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ORIGINAL ARTICLE

Defective NK cell expansion, cytotoxicity, and lack of ability to differentiate tumors from a pancreatic cancer patient in a long term follow-up: implication in the progression of cancer

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Abstract

The majority of the previous reports on NK cells use cross-sectional studies to establish the status of patient NK cell function, however such studies fail to evaluate the immune status of the patients on a continuous basis from the disease-free stage to progression of cancer. In this study, we performed a prospective study of the immune function by continuously monitoring the NK numbers, expansion and function of a pancreatic cancer patient from 1/6/2016 to 2/14/2019. The results indicated that at initial stages of the disease where no overt disease was identified, the patient had consistently higher percentages of NK and B cells and lower percentages of CD3 + T cells in the peripheral blood. The percentages of CD14 + monocytes were similar at the initial stages of the disease, and at the later stages of the disease, it increased and remained higher in the patient when compared to those from healthy donors. The numbers of expanded NK cells and the cytotoxic function, as well as secretion of IFN-γ from primary and osteoclast expanded patient NK cells remained consistently low throughout the years of follow up. Similarly, the majority of cytokines in patient's serum remained lower with the exception of IL-6 which was higher. The IFN-γ secreted from the patients' NK cells had much lower ability to differentiate the poorly differentiated oral tumors as assessed by their lack of ability to upregulate differentiation antigens. Overall, before any evidence of overt disease, patient NK cells exhibited significant dysfunction. Intervention at the stage of no disease or minimal disease may be important for the prevention of pancreatic cancer progression.

Keywords NK cells · Cytotoxicity · IFN-ϒ · Pancreatic cancer · Differentiation

Introduction

Pancreatic cancer is the third leading cause of cancer-related death in the United States and is associated with poor prognosis. Survival rate is considerably low despite the improvements in therapeutic strategies, such as surgery, and the use of chemotherapeutic drugs, radiotherapy, and immunotherapies [1–4]. Pancreatic cancer stem cells (CSCs) were shown to have increased cell growth, migration, clonogenicity, and self-renewal capacity and are found to be resistant to chemotherapy [5–10]. CSCs were shown to express lower MHC-class I on their surface which could be one reason for limited effectiveness and success of T-cell-based immunotherapies in cancer patients [11, 12]. On the other hand, Natural killer (NK) cells were found to be the key effectors in targeting low MHC-class I expressing CSCs, however, NK cells' expansion and function are shown to be defective in pancreatic cancer patients[13–16]. Although it is known that pancreatic cancer patients have diminished NK cell function, it is not clear whether the decrease in NK function is during overt disease and/or during the period when the patient either has a very low disease burden or is disease free based on the imaging studies.

In this case report, we monitored the function and phenotype of peripheral blood mononuclear cells (PBMCs), NK cells, T cells,B cells, and monocytes of a pancreatic cancer patient continuously for four years to determine whether we could find correlations either with treatments that she was receiving or the amount of disease which she had based on

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the imaging studies. In addition, we also present the functional interaction of patient's NK cells with autologous and allogeneic monocytes and osteoclasts. More importantly, we demonstrate the inability of IFN-γ secreted by patient's NK cells to differentiate CSCs, suggesting an important mechanism of cancer progression in the patient.

Results

Increased percentages of NK and B cells, and decreased percentages of T cells in the initial stage, whereas increased percentages of monocytes were observed at the advanced stage of cancer in pancreatic cancer patient.

We determined the percentages of immune cell subsets within CD45 + peripheral blood mononuclear cells (PBMCs) of cancer patient and healthy individuals at different time points. Lower numbers of PBMCs per one ml of blood were obtained in cancer patient in comparison to those of healthy individuals (Fig. S1A). At initial stage of cancer, higher NK and B cells, and decreased T cells percentages were found in cancer patients' PBMCs when compared to healthy individuals (Fig. 1a, b and d). At advanced stage of cancer, percentages of NK and B cells in cancer patients' PBMCs were slightly higher as compared to healthy individuals but progressive decrease in percentages of T cells were seen along with cancer progression in cancer patient (Fig. 1a, b and d). Similar or slightly higher monocyte percentages were seen in cancer patients' PBMCs as compared to healthy individuals at the initial disease stage but significant increases in monocyte percentages were observed at advanced stage in cancer patient (Fig. 1c). Moreover, decreased CD3 + CD4 + T cells, but increased CD3 + CD8 + T cells were obtained at advanced disease stage in cancer patient when compared to healthy individuals (Fig. 1e, f). We also observed decreased NK cell counts at several time points tested, with the exception of those obtained on 11.29.2017, 1.23.2018, and 3.6.2018 (Fig. S1B). Decreased or similar B cell counts (Fig. S1C), and decreased monocyte (Fig. S1D) and T cell counts (Fig. S1E) were seen in cancer patient in comparison to healthy individuals. However, we could only assess the numbers of PBMCs and individual PBMC subtypes per ml of blood until 8/21/2018 since after that period we did not record the amount of blood obtained from either healthy or from patient, but we assessed the percentages of each PBMC subtype until 2/14/2019 (Fig. S1 compared to Fig. 1). These findings indicated imbalance of immune cell subsets in cancer patients' PBMCs depending on the disease stage and/or cancer progression.

Decreased NK cell-mediated cytotoxicity and lower secretion of IFN-γ by cancer patients' PBMCs

Lower NK cell-mediated cytotoxicity was seen by cancer patients' PBMCs as compared to those from healthy individuals (Fig. 2a). Oral supplementation of probiotic bacteria sAJ2 in August 2016 and thereafter, increased NK cell-mediated cytotoxicity in PBMCs for about 11 months after the start of supplementation, however, the effect was lost thereafter during cancer progression (Fig. 2a). We also observed lower amounts of IFN-γ secretion by cancer patients' PBMCs when treated with IL-2 (Table 1) (Fig. 2b, e, and Tables 1 and 3) or the combination of IL-2 and anti-CD16 mAbs (Fig. 2c, f, and Tables 1 and 3) or the combination of IL-2 and sonicated AJ2 (sAJ2) (Fig. 2d, g and Table 3). Decreased IFN-γ secretion by cancer patients' PBMCs was also seen when they were treated with the combination of IL-2 and anti-CD3/CD28 mAbs (Fig. S2A, S2B and Table 3). These findings indicated that cancer patients' PBMCs were defective and had lower ability to lyse tumors and secrete IFN-γ.

Decreased key surface receptors, cytotoxicity and IFN-γ secretion by the patient NK cells

Next, we determined the levels of surface receptors, cytotoxic activity and secretion of IFN-γ by purified NK cells obtained from cancer patient and healthy individuals' PBMCs. As compared to healthy individuals, NK cells from cancer patients were found to exhibit lower Nkp30, Nkp44, Nkp46, CD94, NKG2D, and KIR2, and higher KIR3 receptor expression (Fig. 3a and S3). Lower NK cell-mediated cytotoxicity was seen in cancer patients' NK cells (Fig. 3b). Similar to PBMCs (Fig. 2a), increased NK cell-mediated cytotoxicity was seen when the patient started on the oral supplementation of AJ2, however, this effect did not last long (Fig. 3b). We also observed severe defects in cytotoxicity when patient NK cells were used to lyse MP2 poorly differentiated pancreatic tumors when compared to those from healthy individuals (Fig. 3c). Cancer patients' NK cells secreted lower amounts of IFN-γ when they were treated with IL-2 (Fig. 3d, e, i and Tables 2 and 3), combination of IL-2 and anti-CD16 mAbs (Fig. 3f, i and Tables 2 and 3), and the combination of IL-2 and sAJ2 (Fig. 3g, h and Tables 2 and 3) when compared to those from healthy individuals. NK cells are well known for their ADCC function, therefore, to determine that decline in cytotoxicity also correlates with decrease in ADCC, we differentiated both the MP2 tumors as well as OSCSCs with the NK supernatants generated from activated NK cells from healthy individuals and then used in ADCC assay since we had established previously that the majority of the ADCC function obtained

