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Adoptive Cell Therapy from the Dish: Potentiating Induced Pluripotent Stem Cells

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Abstract
Background: The clinical success of autologous adoptive cell therapy (ACT) is substantial but wide application is challenged by the quality and quantity of the patient's immune cells and the need for personalized manufacturing processes. Induced pluripotent stem cells (iPSCs) can be differentiated into immune effectors and thus provide an alternative, allogeneic cell source for ACT. Here, we compare iPSC-derived immune effectors to their PBMC-derived counterparts and review iPSC-derived ACT products currently under preclinical and clinical development. ***Summary:*** iPSC-derived T cells, NK cells, macrophages, and neutrophils largely mimic their PBMC-derived counterparts in terms of cell-surface marker expression and cytotoxic effector functions. iPSC-derived immune effectors can be engineered with chimeric antigen receptors and other activating receptors to redirect their cytotoxic potential specifically to tumor-associated antigens (TAAs). However, several differences between iPSC- and PBMC-derived immune effectors remain and have inspired additional engineering strategies to enhance the antitumor capacity of iPSC-derived immune effectors. ***Key Messages:*** iPSCs can be engineered to facilitate the generation of immune effectors with homogenous specificity for TAAs and enhanced effector functions. TAA-specific and functionally enhanced iPSC-derived T and NK cells are currently undergoing clinical evaluation in phase 1 trials. Engineered iPSC-derived macrophages and neutrophils are in preclinical development.

Introduction

Adoptive cell therapy (ACT) has provided a treatment option with curative potential for patients with relapsed or refractory hematological malignancies [1]. Lymphocytes are the main cell-type used for ACT, especially αβT and NK cells. T cells can be targeted against tumor-associated antigens (TAA) utilizing their inherent T cell receptor (TCR) specificity via expansion and re-infusion of tumor infiltrating lymphocytes (TILs), or the selection and expansion of antigen-specific T cell clones. Alternatively, they can be redirected to a TAA through the expression of a chimeric antigen receptor (CAR) or TCR [2, 3]. NK cell antitumor potential can be triggered through mismatch of their inhibitory receptors with the tumor HLA [4], antibody-dependent cellular cytotoxicity (ADCC) or CAR expression [5]. More recently, the potential of re-targeting myeloid cells through CAR expression is being explored, and the first CAR-Macrophage clinical trial is currently ongoing [6, 7].

Despite the huge potential of ACT, the autologous nature provides a challenge for the broader application of these treatment strategies. Patient eligibility criteria, the impact of the disease and previous lines of therapy

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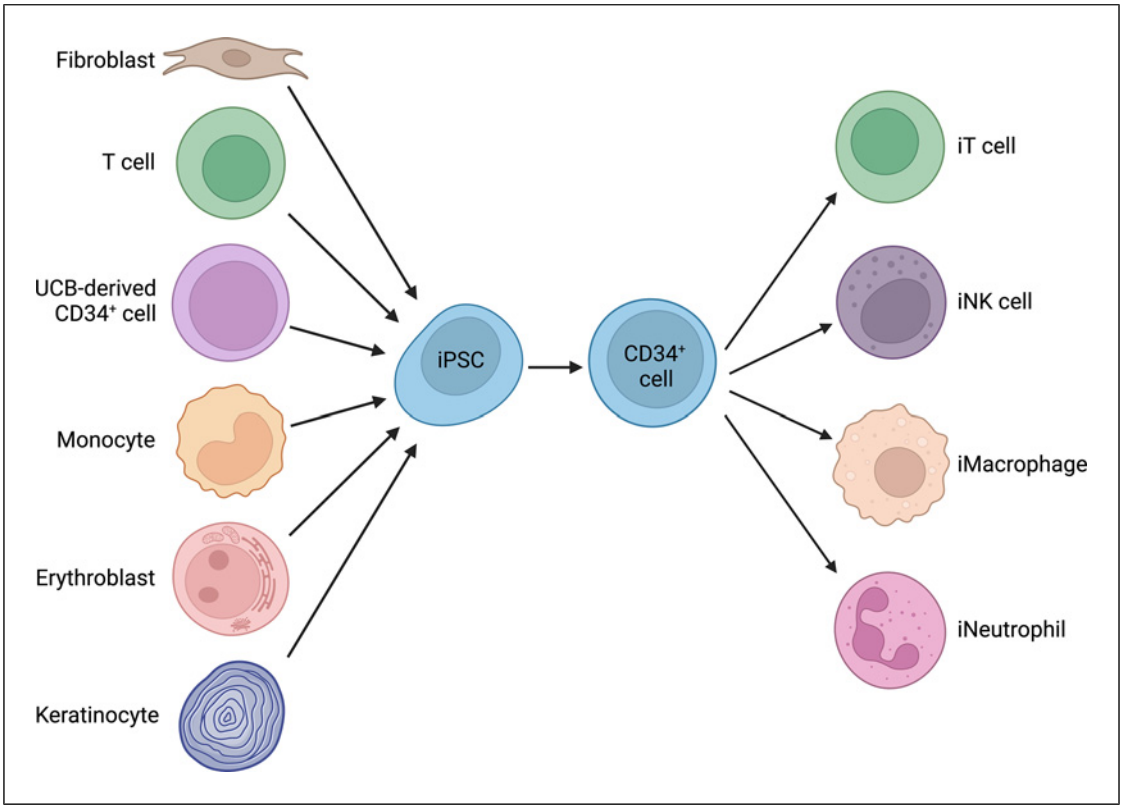

