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Adoptive Immunotherapy: A Human Pluripotent Stem Cell Perspective

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Immunotherapy · Human pluripotent stem cells · Immunoengineering · Bioengineering

Abstract
The past decade has witnessed significant advances in cancer immunotherapy, particularly through the adoptive transfer of engineered T cells in treating advanced leukemias and lymphomas. Despite these excitements, challenges remain with scale, cost, and ensuring quality control of engineered immune cells, including chimeric antigen receptor T, natural killer cells, and macrophages. The advent of human pluripotent stem cells (hPSCs), including human embryonic stem cells and induced pluripotent stem cells, has transformed immunotherapy by providing a scalable, off-the-shelf source of any desired immune cells for basic research, translational studies, and clinical interventions. The tractability of hPSCs for gene editing could also generate homogenous, universal cellular products with custom functionality for individual or combinatory therapeutic applications. This review will explore various immune cell types whose directed differentiation from hPSCs has been achieved and recently adapted for translational immunotherapy and feature forward-looking bioengineering techniques shaping the future of the stem cell field.

Introduction

Cancer immunotherapy utilizes a patient's immune system to treat cancer. With the development of immune checkpoint inhibitors and chimeric antigen receptor (CAR) T cell therapy, which showed many successful clinical cases to fight blood malignancies, immunotherapy has become a firmly established pillar of cancer treatment along with surgery, radiotherapy, and chemotherapy [Esfahani et al., 2020]. Due to its specificity and efficacy in treating refractory blood cancers, CAR T cell therapy has attracted significant attention from the public as well as research groups. As of March 2022, six CAR T cell therapies have been approved by the US Food and Drug Administration (FDA) [National Cancer Institute, 2022], and clinical trials utilizing other immune cells such as natural killer (NK) cells engineered with CARs are also being conducted. The process of CAR T cell therapy includes a collection of the patient's T cells, engineering of the collected T cells to specifically target cancer cells, and infusion of the engineered T cells back into the patient. Such an adoptive therapy is more about the set of multiple individualized procedures requiring special techniques and facilities rather than prepared therapeutic products, which poses problems such as lengthy process, high cost, and limited accessibility. Moreover, the use of autologous

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cells has manufacturing issues including difficulties in getting a sufficient number of immune cells and possible disease-related dysfunction of patients' cells [Rafiq et al., 2020]. The use of allogeneic immune cells has been suggested as an alternative approach to address these limitations. Remarkably, human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), have emerged as a promising source to obtain off-the-shelf allogeneic immune cells due to their capability to expand unlimitedly and differentiate into all types of immune cells (Fig. 1). The advances in genome editing techniques have also allowed broader applications of hPSC-derived immune cells in adoptive immunotherapy. Here, we will review strategies to enhance allogeneic adoptive immunotherapy by genetic modification of hPSCs or hPSC-derived cells, directed differentiation of hPSCs into various antitumor immune cells, and methods for biomanufacturing of hPSC-derived immune cells.

Genome Engineering of hPSCs for Adoptive Immunotherapy

Whereas the use of autologous immunotherapy often suffers from challenges such as primary immune cells' resistance to genome editing, low transduction efficiency, and random integration of inserts, genome modification in hPSCs is relatively easy and versatile, which allows more stable and controllable generation of genetically engineered donor cells. Importantly, clone selection of genetically modified hPSCs enables better quality control for the generation of isogenic donor cells. The advent of genome editing techniques that take advantage of sequence-specific nucleases, such as transcription activator-like effector nucleases, zinc-finger nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing, has revolutionized the field of biological research due to their capacity to precisely edit specific genomic locations for targeted gene knock-in and knock-out, which have been employed to enhance adoptive cellular immunotherapy in terms of their efficacy, function, and safety.

Generation of Hypoimmunogenic Donor Cells

Two major hurdles in allogeneic transplant are the immune rejection by host where a recipient's immune system recognizes grafted cells as foreign subjects and attacks them, and graft-versus-host disease (GvHD) where transplanted T cells recognize the recipient's normal tissue as a target and attack it. Both incidents occur mostly due to variability in the major histocompatibility complex (MHC) molecules which are highly polymorphic cell-surface proteins that present antigen peptides to T cells. The MHC molecules in humans are called human leukocyte antigens (HLAs), whose diversity across different individuals is the main cause of host immune rejection to the allograft. If HLAs do not match between a donor and a recipient, rejection of the graft is likely to occur as the host T cells recognize the donor cells' HLAs as foreign antigens. Since the banking of many donor hPSC lines for HLA matching is a relatively impractical option to achieve off-the-shelf cell therapy [Taylor et al., 2005; Turner et al., 2013], genome engineering of hPSCs to generate hypoimmunogenic hPSC lines has been widely studied as a more practical approach (Fig. 2). Initial efforts to circumvent immune rejection of hPSC-derived cells focused on the elimination of HLA proteins, particularly HLA class I proteins that are expressed on virtually all nucleated cells and responsive to $CD8^+$ cytotoxic T cells. Beta 2 microglobulin (encoded by the *B2M* gene) is a nonpolymorphic subunit of HLA class I proteins and has been a target to remove HLA class I proteins from the surface of hPSCs and hPSC-derived cells [Riolobos et al., 2013; Feng et al., 2014; Karabekian et al., 2015; Wang et al., 2015; Börger et al., 2016]. When *B2M* was knocked out or suppressed in hPSCs, either reduced $CD8^+$ T cell response or protection from anti-HLA antibody-mediated cytotoxicity was observed in hPSCs and hPSC-derived cells. HLA class II proteins are involved in the response of $CD4^+$ helper T cells and thus play a role in immune rejection. While the expression of these proteins is restricted to antigen-presenting cell types such as macrophages, dendritic cells, and B lymphocytes, knocking out class II major histocompatibility complex transactivator (CIITA), a transcription factor that activates HLA class II gene expression, has been proposed to further reduce activation of $CD4^+$ T cells against hPSC-derived cells [Mattapally et al., 2018; Petrus-Reurer et al., 2020]. Notably, a synergistic effect on lowering activation of T cells against retinal pigment epithelial cells derived from HLA class I/II double knock-out hPSC lines was observed compared to single HLA class knock-out lines [Petrus-Reurer et al., 2020]. Eliminating all the HLA class I proteins, however, possibly increases NK cell-mediated cytotoxicity as NK cells attack target cells that lack HLA class I proteins due to a "missing self" response [Liao et al., 1991; Kruse et al., 2015]. As the expression of minimally polymorphic HLA class Ib proteins including HLA-G and HLA-E can protect cells from NK cell-mediated lysis [Pazmany et al.,

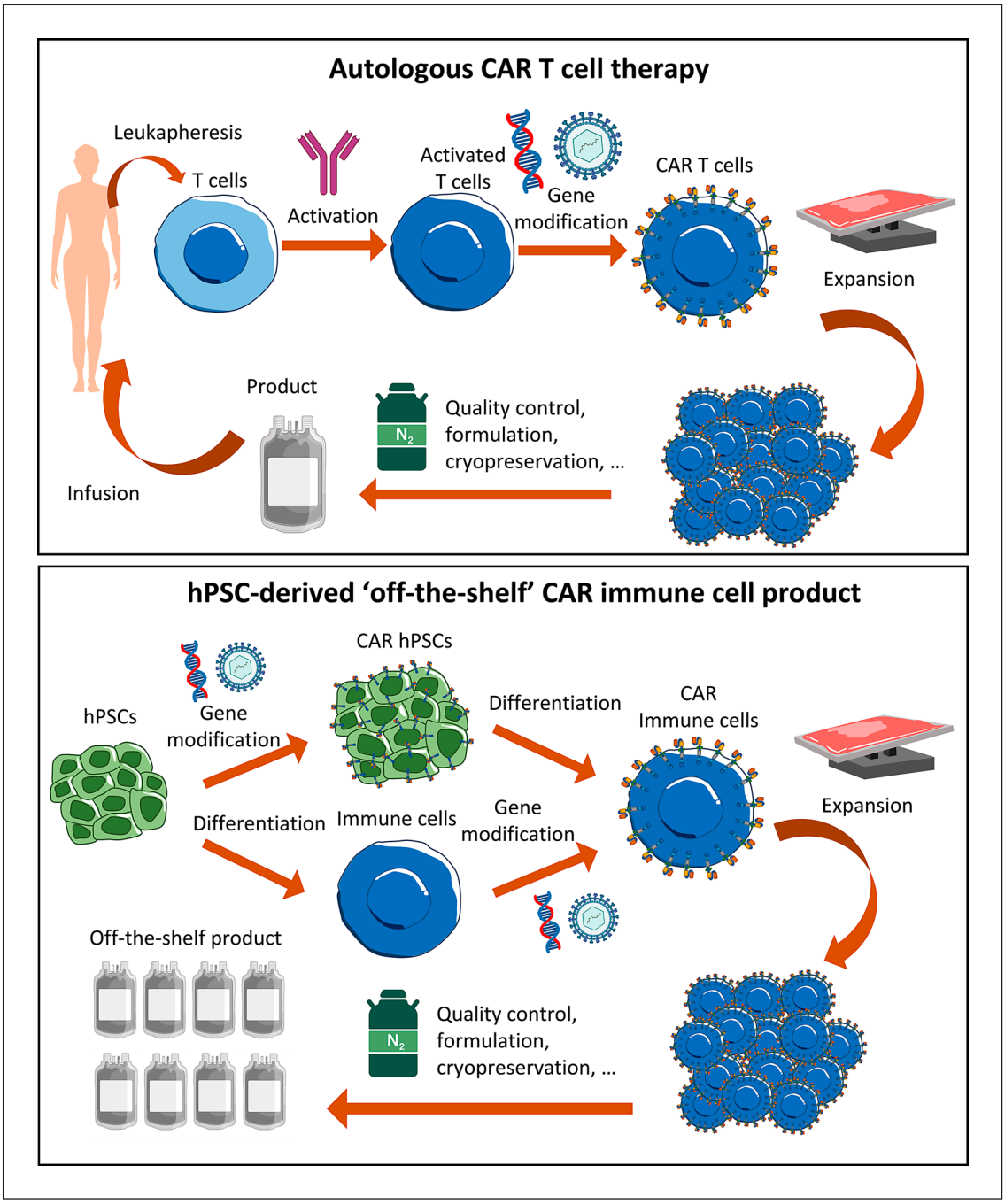

Fig. 1. Schematic summary of the manufacturing process for adoptive cellular immunotherapy. Typical procedures of autologous CAR T cell therapy involve leukapheresis, T cell activation, gene modification, expansion, quality control, formulation, and infusion. The proposed manufacturing process for hPSC-derived immune cells involves additional procedures such as differentiation and large-scale biomanufacturing of the off-the-shelf products.