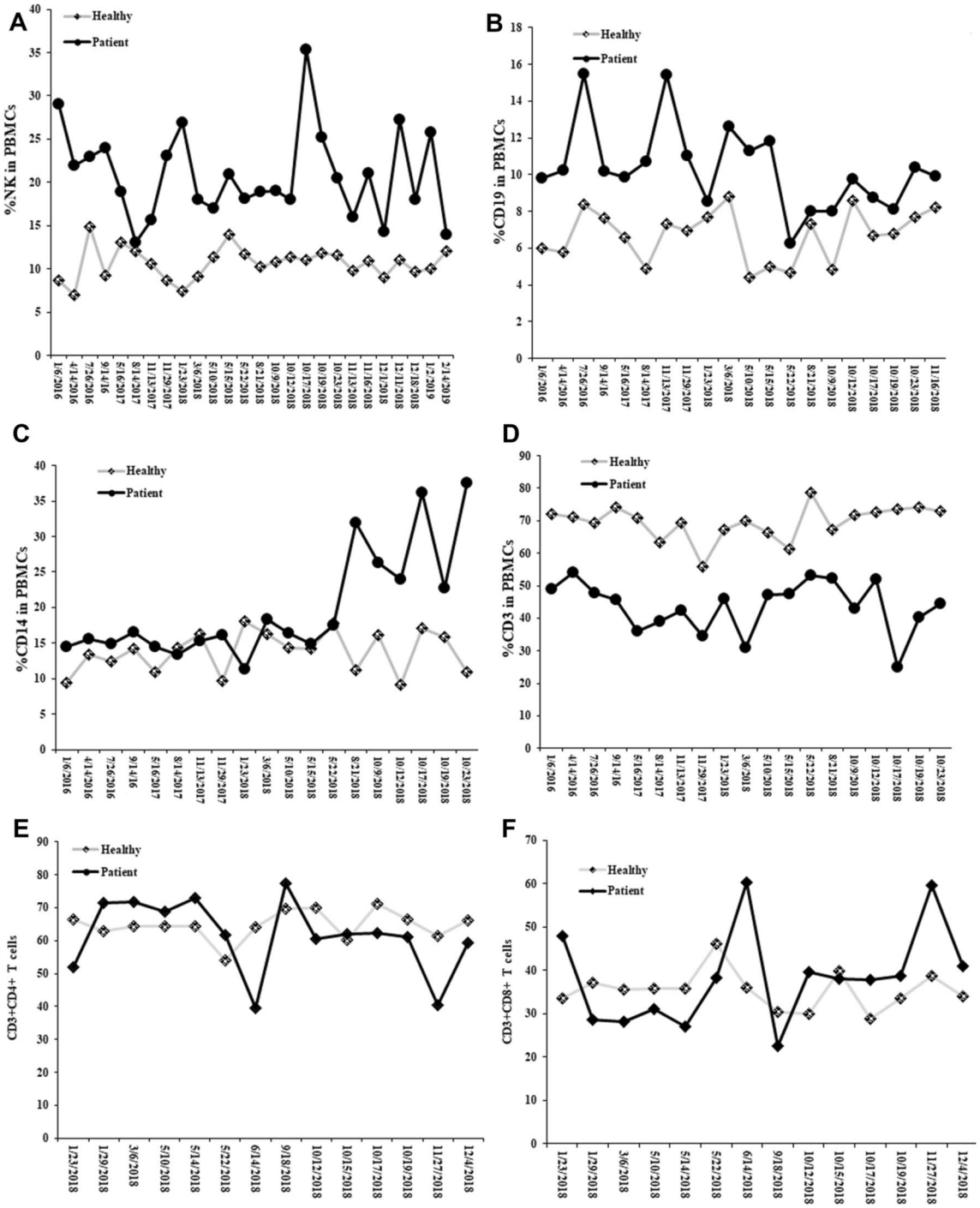
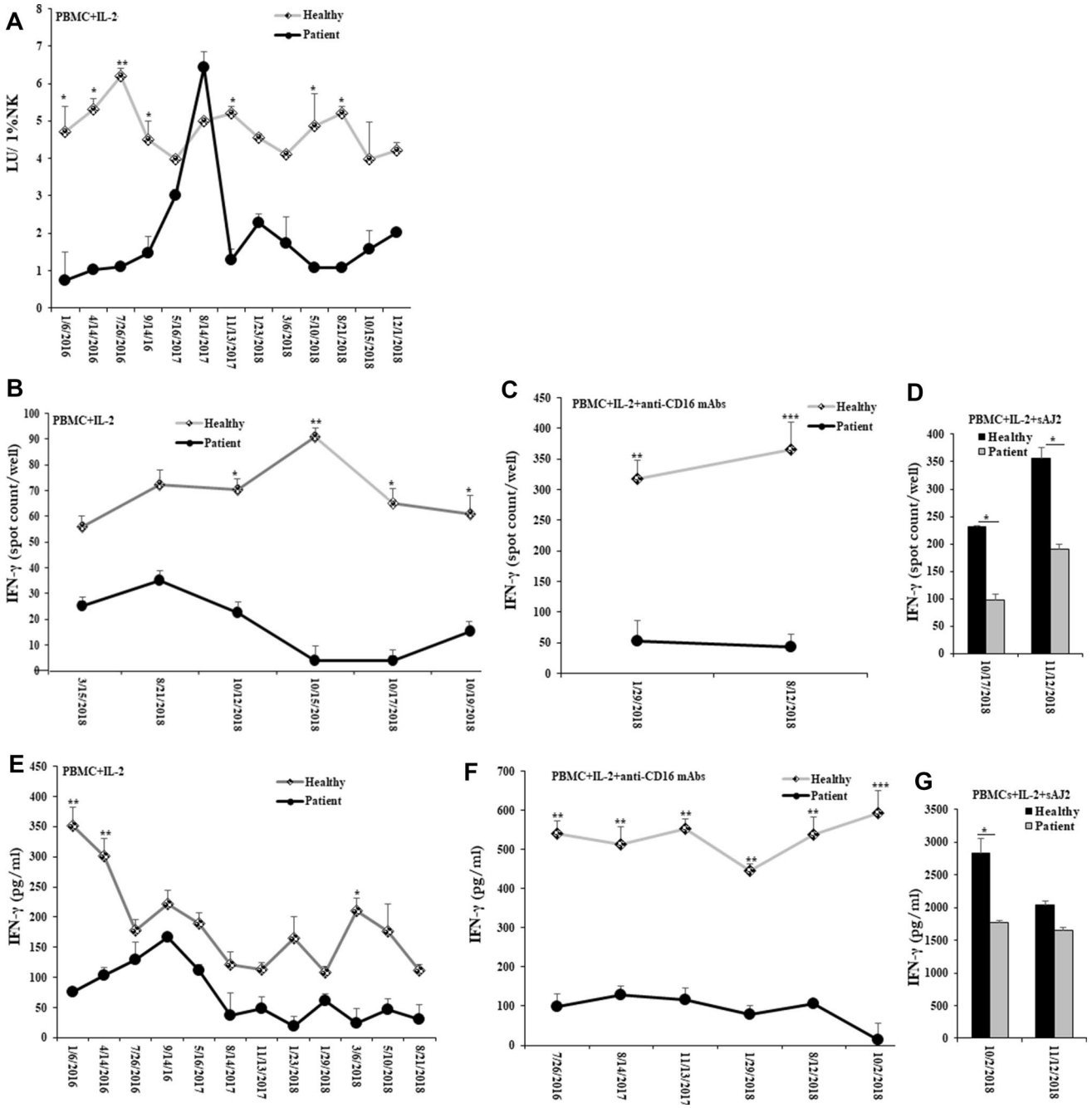


Fig. 1 Determination of immune cell subsets in PBMCs of cancer patient and healthy individuals. PBMCs were isolated from the 30 ml peripheral blood, and similar counts of PBMCs (2×10^5 cells) were used to determine the percentages of NK cells **(a)**, B cells **(b)**, monocytes **(c)**, T cells **(d)**, CD3 + CD4 + T cells **(e)**, and CD3 + CD8 + T cells **(f)**. IgG2 isotype control antibodies were used as controls. The data were obtained at different time points as shown in the figures. PBMCs obtained from one healthy individual was used at each time point. Healthy individuals were different throughout the study but were of same age (52–56 years) and gender (female) group as cancer patient

were seen against differentiated tumors. Patient NK cells mediated poor ADCC against NK-differentiated MP2 (Fig. 3j) and NK-differentiated OSCSCs (Fig. 3k), when compared to those obtained by healthy NK cells. Therefore, both direct cytotoxicity and ADCC function were compromised in the patient.



Decreased levels of cytokines were also seen in the peripheral blood-derived sera of cancer patient when compared to those of healthy individuals (Fig. 4). We also determined the secretion of IFN-γ by T cells when they were treated with IL-2 (Fig. S4A and, Tables 3 and S2), or with the combination of IL-2 and anti-CD3/CD28 mAbs (Fig. S4B and, Tables 3 and S2). Similar levels of IFN-γ secretion were seen by cancer patients' and healthy individuals' T cells (Fig. S4). These results indicated severe functional defects in NK cells of cancer patient.