Fig. 1. Overview of cell types reprogrammed into iPSCs and re-differentiated into immune effectors.

on the quality of the patient's immune cells, the availability, duration and demands of the manual manufacturing in specialized facilities, the need for bridging therapy during manufacturing time and the high costs associated with ACT are all contributing to this challenge [8–11]. To overcome these hurdles, the development of allogeneic, "off-the-shelf" versions of ACT is highly sought after. To achieve this, two major allogeneic resources are currently being pursued, namely the use of healthy donor-derived lymphocytes, and the differentiation of lymphocytes in vitro from induced pluripotent stem cells (iPSCs).

iPSCs can be derived from a variety of cell sources through the expression of four transcription factors, cMyc, Oct4, Sox2, and Klf4 [12]. The unlimited self-renewal capacity of iPSCs allows a single established iPSC line to support repeated production of different immune effectors, without the requirement of continuous donations from healthy subjects. Additionally (multiplexed) gene-engineering can be performed at the iPSC level. Several engineering strategies have effectively been applied in iPSCs, including lentiviral transduction [13], PiggyBack Transposon and CRISPR/Cas9 technologies [14]. Genetic engineering is followed by clonal selection, expansion and banking [15]. The iPSC banks can be used to differentiate the iPSCs into the desired cell type, resulting in a homogenous population with the genetic modifications of choice, and enabling genetic engineering strategies for cell types that are more challenging to genetically engineer and expand at their mature stage, such as macrophages or neutrophils. Multiple immune effectors have been generated from iPSCs for the use of allogeneic ACT, including induced T cells (iT cells), NK cells (iNK cells), macrophages (iMacs), and neutrophils (iNeuts) (Fig. 1). These promising preclinical developments have already triggered phase 1 clinical trials. iT cells engineered to target CD19 and HER2 through a CAR are being tested in patients with, respectively, B cell malignancies and HER2$^+$ solid tumors [16, 17], whilst CAR-engineered iNK cells are currently in four trials for several hematological malignancies [18–21]. However, the realization of iPSC-derived immunotherapy also faces challenges. The differentiation protocols are lengthy, typically ranging from 3 to 6 weeks for lymphoid cells, and 2 to 4 weeks for myeloid cells [13, 22–27]. Additionally, in some cases the phenotype and effector functions of the in vitro generated cells are distinct from the peripheral blood-derived primary cells currently used in the clinic. Here we will discuss the in situ and in vitro differentiation of T cells and NK cells, the engineering strategies deployed to facilitate tumor targeting of in vitro generated cells, and their characteristics compared to their primary counterparts. Additionally, we will discuss the recent development of CAR-iMacs and -iNeuts.

iPSC-Derived T Cells

In situ T Cell Differentiation

During in situ T cell development, hematopoietic stem cell (HSC)-derived thymus seeding progenitors migrate from the bone marrow to the thymus where they undergo an intricate developmental trajectory toward T cell lineage commitment [28]. This development is driven by Notch stimulation and TCR chain rearrangement and subsequent signaling [29]. The thymic environment first induces thymus seeding progenitors to express T/NK cell marker CD7 and subsequently T cell marker CD5. The two main T cell subsets that develop from these thymic $CD7^+CD5^+$ intermediates are the αβT cells and γδT cells [30]. These subsets are defined by the TCR chains expressed on the cell surface. γδT cells express successfully rearranged genes encoding the TCRγ and TCRδ chains (*TRG* and *TRD*). The TCRγ and TCRδ chains heterodimerize and bind to soluble or membrane proteins, or CD1d on antigen-presenting cells, and γδT cells mature toward $CD4^-CD8^-$ double negative (DN) or $CD4^-CD8\alpha\alpha^+$ cells [31].

