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Human pluripotent stem cells to produce cell-based cancer immunotherapy

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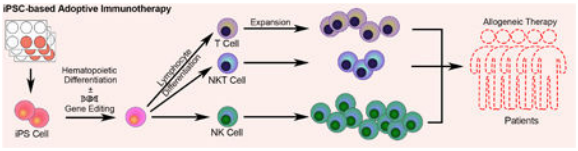
Abstract

Human pluripotent stem cells (PSCs) provide a promising resource to produce immune cells for adoptive cellular immunotherapy to better treat and potentially cure otherwise lethal cancers. Cytotoxic T cells and natural killer (NK) cells can now be routinely produced from human PSCs. These PSC-derived lymphocytes have phenotype and function similar to primary lymphocytes isolated from peripheral blood. PSC-derived T and NK cells have advantages compared to primary immune cells, as they can be precisely engineered to introduce improved anti-tumor activity and produced in essentially unlimited numbers.

Graphical abstract



Keywords

Cancer immunotherapy; Adoptive cell therapy; Human pluripotent stem cell; T cell; Natural Killer cell; Natural Killer T cell

Human pluripotent cells for regenerative medicine

Ever since human embryonic stem cells (hESCs) were first described in 1998 [1] and induced pluripotent stem cells (iPSCs) were first produced from mouse cells in 2006 [2] and human cells in 2007 [3, 4], these pluripotent stem cells have been touted as an important new avenue to produce therapeutic cells to better treat or repair diseased or damaged cells and tissues. Indeed, multiple clinical trials are now ongoing using hESC-derived cells for treatment of spinal cord injury, retinal disease, diabetes and heart failure [5, 6]. For example, hESC-derived retinal pigment epithelial (RPE) cells have been used for patients with late-stage retinal degenerative disease and show no evidence of adverse proliferation, rejection,

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or serious ocular or systemic safety issues related to the transplanted tissue in phase I/II clinical trials[7]. Additionally, recent studies using an autologous iPSC-derived RPE for treatment of age-related macular degeneration illustrates the safety of this approach with no serious adverse events noted at 25 months of follow-up[8]. With this exciting progress, we now highlight the ability to use hESCs and iPSCs for the treatment of cancers that are refractory or resistant to other forms of therapy such as surgery, chemotherapy, or radiation therapy.

Cytotoxic T lymphocytes in cancer immunotherapy

Cytotoxic T lymphocytes (CTLs) play an important role in the immune system with the ability to recognize and kill infected cells and certain tumors. CTLs express a broad repertoire of T cell receptors (TCRs) which recognize foreign antigens presented by the HLA class I complex. TCR engagement triggers signaling pathways to mediate the release of cytotoxic granules, as well as increased expression of Fas-ligand leading to apoptosis initiation in the targeted cells. T cell-mediated immunotherapy has been used in cancer treatment for decades. Ex vivo expansion followed by re-infusion of autologous tumor-infiltrating lymphocytes (TILs) into patients with metastatic melanoma can mediate cancer regression [9]. Recent studies demonstrate TILs still provide an important anti-cancer modality [10]. Refinement of this modality was demonstrated by identifying tumor-specific TCRs from TILs [11, 12]. The re-arranged TCRs against tumor antigen can be cloned and re-introduced into peripheral lymphocytes via viral vectors to provide a more uniform population of anti-tumor lymphocytes [13, 14].

Chimeric antigen receptors (CARs) provide another strategy to enhance T cell-mediated anti-tumor activity [15, 16]. Zelig Eshhar and colleagues are credited with first conceptualizing the CAR as a single chain variable fragment (ScFv) joined to the heavy and light chain variable regions of a monoclonal antibody (mAb) with a linker sequence to directly connect to intracellular signaling domain(s). The initial (first generation) CARs link the scFv with the ζ chain of the T cell signaling complex to endow transduced T cells with an antibody-like specificity capable of effectively transmitting the intracellular signals needed for T-cell activation[17]. More recent studies have improved CAR efficacy and expansion of targets. Second and third generation CARs now use an ScFv coupled with additional co-stimulatory domains (typically CD28 and/or 41BB) and combined with the ζ chain of the TcR complex to improve efficacy. Defining the optimal signaling elements still remains an area of active investigation, as some domains may improve T cell activation while other domains may improve T cell persistence[18].

Currently, more than 150 CAR-T cell related clinical trials are on-going (per ClinicalTrials.gov) to target diverse types of cancer including both hematological malignancies and solid tumors. The most successful results to date for CAR-T cell therapies have been reported with anti-CD19 CAR-T cells used to treat B cell malignancies such as pre-B cell acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and non-Hodgkin's lymphoma[19-22]. Recently, the U.S. Federal Drug Administration (FDA) approved of Novartis' CAR T-Cell Therapy CTL019 for the treatment of relapsed or refractory pediatric and young adult patients with B-cell acute lymphoblastic leukemia