1996; Braud et al., 1998; Lee et al., 1998], HLA-E was inserted into hPSCs at the same time as B2M was disrupted to inhibit immune response by NK cells while minimizing $CD8^+$ T cell response and binding of anti-HLA antibodies against hPSCs [Gornalusse et al., 2017]. Additional knock-out of PVR, a ligand of NK cell-activating receptor DNAM-1, along with HLA-E transduction and HLA class I and II knock-out, allowed better survival of hPSC-

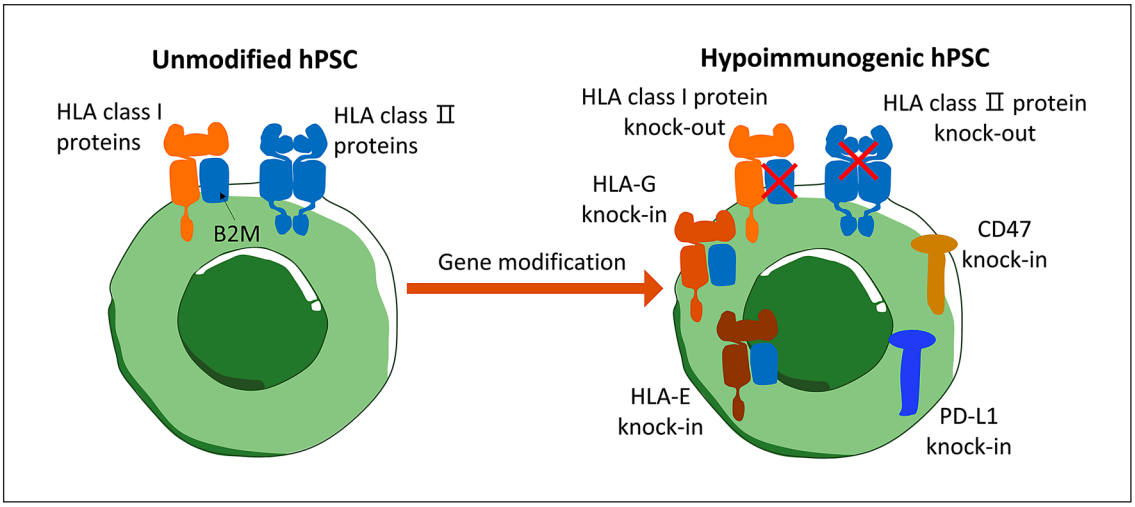

Fig. 2. Representative strategy to generate hypoimmunogenic hPSCs. HLA class I and II proteins are eliminated to reduce host immune rejection caused by HLA mismatching. Immunomodulatory molecules are introduced into hPSCs to minimize host immune response against hPSCs due to the absence of HLA molecules.

derived T cells both in vivo and in vitro by reducing NK cell activity compared to unmodified hPSC-derived T cells [Wang et al., 2021]. Constitutive expression of HLA-G in hPSCs also decreased NK cell-mediated cytotoxicity [Zhao et al., 2014]. In line with the protective effect of HLA class 1b proteins, overexpression of immunomodulatory molecules that give immune tolerance to cells has been explored as a strategy to evade immune rejection of hPSC-derived cells. Candidates for immune inhibitory molecules such as HLA-G, HLA-E, CD47, and PD-L1 are mostly found within fetal-maternal immune tolerance and immune escape mechanisms of "stealth" cancer and are typically used together with HLA knock-out to generate universal donor cells [Malik et al., 2019]. Cytotoxic T lymphocyte antigen 4-immunoglobulin fusion protein that disrupts T cell costimulatory pathways and PD-L1 that activates T cell inhibitory pathway were both knocked into hPSCs, which gave rise to fibroblasts and cardiomyocytes that were protected from allogeneic immune rejection in humanized mice [Rong et al., 2014]. Deuse et al. [2019] reported generation of hypoimmunogenic human iPSCs by inactivating HLA class I and II proteins and overexpressing CD47, which is a membrane protein found in cells at the interface between maternal blood and fetal tissue and inhibits phagocytosis. Instead of eradicating all HLA class I proteins by eliminating B2M, specific ablation of HLA-A, -B, and -C was combined with HLA class II knock-out and HLA-G, PD-L1, and CD47 knock-in to generate universal donor cells [Han et al., 2019]. Specific bi-allelic deletion of HLA-A and HLA-B and mono-allelic deletion of HLA-C also increased immune compatibility of hPSC-derived blood cells by avoiding T and NK cell activity in vitro and in vivo [Xu et al., 2019]. These are valuable approaches to augment therapeutic effects of allogeneic cell-based treatment with prolonged survival of transplanted cells not only for adoptive immunotherapy but also for other allogeneic cell therapies using hPSCs. However, as prolonged presence of hPSC-derived cells may cause problems, such as tumor formation by undifferentiated hPSCs, it is also important to have safeguards with additional genome engineering. One example is the introduction of suicide genes to selectively ablate undifferentiated hPSCs [Li and Xiang, 2013].

Engineering of CARs

CAR therapy involves genetic modification of immune cells to introduce a CAR construct. Expression of CARs on the surface of immune cells is an essential part of adoptive immunotherapy for enhanced cytotoxicity against specific tumor cells. CARs typically consist of an extracellular antigen-binding domain, an extracellular spacer that separates the binding domain and the transmembrane domain, a transmembrane domain that anchors the extracellular region in the cell membrane, and intracellular domains that transmit immune cell activation signals [Guedan et al., 2019]. Extensive studies have been conducted to develop and optimize each domain for improved specificity and efficacy of autologous CAR T

cells [Stoiber et al., 2019]. CAR engineering of hPSC-derived T cells generally adapts common CAR structures developed for autologous CAR T cells, whereas some modifications in intracellular domains were made for CAR engineering of other immune cells such as NK cells and macrophages [Pan et al., 2022]. The first hPSC-derived CAR T cells reported in a preclinical study were generated by transduction of iPSCs using lentiviruses harboring a second-generation CAR construct that consists of CD19-binding extracellular domain and CD28/CD3ζ transmembrane/intracellular domains (19-28z) [Themeli et al., 2013]. CD19 is a surface antigen predominantly expressed in B-cell leukemias and lymphomas and has been the most common target for CAR therapy [June and Sadelain, 2018]. CD3ζ is an intracellular domain that mediates T cell activation signaling upon antigen binding to CAR (recapitulating signal 1), and CD28 is a costimulatory domain that further helps T cell activation (recapitulating signal 2) [Tai et al., 2018; Stoiber et al., 2019]. The same CAR construct (19-28z) was used to generate iPSC-derived CAR T cells by 3D-organoid culture [Wang et al., 2022] and forward programming [Lv et al., 2021]. Jing et al. [2022] reported generation of mature iPSC-derived anti-CD19 CAR T cells by EZH1 repression. In this study, the authors used a CAR construct having 4-1BB costimulatory domain instead of CD28 and this CAR structure was introduced into differentiated iPSC-T cells, but not undifferentiated iPSCs, by a lentiviral vector. In another study, differentiated iPSC-T cells were transduced with the retroviruses containing CD19 single-chain variable fragment (scFv), 4-1BB, and CD3ζ to produce iPSC-derived anti-CD19 CAR T cells [Iriguchi et al., 2021a]. FT819, currently under clinical trial, is the first iPSC-derived CAR T cell product candidate [Chang et al., 2019]. The CD19-targeting CD28/CD3ζ CAR of FT819 has a mutant form of CD3ζ that has a single immunoreceptor tyrosine-based activation motif (ITAM) instead of three ITAMs in the original CD3ζ. Importantly, this mutant, named 1XX CAR, increased animal survival rate in a mouse tumor model, possibly due to the prolonged persistence of CAR T cells in vivo [Feucht et al., 2019; van der Stegen et al., 2022]. Another engineered feature of FT819 is the targeted integration of CD19 1XX CAR structure into the T cell receptor α constant (*TRAC*) locus to provide enhanced efficacy, specificity, temporal CAR expression by an endogenous T cell receptor (TCR) promoter and a reduced risk of GvHD by eliminating TCR expression [Eyquem et al., 2017; Chang et al., 2019].

Since the mode of action is different between immune cell types, the CAR domains designed in the context of T cells might need to be modified for other immune cells [Ahmad and Amiji, 2022]. Indeed, Li et al. [2018] demonstrated that human iPSC-derived anti-mesothelin CAR NK cells mediated strong antigen-specific cytotoxicity when CAR construct bearing the NKG2D transmembrane domain and 2B4 costimulatory domain along with the CD3ζ signaling domain was introduced by lentiviruses. The NKG2D and 2B4 domains were derived from the NK cell-activating receptor (NKG2D) that mediates cytolytic programs and cytokine/chemokine secretion via ITAMs (2B4). FT596, the first iPSC-derived CAR NK cell product candidate that is currently under clinical trial, utilized the same CAR construct to target $CD19^+$ B-cell lymphoma [Goodridge et al., 2019]. On the other hand, CD28 and 4-1BB costimulatory domains and CD3ζ intracellular domain were used to generate anti-EpCAM and anti-GPC3 CAR NK cells derived from human iPSCs [Ueda et al., 2020; Tang et al., 2021]. CD28 and 4-1BB domains, typically utilized for T cells, were still effective in mediating cytotoxicity of NK cells against $EpCAM^+$ and $GPC3^+$ cancer cells. Macrophage-specific CAR containing CD86 and FcγRI intracellular domains along with CD8 transmembrane domain was designed to generate human iPSC-derived anti-CD19 CAR macrophage [Zhang et al., 2020]. These CAR macrophages showed antigen-dependent phagocytosis and anti-cancer cell function in vitro and in vivo. More recently, our laboratory reported generation of chlorotoxin (CLTX)-targeting CAR neutrophils from hPSCs and showed that CD4 transmembrane domain and CD3ζ intracellular domain can mediate cytotoxic effects of neutrophils both in vitro and in vivo [Chang et al., 2022b].

As limited studies have been conducted for optimization of CAR construct in the context of cytotoxic immune cell types derived from hPSCs compared to studies done for primary T cells, improvement in efficacy of hPSC-derived CAR immune cells in cancer immunotherapy is expected, given the previously developed strategies to enhance efficacy of CARs, our increased understanding of killing mechanisms used by different immune cell types, and rapid advances in CAR design. Additionally, the use of precise genome editing techniques such as CRISPR/Cas9 in hPSCs will allow a controlled copy number of CAR construct compared to the conventional viral transduction, thereby generating safer CAR products as the copy number of CAR construct affects cell lysis [Ritchie et al., 2013].

Other Genome Engineering Approaches to Enhance Efficacy and Safety of Immunotherapy

Genome engineering in hPSCs is readily multiplexed to make several different genetic modifications in the same cell source. Through screenings and selections, it is possible to make a clonally derived master off-the-shelf hPSC line with improved functionality. This feature is important as immunotherapy inherently requires the introduction of CARs, but there are many other genome engineering strategies that further improve the efficacy and safety of CAR-based immunotherapy. Even though most genome engineering strategies have been developed for autologous CAR T cell therapy, they are expected to be easily translated into hPSC-based allogeneic CAR therapy due to the versatility of genome engineering of hPSCs.

As mentioned above, GvHD is one of the significant barriers to the allogeneic transplantation of T cells. While HLA matching possibly reduces GvHD, HLA-deleted hypoimmunogenic donor cells may still cause GvHD due to the presence of TCR, which is responsible for the alloreactivity of the donor T cells. As αβTCR is a heterodimer required for the assembly and activity of the TCR complex, ablation of αβTCR by disrupting the *TRAC* locus has been the most straightforward and common gene editing method to prevent GvHD of allogeneic CAR T cells [Depil et al., 2020; Rafiq et al., 2020]. Most of the allogeneic CAR T cell products at preclinical and clinical stages use the TRAC knock-out approach. For example, TRAC is knocked out by incorporating CAR structure into the *TRAC* locus to generate iPSC-derived CAR T cells with reduced risk of GvHD [Chang et al., 2019; van der Stegen et al., 2022]. It should be noted that TCR signaling plays an important role in T cell differentiation [Carpenter and Bosselut, 2010]; thus, the TRAC deletion may affect T cell differentiation from hPSCs. Notably, the use of CAR NK cells reduces the risk of GvHD as NK cells have MHC-unrestricted cytotoxic activity.