Suppressed osteoclast-mediated expansion and functional activation in cancer patient's NK cells compared to those of healthy individuals

To investigate NK cell numbers and function, we used osteoclasts (OCs) as feeder cells in combination with sAJ2 to expand NK cells as previously described [17]. OC-expanded NK cells were counted on different days of cultures as shown in figures (Figs. 5a, d, S5A, S5D, S6A, and S6D). We found decreased levels of the NK expansion in all time-points tested during the initial phases of the disease in the cancer

◂**Fig. 2** Cancer patients' PBMCs exhibited lower levels of NK cell-mediated cytotoxicity and IFN-γ secretion when compared to healthy individuals' PBMCs. PBMCs (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with IL-2 (1000 U/ml) for 18 h before they were used as effectors to determine cytotoxicity against OSCSCs using standard 4-h ^{51}Cr release assay. The Lytic units (LU) 30/10^6 cells were determined using the inverse number of cells required to lyse 30% of OSCSCs × 100. Cytotoxicity mediated by 1% NK cells was determined using flow cytometry obtained percentages of CD16 + NK cells in PBMCs **(a)**. PBMCs (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with IL-2 (1000 U/ml) for 18 h before ELISpot was conducted to determine the numbers of IFN-γ spots **(b)**. PBMCs (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with the combination of IL-2 (1000 U/ml) and anti-CD16 mAbs (3 µg/ml) for 18 h before ELISpot was conducted to determine the numbers of IFN-γ spots **(c)**. PBMCs (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with the combination of IL-2 (1000 U/ml) and probiotic bacteria sAJ2 at a ratio of 1:2 (PBMCs:sAJ2) for 18 h before ELISpot was conducted to determine the numbers of IFN-γ spots **(d)**. PBMCs were treated as described in **b** for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA **(e)**. PBMCs were treated as described in **c** for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA **(f)**. PBMCs were treated as described in **d** for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA **(g)**. The data were obtained at different time points as shown in the figures. Duplicate samples from healthy individual and cancer patient were used at each time point. Healthy individuals were different throughout the study but were of same age (52–56 years) and gender (female) group as the cancer patient

patient (Figs. 5a, S5A, and S6A). In 2018, day 6 to day 15 expansion profile was similar to those seen at the initial stages of the disease, but increased expansion was seen on days 18 to day 25 (Figs. 5a, S5A, and S6A). We have previously demonstrated increased percentages of T cells and decreased percentages of NK cells in cancer patients' OC-expanded NK cells due to faster expansion rate of small numbers of contaminating T cells in the sorted populations of NK cells [17]. In agreement with our previous findings, we also observed decreased NK cells and increased T cells on all time-points at the initial stages of the disease (Figs. 5b–e, S5B-S5E, and S6B-S6E). In 2018, days 6-day 15 expansion profile was similar to those seen at the initial stages of disease, but increased NK cells and decreased T cells were observed on days 18-day 25 (Figs. 5b-5e, S5B-5E, and S6B-S6E). OC-expanded patient NK cells exhibited significantly lower IFN-γ secretion (Figs. 5f, S5F and S6F), and mediated lower cytotoxicity (Figs. 5g, S5G, and S6G-S6H) at all time-points tested when compared to those of healthy individuals. We also determined IFN-γ secretion of OC-expanded T cells and, observed similar or slightly lower IFN-γ secretion by patient's OC-expanded T cells when compared to healthy individuals' OC-expanded T cells (Fig. S7). These results indicated that cancer patient's NK cells exhibited much lower potential to be expanded and exhibited lower function when compared to healthy individuals.

Monocytes from the patient had much lower ability to induce IFN-γ secretion by the NK cells when compared to those from healthy individuals

To investigate the function of monocytes, we cultured the healthy individuals' and cancer patient's NK cells with either autologous or allogeneic monocytes. Patient's monocytes in comparison to those of healthy individuals were less capable of inducing IFN-γ secretion in both healthy individuals' and cancer patient's NK cells (Fig. 6a–d). Cancer patient's monocytes were also found to express lower surface expression of CD16 and MHC-class II when compared to healthy individuals' monocytes (Fig. 6e). These findings suggested defects in the ability of cancer patient's monocytes to activate NK cells.

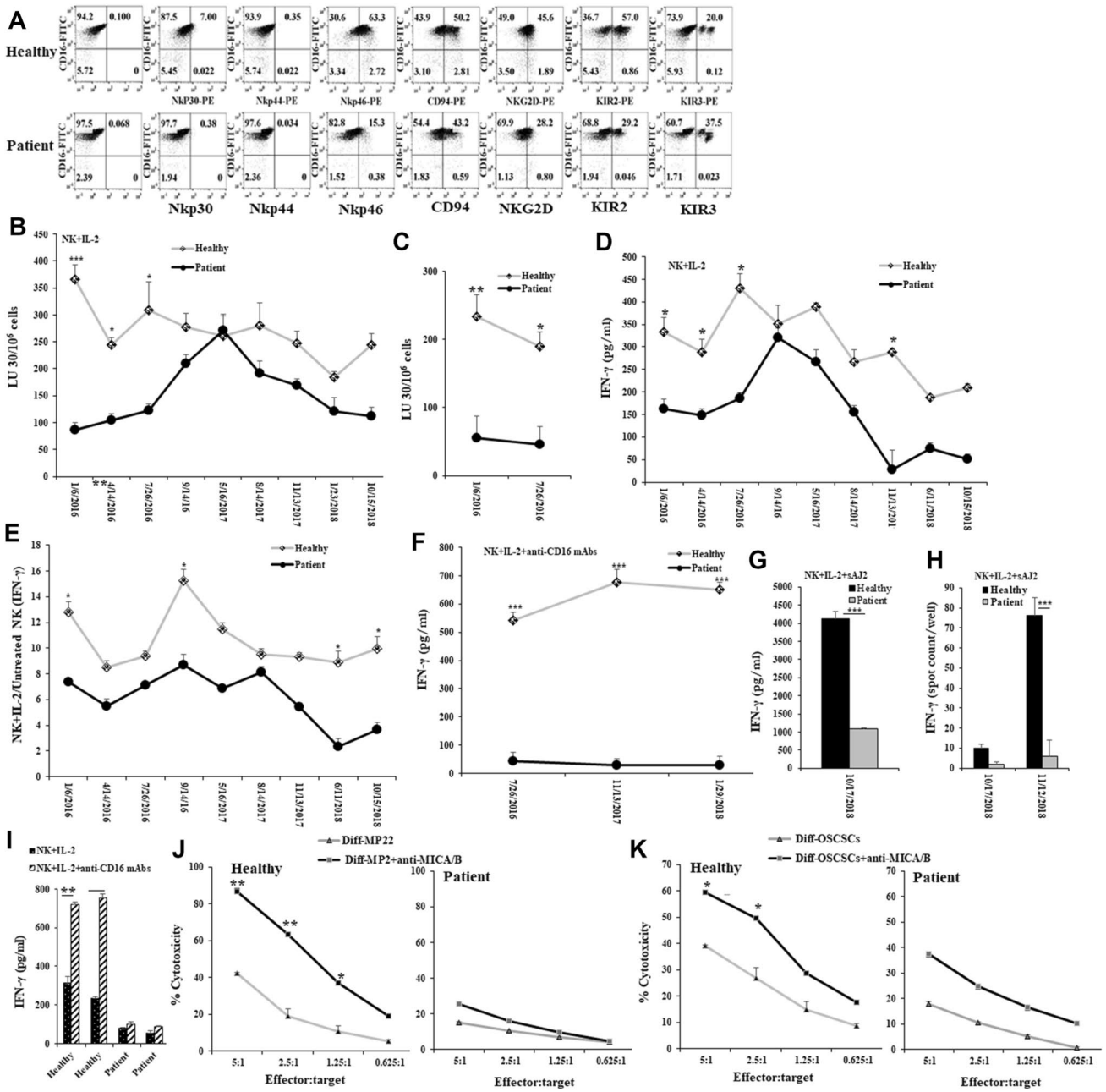
IFN-γ secreted by cancer patients' NK cells was less capable of inducing differentiation of cancer stem cells

When we used the same amounts of IFN-γ to induce differentiation, the IFN-γ secreted by the pancreatic cancer patient's NK cells was less effective in differentiating OSCSC tumors when compared to those from healthy individuals (Fig. 7). The rationale for using OSCSCs is because these tumors are highly sensitive to IFN-γ mediated differentiation [16, 18, 19]. We have previously shown that IFN-γ is responsible for the increased surface expressions of MHC-class I, B7H1, and CD54 on OSCSCs which correlates with the degree of differentiation in these cells ([18, 20]). The magnitude of the increases in the surface expression of MHC-class I, B7H1, and CD54 on OSCSCs by supernatants from patient NK cells' was much less than those observed by the supernatants obtained from the healthy individuals' NK cells (Fig. 7a). In addition, differentiated tumors became resistant to NK cell-mediated cytotoxicity, and the levels of resistance induced by supernatants from the cancer patient's

Table 1 Cancer patients' PBMCs exhibited lower levels of IFN-γ secretion when compared to healthy individual PBMCs

IFN-γ (pg/ml)	Patient	Healthy
11/13/2017		
Untreated PBMCs	4.94	30.01
IL-2 treated PBMCs	68.07	155.04
IL-2 + anti-CD16 mAbs treated PBMCs	104.98	721.7
1/23/2018		
IL-2 treated PBMCs	8.11	56.02
IL-2 + anti-CD16 mAbs treated PBMCs	133.23	457.93

PBMCs (1×10^6 cells/ml) of cancer patient and healthy individuals were left untreated or treated with IL-2 (1000 U/ml) or with the combination of IL-2 (1000 U/ml) and anti-CD16 mAbs (3 µg/ml) for 18 h before the supernatants were harvested to determine IFN-γ secretion using multiplex assay



NK cells were much less than those from healthy individuals' NK cells (Fig. 7b). These findings indicated lower potential of IFN-γ secreted by the cancer patient's NK cells to induce tumor differentiation.