Differentiating αβT cells rearrange the genes encoding the TCRβ and TCRα chains (*TRB* and *TRA*) to form an αβTCR which binds peptides presented by HLA-I or -II complexes. Thymic cells committed to the αβT cell lineage become double positive (DP) for CD4 and CD8αβ. DP cells undergo positive and negative selection, in which their αβTCR is evaluated for successful rearrangement and a lack of response to self-peptide. Only DP T cells that formed an αβTCR able to bind self-HLA, but failing to respond to self-peptide are matured into $CD4^+$ or $CD8\alpha\beta^+$ single positive (SP) T cells. The matured $CD4^+$ and $CD8\alpha\beta^+$ SP T cells then migrate out of the thymus and into the peripheral blood and secondary lymphoid structures [32]. In the periphery, TCR engagement combined with costimulatory and cytokine signals activates the effector functions of matured T cells, including the production of cytokines that further support an immune response and cytolytic granules containing Perforin and Granzyme B [32, 33]. Consistent with their respective helper and cytolytic effector functions, $CD4^+$ T cells produce more IL-2 and TNFα, whilst $CD8\alpha\beta^+$ T cells can accumulate and secrete more Perforin and Granzyme B [33–36].

In most forms of autologous ACT for cancer, mature SP αβT cells are harvested from the patient's tumor or peripheral blood. Adoptive T cell therapy utilizes the antigen-specific nature to trigger the T cells' cytolytic capacity. In TIL-based therapy, T cells are isolated from a resected tumor, expanded ex vivo and then infused back into the patient in large numbers. TIL therapy relies on the existence of endogenously occurring T cell clones that express TAA-specific TCRs [3]. In other approaches, the specificity of peripheral blood-derived T cells is redirected through the transduction with a TCR or CAR targeting TAA [1, 2].

iPSC-To-T Cell Differentiation

Current in vitro T cell differentiation procedures have reported on the induction of $CD34^+$ intermediates in iPSCs derived from fibroblasts, T cells, monocytes, erythroblasts, and keratinocytes [13, 37–40] (Fig. 1). These procedures mimic a selection of the biochemical signals present in the thymus to instruct iPSC-derived $CD34^+$ cells to commit to the T cell lineage [41]. Mesoderm induction and hematopoietic specification resulting in $CD34^+$ cells can be facilitated through iPSC co-culture with OP9 [42] or feeder-free through embryoid bodies combined with Bone Morphogenetic Protein 4 stimulation [13]. T cell lineage commitment requires the engagement of Notch receptors on $CD34^+$ cells, which can be achieved through 2D or 3D co-cultures with feeder cells (OP9 or MS5) engineered to express Notch ligands, or feeder free cultures utilizing recombinant Notch ligands [13, 43–47]. Differentiation cultures are supplemented with the cytokines IL-7, SCF, and Flt3-L [41]. These conditions induce the expression of early T cell lineage markers CD7 and CD5 [13, 39, 48] and when the differentiations are started from fibroblast-derived iPSCs (FiPSCs), they also support the formation of the $CD4^+CD8\alpha\beta^+$ DP cells [40, 49].

However, in differentiations starting from T cell-derived iPSCs (TiPSCs), the successful generation of mature iT cells was challenged by a divergence toward a DN/$CD8\alpha\alpha^+$ phenotype, suggesting commitment to a more innate, γδT cell-like lineage, in lieu of passing through two hallmark steps of αβT cell development: the DP stage followed by positive/negative selection [13, 32, 50, 51]. Recent studies have shown that the relatively early TCR expression of differentiating TiPSCs and the strength of Notch signal induction are among the determining factors that can be optimized to enhance DP formation [49, 52]. Currently reported TiPSC-to-iT procedures result in efficient rates of DP formation, with relative frequencies ranging up to 90% [52–55].