Improving specificity while reducing off-target effects is important for the efficacy and safety of CAR therapy. Targeting multiple antigens either with a single CAR construct having two or more antigen-binding domains or with multiple CAR constructs has been implemented to prevent antigen escape of CARs. For example, dual targeting of B-cell antigens, CD19 and CD20, was reported in many CAR T cell studies [Shah et al., 2019; Tong et al., 2020]. The combination of a CAR, bearing a signaling domain activated by binding to a tumor antigen, and a chimeric costimulatory receptor, having a costimulatory domain activated by binding to a second tumor antigen, was proposed to enhance specificity, as more efficient therapeutic effects of this CAR are only induced when both signaling and costimulatory domain are stimulated [Kloss et al., 2013]. Inhibitory CAR also involves two CAR structures to reduce off-target activity: one with inhibitory signaling activated upon binding of non-tumor cells and the other with normal activation signaling activated upon binding of tumor cells [Fedorov et al., 2013]. Another strategy for targeting multiple antigens is to make CAR T cells secrete bi-specific T cell engagers which have two scFvs that are capable of binding to both bystander T cells and cancer cells to physically link the effector and target cells [Choi et al., 2019a]. A similar approach was applied to CAR NK cells where the antigen-binding domain of CAR construct comprises two scFvs that target NKG2D-expressing effector cells and ErbB2-positive tumor cells simultaneously [Zhang et al., 2021a]. The synNotch system utilizes combinatorial antigen-sensing circuits consisting of a synthetic Notch receptor activated upon binding to a tumor antigen and a CAR construct expressed upon activation of the Notch receptor. The induced CAR recognizes another tumor antigen for more precise tumor killing [Roybal et al., 2016]. A recent study demonstrated that dual targeting in iPSC-derived CAR T cells was achieved with CAR engineering in antigen-specific T cell-derived iPSCs [Harada et al., 2022]. Dual targeting can also be achieved with a combination of a CAR and a monoclonal antibody (mAb). CD38 knock-out and BCMA CAR knock-in were performed to generate FT576, an iPSC-derived CAR NK cell product, to treat multiple myeloma by using BCMA CAR NK cells and anti-CD38 mAbs [Bjordahl et al., 2019a]. This CD38 knock-out was shown to elevate NAD^+ and provide protection against oxidative stress in iPSC-derived NK cells [Woan et al., 2021].

On-target tumor killing can be achieved with the localization of CAR activity to the tumor site, and it has also been suggested as a method to increase efficacy of CAR therapy in solid tumors. A light-inducible nuclear translocation and dimerization system was developed by Huang et al. [2020] to control CAR T cell activation by localized and noninvasive light stimulation which showed tumor cytotoxicity with high spatial resolution in vivo. Spatially controlled CAR T cell activation was also achieved with focused ultrasound that induced gene expression of CAR construct by a heat-inducible heat shock protein promoter [Wu et al., 2021]. It led to less "on-target off-tumor" toxicity than standard CAR T while providing reversibility as a safeguard. Since hypoxia is a hallmark of certain tumors, a fusion of an oxygen-sensitive

domain of HIF1α and CAR construct was used to generate CAR T cells that are responsive to a hypoxic environment to minimize "on-target off-tumor" effects [Juillerat et al., 2017]. Homing of CAR T or NK cells to tumor site by introducing chemokine receptors has provided another option to localize CAR immune cell activity [Di Stasi et al., 2009; Craddock et al., 2010; Moon et al., 2011; Kremer et al., 2017]. Other engineering strategies for enhanced on-target effects and reduced off-target activity of CAR T cell therapy include changing affinity of scFv [Song et al., 2015], inactivating target antigen from normal cells [Kim et al., 2018], and use of a T cell subset such as γδ T which has higher specificity against tumor cells [Fisher and Anderson, 2018].

In vivo persistence of CAR immune cells significantly affects the outcome of CAR therapy. Genome engineering for cytokine and cytokine receptor expression is a popular strategy to enhance persistence and cytotoxic effects of CAR immune cells. IL-7/CCL19 [Adachi et al., 2018], IL-12 [Pegram et al., 2015], IL-18 [Avanzi et al., 2016], IL-15 [Hoyos et al., 2010; Hurton et al., 2016; Liu et al., 2018; Wang et al., 2020], IL-2 [Lee et al., 2010], IL-4αβ chimeric cytokine receptor [Wilkie et al., 2010], IL-4/7 inverted cytokine receptor [Mohammed et al., 2017], IL-4/21 inverted cytokine receptor [Wang et al., 2019], and IL-7 receptor [Shum et al., 2017] have been assessed for their function in not only improving persistence of CAR T and CAR NK cells but also remodeling tumor microenvironment (TME), thereby enhancing efficacy of CAR therapies. For example, membrane-bound IL-15 was used to augment antitumor activity of hPSC-derived CAR T cells [Iriguchi et al., 2021a]. A similar approach was applied to FT538, FT576, and FT596, human iPSC-derived CAR NK cell products that are currently under multiple clinical trials [Bjordahl et al., 2019a, b; Goodridge et al., 2019]. Insertion of IL-15/IL-15 receptor alpha recombinant fusion (IL-15RF) improved persistence, functional maturation, and CAR activity of these NK cell products. Modulation of CAR construct has also been widely studied to extend survival of CAR immune cells. While incorporating costimulatory domains (second- and third-generation CARs) has already been established for most of the preclinical and clinical studies, modification in other CAR domains such as transmembrane domain was also shown to affect CAR T cell persistence [Guedan et al., 2018]. A mutant form of CD3ζ (single ITAM instead of three ITAMs) increased persistence of CAR T cells [Feucht et al., 2019], and notably, it was translated into FT819, a human iPSC-derived CAR T cell product, which is currently under clinical trial [Chang et al., 2019].

Antibody-dependent cellular cytotoxicity (ADCC), a crucial effector mechanism of NK cells, is mediated by the Fc receptor CD16a on NK cells. Zhu et al. [2020b] introduced the high-affinity non-cleavable variant of CD16a (hnCD16) into human iPSCs, and NK cells derived from these hnCD16-modified iPSCs showed enhanced ADCC against multiple tumor targets. This feature was introduced into FT538, FT576, and FT596 along with BCMA (FT576)/CD19 (FT596)-targeting CAR and IL-15RF to improve functionality of the human iPSC-derived CAR NK cells [Bjordahl et al., 2019a, b; Goodridge et al., 2019]. These engineering strategies were recently published in the context of iPSC-derived NK cells without CAR modification [Woan et al., 2021].

Modulation of immune checkpoint-mediated immune cell inhibition is also an important approach to further enhance the efficacy of CAR therapy. Other than administration of immune checkpoint inhibitors along with CAR therapy, genome engineering approaches have been developed to minimize inhibitory checkpoint signaling by introducing genetic factors that interfere with this inhibitory signaling pathway. They include incorporation of PD-1-dominant negative receptor [Cherkassky et al., 2016], a switch receptor construct consisting of a truncated extracellular domain of PD-1 and the transmembrane and cytoplasmic signaling domains of CD28 [Liu et al., 2016], anti-PD-L1 antibody [Suarez et al., 2016], anti-PD-1 scFv [Rafiq et al., 2018], and B7-H3 immune checkpoint molecule for NK cells [Yang et al., 2020]. Controlling toxicity of CAR immune cells with suicide genes provides a safer approach for cancer immunotherapies and has been utilized in many preclinical and clinical studies [Serafini et al., 2004; Hoyos et al., 2010; Di Stasi et al., 2011; Liu et al., 2020; Wang et al., 2020; Harada et al., 2022]. Another engineering approach to increase safety of the CAR T cell therapy is to knock out granulocyte-macrophage colony-stimulating factor to reduce cytokine release syndrome [Sachdeva et al., 2019; Sterner et al., 2019].

Although significant progress has been made in genome engineering of CAR T and CAR NK cells to enhance cancer immunotherapy, only a small portion of these strategies have been evaluated in the context of other immune cell types or hPSC-derived immune cells. More research will be needed to facilitate the development of hPSC-derived allogeneic cellular products with previously developed engineering strategies for adoptive immunotherapy.

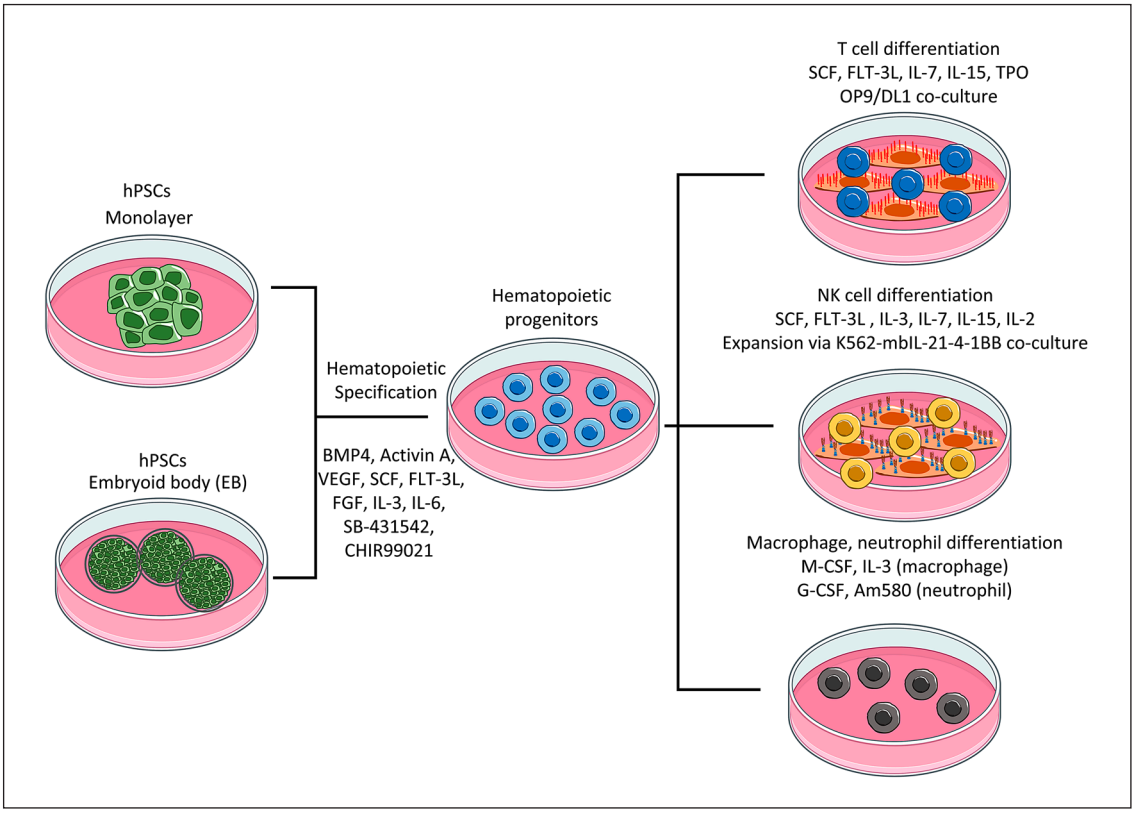

Fig. 3. Simplified differentiation methods for generation of antitumor immune cells from hPSCs. Differentiation protocols typically involve stage-specific treatment of differentiation factors such as growth factors, cytokines, small molecules, and cells. Some of the representative factors and co-culture methods are shown for each immune cell type.

hPSC-Derived Immune Cells for Adoptive Cellular Immunotherapy

Efficient generation of cytotoxic immune cells, such as T, NK cells, and macrophages, from hPSCs is the key to development of off-the-shelf allogeneic cell products for cancer immunotherapy. Significant advances have been made in differentiating hPSCs into hematopoietic progenitors and mature immune cells based on the knowledge and techniques obtained from numerous types of research within the field of developmental biology, stem cell biology, hematopoiesis, biomaterials, and others. Progress in differentiation protocols for each immune cell type was reviewed elsewhere (T cells [Montel-Hagen and Crooks, 2019; Zhou et al., 2022], NK cells [Chang and Bao, 2020; Hsu et al., 2021], macrophages [Lyadova and Vasiliev, 2022]) (Fig. 3). Here, we focus on generation of antigen-specific immune cells, primarily CAR-modified immune cells, derived from hPSCs for cancer immunotherapy (Table 1).