Discussion

The majority of studies performed on patients with pancreatic cancer addresses the immune cell abnormalities using the patient blood on cross sectional studies. Our study is unique since we have performed a longitudinal study in which we tracked the immune cell function of the patient for several years at the regular intervals to determine the changes in the patters of immune cell phenotype and function. Indeed, we have identified several factors that are likely important for disease progression or remission. As shown in Fig. 1, the percentages of NK and CD19 positive B cells remained higher whereas the levels of CD3 + T cells were lower in the patient when compared to healthy individualss. The pattern of the increase in NK cells and the decrease in CD3 + T cells remained consistent throughout the observation period, however, in 5/22/2018 the percentages of CD19 + B cells declined and the levels of CD14 + monocytes

◂**Fig. 3** Cancer patients' NK cells exhibited lower levels of cytotoxicity, IFN-γ secretion, and ADCC when compared to healthy individuals' NK cells. Freshly purified NK cells from cancer patient and healthy individual were analyzed for the surface expressions of CD16, Nkp30, Nkp44, Nkp46, CD94, NKG2D, KIR2, and KIR3 using flow cytometry. IgG2 isotype control antibodies were used as controls. The data were obtained on 9.14.2016 **(a)**. NK cells (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with IL-2 (1000 U/ml) for 18 h before they were used as effectors to determine cytotoxicity against OSCSCs **(b)** and MP2 **(c)** using standard 4-h ^{51}Cr release assay. The LUs were determined as described in Fig. 2a. NK cells (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with IL-2 (1000 U/ml) for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA **(d)**. NK cells (1×10^6 cells/ml) of cancer patient and healthy individuals were left untreated or treated with IL-2 (1000 U/ml) for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA. Ratio of IFN-γ secretion by IL-2-treated vs. untreated NK cells is shown in the figure **(e)**. NK cells (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with the combination of IL-2 (1000 U/ml) and anti-CD16 mAbs (3 μg/ml) for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA **(f)**. NK cells (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with the combination of IL-2 (1000 U/ml) and probiotic bacteria sAJ2 at a ratio of 1:2 (NK:sAJ2) for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA **(g)**. NK cells were treated as described in **g** for 18 h before ELISpot was conducted to determine the numbers of IFN-γ spots **(h)**. NK cells (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with IL-2 (1000 U/ml) or with the combination of IL-2 (1000 U/ml) and anti-CD16 mAbs (3 μg/ml) for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA **(i)**. MP2 and OSCSCs were differentiated as described in the Materials and Methods section. Purified NK cells (1×10^6 cells/ml) from healthy individuals and cancer patients were treated with IL-2 (1000 U/ml) for 18 h before they were used as effectors in chromium release assay. NK cell-differentiated MP2 **(j),** and NK cell-differentiated OSCSCs **(k)** were labeled with ^{51}Cr for an hour before they were washed and used in ^{51}Cr release assay. ^{51}Cr-labeled tumor cells were then left untreated or treated with anti-MICA/B mAb (5 μg/ml) for 30 min. The unbound antibodies were then washed , and the cytotoxicity was determined using a standard 4-h ^{51}Cr release assay. LU 30/10^6 cells were determined as described in Fig. 2a. The data was obtained at different time points as shown in the figures. Duplicate samples from healthy individual and cancer patient were used at each time point. Healthy individuals were different throughout the study but were of same age (52–56 years) and gender (female) group as the cancer patient

increased and remained consistently higher until the end of life. In addition, even though the levels of CD19 + B cells declined, it remained within the range of those from the healthy individuals, and the levels of monocytes were raised sharply and remained higher when compared to healthy individuals until the end of the patient's life. Increase in monocytes is likely due to the decrease in CD19 + B cells since both the percentages of NK and CD3 + T cells remained fairly unchanged. In terms of CD4 + and CD8 + T cell percentages, initially the patient exhibited slightly higher proportions of CD4 + T cells when compared to the healthy individuals, however, later the levels of CD8 + T cells rose moderately in the patient when compared to healthy controls. In general, monocytes were the subsets that deviated the most from the healthy controls. Of interest, the patient had higher percentages of NK cells and lower percentages of CD3 + T cells when compared to healthy individuals throughout the observation period.

We assessed the functions of NK cells in different time points using PBMC-derived NK cells. The findings indicated that NK cells within PBMCs had significantly less NK cell-mediated cytotoxicity in the patient when compared to healthy individuals, and that this profile was observed from the beginning of 2016 and continued until the end of 2018 with the exception of one time point in 2017 in which the patient NK cells had higher cytotoxicity when compared to healthy donors. The NK cell-mediated cytotoxicity was standardized between the donors based on per percent of NK cells in PBMCs to determine the function of NK cells based on the numbers of NK cells in PBMCs. PBMCs from the patient also consistently secreted lower amounts of IFN-γ when compared to those obtained from the PBMCs' of healthy individuals with all different types of treatments. We have also seen the same profile when IFN-γ was measured in ELISpot assay. Similarly, when NK cells were sorted out from both the patient and healthy individuals, they exhibited the same pattern as seen with PBMCs. In addition, both direct cytotoxicity and ADCC were compromised in patient NK cells. Therefore, NK cells from the patient consistently exhibited lower cytotoxicity throughout the years of observation, but it also failed to either demonstrate adequate IFN-γ spots or its secretion in ELISpot and ELISA assays, respectively. Since we have shown previously that NK cells are the key effectors in lysis of cancer stem cells/poorly differentiated cells, and also, have a role in differentiation of the tumor cells through the function of IFN-γ, having defects in several key functions is likely the key mechanisms for the survival and expansion, and eventual invasion of the tumor cells.

We next assessed the ability of NK cells from the patient to be super-charged by the use of IL-2 and anti-CD16 mAb treatment in the presence of osteoclasts and sonicated AJ2 which is the combination of several gram positive probiotic bacterial species [17, 21]. When the ability of sorted/enriched populations of patient NK cells was compared to those of healthy individuals, number of expanding cells in healthy were consistently higher at different days of expansion when compared to the patient as assessed from 1/6/2016 to 1/24/2018. Within the expanding cells the percentages of expanding NK cells were always higher in healthy when compared to patient until 9/2016 in which at later expansion periods (≥ 18 days) started approaching to the healthy donor NK expansion (Figs. 5b, d, S5B, S5D, S6B and S6D). Four weeks before 9/2016 the patient started taking AJ2 probiotic supplement in addition to her regular treatment

Table 2 Cancer patients' NK cells exhibited lower levels of IFN-γ secretion when compared to healthy individual's NK cells

IFN-γ (pg/ml)	Patient	Healthy
11/13/2017		
IL-2 treated NK cells	80.95	98.38
IL-2 + anti-CD16 mAb treated NK cells	57.24	145.29

Freshly purified NK cells (1×10^6 cells/ml) of cancer patient and healthy individual were treated with IL-2 (1000 U/ml) or with the combination of IL-2 (1000 U/ml) and anti-CD16 mAbs (3 µg/ml) for 18 h before the supernatants were harvested to determine IFN-γ secretion using multiplex assay

Table 3 Decreased IFN-γ secretion in by cancer patient's PBMCs, NK, and T cells when compared to those from healthy individual

Cultures	Healthy vs. Patient IFN-γ secretion
PBMCs + IL-2	1.8–3.9
PBMCs + IL-2 + anti-CD16 mAb	4.4–26.9
PBMCs + IL-2 + sAJ2	1.1–1.5
PBMCs + IL-2 + anti-CD3/28	1.25–1.6
NK + IL-2	1.2–11.7
NK + IL-2 + anti-CD16 mAb	18.66–32
NK + IL-2 + sAJ2	3.2–4.1
T cells + IL-2	1.06–1.9
T cells + IL-2 + anti-CD3/28	1.05–2.8