(ALL)[23]. To further advance CAR T cell-based therapy, the next task involves improving availability, consistency, and safety of CAR-T cells. To date, manufacturing of recipient-specific clinical-grade products is done using individualized cell culture technologies. Collection of T cells, expansion and transduction varies from patient to patient, leading to differences in infused cell populations and numbers of therapeutic cells. Potential toxicities associated with this treatment are another roadblock potentially limiting the wide spread use of CAR-T cells. For example, in a recent CAR-T clinical trial (JCAR015) for adults with B cell-ALL, 5 of 38 patients died from cerebral edema following JCAR015 treatment. This led to permanent termination of the trial and highlighted the possible dangers of this new treatment[24]. Another major toxicity of CAR T cells is cytokine release syndrome (CRS) caused by cytokines directly produced by infused CAR T cells or indirectly by other immune cells in response to the treatment. CRS can result in systemic organ dysfunction[20, 25]. Besides CRS and neurotoxicity, other toxicities such as tumor lysis syndrome (TLS) and allergic reactions have occurred form CAR-T cell treatments [26, 27]. While there are treatments to help manage these potential adverse reactions such as tocilizumab (antagonist of IL-6 receptor) for CRS and corticosteroids for neurologic toxicities, these procedures do have considerable risk [28]. Although no adverse effect due to viral vector-induced insertional mutagenesis has been confirmed, at least one study reported that the enhanced clonal expansion of CAR T cells in one of the CLL patients was due to TET2 disruption[29]. While this patient didn't develop into T cell leukemia, these findings raise concerns due to the ability of TET2 to initiate acute myeloid leukemia (AML)[30].

Making T cells in a dish

During development, lymphocytes arise from hematopoietic stem cells that differentiate into a common lymphoid progenitor cell, then into defined lymphocyte sub-populations through a series of differentiation events[31, 32]. Unlike B cell development that takes place within the bone marrow microenvironment, early T cell progenitors (ETPs) enter the thymus and undergo a series of commitment events and developmental checkpoints. Developing thymocytes transition from $CD4^-CD8^-$ double negative (DN) cells to $CD4^+CD8^+$ double positive (DP) cells ending as either $CD4^+$ single positive (SP) or $CD8^+$ SP stage T cells. Mature $CD4^+CD8^-$ and $CD4^-CD8^+$ SP T cells are released to the periphery and perform their roles in the immune system [33]. In vitro T cell maturation was first demonstrated using fetal thymic organ culture (FTOC) to support T differentiation from mouse thymic progenitor cells in vitro [34]. Human T cell development has also been demonstrated using FTOC [35]. More recently, Zúñiga-Pflücker and colleagues developed a more defined co-culture system based on the critical function of the Notch pathway in T cell development. Studies using hematopoietic progenitor cells isolated from different sources demonstrated that overexpressing Notch ligand Delta-like-1 (OP9-DL1) [36] or Delta-like-4 (OP9-DL4) [37] on mouse bone marrow-derived OP9 stromal cells provides a suitable microenvironment to initiate T lymphopoiesis [38]. These studies have used cells from human fetal liver [38, 39], human umbilical cord blood (UCB)[40], and mouse ESC-derived hematopoietic cells [36]. The in vitro-derived $CD8^+$ SP T cells exhibit standard pathway activation upon stimulation and are capable of killing specific target cells if engineered with CAR and TCR[37, 41-43].

In contrast to the successful generation T cells from sources such as mouse ESC-derived hematopoietic cells and human UCB, the generation of T cells from human ESCs and iPSCs has typically been less efficient. Several strategies have been used to produce hematopoietic progenitor cells from hESCs/iPSCs. The first method involves co-culturing hESC/iPSCs with stromal cells such as S17 or OP9 cells in serum-containing medium[44, 45]. In the second method, hESCs or iPSCs are used to generate embryoid bodies (EBs), either in suspension or aggregated in round-bottom wells [46, 47]. Initial studies to produce hESC-derived hematopoietic progenitor cells capable of completing the differentiation program of CD4 and CD8 T cells injected these progenitor cells into an in vivo SCID-hu (Thy/Liv) mouse system. This model is constructed by implantation of small pieces of human fetal liver and thymus under the renal capsule to provide a microenvironment for T cell development. hESCs-derived $CD34^+$ cells were shown to give rise to phenotypically and functionally mature T cell progeny after 8 weeks in vivo[48, 49]. While an interesting system, this SCID-hu model precludes more careful dissection of key signals that mediate T cell development or to obtain large numbers of T cells for subsequent functional testing or clinical translation.

Subsequent studies aimed to differentiate hESCs and iPSCs into T cells using different in vitro methods. One study was able to regulate the WNT–βcatenin and Nodal–activin pathways during EB formation to produce an hESC/iPSC derived $CD34^+CD43^-$ population that were thought to represent a “definitive” hematopoietic progenitor cell capable of T cell, erythroid and myeloid potential while characterizing an hESC/iPSC derived $CD34^+CD43^+$ population as the “primitive” progenitor cells that can only produce restricted erythroid, macrophage and megakaryocytic lineages [47]. However, it is likely these $CD34^+CD43^-$ hESC/iPSC-derived cells still represent a primitive hematopoietic lineage, possibly similar to a second wave of hematopoietic cells that develop in the yolk sac during early embryogenesis [50, 51]. Indeed, these hESC/hiPSC-derived $CD34^+CD43^-$ cells cultured on OP9-DL1 were able to produce $CD3^+$ TCRαβ T cells but failed to mature to functional $CD4^+$ or $CD8^+$ T cells [47, 52]. Other studies have used the OP9 co-culture system to produce lin $^-CD34^+CD43^+CD45^-$ hematopoietic progenitor cells with a broad differentiation potential[53]. These hematopoietic cells are able to produce both lymphoid and myeloid cells, including $CD8^+$ T cells by co-culture with OP9 or OP9-DL4 cells [54-56]. Additional studies demonstrate intrinsic characteristics of HSPCs generated in different system have been suggested to determine the capability of lineage specification. For instance, unsuccessfully silencing of ID gene expression was shown to promote NK differentiation and expansion while inhibiting the activity of E-proteins that are required for T- and B-cell development in HSPCs derived from human ESC [57].