Cytotoxic T Cells

Cytotoxic T lymphocytes (CTLs) recognize foreign antigens via TCRs and selectively exert cytotoxic effects on the target cells. This characteristic is utilized to generate antigen-specific "rejuvenated" T cells from human iPSCs. To make "rejuvenated" T cells, iPSCs are reprogrammed from antigen-specific CTLs which have antigen-targeting TCRs and then re-differentiated into CTLs with the retained TCR expression [Nishimura et al., 2013]. Vizcardo et al. [2013] reported generation of MART-1-specific $CD8^+$ T cells from human iPSCs, which exhibited immune reactivity upon the binding of MART-1 antigen. These iPSCs were reprogrammed from MART-1-specific $CD8^+$ T cell line JKF6 that has a TCR specific for MART-1 epitope. To induce more specific cytotoxicity, $CD8\alpha\beta^+$ CTLs were differentiated from WT1-specific CTL-derived human iPSCs [Maeda et al., 2016]. Since the TCR rearrangement during T cell differentiation from iPSCs can prevent retention of antigen-specific TCRs,

Table 1. hPSC-derived immune cells for cancer immunotherapy

Cell type	hPSC type/ iPSC source	Differentiation method	Target antigen/ cancer (cancer cell line)	Genome engineering method	CAR design	Therapeutic outcome	Features	Reference
$CD8^+$ T cell	hiPSCs/$CD8^+$ T cells	OP9 and OP9/DL1 stromal cell co-culture [Vodyanik and Slukvin, 2007; Timmermans et al., 2009]	MART-1/melanoma	Sendai virus (iPSC reprogramming)	–	IFNγ secretion	Rejuvenation (antigen-specific T cell-derived iPSC)	[Vizcardo et al., 2013]
$CD8^+$ T cell	hiPSCs/$CD8^+$ T cells	OP9 and OP9/DL1 stromal cell co-culture [Schmitt et al., 2004; Timmermans et al., 2009]	WT1/leukemia	Sendai virus (iPSC reprogramming)	–	In vitro and in vivo cytotoxicity	Rejuvenation $CD8\alpha\beta^+$ innate type T cells	[Maeda et al., 2016]
$CD8^+$ T cell	hiPSCs/$CD8^+$ T cells, monocytes	OP9 and OP9/DL1 stromal cell co-culture, modified from the previous method [Nishimura et al., 2013]	GPC3/hepatic cancer WT1/leukemia	Sendai virus (iPSC reprogramming) CRISPR/Cas9 (RAG knock-out) Lentivirus (TCR transduction in iPSCs)	–	In vitro and in vivo cytotoxicity	Rejuvenation RAG knock-out to prevent TCR rearrangement Antigen-specific TCR knock-in	[Minagawa et al., 2018]
$CD8^+$ T cell	hESCs	EMO and PSC-ATO-based differentiation, MS5-hDLL4 cells [Montel-Hagen et al., 2019]	NY-ESO-1/leukemia	Lentivirus (TCR transduction in ESCs)	–	In vitro and in vivo cytotoxicity	Antigen-specific TCR knock-in	[Montel-Hagen et al., 2019]
$CD8^+$ T cell	hiPSCs/ monocytes	OP9 and OP9/DL1 stromal cell co-culture, modified from the previous method [Maeda et al., 2016]	WT1/leukemia	Lentivirus (TCR transduction in iPSCs)	–	In vitro cytotoxicity	Antigen-specific TCR knock-in	[Maeda et al., 2020]
$CD8\alpha^+$ T cell	hiPSCs/ peripheral blood lymphocytes	EBs, OP9/DL1 stromal cell co-culture [Themeli et al., 2013]	CD19/lymphoma	Retrovirus (iPSC reprogramming) Lentivirus (CAR transduction in iPSCs)	CD19 (antigen binding) CD3ζ (signaling) CD28 (costimulatory)	In vitro and in vivo cytotoxicity	γδ T cell-like phenotype	[Themeli et al., 2013]
$CD8^+$ T cell/ $CD4^+$ T cell	hiPSCs	Inducible expression of RUNX1 and HOXA9 OP9 and OP9/DL1 stromal cell co-culture [Schmitt et al., 2004]	CD19/lymphoma	Retrovirus (CAR transduction in iPSC-derived T cells)	CD19 (antigen binding)	In vitro and in vivo cytotoxicity	Forward programming	[Lai et al., 2021]
$CD8^+$ T cell	hiPSCs/PB T cells	Feeder-free, DL4 and RN culture [Iriguchi et al., 2021a, b]	GPC3/hepatic cancer WT1/lung cancer CD19/leukemia	Sendai virus (iPSC reprogramming) Lentivirus (TCR transduction in iPSCs) Retrovirus (CAR transduction in iPSC-derived T cells)	CD19 (antigen binding) CD3ζ (signaling) 4-1BB (costimulatory) CD8 (transmembrane)	In vitro and in vivo cytotoxicity	Rejuvenation Membrane-bound IL-15 RAG knock-out Antigen-specific TCR knock-in	[Iriguchi et al., 2021a]

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Table 1 (continued)

Cell type	hPSC type/ iPSC source	Differentiation method	Target antigen/ cancer (cancer cell line)	Genome engineering method	CAR design	Therapeutic outcome	Features	Reference
$CD8^+$ T cell	hiPSCs/$CD8^+$ T cells	C3H10T1/2, DL1/4-expressing C3H10T1/2 feeder cell co-culture [Harada et al., 2022]	LMP1, LMP2, CD19/ Epstein-Barr virus-associated lymphomas	Sendai virus (iPSC reprogramming) Lentivirus (CAR transduction in iPSCs)	CD19/LMP1 (antigen binding) CD3ζ (signaling) CD28, OX40/4-1BB (costimulatory)	In vitro and in vivo cytotoxicity	Rejuvenation Suicide gene (inducible caspase-9)	[Harada et al., 2022]
$CD8^+$ T cell	hiPSCs/$CD8^+$ T cells	OP9 and OP9/DL1 stromal cell co-culture, modified from the previous method [Minagawa et al., 2018]	CD19, CD20/ leukemia, lymphoma	CRISPR/Cas9 (B2M, CIITA, PVR knock-out in iPSCs) Lentivirus (HLA-E transduction in iPSCs) Retrovirus (CAR transduction iPSC-derived T cells)	CD20 (antigen binding)	In vitro and in vivo cytotoxicity	Hypoimmunogenic iPSCs	[Wang et al., 2021]
$CD8^+$ T cell	hiPSCs/$CD62L^+$ naïve and memory T cells	3D-organoid culture, MS5-hDLL4 cells [Wang et al., 2022]	CD19/leukemia	Episomal plasmids (iPSC reprogramming) Lentivirus (CAR transduction in iPSCs)	CD19 (antigen binding) CD3ζ (signaling) CD28 (costimulatory)	In vitro and in vivo cytotoxicity	3D-organoid culture	[Wang et al., 2022]
$CD8^+$ T cell	hiPSCs/ erythroblast	EZH1 repression, OP9 and OP9/DL1 stromal cell co-culture [Shukla et al., 2017; Jing et al., 2022]	CD19/lymphoma	CRISPR interference (EZH1 repression) Lentivirus (CAR transduction in iPSC-derived T cells)	CD19 (antigen binding) CD3ζ (signaling) 4-1BB (costimulatory) CD8 (transmembrane/ costimulatory)	In vitro and in vivo cytotoxicity	EZH1 repression	[Jing et al., 2022]
$CD8^+$ T cell	hiPSCs/$CD8^+$ T cells	Original T cell differentiation protocol with GMP system [Chang et al., 2019]	CD19/leukemia	Not available	CD19 (antigen binding) Mutant CD3ζ 1XX (signaling) CD28 (costimulatory)	In vitro and in vivo cytotoxicity	CAR knocked into TRAC locus for TCR knock-out	[Chang et al., 2019]
$CD8^+$ T cell	hiPSCs/PB T cells hESCs	OP9 and OP9/DL1 stromal cell co-culture [Themeli et al., 2013; Valamehr et al., 2014; Cichocki et al., 2020; van der Stegen et al., 2022]	CD19/leukemia	Retrovirus (iPSC reprogramming) AAV6 (CAR transduction in PB T cells) CRISPR/Cas9 (TRAC knock-out in iPSCs)	CD19 (antigen binding) Mutant CD3ζ 1XX (signaling) CD28 (costimulatory)	In vitro and in vivo cytotoxicity	CAR knocked into TRAC locus for TCR knock-out	[van der Stegen et al., 2022]
NK cell	hESCs	S17 and AFT024 stromal cell co-culture [Miller et al., 1999; Kaufman et al., 2001; Tian and Kaufman, 2005]	Leukemia	–	–	In vitro cytotoxicity	Both direct cell-mediated cytotoxicity and ADCC	[Woll et al., 2005]

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Table 1 (continued)

Cell type	hPSC type/ iPSC source	Differentiation method	Target antigen/ cancer (cancer cell line)	Genome engineering method	CAR design	Therapeutic outcome	Features	Reference
NK cell	hESCs	M210-B4 and AFT024 stromal cell co-culture [Kaufman et al., 2001; Woll et al., 2005]	Leukemia	–	–	In vitro and in vivo cytotoxicity	Superior in vivo antitumor effects compared to UCB-derived NK cells	[Woll et al., 2009]
NK cell	hESCs hiPSCs/UCB cells	Spin EBs, EL08-1D2 stromal cell, and membrane-bound IL-21-expressing artificial antigen-presenting cell (K562-mbIL-21-4-1BB) co-culture [Denman et al., 2012; Knorr et al., 2013]	Leukemia, multiple myeloma, pancreatic cancer	–	–	In vitro cytotoxicity	NK cell differentiation without sorting and xenogeneic stromal cell co-culture Membrane-bound IL-21 in artificial antigen-presenting cells	[Knorr et al., 2013]
NK cell	hESCs	EBs, MS-5/SP-HOXB4 stromal cell co-culture [Haddad et al., 2007; Larbi et al., 2012]	Leukemia	–	–	In vitro cytotoxicity	NK cell enrichment by HOXB4 protein	[Larbi et al., 2012]
NK cell	hiPSCs/UCB cells	Spin EBs, K562-mbIL-21-4-1BB co-culture [Ng et al., 2008; Woll et al., 2009; Knorr et al., 2013]	Ovarian cancer	–	–	In vitro and in vivo cytotoxicity	Comparison between PB-NK and iPSC-derived NK cells	[Hermanson et al., 2016]
NK cell	hiPSCs/PB cells	OP9 and OP9/DL1 stromal cell co-culture [Zeng et al., 2017]	Leukemia, myeloma, and others	Sendai virus (iPSC reprogramming)	–	In vitro cytotoxicity	PB cell-derived iPSCs	[Zeng et al., 2017]
NK cell	hESCs hiPSCs/CB cells	Chemically defined differentiation modified from the previous method, feeder-free [Niwa et al., 2011; Yanagimachi et al., 2013; Ohta et al., 2016]	Leukemia	–	–	In vitro and in vivo cytotoxicity	Chemically defined differentiation	[Matsubara et al., 2019]
NK cell	hiPSCs/ fibroblasts	EL08-1D2 stroma cell and K562-mbIL-21-4-1BB co-culture [Cichocki and Miller, 2010; Wang et al., 2013; Cichocki et al., 2020]	Lung cancer, hepatocyte cancer, ovarian cancer, leukemia, melanoma	Lentivirus (iPSC reprogramming)	–	In vitro and in vivo cytotoxicity	Synergistic cytotoxic effect with T cells and anti-PD-L1 antibody	[Cichocki et al., 2020]

