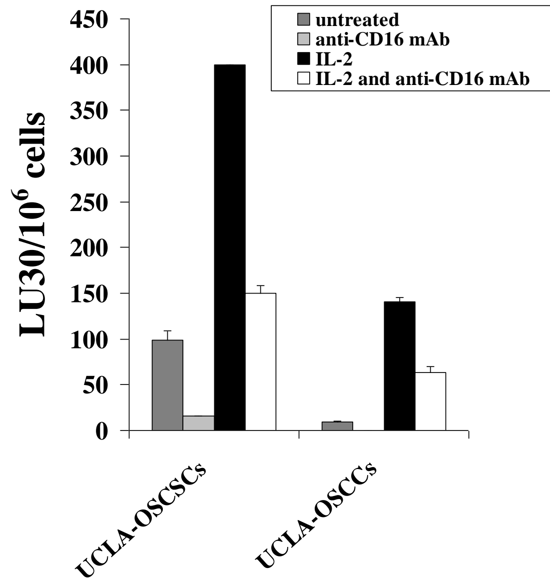


Fig. 1B

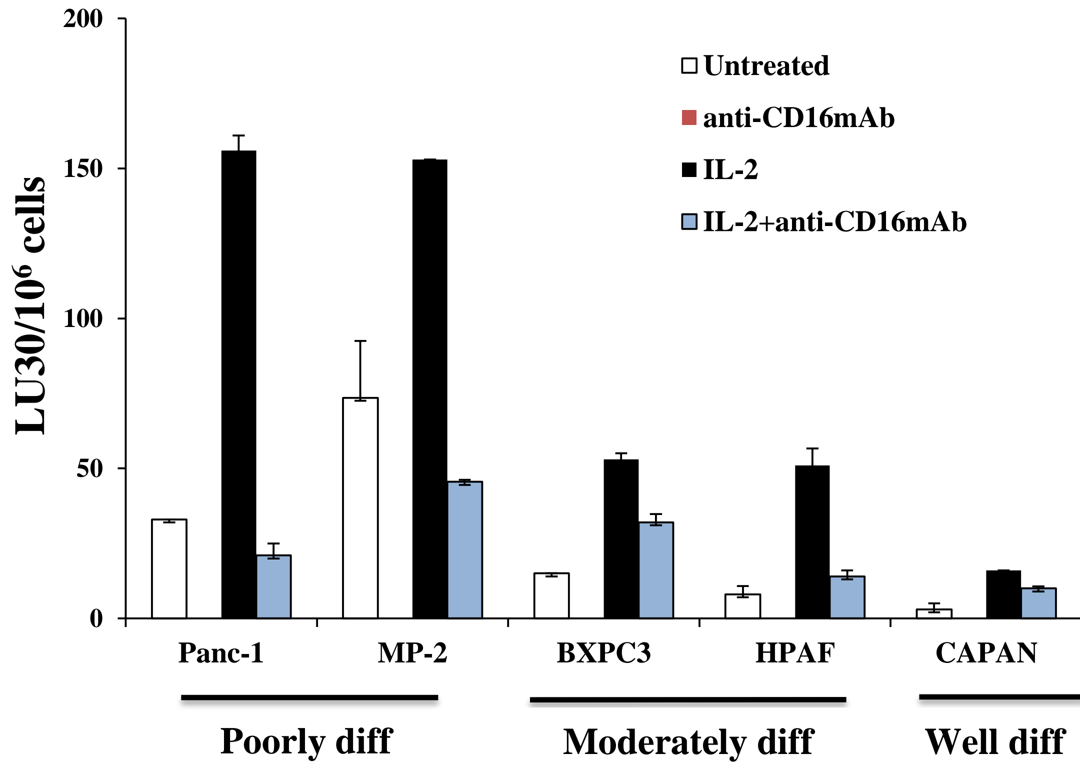


Fig. 1C