Additional studies have shown that reprogramming mature T cells into iPSCs (T-iPSC) and then differentiating those cells back into T cells leads to more effective generation of mature, antigen-specific cytotoxic iPSC-derived T cells. In addition to the clinical benefits of producing unlimited cytotoxic T cells against a specific target e.g. tumor, developing T cells with a pre-rearranged TCR may suppress the initiation of TCR rearrangement and early expression of TCR may accelerate T cell maturation [58]. Kawamoto's group derived $CD8^+$ T cells from iPSC which were derived from mature cytotoxic T cells specific for the melanoma epitope MART-1. Most of these $CD8^+$ T cells were specific for the original

MART-1 epitope[59]. As another example of this strategy, Nakauchi and colleagues reprogrammed antigen-specific $CD8^+$T cells from an HIV-1-infected patient to iPSC. Then they redifferentiated T-iPSC into $CD8^+$ T cells with high proliferative capacity and elongated telomeres which also maintained antigen-specific killing activity[60].

Another strategy has utilized direct reprograming of somatic cells using defined transcription factors to produce hematopoietic stem/progenitor cells capable of in vivo engraftment and differentiation into functional T cells. Lis and colleagues successfully reprogrammed adult mouse endothelial cells to hematopoietic stem cells (rEC-HSCs) via transient expression of 4 transcription factors (Fosb, Gfi1, Runx1, and Spi1) and vascular-niche-derived angiocrine factors. T cell proliferation and antigen-specific T cell immune responses were demonstrated in mice engrafted with these rEC-HSCs [61]. In another recent study, Daley's group demonstrated that seven transcription factors (ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1 and SPI1) were able to convert hemogenic endothelium derived from human pluripotent stem cells into hematopoietic stem and progenitor cells able to maintain engraftment when transplanted into immunodeficient mice [62]. These mice displayed human T cells with a diverse TCR repertoire, as well as IFN-γ production from human $CD3^+$ cells isolated from bone marrow of NSG mice engrafted with these HE-7TF cells. Therefore, this direct reprograming approach provides another strategy to produce human T cells, though to date, this approach has only been successful in T cell development via in vivo transplantation studies.

T-iPSC cells can be engineered to provide a powerful tool for cancer immunotherapy. For example, T-iPSCs stably transduced with a lentiviral vector encoding a second generation anti-CD19 CAR were utilized to produce T cells via OP9-DL1 co-culture. Although these cells primarily developed into γδ T cells, the T-iPSC-derived, CAR-expressing T cells were able to potently inhibit growth of human Burkitt's ($CD19^+$ B cell) lymphoma in a xenogeneic tumor model [43]. The importance of precisely regulating CAR expression was shown as targeting the CAR coding sequence to the T-cell receptor α constant (TRAC) locus not only results in uniform CAR expression in human primary T cells, but also enhances T-cell potency. By testing CAR expression driven by different promoters and in different genomic loci, subsequent studies demonstrated maintenance of CAR expression under the control of endogenous regulatory elements reduces tonic signaling and increases the therapeutic potency of engineered T cells [63]. However, it will be necessary to pay particular attention when using the endogenous promoter to drive transgene expression in T-iPSCs, as endogenous regulatory elements may still be active in iPSC status could affect the T cell differentiation. To address this concern, an inducible expression system might be employed.

In summary, the combined power of somatic cell reprogramming, CAR engineering and gene editing technology can be used to produce cytotoxic T cells with antigen specificity. For clinical translation, additional modifications such as genetic deletion of HLA expression and endogenous TCR expression may be necessary to reduce allogeneic response against these cells and prevent graft-versus-host disease due to T cells non-specifically attacking recipient cells. However, deletion of HLA expression leaves the cells vulnerable to lysis by NK cells, a process defined as ‘missing self’ response, thus reducing the persistence of

CAR-T cells. This problem can be solved by forced expression of minimally polymorphic HLA-E molecules in HLA deletion T cells. A recent study indicated that over-expression of HLA-E in beta-2 microglobulin (B2M) gene knockout (KO) pluripotent stem cells prevent allogeneic reaction by CD8+ T cells and make these cells resistant to NK cell-mediated lysis[64]. Additional modification such as deletion of inhibitory receptors such as PD1 can improve anti-tumor activity [65]. Allogeneic CAR-T cells have been demonstrated clinically beneficial for patients with relapsed B cell malignancies after allogenic hematopoietic stem cell transplantation (HSCT) with low toxicities and complications[66]. Allogeneic CAR-T therapy has recently been tested in a clinical trial for patients with refractory ALL [67]. Therefore, with these advances, we should eventually be able reach the goal of using human hESC and/or iPSC-derived T cells as a novel, standardized anti-cancer therapy.