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Table 1 (continued)

Cell type	hPSC type/ iPSC source	Differentiation method	Target antigen/ cancer (cancer cell line)	Genome engineering method	CAR design	Therapeutic outcome	Features	Reference
NK cell	hiPSCs	Spin EBs, K562-mbIL-21-4-1BB co-culture [Knorr et al., 2013; Hermanson et al., 2015; Zhu and Kaufman, 2019]	Leukemia, lymphoma, and others	–	–	In vitro cytotoxicity	Comparison between UCB-NK and iPSC-derived NK cells	[Goldenson et al., 2020]
NK cell	hiPSCs	Spin EBs, K562-mbIL-21-4-1BB co-culture [Knorr et al., 2013; Hermanson et al., 2015]	Lymphoma, ovarian cancer	Lentivirus (hnCD16 transduction)	–	In vitro and in vivo cytotoxicity	hnCD16 knock-in	[Zhu et al., 2020b]
NK cell	hiPSCs	Spin EBs, K562-mbIL-21-4-1BB co-culture [Zhu and Kaufman, 2019]	Leukemia, ovarian cancer	CRISPR/Cas9 (CISH knock-out)	–	In vitro and in vivo cytotoxicity	CISH knock-out	[Zhu et al., 2020a]
NK cell	hiPSCs/ fibroblasts	EBs, OP9/DL1 stromal cell co-culture [Ni et al., 2013; Euchner et al., 2021]	Leukemia	Sendai virus (iPSC reprogramming)	–	In vitro cytotoxicity	Molecular characterization of iPSC-derived NK cells	[Euchner et al., 2021]
NK cell	hiPSCs	OP9 and K562-mbIL-21-4-1BB co-culture [Cichocki and Miller, 2010; Cichocki et al., 2020]	Leukemia, multiple myeloma	CRISPR/Cas9 (IL-15RF and hnCD16 knock-in into CD38 locus)	–	In vitro and in vivo cytotoxicity	IL-15RF and hnCD16 knock-in CD38 knock-out	[Woan et al., 2021]
NK cell	hiPSCs/CB cells	EBs [Ng et al., 2008; Knorr et al., 2013]	Leukemia, lymphoma, breast cancer	Nucleofection (iPSC reprogramming)	–	In vitro cytotoxicity	Cryopreserved CB mononuclear cell-derived iPSCs	[Du et al., 2022]
NK cell	hESCs	Temporal ID2 induction, modified from the previous method [Matsubara et al., 2019; Jung et al., 2022]	Brain cancer, prostate cancer, breast cancer	CRISPR/Cas9 (ID2 knock-in)	–	In vitro cytotoxicity	Inducible ID2 expression	[Jung et al., 2022]
NK cell	hESCs hiPSCs	Spin EBs, K562-mbIL-21-4-1BB co-culture [Knorr et al., 2013]	CD19, mesothelin/ leukemia, ovarian cancer	Sleeping Beauty transposon system (CAR transfection in hPSCs)	CD19, mesothelin (antigen binding) CD3ζ (signaling) 4-1BB, CD28 (costimulatory, transmembrane) CD8α (hinge)	In vitro cytotoxicity	Sleeping Beauty transposon system	[Hermanson et al., 2013]

Table 1 (continued)

Cell type	hPSC type/ iPSC source	Differentiation method	Target antigen/ cancer (cancer cell line)	Genome engineering method	CAR design	Therapeutic outcome	Features	Reference
NK cell	hiPSCs	Spin EBs, K562-mbIL-21-4-1BB co-culture [Ng et al., 2008; Knorr et al., 2013; Ni et al., 2013; Bachanova et al., 2014]	Mesothelin/ leukemia, ovarian cancer	PiggyBac transposon vectors (CAR transfection in iPSCs)	Mesothelin (antigen binding) CD3ζ (signaling) 2B4 (costimulatory) CD8α (hinge) NKG2D (transmembrane)	In vitro and in vivo cytotoxicity	Optimization of CAR construct for NK cells	[Li et al., 2018]
NK cell, innate lymphoid cell	hiPSCs/blood mononuclear cells	EBs, FcDLL4-coated plates [Ueda et al., 2020]	GPC3/hepatic cancer	Lentivirus (CAR transduction in iPSCs)	GPC3 (antigen binding) CD3ζ (signaling) 4-1BB, CD28 (costimulatory, transmembrane) CD8α (hinge)	In vitro and in vivo cytotoxicity	Clinically relevant manipulation of NK cell production	[Ueda et al., 2020]
NK cell	hiPSCs/PB mononuclear cells (PBMCs)	OP9 and OP9/DL1 stromal cell co-culture [Zeng et al., 2017]	EpCAM/breast cancer	ZFN (CAR integration in iPSCs)	EpCAM (antigen binding) CD3ζ (signaling) 4-1BB, CD28 (costimulatory, transmembrane) CD8α (hinge)	In vitro cytotoxicity	CAR integration into AAVS1 safe harbor locus	[Tang et al., 2021]
Macrophage	hiPSCs/ fibroblasts, epithelial cells, PB erythroblasts	Modified from the previous method [Uenishi et al., 2014; Cao et al., 2019]	Leukemia	–	–	In vitro cytotoxicity	M1, M2 polarization	[Cao et al., 2019]
Myeloid cell/ macrophage	hiPSCs	Previously described [Senju et al., 2011]	Melanoma	Lentivirus (IFNα and IFNβ transduction in iPSC-derived myeloid cells)	–	In vitro and in vivo cytotoxicity	cMYC, BMI1, MDM2, EZH2 transduction for proliferation of iPSC-myeloid precursor cells	[Miyashita et al., 2016]
Macrophage	hiPSCs/ fibroblasts	OP stromal cell co-culture or xeno-free culture, fetal calf serum [Senju et al., 2011]	Leukemia	Lentivirus (iPSC reprogramming) Plasmid electroporation (scFv transection in iPSCs)	–	In vitro and in vivo cytotoxicity	scFv targeting amyloid β	[Senju et al., 2011]
Myeloid cell/ macrophage	hiPSCs	Previously described [Haruta et al., 2013]	Gastric cancer, pancreatic cancer	Plasmid electroporation (scFv transection in iPSCs) Lentivirus (additional genes)	–	In vitro and in vivo cytotoxicity	IFNα IFNβ, IFNγ, TNFα, FAS-ligand, or TRAIL expression in iPSC-myeloid cells	[Koba et al., 2013]

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Table 1 (continued)

Cell type	hPSC type/ iPSC source	Differentiation method	Target antigen/ cancer (cancer cell line)	Genome engineering method	CAR design	Therapeutic outcome	Features	Reference
Macrophage	hiPSCs/PBMC	EBs [Zhang et al., 2020]	Leukemia, ovarian cancer	Plasmid electroporation (iPSC reprogramming) Lentivirus (CAR transduction in iPSCs)	CD19, mesothelin (antigen binding) CD86 and FcγRI (signaling, costimulatory) CD8α (transmembrane)	In vitro and in vivo cytotoxicity	hPSC-derived CAR macrophages	[Zhang et al., 2020]
Neutrophils	hESCs	Chemically defined, feeder-free [Chang et al., 2022a; Chang et al., 2022b]	CLTX/glioblastoma	CRISPR/Cas9 (CAR insertion in hESCs)	CLTX (antigen binding) CD3ζ (signaling) CD4 (transmembrane)	In vitro and in vivo cytotoxicity	hPSC-derived CAR neutrophils	[Chang et al., 2022b]
iNKT cell	hiPSCs/iNKT cell	C3H10T1/2, OP9 and OP9/DL1 stromal cell co-culture, EBs modified from the previous method [Nishimura et al., 2013; Kitayama et al., 2016]	Leukemia, lymphoma	Sendai virus (iPSC reprogramming)	–	In vitro cytotoxicity	TCR-independent cytotoxicity via NKG2D and DNAM-1	[Kitayama et al., 2016]
iNKT cell	hiPSCs/iNKT cell	OP9 and OP9/DL1 stromal cell co-culture, modified from the previous method [Vizcardo et al., 2013; Yamada et al., 2016]	Leukemia and others	Sendai virus (iPSC reprogramming)	–	In vitro and in vivo cytotoxicity	Adjuvant activity to activate autologous NK cells	[Yamada et al., 2016]
Eosinophil	hESCs hiPSCs	[Lai et al., 2021]	Colon cancer, breast cancer, liver cancer	–	–	In vitro and in vivo cytotoxicity	Combinatorial cytotoxic effects with CAR T cells	[Lai et al., 2021]

CLTX, chlorotoxin; EMO, embryonic mesoderm organoids; PSC-ATO, PSC-artificial thymic organoid; GMP, good manufacturing practice; AAV6, adeno-associated virus 6; UCB, umbilical cord blood; EB, embryoid body; ZFN, zinc-finger nuclease; CB, cord blood.

RAG2, a protein involved in TCR rearrangement, was knocked out in iPSCs to preserve antigen-specific TCRs [Minagawa et al., 2018]. CDαβ$^+$ T cells differentiated from GPC3-specific CTL-derived RAG2$^-$ iPSCs exhibited GPC3-specific cytotoxicity both in vitro and in vivo. The authors also tested introduction of antigen-specific TCRs into monocyte-derived iPSCs as an approach to confer antigen specificity to iPSC-derived CTLs. Montel-Hagen et al. [2019] demonstrated that generation of hESC-derived antigen-specific T cells was achieved by introducing antigen-specific TCR to hESCs and differentiating these hESCs into conventional T cells with their organoid-based differentiation protocol. In another study, iPSCs were also transduced with WT1-specific TCR to make antigen-specific CTLs [Maeda et al., 2020].

The first hPSC-derived CAR T cells were reported by Themeli et al. [2013], where they generated CD19-targeting CAR T cells from peripheral blood (PB) T lymphocyte-derived iPSCs (1928z-T-iPSC-T cell). The CAR construct was introduced into iPSCs by lentiviruses, and successfully transduced iPSCs were sorted based on a fluorescent marker expression followed by T cell differentiation. These iPSC-derived CAR T cells showed a phenotype of γδ T cell, a T cell subpopulation enriched in peripheral tissues, as well as cytotoxicity against CD19$^+$ lymphoma in vitro and in vivo. Forward programming with inducible expression of *RUNX1* and *HOXA9* was used to generate hematopoietic-endothelial cells and subsequent T lymphocytes from iPSCs [Lv et al., 2021]. These induced T cells were further transduced with retroviruses for incorporation of CD19 CAR, and the CAR-transduced T cells exhibited in vitro and in vivo cytotoxicity against B-cell lymphoma cells. Iriguchi et al. [2021a] proposed an improved T cell differentiation protocol in the absence of a feeder layer to differentiate iPSCs into antigen-specific T cells. This differentiation method was applied to the generation of antigen-specific T cells with three different approaches: T cells differentiated from antigen-specific CTL-derived iPSCs, T cells differentiated from TCR-engineered iPSCs, and CAR T cells engineered from differentiated T cells. All these antigen-specific T cells showed cytotoxicity against their targets. In another independent study, "rejuvenated" T cells from antigen-specific CTL-derived iPSCs were further engineered with a CAR to generate dual antigen-targeting iPSC-derived T cells [Harada et al., 2022]. The capability of hypoimmunogenic hPSC-derived T cells in escaping immune rejection was evaluated along with CAR engineering [Wang et al., 2021]. T cells differentiated from hypoimmunogenic iPSCs with HLA class I, HLA class II, or CD155 knockout, and HLA-E knock-in were further engineered with either anti-CD19 or anti-CD20 CAR constructs. These iPSC-derived CAR T cells exhibited antitumor potency with minimal recognition by the host NK and T cells. A recent study demonstrated that 3D-organoid culture supports differentiation of CD19-targeting CAR T cells from iPSCs [Wang et al., 2022]. These iPSCs were derived from CD62L$^+$ naïve and memory T cells which showed promising therapeutic effects in CAR T cell therapy, transduced with the lentiviruses encoding 19-28z, and differentiated into CD8$^+$ T cells. The resulting CAR T cells showed antitumor activity against CD19$^+$ tumor cells in vitro and in vivo. Repression of EZH1, a negative epigenetic regulator of lymphoid commitment, in iPSCs along with a feeder-free T cell differentiation led to the generation of primary αβ T cell-like cells capable of differentiating into cytotoxic T cells and memory T cell-like cells [Jing et al., 2022]. Importantly, these T cells exhibited superior antitumor activities when transduced with a CD19 CAR compared to the CAR T cells differentiated without EZH1 repression. Fate Therapeutics reported the generation of an iPSC-derived CAR T cell product, FT819, which is in phase 1 clinical study, with their original and modified T differentiation protocols [Chang et al., 2019]. In this product, CD19 1XX CAR was introduced into the *TRAC* locus of T cell-derived iPSCs for TCR knock-out and endogenous TCR promoter-regulated CAR expression, and the CD19 1xx CAR iPSCs were efficiently differentiated into CTLs (>95% CD45$^+$, CD7$^+$, CD3$^+$, CAR$^+$/>70% CD8αβ$^+$) with cytotoxic activities against CD19$^+$ tumor cells in vitro and in vivo. Generation of iPSC-derived CD19 1XX CAR T cells with the same engineering features was recently reported [van der Stegen et al., 2022].