PBMCs (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with IL-2 (1000 U/ml) or with the combination of IL-2 (1000 U/ml) and anti-CD16 mAb (3 µg/ml) or with the combination of IL-2 (1000 U/ml) and probiotic bacteria sAJ2 at a ratio of 1:2 (PBMCs:sAJ2) or with the combination of IL-2 (1000 U/ml) and anti-CD3/CD28 (3 µg/ml) for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA. NK cells (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with IL-2 (1000 U/ml) or with the combination of IL-2 (1000 U/ml) and anti-CD16 mAb (3 µg/ml) or with the combination of IL-2 (1000 U/ml) and probiotic bacteria sAJ2 at a ratio of 1:2 (NK:sAJ2) for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA. T cells (1×10^6 cells/ml) of cancer patient and healthy individuals were treated with IL-2 (100 U/ml) or with the combination of IL-2 (100 U/ml) and anti-CD3/CD28 mAbs (3 µg/ml) for 18 h before the supernatants were harvested to determine IFN-γ secretion using single ELISA. For all these treatments, ratios of IFN-γ secreted by healthy vs. patient cells are shown in the table

regimen. Even though both patient and healthy individuals started with similar levels of purity for NK cells, the patient lost the ability to expand NK cells initially and expanded CD3 + T cells until later days of expansion (≥ 18 days) after which the patient started expanding NK cells. Overall, patient expanded less total cells, more CD3 + T cells and less CD16 + NK cells in the majority of time points tested. More importantly, the levels of NK cell-mediated cytotoxicity and also ability to produce IFN-γ was severely compromised at all time points in expansion, and all the dates tested. We have reported previously that the function of NK cells is significantly compromised at the pre-neoplastic stage of pancreatic cancer in the mouse model suggesting that establishment of overt tumors may be the reason for the progression of pancreatic cancer since NK cells are the key cells in targeting cancer stem cells/poorly differentiated tumors which seed the tumors [22, 23]. The results obtained from the PBMCs, sorted primary NK cells and super-charged NK cells in the patient, all point to the severe inhibition of NK cytotoxicity and ability to secrete IFN-γ under different experimental conditions. Indeed, when comparing the levels of cytokines and chemokines, patient's peripheral blood-derived sera exhibited lower levels of all cytokines and chemokines including IFN-γ and TNF-α with the exception of IL-8 and MIP-3a, which were similar in both patient and healthy. Moreover, the levels of IL-6 were elevated in the serum from patient when compared to that obtained from the healthy individuals, suggesting a role for IL-6 in the disease progression. Indeed, it was shown previously that IL-6 is likely induced by the pancreatic tumor cells, which participate in tumor growth as well as inhibition of IFN-γ secretion by the NK cells [23].

We have previously shown that IFN-γ and TNF-α synergize to increase the levels of differentiation in the tumors as assessed by increased expressions of CD54, MHC-class I and PDL-1 and downmodulation of CD44 in tumor cells and tumor tissues [21]. Accordingly, when OSCSCs were treated with the supernatants obtained from the primary NK cells or super-charged NK cells, patient derived NK supernatants at the same amounts of IFN-γ had significantly lower ability to differentiate tumors when compared to those from healthy controls (Fig. 7), whereas differentiation by the activated T cell supernatants were relatively better but not similar between the healthy individual and the patient (data not shown). In addition, we have recently shown that super-charged NK cells are important in expansion of CD8 + T cells selectively, since they can target and kill the CD4 + T cells directly [24]. Thus, decreased ability of NK cells to target CD4 + T cells may be one reason why T regulatory cells survive and inhibit the function of NK and T cells in pancreatic cancer patients [24].

We demonstrated that at the latest dates, patient exhibited increased percentages of monocytes and decreased percentages of B cells. Certain subsets of monocyte/macrophages and MDSCs are known to inhibit the function of NK and T cells, therefore, we analyzed the interaction of NK cells with monocytes and found monocytes from the patients to have much lower ability to induce synergistic induction of either IFN-γ spots or secretion of IFN-γ in NK cells. These results indicated that although the patient had higher percentages

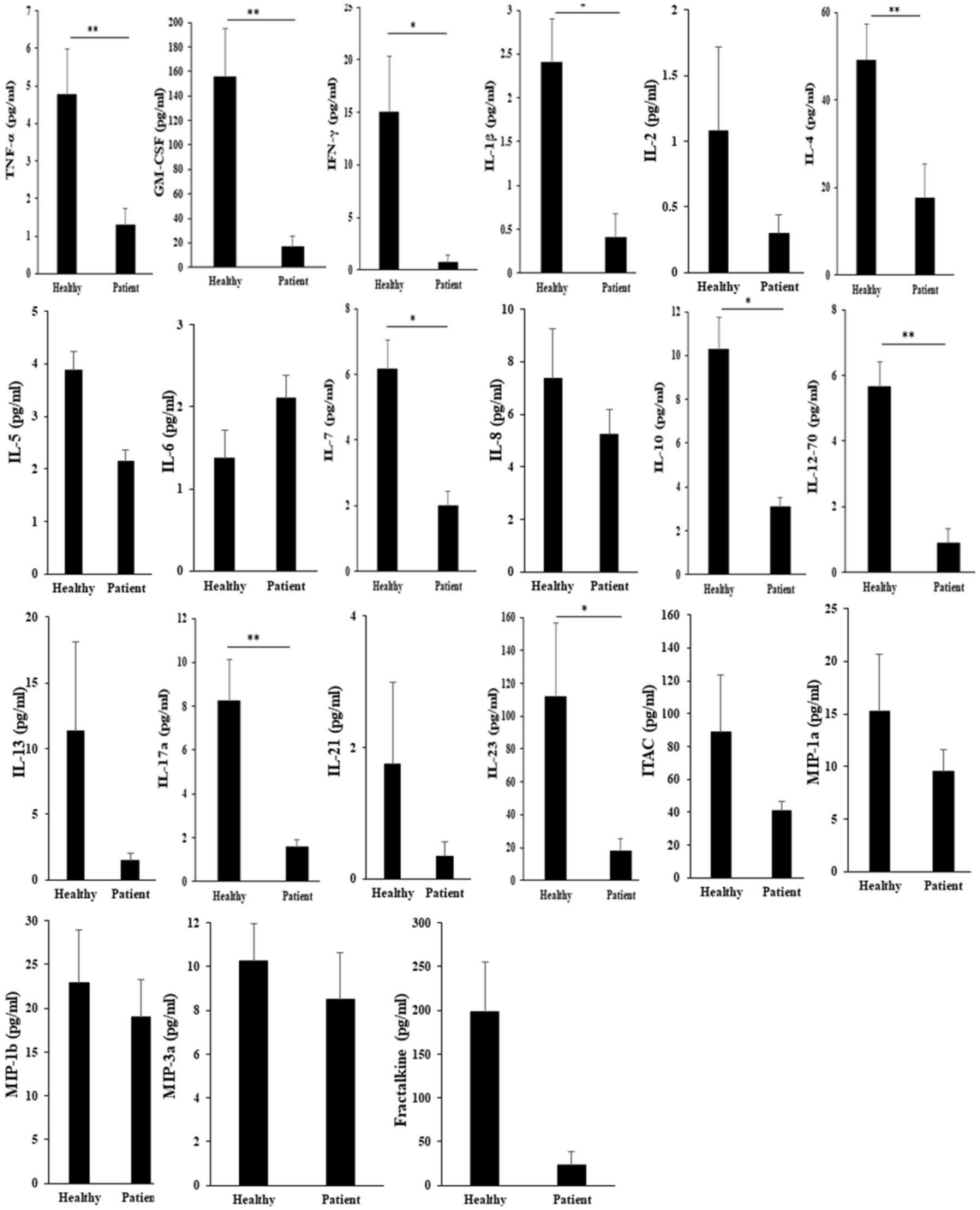


Fig. 4 Decreased cytokine, chemokine, and growth factor secretion in cancer patients' peripheral blood-derived serum. Sera were obtained from the peripheral blood of healthy individuals ($n=4$; two healthy individuals at each time point) and cancer patient ($n=2$), and analyzed for the levels of cytokines, chemokines, and growth factors using a multiplex array kit. Figures present the average of two time points (1/6/2016 and 5/16/2017). Healthy individuals were different but were of same age (52–56 years) and gender (female) group as the cancer patient

of monocytes/macrophages, their ability to induce activation of NK cells was compromised. Patient monocytes had decreased ability to upregulate key surface receptors such as CD16 receptors. Decreased receptor expression, compounded by decreased functional activation of these cells through CD16 receptor could be one reason why the monocytes were not able to activate NK cells (Fig. 6). Such