Natural killer cells for cancer immunotherapy

Natural killer (NK) cells are lymphocytes of the innate immune system able to kill virally infected and transformed cells without prior sensitization [68] [69, 70]. NK cells were first identified as a novel sub-lymphocyte population able to lyse tumor cells in mice by 2 independent research groups in 1975 [71, 72]. In humans, NK cells comprise 5–15% of circulating lymphocytes and can be categorized within two subpopulations based on CD56 expression [73]. Most peripheral NK cells express lower levels of CD56 and demonstrate high cytotoxicity toward target cells. About 10% of peripheral NK cells express high levels of CD56 and have lower cytotoxic activity while displaying a high capacity to produce immunoregulatory cytokines[69, 74, 75]. More recent studies have demonstrated additional sub-populations of human NK cells [76, 77].

NK cell activity is regulated by integrating signals from multiple activating and inhibitory receptors to distinguish healthy cells from infected or tumor targets [69, 78, 79]. One class of NK cell inhibitory receptors are killer immunoglobulin-like receptors (KIRs) that bind human leukocyte antigen (HLA) class I proteins on target cells [70]. Healthy cells express normal levels of self-HLA, which suppresses NK cell cytotoxic activity. As stipulated by the “Missing-self hypothesis”, when cells down-regulate expression of HLA (as can occur in tumor cells), NK cell inhibitory signaling is eliminated leading to NK cell activation[80]. NK cytolytic function also requires engagement of strong activating signals. These activating receptors include natural cytotoxicity receptors (NCRs), NKG2D and CD16, the receptor that mediates antibody dependent cellular cytotoxicity (ADCC) to direct NK cells' activity to target cells [69, 81-83].

Adoptive NK cell therapy

NK cell-based adoptive immunotherapy has been tested clinically for the treatment of multiple types of cancer [84, 85]. In the past decade, several clinical trials have demonstrated NK cells have a potent ability to treat hematological malignances [85-87]. Several trials demonstrate that 30-50% of patients with refractory or relapsed acute myelogenous leukemia (AML) can achieve complete remission after receiving allogenic peripheral blood NK cells (PB-NK) [88, 89]. These exciting results have led to increased interest on NK cell-based adoptive immunotherapy. Many NK cell-based clinical trials are

now recruiting patients from a range of institutions for a variety of both hematological malignancies and solid tumors [85-87].

A critical step to enable expanded therapeutic use of NK cells requires a strategy to efficiently produce a large number of cells suitable for clinical trials. There are different sources of NK cells that have been used for adoptive NK cell therapy (Table 1). These include: isolation and ex vivo expansion of primary NK cells from peripheral blood (PB-NK), from umbilical cord blood (UCB-NK)[90, 91], differentiation from human embryonic stem cells (hESC-NK) or induced pluripotent stem cells (iPSC-NK) [92]and cell lines such as NK-92[93]. NK cells can be obtained from either autologous or allogenic sources for adoptive cell therapy (Figure 1). In autologous NK cell therapy, primary NK cells from patients' peripheral blood are isolated and stimulated with cytokines (typically IL-2 or IL-15), then injected back to the patient. Several clinical trials have been conducted with autologous NK cells for the treatment of different cancers, including lymphoma, multiple myeloma, breast cancer, Colorectal carcinoma, non-small cell lung cancer and gastric cancers [94-96]. These trials demonstrated safety with no toxic side effects. However, the clinical benefits are typically limited, likely due to inhibitory KIRs recognizing self HLA molecules. Thus, research has shifted toward allogenic NK cell therapy[97]. Some recent clinical studies demonstrated that allogenic NK cells contribute to the graft versus tumor (GVT) effect without GVHD [97-99]. One possible reason for the superior efficacy of using allogenic NK cells is they are not susceptible to the KIR-mediated inhibition caused by self-HLA Class I recognition [100, 101].

Allogeneic NK cell-based therapy can be delivered in either a non-transplant or transplant setting [102]. In a non-transplant setting, adoptive transfer of ex vivo expanded HLA-haploidentical NK cells were used to treat patients with relapsed/refractory AML followed up with administration of IL-2 [88]. Greater tumor-killing activity was observed in patients who received NK cells with KIR-HLA mismatches without causing apparent GVHD [100, 103]. Another pioneering clinical study also demonstrated that the GVT effect of allogeneic NK cells is significantly improved when the HLA class I ligands of the patient and certain KIRs of donor are incompatible [99].

In addition to treating hematological malignancies, allogeneic NK cell therapy has been used for the treatment of solid tumors[103], such as renal cell carcinoma, colorectal, hepatocellular [104], ovarian, breast [105]and lung cancers[106]. These studies indicate NK cells can also play a therapeutic role in the treatment of solid tumors. However, the efficacy of NK cell-based therapy against solid tumors is typically less than that seen in AML. These clinical trials do demonstrate that adoptive transfer of allogeneic NK cell transplantation is safe and holds promise as a therapeutic adjuvant.

Although the peripheral blood provides a promising source of NK cells for clinical use, there are some shortcomings. First, the yield of NK cells in leukapheresis collections is highly donor-dependent [107]. Second, PB-NK cells remain a heterogeneous cell population varying from donor-to-donor [88]. Moreover, it is challenging to genetically engineer PB-NK cells[108]. These disadvantages of PB-NK cells may limit the potential for developing an off-the-shelf PB-NK cell product.