In vitro differentiating iPSCs that are not derived from T cells, such as FiPSCs, undergo rearrangement of their *TRB* and *TRA* loci and thereby form a diverse TCR repertoire [37, 54]. Whilst rearrangement and the creation of a diverse TCR repertoire is akin to thymic T cell differentiation, most clinical applications require the formation of an iT cell pool with known specificity [56]. TiPSCs have been shown to typically maintain their original rearranged *TRB* and *TRA* genes through the processes of reprogramming and redifferentiation, and to give rise to pools of iT cells with the same antigen specificity as the T cell from which the respective TiPSCs were derived. Utilizing this conservation of specificity, groups have isolated T cell clones specific to TAA (e.g., WT1 or MART1 epitopes) and virus-derived epitopes, and used these to create TiPSC lines that can give rise to large pools of antigen-specific iT cells [22, 57–59].

Table 1. The expression of phenotype markers and antigen-induced effector molecules by iT cells compared to in situ differentiated T cells

Phenotype marker distribution		
CD7	=	[49, 61]
CD5	▼	[61]
TCRαβ	=	[61]
DN	▲	[13, 49, 51]
CD8αα	▲	[13, 49, 51]
CD8αβ	=	[13, 49, 51]
CD4	▼/absent	[13, 49, 51]
Functionality		
Perforin	=	[37, 50]
Granzyme B	=	[37, 49, 50]
IFNγ	=/▼	[39, 49–51]
TNFα	▼	[37, 39, 49]
IL-2	▼	[37, 39, 49]

However, in some studies it was found that differentiating TiPSCs can undergo a limited form of further *TRA* rearrangement, resulting in a loss of specificity in a minority of the generated iT cells [38, 57].

For the final stage of T cell development, positive/negative selection, in vitro procedures mimic positive selection by delivering a TCR-signal through anti-CD3 antibodies, phytohemagglutinin, or antigen-presenting cells, often combined with a costimulatory signal engaging CD28 or 4-1BB and always in the presence of one or more common γ-chain cytokines (such as IL-2, IL-7, IL-15 or IL-21) [13, 39, 49, 52, 53, 60]. The resulting cells maintain CD7 and partially CD5 expression, and are typically a heterogeneous mix of $CD4^{-}CD8\alpha\beta^{-}$ DN, $CD8\alpha\alpha^{+}$ SP cells, and $CD8\alpha\beta^{+}$ SP cells, notably lacking a profound $CD4^{+}$ SP population [46, 49, 52, 53, 61] (Table 1).

Several groups reported a high efficiency of $CD8\alpha\beta^{+}$ SP generation, with some protocols achieving frequencies over 80% [46, 53]. Similar to in situ differentiated $CD8\alpha\beta^{+}$ T cells derived from peripheral blood mononuclear cells (PBMCs), iT cells produce Perforin and Granzyme B and are able to lyse TCR-antigen expressing target cells [50, 57]. Furthermore, iT cells produce and secrete IFNγ in an antigen-dependent manner, with some studies reporting similar and others reporting lower levels compared to their PBMC-derived counterparts [49–51]. TCR-activated iT cells also produce TNFα and IL-2, but it remains to be determined how these levels compare to those produced by PBMC-derived $CD8\alpha\beta^{+}$ T cells [51]. However, studies of CAR-engineered iT cells (discussed further below) show that CAR-iT cells secrete less IL-2 and TNFα compared to PBMC-derived $CD8\alpha\beta^{+}$ CAR-T cells [39, 49] (Table 1).

Differences in the cytokine-secretion profile of iT cells compared to PBMC-derived T cell products are further enhanced by the absence of $CD4^{+}$ cells in almost all currently reported iPSC-derived products. Notably, one group recently reported the generation of $CD4^{+}$ iT cells by knocking out a not-yet disclosed gene in TiPSCs derived from an HLA-I restricted $CD8\alpha\beta^{+}$ T cell clone recognizing an Epstein-Barr Virus (EBV)-derived peptide. In in vitro tests, these cells retained their affinity for the HLA-I-presented EBV-peptide, and when exposed to the peptide produced less IFNγ and TNFα than $CD8\alpha\beta^{+}$ iT cells, and more IL-2, IL-4, and IL-10. The $CD4^{+}$ iT cells produced Perforin and Granzyme B and were able to lyse an EBV-associated Lymphoma cell line [62]. However, it remains to be elucidated if and how the CD8αβ-derived TCR expressed on these $CD4^{+}$ iT cells was able to mediate activation in the absence of a CD8αβ-cofactor, or if other activation mechanisms were in place.