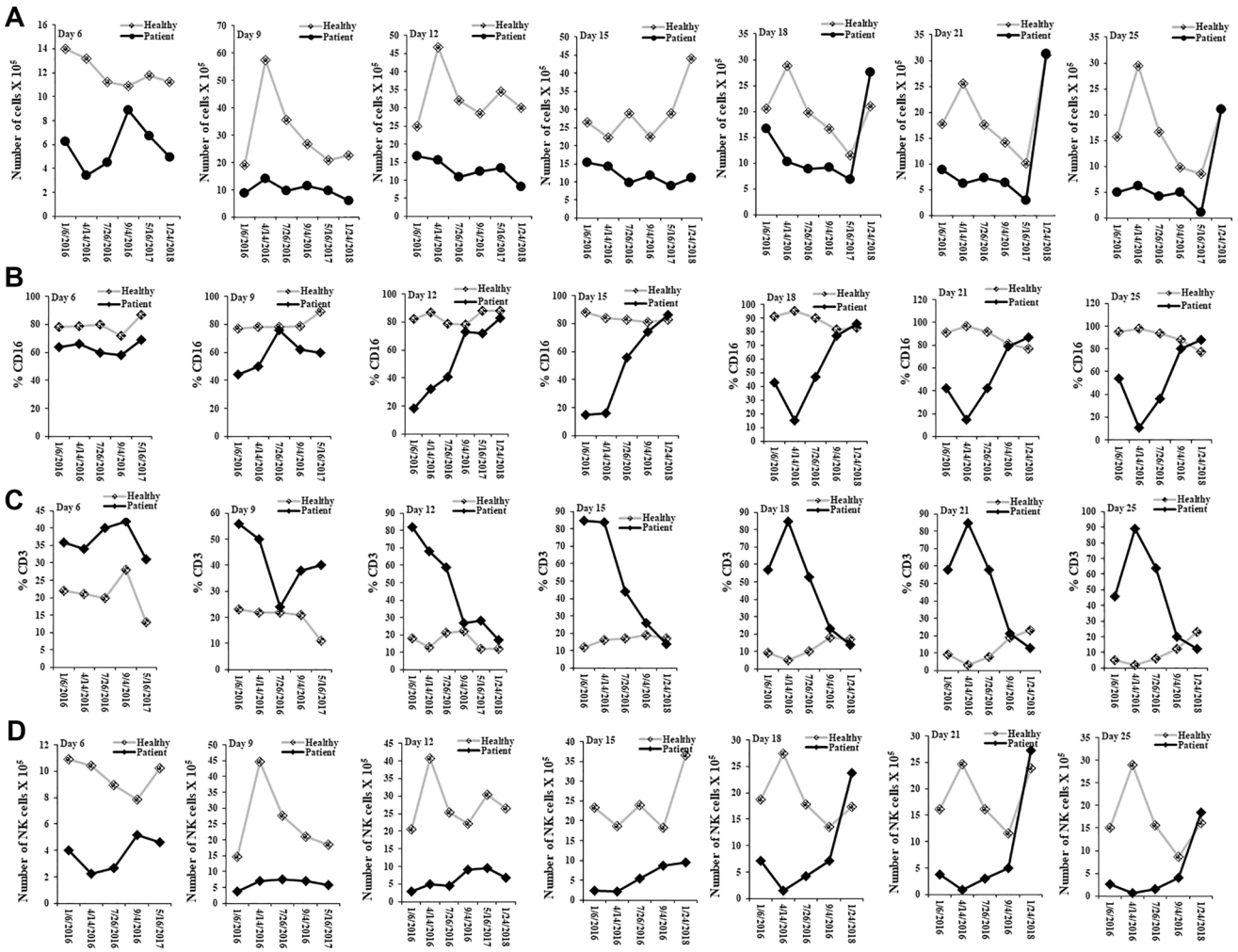


Fig. 5 Decreased capacity to expand, mediate cytotoxicity and secrete IFN-γ in OC-expanded NK cells from cancer patient when compared to healthy individuals. OCs were generated as described in Materials and Methods section. NK cells (1×10^6 cells/ml) from healthy individuals and cancer patient were treated with a combination of IL-2 (1000 U/ml) and anti-CD16 mAb (3 µg/ml) for 18 h before they were co-cultured with the healthy individuals' OCs and sAJ2 at a ratio of 1:2:4 (OCs:NK:sAJ2). On days 6, 9, 12, 15, 18, 21 and 25 of co-culture, the numbers of lymphocytes were counted using microscopy **(a)**. NK cells were treated and cultured as described in **a**. Surface expression of CD16 **(b)**, and CD3 **(c)** were determined using flow cytometry. IgG isotype control was used to assess non-specific binding. NK cells were treated and cultured as described in **a**. Using the percentage of CD16 and CD3 as determined in **b** and **c**, respectively, the numbers of NK cells **(d)**, and T cells **(e)** were determined in total expanded cells. NK cells were treated and cultured as described in **a**. On days 6, 9, 12, 15, 18, and 21, the supernatants were harvested from the co-cultures to determine IFN-γ secretion using single ELISA **(f)**. NK cells were treated and cultured as described in **a**. Cytotoxicity of days 12 and 18 cultured NK cells was determined using standard 4-h ^{51}Cr release assay against OSCSCs. The LUs were determined as described in Fig. 2a, and were determined for 1% NK cells using CD16 percentages as shown in **b** (**g**). The data were obtained at different time points as shown in the figure. Duplicate samples from healthy individual and cancer patient were used at each time point. Healthy individuals were different throughout the study but were of same age (52–56 years) and gender (female) group as the cancer patient

lack of CD16 expression was also seen in other pancreatic cancer patients.

Overall, due to the major defect in expansion and function of NK cells, even at the stage where overt cancer could not be detected in the patient is likely an important underlying mechanism for the survival and progression of pancreatic cancer. Lack of significant defect in patient T cells in the presence of dysfunctional NK cells provides the rationale for treatment strategies to reverse the inactivation of NK cells in pancreatic cancer patients. In addition, treatment strategies should be designed at the pre-neoplastic stage of the disease, if a number of informative biomarkers can be identified and designed to predict the progression in susceptible individuals. One such biomarker is the use of NK diagnostics to

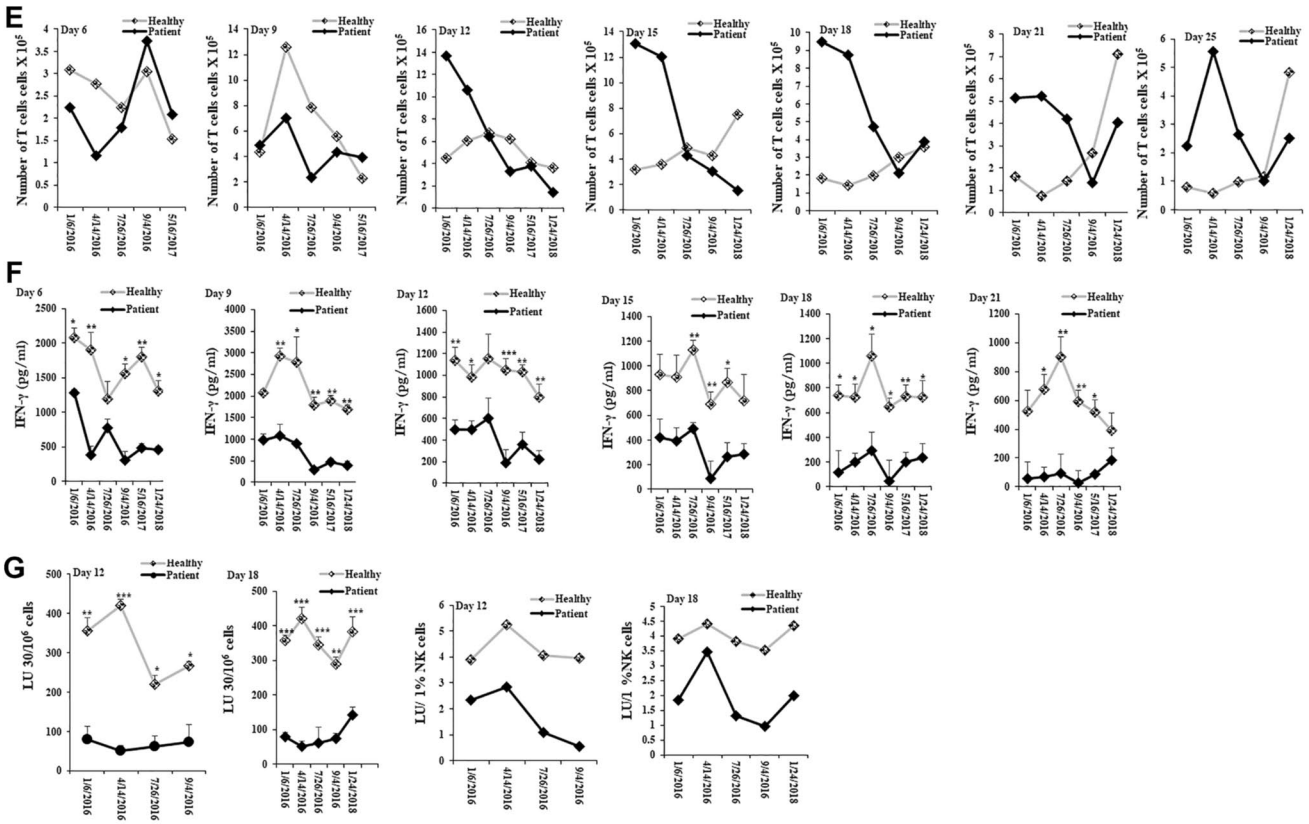


Fig. 5 (continued)