In addition to PB-NK cells, NK cells obtained from umbilical cord blood (UCB-NK cells) have been used in clinical trials to treat AML, myeloma, and other hematological malignancies [109, 110]. While these studies have been rather limited, some efficacy has been seen in these trials and UCB-NK cells provide another alternative for NK cell-based immunotherapy. However, wide-spread translation of UCB-NK cells is challenging due to the difficulty of expanding these cells, and that upon derivation, they yield a more immature and less cytotoxic NK product [111].

NK Cell lines

NK cell lines such as NK-92, NKG, NKL, and KHYG-1 are homogenous, relatively easy to genetically modify and have the potential to develop into off-the shelf NK cell therapy products [112-114]. NK-92 cells have been studied in clinical trials where they demonstrate an excellent safety profile and some anti-tumor efficacy [115, 116]. However, because NK-92 cells are aneuploidy and can proliferate in an uncontrolled manner, these cells the need to be irradiated NK-92 cells prior to administration. This irradiation limits the ability of these cells to survive and expand in vivo [116] which may limit the anti-tumor potential of these cells as studies of PB-NK cells clearly show that expansion and survival of the NK cells in patients is closely correlated to treatment outcome[88, 89, 105, 117]. However, NK-92 cells can be easily modified to express CARs as strategies to improve efficacy [82, 118].

NK cells from human pluripotent stem cells

hESC-derived NK cells (hESC-NK) and hiPSC-derived NK cells (hiPSC-NK) provide a homogenous, genetically defined population for NK cell adoptive therapy, eliminating the donor-to-donor variability when using PB-NK cells [81]. Additionally, it is feasible to genetically engineer hESC-NK or hiPSC-NK by modifying the stem cells using various genome-editing technologies, such as, viral vectors, transposons, or the CRISPR-Cas9 system [6, 119-122]. These properties potentially make hESC-NK and/or iPSC-NK cells an ideal cell population for “off-the-shelf” immunotherapy that can be used to treat hundreds or thousands of patients with otherwise relapsed or refractory malignancies.

In the last decade, a series of studies have advanced the development of mature NK cells from both hESCs and hiPSCs [57, 123-125]. Initial studies used a two-stage differentiation system that utilized murine stromal cell lines. First, hESCs were cultured on one stromal cell line (S17 or M2-10B4) using FBS-containing media to derive $CD34^{+}CD45^{+}$ hematopoietic progenitor cells. These cells were then sorted and cultured on a second stromal cell line (AFT024 or EL08-1D2) in media supplemented with interleukin-3 (IL-3), stem cell factor (SCF), IL-15, IL-7 and Fms-like tyrosine kinase 3 ligand (FLT3L) to produce $CD45^{+}CD56^{+}$ NK cells [111]. These hESC-derived NK cells were able to kill diverse hematologic and solid tumor cell lines in vitro [111, 123]. More remarkably, these hESC-derived NK cells effectively eliminated leukemia cells (K562 cells) xenografted in immune-deficient mice [111].

With these exciting findings, the next goal became to produce these hESC-derived NK cells in more clinically compatible conditions, and at a scale suitable for clinical trials. Specifically, our group aimed to eliminate the stromal cell lines and FBS and increase expansion of the hESC-NK cells. For thesew subsequent studies, we have adopted a "spin-EB" protocol [92, 124, 126] to produce CD34+CD45+ hematopoietic progenitor cells in serum-free, stromal-free conditions using media containing IL-3, SCF, IL15, IL-7 and FLT3L. Indeed, these conditions produce approximately 10-fold more progenitor cells than the stroma plus FBS conditions used previously [92]. Initially, the progenitor cells were sorted and cultured with stromal cells to produce mature NK cells. However, subsequent studies demonstrated that the intact EB could be transferred to uncoated plates in serum-free media supplemented with cytokines to effectively produce mature $CD45^+CD56^+$ NK cells. To scale up these NK cells to a clinical-scale, a membrane bound IL-21 expressing cell line that had previously been shown to effectively expand PB-NK cells was used[127]. These conditions can routinely produce $>10^9$ $CD45^+CD56^+$ NK cells from 10^6 starting undifferentiated hESCs. Since clinical protocols of PB-NK cells typically treat patients with approximately 10^7 NK cells per kg, this 3-stage system can effectively be used for clinical-scale NK cell production[92]. Using these conditions, NK cells have been effectively produced from multiple different hESC and iPSC lines [92, 128]. Both these hESC- and iPSC-derived NK cells express a typical repertoire of activating and inhibitory receptors found on PB-NK and UCB-NK [92, 124]. More importantly, these hESC-NK or hiPSC-NK cells effectively kill both hematological malignancies and solid tumor cells through direct and antibody-dependent cell-mediated cytotoxicity (ADCC), as well as produce cytokines, such as interferon-γ (IFN-γ) [123]. Additional studies using tumor xenograft models demonstrated hESC-NK and hiPSC-NK are effective against leukemia and ovarian cancer in vivo without any indication of teratomas or other untoward effects [129, 130]. Moreover, using a dual-bioluminescent imaging model, we were able to show trafficking hESC-NK cells to the site of tumor to demonstrate hESC-derived NK cells can directly kill tumor cells in vivo[124].