Engineered iT Cells

TCR- and CAR-Engineered iT Cells

To redirect the specificity of iT cells, iPSCs have been engineered to express αβTCRs or CARs against TAA [13, 49, 58, 63]. Two groups have reported DP formation and subsequent $CD8\alpha\beta^{+}$ iT generation from iPSCs transduced with an αβTCR against a peptide derived from Wilms tumor 1 (WT1). WT1-TCR-iT cells showed target specific cytotoxicity and IFNγ production in vitro [58], and upon repeated dosing were able to significantly slow down the outgrowth of an intraperitoneally implanted lung cancer cell line in vivo [53].

Whilst the αβTCR dimer associates with CD3 subunits to form a signaling complex, CARs comprise their own intracellular signaling domains derived from CD3ζ alone (first generation), CD3ζ and one costimulation domain, generally CD28 or 4-1BB (second generation) or CD3ζ and two costimulation domains (third generation) [64]. Three studies have shown that the design of the intracellular signaling domains of the CAR affects the efficiency at which the iPSCs can reach the DP stage, with CAR designs that induce less tonic (i.e., antigen-independent) signaling allowing for the most DP formation [49, 52, 65]. Abating tonic signaling through timed expression of an Immunoreceptor Tyrosine-based Activation Motif (ITAM)-calibrated CAR from the *TCR Alpha Constant*-locus (TRAC-1XX), facilitated differentiating iPSCs to reach the DP and $CD8\alpha\beta^{+}$ SP stages, resulting in TRAC-1XX-iT cells that show antigen-specific cytotoxicity and proliferation in vitro. Compared to PBMC-derived TRAC-1XX $CD8\alpha\beta^{+}$ T cells, TRAC-1XX-iT cells secrete less IFNγ and TNFα, and no IL-2. TRAC-1XX-iT cells are able to induce long-term tumor control in an intravenous murine model for acute lymphoblastic leukemia [49]. Comparing the effects of first, second and third generation CARs transduced into hematopoietic stem and progenitor cells (HSPCs) revealed that 4-1BB-containing second and

third generation CARs allowed for the most efficient DP formation. Constitutive expression of a third generation, GPC3-targeting CAR (GC28BBz) in iPSCs resulted in iT cells exhibiting antigen-specific cytotoxicity in vitro but producing less IFNγ and TNFα than PBMC-derived CD8αβ$^+$ T cells equipped with the same CAR. GC28BBz-iT cells were able to slow down the outgrowth of an intraperitoneally implanted ovarian cancer cell line in vivo, but less so than their PBMC-derived counterparts. Diacylglycerol kinase (encoded by *DGK*) downregulates TCR and CAR signaling by phosphorylating diacylglycerol, which disables diacylglycerol to activate the MEK/ERK pathway. A knockout of *DGK* at the iPSC stage combined with the expression of an IL-15/IL-15 receptor fusion (IL15-RF) molecule in mature *DGK*$^{-/-}$ GC28BBz-iT cells enhanced their in vivo efficacy [52]. Although CAR-iT cells can induce remission in multiple in vivo models (both intraperitoneal and intravenous), they often require higher and/or repeated doses, as well as exogenous cytokine support to reach the same therapeutic effect as similarly engineered CAR-T cells derived from healthy-donor PBMCs [39, 49, 52, 53, 63].

Engineering Strategies to Avoid Alloreactivity

The clinical safety profile of iT cells will depend on their lack of graft-versus-host potential [56]. Because in vitro differentiated iT cell products will not have undergone intrathymic negative selection, they have the potential to contain TCRs directed against self-antigens of the recipient. The graft-versus-host potential of iT cells can be abated by the knockout of *Recombination Activating Gene 2* (*RAG2*) in TiPSCs derived from an antigen-specific T cell clone and in TCR-engineered iPSCs [38]. *RAG2* encodes one of the two proteins that form the RAG1-RAG2 recombinase complex initiating the double strand breaks between the V(D)J fragments and thus *TRB* and *TRA* rearrangement [66]. A CRISPR-mediated knockout of *RAG2* prevents additional *TRA* rearrangement in TiPSCs and the formation of a diverse αβTCR repertoire in TCR-engineered iPSC, and thus renders iT cells with homogenous specificity [38]. This finding was recently corroborated in an in vitro platform for T cell generation from TCR-engineered embryonic stem cell lines [67]. When iT cell specificity is redirected through a CAR, graft-versus-host disease can be prevented by the knockout of the *TRAC* locus, which results in homogeneously αβTCR$^-$ iT cells [49].