determine the levels of NK expansion and function. Such comprehensive study presented with one patient could be extended to more patients to observe the similarities and potential differences in the manifestations of the disease and their correlation with the status of NK cell numbers and function.

Materials and methods

Case presentation

A 51-year-old female with metastatic pancreatic adenocarcinoma (PDAC) was initially diagnosed as colloid carcinoma and mucinous non-cystic carcinoma. After diagnosis, in May of 2015 she underwent distal pancreatectomy . Two of the thirteen lymph nodes were found to be involved. However, no visible disease could be seen on imaging. Her tumor was found to be microsatellite Stable with KRAS, TP53 and MED 12 mutations. She received a number of different chemotherapeutic drugs in different combinations from Nov, 2016 to June 2019 which included 12 cycles of Folfirinox, anti-PD-1 and anti-IL6R antibody therapy. The list of the medications used is presented in Table S1. The CT scan of abdomen performed in April, 2018 suggested serosal liver metastasis, cholelithiasis, very minimal ascites, and a small right adnexal cyst. In Nov, 2018 she underwent debulking surgery due to the tumor recurrence.

Cell lines, reagents, and antibodies

RPMI 1640 (Life Technologies, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA) was used for the cultures of NK cells, T cells, monocytes, and peripheral blood mononuclear cells (PBMCs). Oral squamous carcinoma stem cells (OSCSCs) were isolated from patients with tongue tumors at UCLA [20, 25–27] and were cultured in RPMI 1640 supplemented

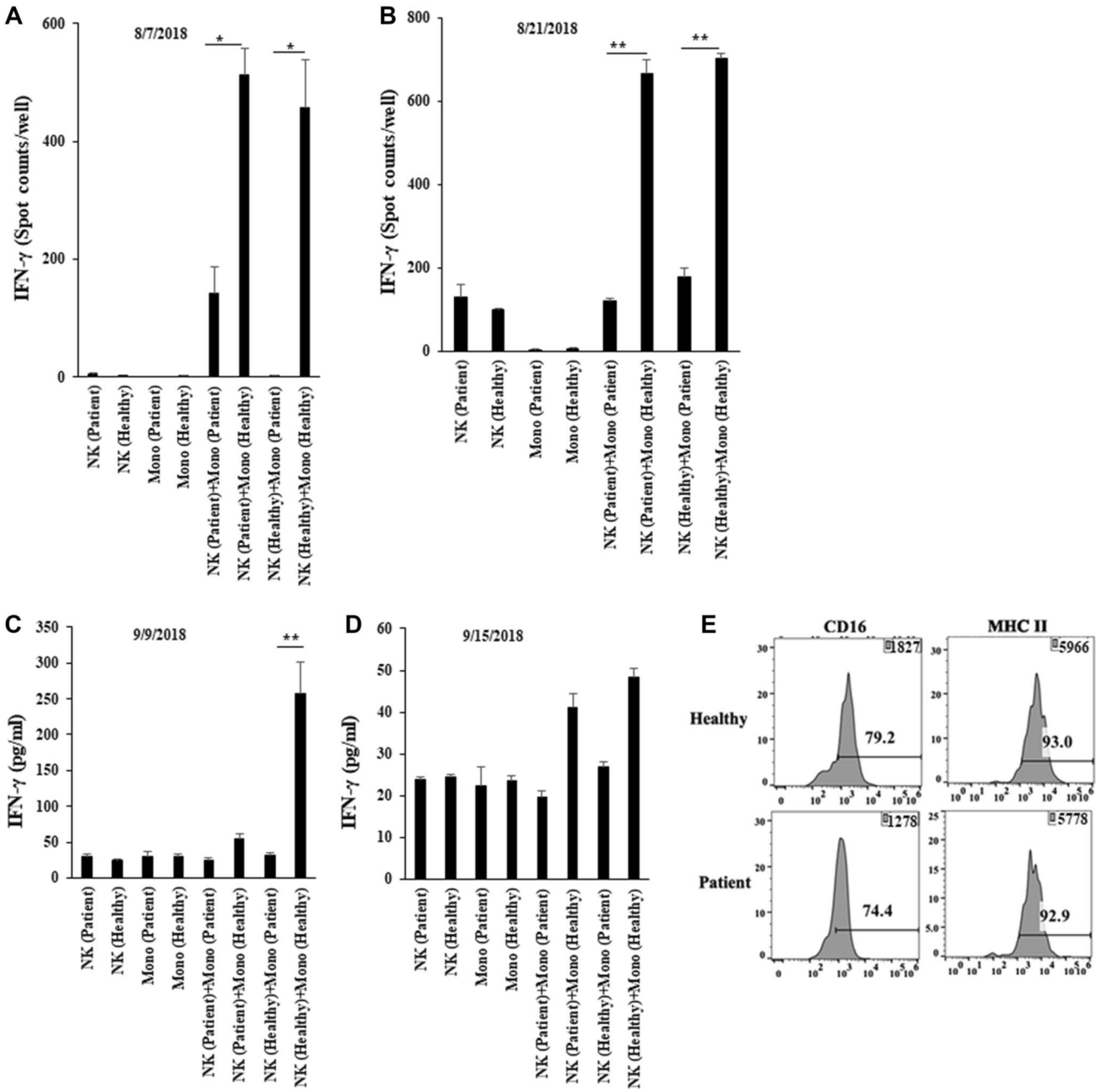


Fig. 6 Patient monocytes are less capable of activating NK cells to secrete IFN-γ, and exhibit lower surface expression of CD16. NK cells from cancer patient and healthy individuals were treated with the combination of IL-2 (1000 U/ml) and anti-CD16 mAb (3 µg/ml) and were co-cultured with monocytes in a crisscross manner (NK:monocytes; 1:1) as shown in the figures. After 18 h of coculture, ELISpot was conducted to determine the numbers of IFN-γ spots **(a**, **b)**. NK and monocytes were treated and co-cultured as described in **a**, **b** for 18 h before the supernatants were harvested to measure IFN-γ secretion using single ELISA **(c**, **d)**. A representative experiment is shown and data are presented as Mean ± SD (**a–d**). Duplicate samples from healthy individual and cancer patient were used at each time point. Healthy individuals were different throughout the study but were of same age (52–56 years) and gender (female) group as the cancer patient. Freshly purified monocytes from cancer patient and healthy individuals were analyzed for the surface expression of CD16 and MHC-class II using flow cytometry. IgG2 isotype control antibodies were used as controls **(e)**. One of 4 representative experiments is shown in the figure

with 10% FBS. Alpha-MEM (Life Technologies, CA, USA) supplemented with 10% FBS was used for osteoclast (OCs) cultures. RANKL was purchased from PeproTech, NJ. Recombinant IL-2 was obtained from NIH- BRB. Flow