Human pluripotent stem cells as a platform to produce NK cells with enhanced cytotoxic activity

Generally, NK cell-mediated killing is determined by a summation of positive and negative intracellular signaling [78]. One key activating receptor is the Fc receptor CD16a which activates NK cell killing by binding the Fc portion of IgG molecules resulting in ADCC[79] [83]. This potent anti-tumor response is already of routine clinical relevance, as there are a number of cancers already being treated by therapeutic antibodies [131, 132]. CD16a comes in two single nucleotide polymorphism (SNP) variants, the high Fc affinity FcγRIIIa158V and low affinity FcγRIIIa158F [133]. It has been shown that lymphoma patients with the higher affinity allele exhibited improved clinical responses when treated with the therapeutic monoclonal antibody rituximab [134-136]. Likewise, in patients with ovarian cancer, CD16a expression has been shown to be decreased on NK cells [137, 138]. The decrease in expression is due to the effects of a metalloprotease called ADAM17 [139]. In response to activating signals from CD16a, ADAM17 cleaves CD16a from the cell surface [82, 139]. Since CD16a downregulation leads to a decrease in NK cell cytolytic activity, we have

utilized a high-affinity, non-cleavable version of CD16a that is not susceptible to cleavage by ADAM17 [82]. By stably integrating this construct into hiPSCs, we can derive NK cells with improved ADCC for clinical applications [82].

CARs have been also used to enhance NK cell-mediated anti-tumor activity against diverse malignances [140]. Currently, at least 4 clinical trials are utilizing CAR-expressing NK cells (NCT02839954, NCT02944162, NCT02742727 and NCT02892695) for the treatment of both hematological malignancies and refractory solid tumor. CARs recognizing tumor antigens (CD19 and mesothelin) have also been effectively expressed in hESC/iPSC-NK cells [140, 141]. Use of pluripotent stem cells for CAR engineering NK cells has the advantage of being able to genetically modify at the stem cell stage, rather than in the mature NK cell. CAR expression in hESCs/iPScs can be done either by viral vectors, or a non-viral transposon system (piggybac or *Sleeping Beauty*) that have been previously utilized for stable transgene expression in hESC/iPSC-derived blood cells[120, 142-144]. Together, these strategies of CD16 and CAR-engineered hESC and/or iPSC-derived NK cells provide an important strategy to create a universal, off-the-shelf, targeted immunotherapy against diverse malignancies.

NKT cells in cancer immunotherapy

Natural killer T (NKT) cells are a subset of T cells which share properties of both T cells and natural killer cells, and constitute about 0.1% of all peripheral blood T cells [145, 146]. NKT cells are a heterogeneous cell population which can be classified into 3 groups based on CD1b dependence, invariant or diverse TCR and reactivity to α-galactosylceramide (αGalCer): type I NKT or invariant NKT (iNKT), type II NKT and NKT-like cells. iNKT cells are the most studied and best-characterized subset which are CD1d-restricted, immunoregulatory T lymphocytes characterized by the expression of an invariant TCR α chain encoded by Vα14-Jα18 in mice and by Vα24-Jα18 in humans. NKT cells have been reported to augment anti-tumor responses[147], maintain immune tolerance and inflammatory diseases in liver [148, 149], and involve in pathogenesis of some autoimmune diseases[149]. The role of NKT cells to augment anti-tumor responses was first elucidated in animal studies. NKT cell-deficient mice show a higher incidence of tumor development or advancement[147]. Stimulation or administration of NKT cells can be beneficial in advanced non-small cell lung cancers and head and neck tumors [149, 150]. In cancer patients, NKT cell number is reported to correlate positively with prognosis [151]. One of the working hypothesis for anti-tumor effect of these cells is that NKT cells, upon activation, can secrete large amounts of IFN-γ, which in turn stimulate NK cells, dendritic cells and neutrophils to kill HLA Class I-negative tumor cells[147]. IFN-γ also actives CD8 cytotoxic T cells and CD4 Th1 cells to target HLA Class I-expressing tumor cells. Importantly, a phase II clinical study in patients with head and neck squamous cell carcinoma (HNSCC), adoptive transfer of ex vivo expanded NKT cells αGalCer-loaded APCs demonstrated moderate clinical benefit [147, 152]. Although the detailed role of NKT cells in these diseases remains unclear, NKT cell-based therapies hold promise as novel treatments [149].

NKT cells derived from human pluripotent stem cells

As with T cells and NK cells, NKT cells can be also be derived from iPSCs. Mouse NKT cells were derived from both ESC and iPSC via co-culture with OP9-DL1 stroma cells in the presence of FLT3L, IL7[153]. Function of these iPSC/ESC-derived NKT cells was demonstrated in vivo using the experimental model of OVA as an artificial tumor antigen [153]. To increase the number of iNKT cells in mice, Smith et. al. cloned iNKT TCR genes and engineered HSC with these intrinsic TCR. They further demonstrated iNKT TCR engineered HSC can generate large numbers of functional iNKT cells after adoptive transfer to B6 mice[154]. Recently, Human iNKTs were obtained successfully in vitro via a multi-step method[155]. Specifically, $CD3^+$ cells were first generated and sorted from bone marrow-derived adult hematopoietic stem-progenitor cells (HSPC) via co-culture with OP9-DL1 stroma cells in the presence of FLT3L, IL7. This was followed by co-culture with CD1d-Ig-based aAPC to induce and expand functional iNKT cells [155]. However, obtaining clinical scale iNKT cells remains challenging as the differentiation and expansion of iNKT cells are not standardized or adopted for clinical use, and how NKT cells are naturally responding to cancer in patients is far from clear, hampering the advancement of NKT cell based therapies[149, 150].