It is noteworthy that almost all antigen-specific T cells are derived from human iPSCs that were reprogrammed from T lymphocytes or from iPSCs and hESCs that were transduced to incorporate antigen-specific TCRs, suggesting the importance of epigenetic memory [Kim et al., 2010] and TCR signaling [Carpenter and Bosselut, 2010] during differentiation of hPSCs into cytotoxic T cells. Unlike this seemingly positive role of an antigen-specific TCR at the pluripotent stem cell stage in the development of CD8αβ$^+$ single-positive cytotoxic T cells [Montel-Hagen et al., 2019], a recent study demonstrated that the premature expression of rearranged αβTCR genes in T cell-derived iPSCs interferes with the formation of CD4$^+$CD8αβ$^+$ double-positive T cells, thus the following formation of CD8αβ$^+$ single-positive cytotoxic T cells [van der Stegen et al., 2022]. As these iPSCs were not derived from antigen-specific T lymphocytes, their TCR re-

arrangement may differ from the previous study, resulting in a different differentiation pathway. Delta-like ligand 4 (DLL4), a Notch ligand, offset this effect of premature TCR expression and facilitated the formation of $CD4^+CD8\alpha\beta^+$ double-positive T cells from the wild-type iPSCs. More importantly, they found that the constitutive expression of anti-CD19-28z CAR in T cell-derived iPSCs interfered with the double-positive T cell formation but led to the generation of $CD4^-CD8^-$ double negative or $CD8\alpha\alpha^+$ T cells, even in the presence of DLL4-mediated Notch signaling. When CAR expression was induced in the later stage of T cell differentiation by the TRAC promoter, it facilitated the formation of $CD4^+CD8\alpha\beta^+$ double-positive T cells and then maturation to $CD8\alpha\beta^+$ single-positive cytotoxic T cells. This study suggests that the expression of CARs in hPSC stage can negatively affect the differentiation capability of hPSCs into cytotoxic T cells; thus, other strategies such as inducible expression of CARs are needed to generate hPSC-derived CAR T cells. More research will be needed to reveal the role of epigenetic memory, TCR arrangement, CAR expression, and Notch signaling in T cell differentiation and subsequent subtype specification, thereby developing a more robust and scalable differentiation protocol for CAR-engineered cytotoxic T cells from hPSCs.

NK Cells

NK cells are a type of cytotoxic lymphocyte that is part of the innate immune system. In contrast to the cytotoxic T cells, they recognize and kill pathogens and stressed cells in an MHC-independent manner, making them a promising allogeneic cell source for cellular immunotherapy. Not only does an allogeneic NK cell transplant not require HLA matching to prevent GvHD but NK cells can also be armed with a CAR construct for improved antigen-specific cytotoxicity. Many studies at preclinical and clinical levels have been conducted to evaluate the efficacy of allogeneic NK cells in cancer immunotherapy, where NK cells used are mainly NK-92 cell line or derived from PB and cord blood. This immortalized line and primary cell sources have several disadvantages such as low transduction efficiency, heterogeneity, and the need for irradiation before infusion [Gong et al., 2021]. Recently, hPSCs have emerged as an alternative cell source for NK cells to address these issues. Due to their innate killing ability, many studies have shown the superior antitumor activities of hPSC-derived NK cells without additional engineering approaches that confer antigen-specific activity [Woll et al., 2005, 2009; Larbi et al., 2012; Knorr et al., 2013; Hermanson et al., 2016; Zeng et al., 2017; Matsubara et al., 2019; Zhu et al., 2020a, b; Cichocki et al., 2020; Goldenson et al., 2020; Euchner et al., 2021; Woan et al., 2021; Du et al., 2022; Jung et al., 2022]. A few strategies were developed to enhance in vivo persistence and antitumor activities of hPSC-derived NK cells. Elimination of cytokine-inducible SH2-containing protein (CIS, encoded by *CISH*), a negative regulator of IL-15 signaling in NK cells, improved in vivo persistence and cytotoxic efficacy of hPSC-derived NK cells as IL-15 stimulates NK cell functions such as differentiation, proliferation, activation, and survival [Zhu et al., 2020a]. Another study from the same group reported that hnCD16 expression on iPSC-derived NK cells enhanced ADCC against tumor cells [Zhu et al., 2020b]. Woan et al. [2021] combined these concepts to produce triple-gene-edited iPSC-derived NK cells, termed iADAPT NK cells, for enhanced immunotherapy. hnCD16 and membrane-bound IL-15/IL-15R fusion protein (IL-15RF) were inserted into the *CD38* locus to knock out CD38 using CRISPR/Cas9. Not only did overexpression of hnCD16 and IL-15RF augment ADCC and persistence as reported in the previous studies but CD38 knock-out also provided additional protection of iPSC-derived NK cells against oxidative stress. These engineering features were used to produce FT538 and FT576, iPSC-derived NK cell products, which are currently under multiple clinical trials [Bjordahl et al., 2019a, b].

CAR modification for hPSC-derived NK cells was reported in a limited number of preclinical studies compared to the studies without CAR engineering. Based on a previously developed NK cell differentiation method [Knorr et al., 2013], an anti-CD19 or an anti-mesothelin CAR was introduced into hPSC-derived NK cells [Hermanson et al., 2013]. Even with the CD8α hinge region, CD28 costimulatory/transmembrane domain, 4-1BB costimulatory domain, and CD3ζ activating domain, which are typically employed for CAR T cells, hPSC-derived NK cells engineered with these CAR constructs still exhibited antitumor activities against targeted cancer cells in vitro. Li et al. [2018] optimized the CAR design in the context of NK cells by exploiting the mechanism triggering cytolytic programs in NK cells to enhance antitumor activities of hPSC-derived NK cells. The transmembrane domain of NKG2D, a NK cell-activating receptor, costimulatory domain of 2B4, a mediator of cytotoxicity in NK cells, and a CD3ζ signaling domain improved the efficacy of CAR NK cells in tumor killing. Anti-GPC3 [Ueda et al., 2020] and anti-EpCAM [Tang et al., 2021] CAR NK cells derived from human iPSCs also exhibited specific cytotoxicity against targeted tumor cells.

Table 2. hPSC-derived immune cells under clinical trials

Cell type/additional drugs	hPSC type	Target cancer/CAR (if applicable)	Features	Region/primary sponsor	Main ID/phase	Date of registration
NK T cell	hiPSCs	Head and neck cancer	–	Japan/Iinuma Tomohisa	JPRN-jRCT2033200116	Sep 11, 2020
NK cell	hiPSCs	Advanced metastatic breast cancer	–	Iran/International Center for Personalized Medicine (ICPM)	IRCT20200429047241N1	Aug 10, 2020
Innate lymphoid/NK cell	hiPSCs	Advanced ovarian clear cell carcinoma	GPC CAR	Japan/Harano Kenichi	JPRN-jRCT2033200431	Mar 18, 2021
NK cell (FT500)/immune checkpoint inhibitors, IL-2	hiPSCs	Advanced solid tumors	–	USA/Fate Therapeutics	NCT03841110 (FT500-101) NCT04106167 (FT003)	Sep 16, 2019
NK cell (FT516)/enoblituzumab (anti-B7-H3 mAb), IL-2	hiPSCs	Ovarian cancer	hnCD16 expression	USA/Masonic Cancer Center, University of Minnesota	NCT04630769	Nov 11, 2020
NK cell (FT516)/anti-CD20 mAbs	hiPSCs	Acute myeloid leukemia, B-cell lymphoma	hnCD16 expression	USA/Fate Therapeutics	NCT04023071 (FT516-101)	Jul 9, 2019
NK cell (FT516)/mAbs	hiPSCs	Advanced solid tumors	hnCD16 expression	USA/Fate Therapeutics	NCT04551885 (FT516-102)	Sep 9, 2020
NK cell (FT538)/daratumumab (anti-CD38 mAb)	hiPSCs	Acute myeloid leukemia	hnCD16 expression IL-15RF expression CD38 knock-out	USA/Masonic Cancer Center, University of Minnesota	NCT04714372	Jan 14, 2021
NK cell (FT538)	hiPSCs	Acute myeloid leukemia	hnCD16 expression IL-15RF expression CD38 knock-out	USA/Fate Therapeutics	NCT04614636 (FT538-101)	Oct 29, 2020
NK cell (FT538)/mAbs	hiPSCs	Advanced solid tumors	hnCD16 expression IL-15RF expression CD38 knock-out	USA/Fate Therapeutics	NCT05069935 (FT538-102)	Sep 10, 2021
NK cell (FT576)/daratumumab (anti-CD38 mAb)	hiPSCs	Multiple myeloma	BCMA CAR hnCD16 expression IL-15RF expression CD38 knock-out	USA/Fate Therapeutics	NCT05182073 (FT576-101)	Oct 15, 2021
NK cell (FT596)/rituximab (anti-CD20 mAb)	hiPSCs	Relapse prevention after autologous hematopoietic stem cell transplantation for non-Hodgkin lymphoma	CD19 CAR hnCD16 expression IL-15RF expression	USA/Masonic Cancer Center, University of Minnesota	NCT04555811	Sep 14, 2020
NK cell (FT596)/rituximab and obinutuzumab (anti-CD20 mAbs)	hiPSCs	B-cell lymphoma or chronic lymphocytic leukemia	CD19 CAR hnCD16 expression IL-15RF expression	USA/Fate Therapeutics	NCT04245722 (FT596-101)	Jan 18, 2020
T cell (FT819)/IL-2	hiPSCs	B-cell lymphoma, chronic lymphocytic leukemia, and precursor B-cell acute lymphoblastic leukemia	CD19 1XX CAR TCR knock-out	USA/Fate Therapeutics	NCT04629729 (FT819-101)	Nov 10, 2020
T cells	hiPSCs	Breast cancer, gastrointestinal cancers, lung cancer, melanoma, pancreatic cancer	Antigen-specific T cells	USA/National Cancer Institute (NCI)	NCT03407040	Jan 20, 2018

mAb, monoclonal antibody.