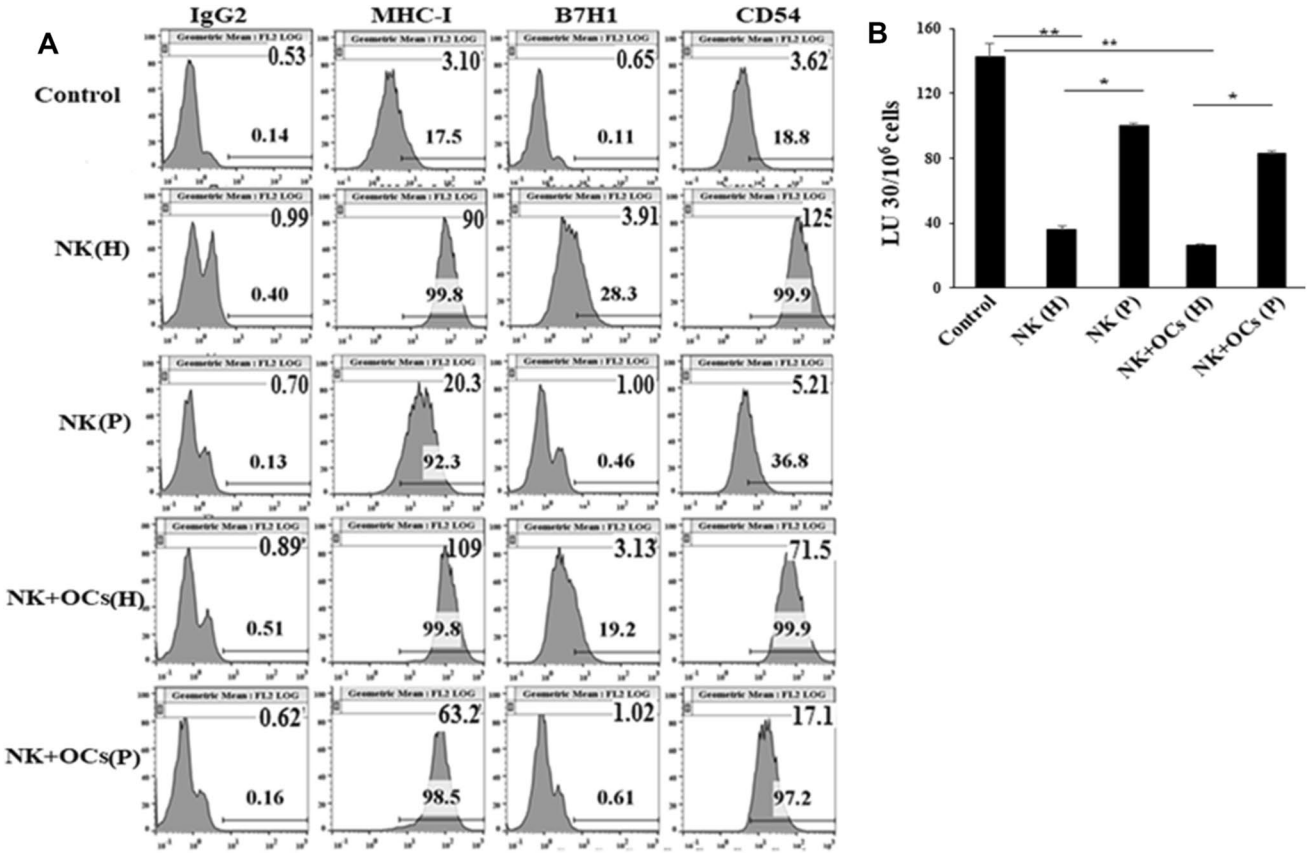


Fig. 7 IFN-γ secreted by cancer patient NK cells in comparison to healthy individuals induced lower levels of differentiation in oral cancer stem-like tumors. Supernatants containing equal amounts of IFN-γ from healthy individual' and pancreatic patient' NK cells treated with IL-2 (1000 U/ml) and anti-CD16 mAb (3μg/ml) for 18 h, or from NK cells co-cultured with OCs for 12 days were added to OSCSCs for 4 days to induce differentiation. Thereafter, expression of MHC-class I, B7H1 and CD54 were determined on the surface of OSCSCs. IgG2 isotype control antibodies were used as controls **(a)**. Allogeneic healthy NK cells were treated with IL-2 (1000 U/ml) for 18–24 h before they were used in cytotoxicity against untreated and healthy and patient NK-supernatant differentiated OSCSCs, generated as described in **a**. Tumors were ^{51}Cr labeled and used in the cytotoxicity assay, and LUs 30/10^6 cells were determined as described in Fig. 2a **(b)**. One of two representative experiments (different healthy individuals for each experiment) is shown in the figure

cytometry antibodies, M-CSF, and anti-CD16 mAb, used in the study were obtained from Biolegend (San Diego, CA). Antibodies to CD3 and CD28 were purchased from Stem Cell Technology (Vancouver, BC, Canada). Probiotic bacteria, sAJ2 is a combination of 7 different strains, and is prepared as described previously [28], and RPMI 1640 supplemented with 10% FBS was used to re-suspend AJ2. Human ELISA kits for IFN-γ were purchased from Biolegend (San Diego, CA).

Purification of human NK cells, T cells, and monocytes

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy individuals and cancer patient, and all procedures were approved by the UCLA-IRB. Healthy individuals were donors with no medical history of cancer, and they were different individuals throughout the study but were of the same age (52–56 years) and gender (female) as the cancer patient. Briefly, PBMCs were isolated from peripheral blood as described before [29]. PBMCs were used to isolate NK cells, T cells, and monocytes using the EasySep® Human NK cell, EasySep® Human T cell, EasySep® Human monocytes enrichments kits, respectively, purchased from Stem Cell Technologies (Vancouver, BC, Canada). Isolated NK cells, T cells, and monocytes were stained with anti-CD16/CD56, anti-CD3, and anti-CD14 antibodies respectively to measure the cell purity using flow cytometric analysis.

Generation of osteoclasts and, expansion of NK and T cells

To generate OCs, monocytes were cultured in alpha-MEM media supplemented with M-CSF (25 ng/mL) and RANKL (25 ng/mL) for 21 days and media was replenished every three days. NK cells were activated with rh-IL-2 (1000 U/ml) and anti-CD16 mAb (3 µg/ml) for 18–20 h before they were co-cultured with OCs and sAJ2 (OCs:NK:sAJ2; 1:2:4) in RPMI 1640 medium containing 10% FBS. The medium was refreshed every three days with RPMI containing rh-IL-2 (1500 U/ml). T cells were activated with rh-IL-2 (100 U/ml) and anti-CD3 (1 µg/ml)/anti-CD28 (3 µg/ml) for 18–20 h before they were co-cultured with OCs and sAJ2 (OCs:T:sAJ2; 1:2:4) in RPMI 1640 medium containing 10% FBS. The culture media was refreshed with rh-IL-2 (150 U/ml) every three days.

Enzyme-linked immunosorbent assays (ELISAs), Enzyme-linked immunospot (ELISpot), and multiplex cytokine assay

Single ELISAs and multiplex arrays were performed as previously described [29]. To analyse and obtain the cytokine and chemokine concentrations, a standard curve was generated by either two- or three-fold dilution of recombinant cytokines provided by the manufacturer. The ELISpot was conducted according to manufacturer's instructions. The number of IFN-γ secreting cells was determined by using Human IFN-γ Single-Color Enzymatic ELISpot Assay, and analyzed by the ImmunoSpot® S6 UNIVERSAL analyzer and ImmunoSpot® SOFTWARE (all CTL Europe GmbH, Bohn, Germany). The levels of cytokines and chemokines were also determined by multiplex cytokine arrays as suggested by the manufacturer. Analysis was performed using a Luminex multiplex instrument (MAGPIX, Millipore, Billerica, MA), and data were analyzed using the proprietary software (xPONENT 4.2, Millipore, Billerica, MA).

Surface Staining

Staining was performed by labeling the cells with antibodies as described previously [29–31]. Flow cytometric analysis was performed using Attune^{TM} NxT Flow cytometer (Thermo Fisher Scientific, Waltham, MA) , and the results were analyzed by the FlowJo vX software (Ashland, OR).

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [32]. Briefly, different numbers of effector cells were incubated with ^{51}Cr–labeled target cells. After a 4-h incubation period, the supernatants were harvested from each sample, and the released radioactivity was counted 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 ×100.

OSCSCs and MP2 differentiation with NK cell supernatants

Differentiation of OSCSCs and MP2 poorly differentiated tumors was conducted as described previously [25]. On average, a total of 3000–5000 pg of IFN-γ containing supernatants obtained from IL-2 + anti-CD16 mAbs treated NK or OC-expanded NK cells were added for 5 days to induce differentiation of OSCSCs and MP2 tumors.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism-8 software. An unpaired or paired, two-tailed student's t-test was performed for the statistical analysis for experiments with two groups. One-way ANOVA with a Bonferroni post-test was used to compare different groups for experiments with more than two groups. Duplicate or triplicate samples were used for each assessment. The following symbols represent the levels of statistical significance within each analysis: ***(p value < 0.001), **(p value 0.001–0.01), *(p value 0.01–0.05).

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ucla.edu/Jewett. The authors are also grateful to funding agencies and donors for supporting the work.

Author contributions KK and MWK generated data. KK wrote, reviewed, and edited the case report. FC performed supporting experiments. AJ oversaw the studies, conceptualization of the report, reviewed and edited the report, and acquired funding.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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