Conclusion

T cell and NK cell-based adoptive immunotherapy can now be considered nearly routine for the treatment of a variety of malignancies [69, 97, 156-159]. T cell production from human pluripotent stem cells provides an important resource to produce an essentially unlimited source of anti-tumor lymphocytes. By combining the power of somatic cell reprogramming, CAR engineering and gene editing technologies, we can facilitate production of engineered cytotoxic T cells with antigen specificity. With additional genetic modifications such as elimination of HLA molecules and inhibitory receptors such as PD1, we can now envision use of hESC or iPSC-derived T cells as the “off-the-shelf” cellular therapy.

Allogeneic NK cells from PB and UCB have been proven to be both safe and effective in multiple clinical studies [88, 97-99, 102-105, 117, 160]. Optimization of NK cell development from hESC and iPSCs and clinical scale expansion of NK cells provides a platform for future clinical studies [6, 92]. However, there remain hurdles before clinical application of NK cell-based adoptive therapy [161]. In most clinical trials using NK cells for treatment of solid tumors, only limited anti-tumor effects has been demonstrated, potentially due to the poor infiltration, inefficient homing and less persistence of the NK cells in these patients with solid tumors[103]. In this context, strategies can be developed to increase mobility and homing of adoptive NK cells in the host [162]. In addition, tumor cells are known to develop several mechanisms to decrease NK cell activity by modifications of the tumor microenvironment[70]. For example, some cytokines, metabolites or enzymes produced by tumor cells such as TGF-β[163], adenosine[164], and IDO [165] were reported to suppress the proliferation and function of NK cells. Additionally, some tumor-induced immune cells (such as Treg cells) can also inhibit the activation and function of NK [166]. Genetically modified NK cells with improved infiltration and killing ability might overcome

the suppression by tumor microenvironment and thus more effective for treating solid tumors.

The differences or similarities of NK cells isolated or derived from different sources (e.g. PB, UCB, hESCs, and iPSCs) still needs to be better defined. One interesting study revealed a remarkable degree of human NK cell diversity by mass cytometry, with over 6000 phenotypic populations within an individual and phenotypes in a donor panel that consisted of 5 sets of monozygotic twins and 12 unrelated donors[77]. Using mass cytometry, Romee et al. were able to provide a more detailed phenotype of cytokine induced memory-like NK cells based on the expression diverse antigens the viSNE map, which allows to map high-dimensional cytometry data onto two dimensions[117, 167]. Moreover, Schlums, Cichocki, and colleagues have demonstrated that cytomegalovirus infection can induce a genome-wide epigenetic diversification of NK cell subsets which affect NK functions[76, 168]. Whether hESC/iPSC-NK cells have the same epigenetic diversifications as PB-NK cells need to be further defined. Better understanding how these phenotypes may be represented in other NK cell populations can provide clues to identify optimal NK cell characteristics for more effective clinical translation.

Pluripotent stem cell-derived lymphocytes provide an optimal approach to produce standardized, cell-based therapies that can be scaled to treat thousands of patients with malignancies that have relapsed or are refractory to traditional surgery, chemotherapy, radiation therapy, and even the new immuno-oncology drugs that have demonstrated exciting ability to treat melanoma, head and neck cancer, lung cancer, and other challenging tumors. Patient-derived CAR-T cells and allogeneic donor-derived NK cells demonstrate the powerful therapeutic potential of adoptive cell therapies. However, these therapies remain largely patient-specific. Therefore, they can be time consuming and expensive to produce. Perhaps more problematic is that these are not a standardized product and vary from donor-to-donor or patient-to-patient. Use of pluripotent stem cells to produce therapeutic T cells and NK cells can overcome this problem of patient/donor variability. Specifically, these cells can be produced on a large scale from a standardized and uniform starting cell population. The therapy can be banked as stable, homogeneous cell products suitable for treatment wherever needed. The current challenges to produce therapeutic pluripotent stem cell-derived lymphocytes is roughly analogous to monoclonal antibody-based therapies that had to overcome significant manufacturing challenges 2-3 decades ago. However, antibody-based therapies have now become ubiquitous to treat not only cancer, but also autoimmune diseases and infection diseases etc. Similar advances in engineering and process development will enable wide-spread therapeutic use of hESC and/or iPSC-derived lymphocytes to treat cancer and possibly refractory chronic infectious diseases in the near future.

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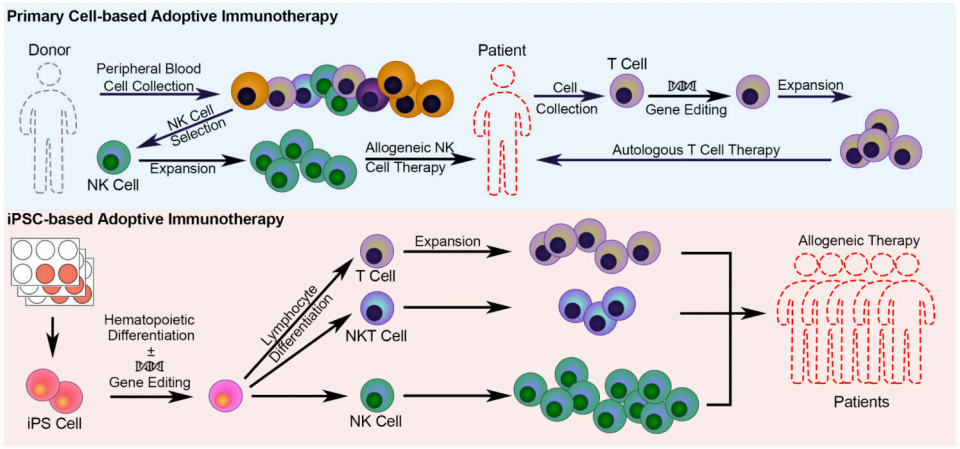