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More than 10 clinical trials are ongoing for hPSC-derived NK cells with or without CAR engineering (Table 2). The majority of them utilize combinatorial approaches with additional drug treatment such as mAbs and IL-2. Given previously developed strategies for immunotherapy in the context of immortalized NK cell lines, PB/cord blood-derived NK cells, CAR T cells [Gong et al., 2021], and possible combinations of these approaches, more studies are expected to be conducted to enhance the efficacy and safety of hPSC NK cell-based cancer immunotherapy.

Macrophage, Neutrophils, and Other Innate Immune Cells

Macrophages are phagocytic cells present in tissues and blood. Due to their ability to infiltrate into solid tumors and abundance in TME as well as their innate capability to phagocytose pathogens, macrophages are becoming increasingly investigated for their roles in treating solid cancers [Pan et al., 2022]. A clinical trial for an autologous CAR macrophage therapy, CT-0508, was recently initiated [Reiss et al., 2022]. The use of allogeneic macrophages, either derived from cell lines or hPSCs, has also been evaluated as an approach to develop off-the-shelf cancer immunotherapy. Similar to NK cells, the innate phagocytic activity of macrophages was exploited to target cancer cells using hPSC-derived macrophages without additional genome engineering [Cao et al., 2019]. Miyashita et al. [2016] engineered iPSC-derived myeloid cells to express type 1 interferons (IFNs) to target human melanoma cells. These iPSC-derived myeloid cells expressed macrophage markers, and the expression of type 1 IFNs promoted pro-inflammatory M1 polarization and antitumor activities against SK-MEL28 melanoma cells both in vitro and in vivo. Interestingly, introduction of antigen-specific scFv was used to confer antitumor specificity to hPSC-derived macrophages. Anti-CD20 scFv was linked to a C-terminal fragment of mouse FcγRI and introduced into iPSCs [Senju et al., 2011]. The macrophages derived from these genetically modified iPSCs exhibited antitumor activities in vitro and in vivo. In another study, anti-HER2 scFv was introduced to generate iPSC-derived macrophages targeting gastric and pancreatic cancers [Koba et al., 2013]. Incorporation of a macrophage-specific CAR construct into macrophage cell lines was reported to generate antitumor macrophages. Intracellular domains such as MEGF10 (human) [Morrissey et al., 2018], mouse CD147 [Zhang et al., 2019], and MerTK (mouse) [Niu et al., 2021] were tested for their functions in mediating cytotoxicity of macrophages. Other studies demonstrated that CD3ζ is still effective in mediating antitumor activity of human macrophages, possibly due to its similarity to the Fc common γ-chain, FcεRI-γ, a signaling mediator for antibody-dependent phagocytosis in macrophages [Klichinsky et al., 2020; Chen et al., 2022]. Zhang et al. [2020] reported generation of hPSC-derived CAR macrophages using CD86 and FcγRI as an intracellular part of CAR targeting CD19 or mesothelin. These cells showed antigen-specific cytotoxicity against cancer cells in vitro and in vivo. Interestingly, an increase in M1 pro-inflammatory cytokine expression was observed after they were incubated with tumor cells, possibly suggesting that antigen binding to CAR induces pro-inflammatory activity in hPSC-derived CAR macrophages.

Neutrophils are phagocytes and the most abundant white blood cells. Their cytolytic function was shown by neutrophils engineered with CD4ζ chimeric immune receptor against HIV envelop-transfected cells in vitro, but the lysis efficiency was minimal [Roberts et al., 1998]. While they possess cytotoxicity, antitumor, and protumor function and play a role in other cancer therapies [Stockmeyer et al., 2000; Guettinger et al., 2010; Nakagawa et al., 2010; Zilio and Serafini, 2016], autologous or allogeneic neutrophils have not been proposed as effector cells for cancer immunotherapy possibly due to their short circulating half-life (6–8 h) [Summers et al., 2010]. Given their similar innate antitumor response and tumor-infiltrating ability to macrophages, neutrophils are expected to be effector cells in cancer immunotherapy [Zilio and Serafini, 2016]. A recent study from our laboratory showed chemically defined, feeder-free generation of neutrophils from hPSCs along with additional CAR engineering to target glioblastoma cells by hPSC-derived CAR neutrophils [Chang et al., 2022b]. CD3ζ signaling domain and CD4 transmembrane domain were able to mediate cytotoxic activity of CAR neutrophils both in vitro and in vivo, and this tumor lysis involved phagocytosis, reactive oxygen species generation, and neutrophil extracellular trap formation. As primary neutrophils are short-lived, hPSC-derived neutrophils might serve as a safer allogeneic cell source for cancer immunotherapy.

Innate immune cells react to microbes and injured cells by causing inflammation and directly killing pathogens. This property has been utilized for hPSC-derived innate immune cells such as NK cells to target tumors without engineering of antigen-specific elements. Mucosal-associated invariant T (MAIT) cells are an abundant subset of T cells in humans and play an important role in antimicrobial functions [Le Bourhis et al., 2011]. Differ-

entiation of hPSCs into MAIT cells, mostly by redifferentiation of reprogrammed MAIT cell-derived iPSCs, has been reported [Wakao et al., 2013; Sugimoto et al., 2016; Saito et al., 2017]. Recently, tumor inhibitory activity of mouse iPSC-derived MAIT cells was evaluated against $MR1^+$ cancer cells [Sugimoto et al., 2022]. The authors not only showed the activated MAIT cells produced T helper (Th)1, Th2, and Th17 cytokines and inflammatory cytokines but also inhibited tumor growth via NK cell-mediated reinforcement of cytotoxicity. It indicates the indirect and collaborative antitumor activity of MAIT cells as an innate immune cell type. $V\alpha24^+$ invariant natural killer T (iNKT) cells, a subtype of T cells that express a single invariant TCRα chain (Vα24-Jα18) and limited TCRβ chains (Vβ11), exhibit antitumor immunity by activating other effector cells and directly mediating NK cell-like cytotoxicity [Wingender et al., 2010; Fujii et al., 2013]. Like macrophages and neutrophils, iNKT cells are tumor-infiltrating innate immune cells [Lança and Silva-Santos, 2012]. These characteristics of iNKT cells have been exploited in several clinical trials for cancer immunotherapy [Motohashi et al., 2006; Yamasaki et al., 2011; Richter et al., 2013; Cheng et al., 2022]. Human iPSC-derived iNKT cells also displayed antitumor effects via their adjuvant and direct cytotoxic activity [Kitayama et al., 2016; Yamada et al., 2016]. Similar to MAIT cells, these iNKT cells were differentiated from iNKT cell-derived iPSCs. This reprogramming-redifferentiation approach along with unique invariant TCR chains found in MAIT and iNKT cells suggests the importance of TCR rearrangement during T cell subset differentiation [Yamada et al., 2016]. Eosinophils are granulocytes that play a role in fighting against parasites and inducing allergies. Like other innate immune cells such as macrophages and neutrophils, eosinophils display antitumor or protumor effects by either direct cytotoxic function or indirect regulatory function toward other immune cells [Simon et al., 2019]. Lai et al. [2021] demonstrated that hPSC-derived eosinophils had cytotoxicity against various solid tumors both in vitro and in vivo. Importantly, these cells exhibited synergistic antitumor activities when they were combined with CAR T cells, possibly suggesting their adjuvant function in immunotherapy.

As described above, the role of innate immune cells within TME is largely biphasic. However, these antitumor and protumor phenotypes can be directed by cell-extrinsic factors or feasibly by other engineering approaches. For example, macrophages can be "educated" to have the pro-inflammatory M1 or anti-inflammatory M2 under the presence of IFN-γ or TGF-β, respectively, and this M1/M2 polarization is reversible [Lyadova and Vasiliev, 2022]. More studies are needed to be conducted to reveal the mechanisms of innate immune cell polarization and develop strategies to sustain their antitumor phenotype within TME. It is encouraging that CAR engineering can be applied to prime hPSC-derived macrophages and neutrophils for their directed cytotoxicity toward target tumors [Zhang et al., 2020; Chang et al., 2022b], which opens the possibility for targeted cancer immunotherapy using other innate immune cells. Another aspect of innate immune cell-mediated cancer treatment is to utilize their adjuvant activity such as secretion of inflammatory cytokines to activate other effector immune cells [Yamada et al., 2016; Cichocki et al., 2020; Lai et al., 2021]. Particularly, the use of hPSCs will allow generation of various immune cell types with the same genomic profiles, enabling researchers to study interactions between different immune cell types in an isogenic manner during cancer immunotherapy. This will lead to the development of strategies that further enhance cancer immunotherapy via combinatorial approaches using a mixture of different immune cell types.

Biomanufacturing of hPSC-Derived Immune Cells for Cancer Immunotherapy

Large-scale production of hPSC-derived immune cells under current good manufacturing practices is one of the most important elements to achieve bona fide off-the-shelf cellular products for allogeneic cancer immunotherapies. Given the self-renewing and differentiation capabilities of hPSCs and the requirement of scalable production of hPSC-derived cells for their applications in drug development and cell therapy, numerous studies have been conducted for hPSC manufacturing (previously reviewed elsewhere [Adil and Schaffer, 2017; Vassilev and Oh, 2021; Tannenbaum and Reubinoff, 2022]). Such advances include development of chemically defined, xeno-free substrate and medium that support hPSC self-renewal as well as the use of 3D culture systems to expand hPSCs [Adil and Schaffer, 2017]. However, the expansion of hPSCs and subsequent differentiation require an increase in differentiation factors such as growth factors and cytokines, which may lead to a decrease in the robustness of differentiation, since most of the protocols were developed based on laboratory-scale experiments and may not be well translated into scaled-up platforms. Thus, the expansion of undifferentiated hPSCs may not be appropriate to achieve cost-effective and robust manufac-

turing of hPSC-derived cell products. Instead, the expansion of progenitor cells or fully differentiated cells with robust differentiation protocols may serve as a more practical approach. Whereas limited studies have been conducted for biomanufacturing hPSC-derived immune cells, some of the manufacturing strategies developed for primary autologous and allogeneic immune cells are expected to be translated into the context of hPSCs. In this section, we will review not only clinical-scale derivation of immune cells from hPSCs but also representative methods for the expansion of autologous and allogeneic immune cells. Other aspects of biomanufacturing such as quality control and manufacturing devices (bioreactors) were extensively reviewed elsewhere with a focus on lymphocytes [Koehl et al., 2016; Vormittag et al., 2018; Garcia-Aponte et al., 2021].

Expansion of T Cells

While the expansion of primary T cells has been actively studied due to its application in CAR T cell therapy, most of the studies about differentiation of hPSC-derived T cells focused on their functional activity rather than the manufacturing aspect. Recently, Iriguchi et al. [2021a] developed a clinically applicable and scalable differentiation method to generate iPSC-derived T cells. The authors showed that immobilized DLL4 protein, lymphopoietic cytokines, and supplements induce robust T cell differentiation from iPSC-derived hematopoietic progenitor cells which are generated by embryoid body-based differentiation. While this feeder-free differentiation platform is a promising advance in the generation of T cells, expansion of fully differentiated hPSC-derived T cells may be a more practical alternative as numerous studies have already been conducted to expand primary T cells. Indeed, a modified rapid expansion method (REM) [Wang et al., 2011], which was originally developed for primary T cells, was applied to expand iPSC-derived T cells differentiated by 3D-organoid culture. It achieved about 75-fold expansion in 2 weeks during REM expansion and yielded clinically relevant cell numbers (600 million CAR T cells per 1 million iPSCs) [Wang et al., 2022].