Engineered iT Cells in the Clinic

The first iPSC-derived CAR-T cell clinical trial is ongoing and tests TRAC-1XX-iT cells in patients suffering from B cell malignancies, including B cell Lymphoma and Chronic Lymphocytic Leukemia. This phase 1 trial has a dose-escalation design and tests both a single dose (starting at 90E6 cells per dose) and a fractioned dosing regimen (3 doses in 5 days, starting with 30E6 cells per dose). All patients undergo lympho-conditioning by a combination of cyclophosphamide, fludarabine, and bendamustine and in extra arms of the study the CAR-iT cells are combined with IL-2 administration [16, 68]. In an interim report on the first 15 treated patients, 1 patient achieved a partial response and 3 a complete response [68]. The same TRAC-1XX-iT cells are being tested in a second trial that enrolls patients with the B cell-mediated autoimmune disorder systemic lupus erythematosus [69, 70]. A third trial in patients with HER2-expressing solid tumors has been initiated recently and uses a similar lympho-conditioning regimen, as well as chemotherapeutic agents cisplatin and docetaxel. In addition to a HER2-targeting CAR and a *TRAC* knockout, the iT cells are equipped with an IL-7/IL-7 receptor fusion (IL7-RF) molecule, which is hypothesized to enhance persistence in lieu of robust IL-2 production and CD4 help [17, 71].

iPSC-Derived NK Cells

In situ NK Differentiation

Whilst in vitro differentiation of HSCs into NK cells was already achieved in the 1990s, the exact cellular localization and molecular orchestration of NK development in situ has remained only partially understood [72]. NK developmental intermediates (NKDI) have been found not only in the bone marrow, but also in peripheral blood and in numerous other tissues such as lymph nodes, tonsils, the thymus, the gastrointestinal tract, the liver, and the uterus, where they are thought to differentiate into tissue-resident NK populations [72, 73]. Although tissue-specific differences have been reported, there are common expression patterns associated with the differentiation of NK precursors through immature NKDI stages into mature NK cells. Generally, NK precursors lose the HSC-marker CD34 and induce and maintain CD7 expression upon their transition into the NKDI stages. The expression of IL-15 receptor subunit B (IL15RB) on NK precursors demarcates their final commitment to the NK lineage and is maintained throughout the differentiation into complete maturation. During the NKDI stages the cells will temporarily express relatively high levels of CD117 (c-KIT, SCF receptor), Natural Killer Group 2 member A (NKG2A) and eventually CD56 (NCAM, with an unknown role in NK biology). Fully matured NK cells are CD117$^-$, NKG2A$^{+/-}$, and CD56^{dim} or CD56^{high}. During their maturation, NK cells also acquire the expression NKG2D, Natural Cytotoxicity Receptors (NCRs), Killer-cell Immunoglobulin-like Receptors (KIRs), and CD16 (FcRIII) [74, 75].

iPSC-To-NK Differentiation

Published in vitro iPSC-to-NK differentiation protocols are similar to iPSC-to-T cell procedures and start from iPSCs derived from CD34$^+$ cells isolated out of

Table 2. The expression of phenotype markers and components of the extrinsic and intrinsic pathway by iNK cells compared to in situ differentiated NK cells

Phenotype marker distribution		
CD7	=	[84]
CD56	=	[80, 85]
Extrinsic pathway		
TRAIL	=	[5, 80]
FASL	=	[80]
Intrinsic pathway		
NKG2D	=	[80, 82, 85]
NCRs	=	[80, 82, 85, 86]
KIRs	▼	[23, 78, 84]
CD16	▼	[23, 81, 85]

umbilical cord blood (UCB) or fibroblasts [23] (Fig. 1). After hematopoietic specification, the differentiating cells are co-cultured with stromal cells (e.g., EL08-1D2 or OP9) in the presence of cytokines including IL-3, IL-7, IL-15, SCF, and Flt3-L [41]. Unlike T cells, NK cells can differentiate in the absence of Notch signaling, but several studies have shown that the lymphoid commitment and NK differentiation of UCB-derived $CD34^+$ cells are enhanced by Notch signaling [76, 77]. Therefore, several reported iPSC-to-NK procedures use Notch-ligand expressing OP9 [78, 79]. The differentiating iPSCs are typically cultured in these conditions for 3 weeks, during which they lose CD34, and acquire CD7 and CD117 [79].