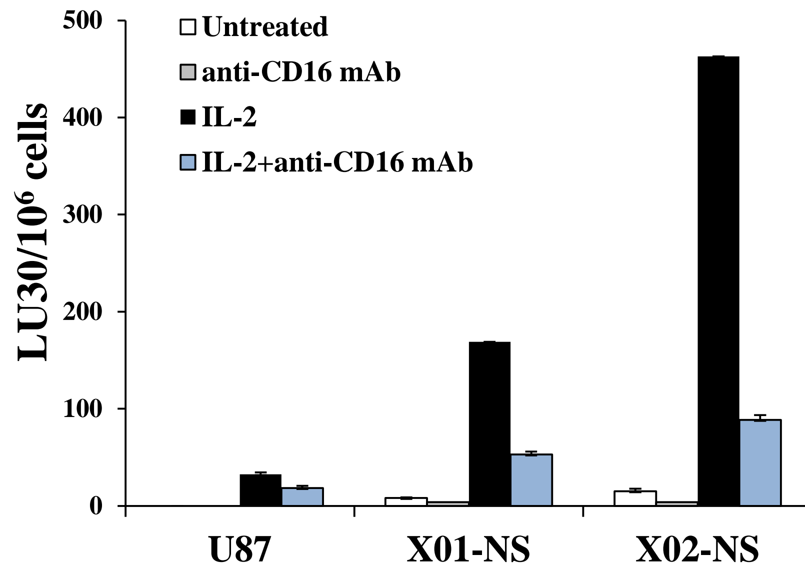


Fig. 2A

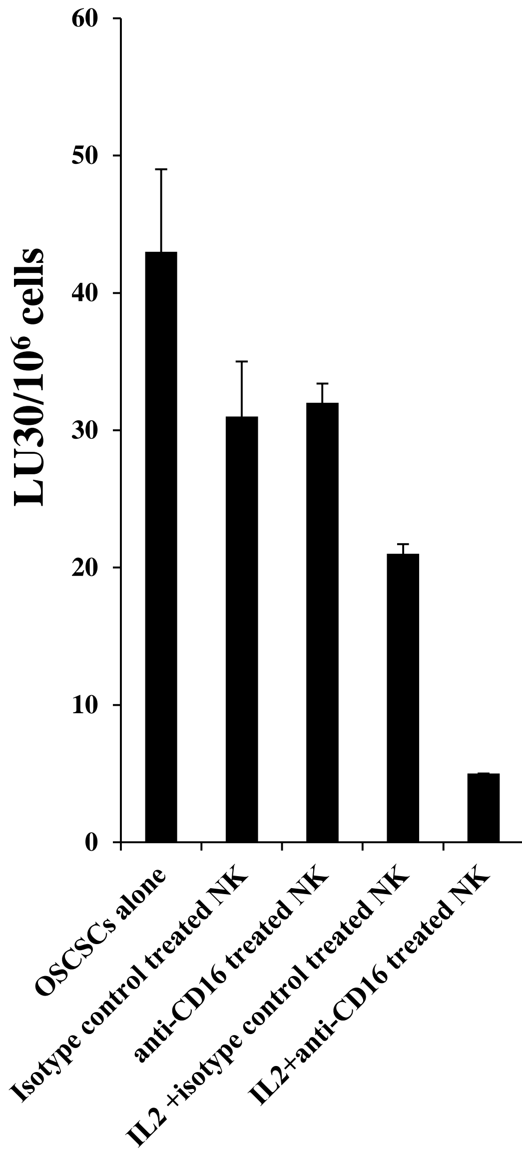


Fig. 2B

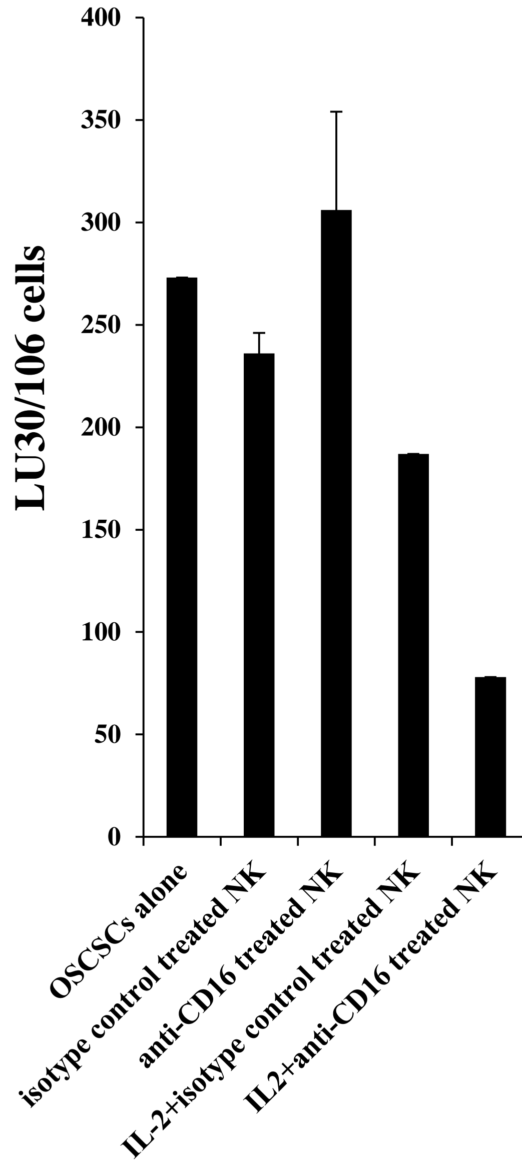