Figure 1. Cellular adoptive immunotherapy

Cellular adoptive immunotherapy utilizes either autologous or allogeneic immune cells that have been activated and/or engineered to augment the anti-tumor response. For autologous therapies, cells are sourced from the patients, while in an allogeneic therapy the cells are obtained from donors who may be related or unrelated. The cells in these therapies can be isolated from primary cells (peripheral blood), or derived from human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs). For primary cell based-adoptive immunotherapy, specific immune cell populations are isolated from peripheral blood using phenotypic selection, such as magnetic positive and/or negative selection for the cell population of interest. Primary NK cells (usually allogeneic), and primary T cells (usually autologous) are then cultured and expanded. Primary T cells are amenable to genome editing such as modifications of chimeric antigen receptors (CARs) and TCRs. However, modifying primary NK cells remains a challenge. As an alternative, immune cells can be derived from hESCs and iPSCs. These pluripotent stem cells are amenable to gene editing technologies that allow the expression of constructs such as CARs, TCRs, stabilized Fcγ receptors, etc. hESCs and iPSCs, with or without genome editing and selection, can be differentiated into hematopoietic and specific immune cell populations such as T cells and NK cells on a scale suitable for clinical therapies.

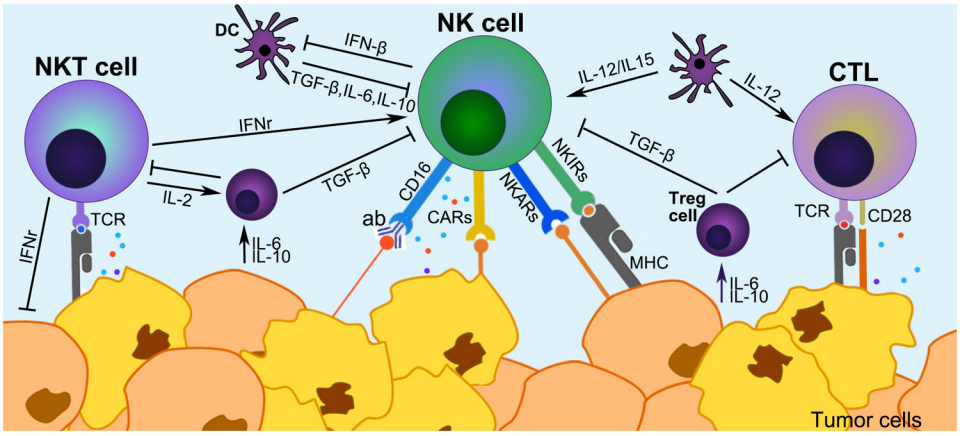

Figure 2. Mechanism of NK cells, cytotoxic T cell, NKT cells cytotoxicity and their regulatory network

NK cells activity is regulated by the balance of signals from surface receptors including activating receptors (NKARs), inhibitory receptors (NKIRs) and CD16. Cytotoxic T cells are activated when TCR binds foreign antigenic protein and co-activating molecules (such as CD28) bind costimulatory proteins on the cell surface of an antigen-presenting cell (APC) or a target cell. NKT cells activation occur when NKT TCR binds foreign antigen presented on CD1d of APC or a target cell, or binds self lipid antigens (such as β-galactosylceramide) in conjugation with exposure to TLR-induced inflammatory cytokines. Upon activation, NK cells, CTLs and NKT cells are able to directly lyse tumor cells by using mechanisms which include perforin, granzyme, and FasL. NK cells, NKT cells and T cells form a regulatory network of immune cells that influence each other by secretion of various cytokines and modulating activity of other cells such as Treg cells that have their own immune-regulatory functions.

Table 1

Comparison of allogeneic NK cell adoptive immunotherapy approaches. NK cells suitable for allogeneic adoptive immunotherapy can be obtained from different sources: peripheral blood (PB-NK cells), hEScC and iPSCs (hESC/iPSC-NK cells, and NK cell lines such as NK-92. The relative strengths and weakness are summarized.

Type of NK cells	Pros	Cons
PB-NK	+ Relatively easy to collect + Good in vivo expansion + Good clinical track record	- Heterogeneous cell population - Differs for each donor/patient - Challenging to genetically modify - Can only give one dose
hESC/iPSC -NK cells	+ Defined, homogeneous cell population + Easy to expand +Easy to genetically modify (as hESCs/iPSCs) + Can engineer multiple enhancements + Can give multiple doses + Don't need to irradiate- should give good in vivo expansion and survival	- More complicated to produce
NK-92 cells	+Defined homogeneous cell population + Easy to expand + Easy to genetically modify + Can give multiple doses	- Aneuploid tumor cells - Irradiated for clinical use limits in vivo expansion