To expand the newly differentiated NK cells, current protocols expose the cells to IL-2, IL-21, and 4-1BB-L [41]. Throughout the differentiation process, the fraction of $CD56^+$ cells goes up gradually [24, 79]. Phenotypical comparison to PBMC-derived NK cells showed that end-stage iPSC-derived NK (iNK) cells express similar levels of CD56 [80–82]. To our knowledge, the timing of the induction of the NK lineage commitment marker IL15RB has not been addressed for iPSC-to-NK differentiations, but one study found it to be expressed post-expansion, which is akin to mature PBMC-derived NK cells [83].

iNK Cell Function

NK cells can induce cytotoxicity by activating extrinsic apoptotic pathways and by the release of cytolytic granules containing Perforin and Granzyme B. The extrinsic pathway is mediated by NK-expressed ligands of death receptors, including TNF-Related Apoptosis-Inducing Ligand (TRAIL) and Fas Ligand (FASL). The expression of the TRAIL and FASL receptors on virally infected and tumor cells can be increased by IFNγ secreted by NK cells. The engagement of these receptors initiates a caspase cascade and the disruption of mitochondrial integrity, ultimately resulting in apoptosis [74]. iNK cells express TRAIL and FASL at similar levels as PBMC-derived NK cells [5, 80] (Table 2) and produce IFNγ in response to exposure to target cells [78, 79], indicating that they mediate their cytotoxicity at least partially through the extrinsic pathway.

Similar to PBMC-derived NK cells, iNK cells produce Perforin and Granzyme B and show degranulation when exposed to tumor cells, although no direct comparisons of secretion levels between iNK and PBMC-derived NK cells have been reported [24, 79]. The release of cytolytic granules is regulated by a wide range of activating and inhibiting receptors, including the NKG2, NCR and KIR families, and Fc receptors (FcRγ) [74] (Table 2). NKG2D is an activating receptor that recognizes ligands that are normally expressed at low levels but induced by physiological stress or malignant transformation, including MICA [87]. In phenotypical comparisons to PBMC-derived NK cells, most studies showed that iNK cells have similar levels of NKG2D [80, 82, 85], although in some instances lower levels were reported [23, 81]. NKG2A dimerizes with CD94 to form an inhibitory receptor that engages HLA-E and prevents NK cells from hypertoxicity and thus the destruction of healthy tissue [88]. Compared to PBMC-derived NK cells, most studies reported similar levels of NKG2A on iNK cells [80–82], although some studies found that iNK cells express relatively less [84] or more [85] NKG2A.

The NCR family has three members (NKp46, NKp44, and NKp30) and was discovered for the ability to mediate NK responses to tumor cells. NCRs recognize a wide range of endogenous and exogenous, non-MHC-restricted ligands which can be activating or inhibitory [89]. Compared to PBMC-derived NK cells, most studies reported similar levels of NKp46 and NKp44 on iNK cells [23, 24, 80–85, 90]. Only one study compared NKp30 expression by iNK cells and found levels similar to those of PBMC-derived NK cells [86].

KIRs constitute a polymorphic class of receptors engaging HLA-I [91]. The expression of activating and stimulatory KIRs is consistently lower in iNK cells compared to PBMC-derived NK cells [23, 78, 81, 83–85, 90]. This has been suggested to reflect a lack of terminal maturation of iNK cells in vitro, but the ultimate effect of lower KIR expression on the effector function of iNK cells remains to be understood [92]. One study compared the cytotoxicity of KIR^+ and $KIR^{-/low}$ iNK cells against neuroblastoma cell lines in vitro. It found that in short term assays KIR^+ cells were significantly more cytotoxic against three out eight tested cell lines [93], suggesting that in certain contexts iNK cells could benefit from higher KIR levels.