Cytokines are cell signaling proteins secreted by immune cells that modulate physiological function of other cells. IL-2 is commonly used as a cytokine to promote T cell proliferation [Liao et al., 2013]. Activation of IL-2 signaling induces initiation of three main intracellular signaling pathways: phosphoinositide 3-kinase, mitogen-activated protein kinase, and signal transducer and activator of transcription 5 pathways which are involved in cell cycle entry, growth, survival, and differentiation of T cells [Cheng et al., 2011]. Other cytokines such as IL-6, IL-7, IL-15, and IL-21 have been utilized to expand cytotoxic T cells [Zeng et al., 2005; Xu et al., 2014; Zhou et al., 2019; Jiang et al., 2021]. It is important to note that these cytokines can also induce differentiation and subtype specification of T cells which possibly affect the efficacy of immunotherapy; thus, uncoupling of expansion and differentiation is necessary when using these cytokines [Crompton et al., 2014]. In the case of T cell differentiation from hPSCs, appropriate time points for the treatment of these cytokines should be chosen to generate T cells with desired functions and proper expansion. Even though cytokine activation is typically achieved by simply adding cytokines to the culture medium, engineering approaches can be used to exploit the cytokine signaling for expansion of T cells. For example, Zhang et al. [2021b] engineered T cells with a human orthogonal IL-2 receptor along with CD19 CAR and observed a 1,000-fold increase in in vivo expansion of CAR T cells upon administration of human orthogonal IL-2. Recombinant human IL-7 fused with a hybrid constant fragment (Fc) for a prolonged in vivo serum half-life promoted in vivo proliferation, persistence, and cytotoxicity of human CAR T cells [Kim et al., 2022]. Overexpression of IL-7 by introducing IL-7 transgene into the CAR backbone also increased proliferation of $CD8^+$ T cells as well as increased cytotoxicity against targeted cancer cells [He et al., 2020].

TCR signaling is a critical element in modulating T cell function. Upon binding to an antigen, TCR and costimulatory molecules are activated to induce differentiation, cytokine production, proliferation, and cell-mediated cytotoxicity [Courtney et al., 2018]. One of the well-known mechanisms of TCR signaling in promoting expansion of T cells is via secretion of IL-2 [Kemp et al., 2007], but IL-2-independent proliferation mechanisms were also reported [Appleman et al., 2000; Colombetti et al., 2006]. To induce activation and subsequent expansion of T cells via TCR signaling, mAbs bind to TCR co-receptors or costimulatory molecules have been used. Anti-CD3 and anti-CD28 antibodies either immobilized to a substrate such as beads or as a soluble form were shown to promote expansion of antigen-specific T cells [Riddell and Greenberg, 1990; Hollyman et al., 2009; Keskar et al., 2020]. The use of an OX40 agonist and a CD83 ligand also induced T cell expansion via TCR signaling [Hirano et al., 2006; Ruby et al., 2009]. Another commonly employed T cell expansion method based on TCR activation is to recapitulate the activity of antigen-presenting cells via artificial antigen-presenting cells (aAPCs). aAPCs are gener-

ated by introducing receptors for antibody binding and/or stimulatory ligands into feeder cells along with a subsequent coating of feeder cells with antibodies targeting TCR signaling molecules. For example, Maus et al. [2002] engineered K562 erythromyeloid cell line to express the low-affinity Fcγ receptor, CD32, and 4-1BB ligand for binding of anti-CD3 and anti-CD28 antibodies to CD32 and for activation of 4-1BB costimulatory molecule by 4-1BB ligand to promote expansion of cytotoxic T cells. Incorporation of CD64, a high-affinity Fcγ receptor, for antibody coating, and CD80/CD86, ligands for CD28 activation, into K562 was also shown to induce T cell expansion [Suhoski et al., 2007; Gong et al., 2008; Ye et al., 2011]. As described above, this TCR signaling is exploited to develop CAR T cells that mediate antigen-specific cytotoxicity. Similarly, CAR signaling is employed to promote proliferation of T cells upon antigen binding. Addition of costimulatory domains and other engineering approaches to modify CAR construct for T cell expansion have been widely studied [Ghorashian et al., 2019; Guedan et al., 2019]. Activation of CAR signaling can also be achieved by an additional antigen presentation to the cells [Reinhard et al., 2020; Ukrainskaya et al., 2021]. For example, Ukrainskaya et al. [2021] developed antigenic vesicles displaying recombinant antigens to CAR T cells and demonstrated that CAR T cells incubated with these vesicles resulted in proliferation and an increase in cell population. It should be noted that certain engineering strategies for hPSCs alter TCR signaling and expression; thus, precautions are needed when exploiting TCR signaling to induce proliferation in the context of engineered hPSCs.

A recent study reported the use of oncolytic virus-loaded CAR T cells led to an increased in vivo expansion and reactivation of these cells [Evgin et al., 2022]. Development of expansion medium [Medvec et al., 2018] and the use of biomaterials (extensively reviewed elsewhere [Isser et al., 2021]) are also reported to enhance expansion of T cells for cancer immunotherapy. These strategies are typically combined together to further support T cell expansion [Marchingo et al., 2014; Wölfl and Greenberg, 2014]. For example, the REM, one of the T cell expansion standards, involves the use of anti-CD3 antibody and IL-2 in the presence of irradiated allogeneic feeder cells [Dudley et al., 2003; Baudequin et al., 2021].

Expansion of NK Cells

Similar to T cells, most of the studies on hPSC-derived NK cells for cancer immunotherapy emphasized their cytotoxic activity and adopted existing expansion methods developed for primary NK cells. The most common expansion method employed for hPSC-derived NK cells is the use of membrane-bound IL-21-expressing aAPC (K562-mbIL-21-4-1BB) [Knorr et al., 2013; Hermanson et al., 2016; Zhu and Kaufman, 2019; Zhu et al., 2020a, b; Cichocki et al., 2020; Goldenson et al., 2020; Woan et al., 2021]. When primary NK cells were co-cultured with K562-mbIL-21-4-1BB and low concentration of IL-2 for 3 weeks, a 47,967-fold expansion was achieved [Denman et al., 2012]. aAPCs were also engineered with membrane-bound IL-15 for NK cell expansion [Fujisaki et al., 2009; Gong et al., 2010]. Other feeder cells such as irradiated human feeder cell line (HFWT) [Ishikawa et al., 2004], NK^- fraction of PBMCs [Kim et al., 2013], Epstein-Barr virus-transformed lymphoblastoid cell line [Granzin et al., 2016], irradiated tumor cells (Jurkat) [Lim et al., 2013], and irradiated PBMCs [Min et al., 2018] were employed to promote NK cell proliferation. The REM standard that was originally developed for T cells by exploiting irradiated PBMCs, IL-2, and anti-CD3 antibody also worked for NK cell expansion [Min et al., 2018]. This similarity applies to the use of cytokines for NK cell expansion. Like T cells, IL-2 is the most common cytokine to induce the expansion of NK cells. Other cytokines including IL-15, IL-18, IL-21, and IL-27 were also used for NK cell expansion [Koehl et al., 2013; Granzin et al., 2016; Choi et al., 2019b; Heinze et al., 2019; Tanaka et al., 2019].

Larbi et al. [2012] proposed the use of HOXB4 homeoprotein, an important regulator of hematopoietic stem cell expansion, to enrich hPSC-derived NK cells. HOX4B protein was actively secreted by engineered MS5 mouse stromal cells and acted on differentiating progenitor cells rather than on terminally differentiated NK cells. This result suggests alternative approaches for expansion of hPSC-derived immune cells where the expansion of progenitors is targeted during differentiation. A recently patented method for hPSC-derived NK cell expansion utilizes a 3D bioreactor platform which allows generation of $\sim 10^{10}$ pure NK cells with a 300 mL bioreactor [Feng et al., 2021; Lu and Feng, 2021].

Expansion of Macrophages and Other Immune Cells

Very limited studies have been done for exploring the expansion methods of other immune cells derived from hPSCs. Available information for the proliferation of these cell types is mainly obtained from the studies on their physiological states rather than induced expansion for therapeutic purposes. For example, G-CSF and IL-4 have been identified as cytokines promoting proliferation of neutrophils and macrophages, respectively [Von Vietinghoff and Ley, 2008; Rückerl and Allen, 2014].

Ackermann et al. [2018, 2022] reported massive production of iPSC-derived macrophages in a bioreactor. First, the authors evaluated the feasibility of massive production in a small-scale suspension culture on an orbital shaker. They found embryoid body-derived myeloid cell forming complexes continuously produced macrophages up to 3 months with weekly harvesting. When they translated this differentiation method into stirred tank bioreactor, production of ~1–3 × 10^7 macrophages per week was observed as early as in week 3 and this production rate was maintained for more than 5 weeks. The resulting iPSC-derived macrophages rescued mice from *Pseudomonas aeruginosa*-mediated acute infections of the lower respiratory tract upon transplantation. This is an interesting result as a translation from a small-scale differentiation to a bioreactor-scale differentiation was achieved without a significant modification in the differentiation protocol as well as a significant impact on outcomes.

Conclusion

The impact of CAR T cell therapy is revolutionizing the field of cancer therapy. Successful clinical cases in the treatment of relapsed and refractory leukemia and lymphomas have encouraged researchers to apply the CAR platform to other malignancies. Over 1,000 CAR-related clinical trials are ongoing, employing not only CAR T cells but also other immune cells engineered with CARs [Pan et al., 2022; Zhou et al., 2022]. Furthermore, CAR-based therapy is expanding its realm of applications into other diseases such as infection, autoimmunity, and inflammation [Maldini et al., 2018; Aghajanian et al., 2019; Rurik et al., 2022]. Even with this excitement, challenges remain for CAR therapy, including the poor efficacy against solid tumors, high cost, and manufacturing problems. Development of off-the-shelf CAR-engineered immune cells is gaining attention to address the existing problems of CAR therapy, and hPSCs have emerged as a promising cell source to achieve this. Advances in engineering strategies, such as generation of hypoimmunogenic hPSCs, have provided more opportunities for hPSC-derived immune cells to be used as therapeutic agents. Unlike primary immune cells, the use of hPSCs can generate a wider variety of engineered immune cell types with the same genomic profile, which may be combined to induce synergistic antitumor effects. For example, whereas primary neutrophils are short-lived and generally resistant to genome editing, hPSC-derived neutrophils can be genetically engineered to target specific tumor cells, being another option as tumor-infiltrating immune cells [Chang et al., 2022b]. Additionally, many engineering strategies to enhance efficacy and safety of immunotherapy have not yet been assessed for hPSC-derived immune cells. This means potential improvement in efficacy and safety of hPSC-derived immune cells may be readily achieved with previously developed approaches. Cell-based therapies typically require a large number of cells (10^8–10^{10}) per patient [Garcia-Aponte et al., 2021], and these numbers are not easily achievable using laboratory-scale differentiation and expansion. For bona fide generation of off-the-shelf cellular products, translation of laboratory-scale studies into bioreactor-based expansion is needed. Some of the expansion methods of immune cells still involve the use of feeder cells or a ligand-presenting substrate, and this substrate-mediated signaling may not work well in a 3D suspension bioreactor. Genome engineering techniques and improved understanding of the extrinsic factors that activate expansion-signaling pathways may permit feeder-free methods more suitable in a bioreactor. Another growing field of immunotherapy is to use extracellular vesicles (EVs), such as exosomes, harvested from immune cells. NK EVs were shown to induce antitumor effects both in vitro and in vivo [Lugini et al., 2012; Zhu et al., 2017], and hPSC-derived immune cells can be used to produce therapeutic EVs. Collectively, hPSC-derived immune cells will provide more opportunities to realize off-the-shelf cellular products which will transform the whole field of cancer immunotherapy from an individualized and costly treatment to a universal and affordable manner.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

X. Bao designed and established the conception of the work. G. Jin did research and drafted the manuscript with interpretation and discussion points. G. Jin, X. Bao, J. Harris, and Y. Chang wrote and revised the manuscript.

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