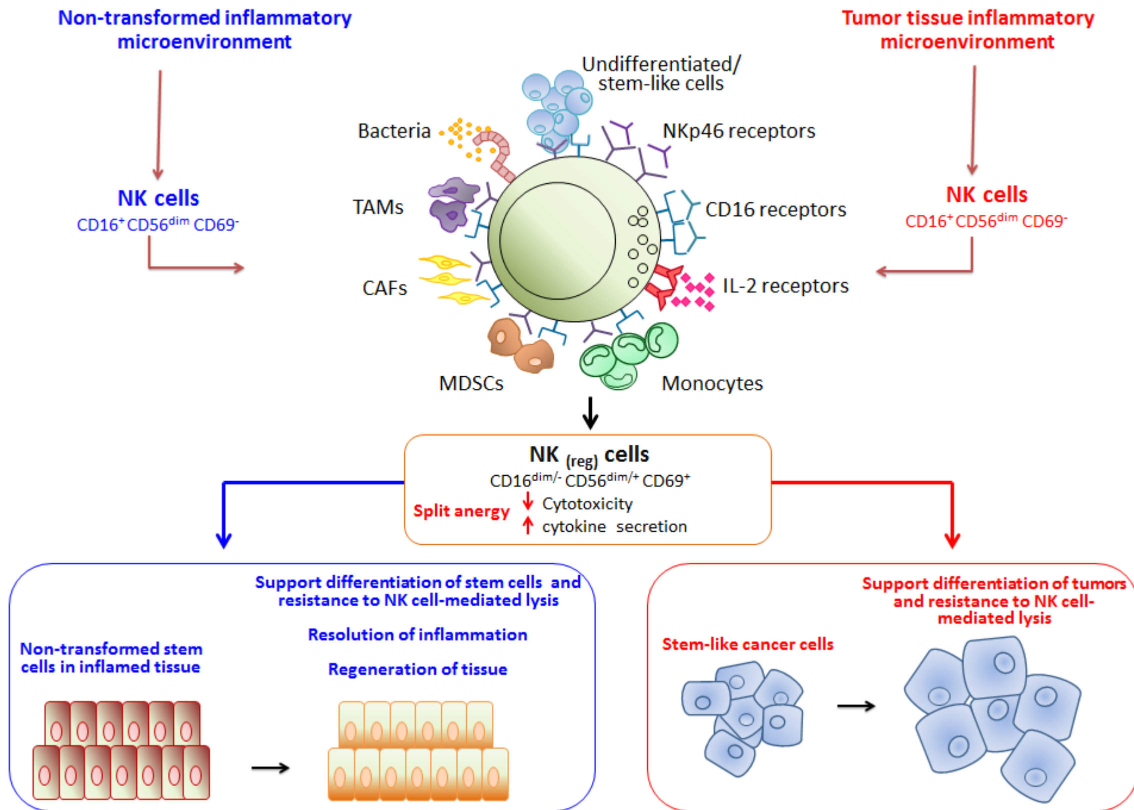


Fig. 3



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