Notably, another difference between iNK and PBMC-derived NK cells is that iNK cells express less of FcRγ CD16 [23, 81, 82, 84, 85, 90]. Expression of CD16 is not only a marker of mature NK cells, but functionally it facilitates antibody-dependent cellular cytotoxicity by

binding the Fc tail of endogenous or therapeutic IgG antibodies bound to a target cell and transducing the signal intracellularly through the phosphorylation of the ITAM-containing adapter proteins CD3ζ or the common γ subunit of FcRγ [94, 95]. The most immediate effects of CD16 engagement are the stabilization of the immunological synapse and the subsequent release of Perforin and Granzyme B, lysing the target cell and thus completing the ADCC [96–98]. In addition, CD16 signals drive the proliferation of NK cells and enhance their cytotoxic potential, priming the NK cells for repeated lysis of target cells [99]. Given the importance of CD16 signals, the relatively low level of CD16 on iNK cells has the potential to limit their cytotoxic clinical capacity. Indeed, in an in vitro comparison with PBMC-derived NK cells, iNK cells showed lower rates of ADCC [5].

Engineered iNK Cells

High-Affinity Non-Cleavable CD16

Addressing the relatively low level of CD16 on iNK cells, several groups have engineered iPSCs to derive NK cells with stronger or sustained CD16-signaling [5, 84, 100]. The affinity of CD16 for IgG antibodies is dependent on a single nucleotide polymorphism in the encoding gene (*FCGR3A*). This single nucleotide polymorphism translates into a substitution of phenylalanine (F) by valine (V) at residue 158. V158 has a higher affinity for IgG antibodies than F158 [101]. Underscoring the importance of CD16 engagement in the context of cancer immunotherapy, 3 studies found that patients who homozygously carried the allele encoding the high-affinity V158, responded better to monoclonal antibody treatments targeting CD20 in non-Hodgkin lymphoma, HER2 in breast cancer and EGFR in colorectal cancer [5, 98].

Zhu et al. [5] engineered iPSCs to express a high-affinity (V158) and non-cleavable version of CD16 (hnCD16). The extracellular domain of CD16 is cleaved of the membrane of activated NK cells by A Disintegrin And Metalloprotease-17 (ADAM17), a process that has been shown to contribute to the disassembly of the immunological synapse [102]. CD16 cleavage can be prevented by mutating the ADAM17 cleavage site, which results in the sustained expression of intact CD16 on PBMC-derived NK and iNK cells upon activation [103]. While non-engineered iNK cells performed worse than PBMC-derived NK cells in an in vitro ADCC assay, hnCD16-iNK cells outperformed PBMC-derived NK cells. In a murine model for B cell lymphoma, hnCD16-iNK cells combined with an anti-CD20 antibody induced improved tumor control and survival, outperforming both non-engineered iNK and PBMC-derived NK cells. hnCD16-iNK cells also showed efficacy against intraperitoneally implanted ovarian cancer cells, mediated by an anti-HER2 antibody [5]. To target hnCD16-iNK cells with an anti-CD38 antibody (daratumumab) without inducing fratricide, hnCD16-engineering can be combined with a *CD38* knockout in iNK cells [104].

CAR-Engineered iNK Cells

To redirect the specificity of NK cells without the need for a mediating antibody, PBMC- and iNK cells have been engineered to express CARs [105]. The first generations of CARs tested in (i)NK cells were originally designed for T cells and contained ITAM signaling domains derived from CD3ζ [64]. Because CD16, NCRs and some KIRs transduce their signal through ITAM-containing adapter proteins, including CD3ζ and FcRγ [94, 95, 106], T cell-tailored CARs were able to mediate antigen-specific activation of NK cells [105, 107].

Li et al. [82] hypothesized that CAR designs could be optimized for their application in NK cells, and engineered iPSC lines to express an array of CAR designs targeting mesothelin. They found that iNK cells expressing a CAR comprised of the transmembrane domain of NKG2D and the signaling domains of 2B4 and CD3ζ showed superior efficacy compared to iNK cells that were not engineered or engineered to express a CAR designed for T cells in an in vivo model of ovarian cancer. iPSCs expressing a BCMA-targeting CAR with the same transmembrane and signaling domains gave rise to CAR-iNK cells that showed antigen-specific TNFα and IFNγ secretion, and cytotoxicity against tumor cell lines and primary multiple myeloma cells. These cells were also able to significantly delay tumor outgrowth in an intravenous in vivo model for multiple myeloma [86].

Engineered iNK Cells in the Clinic

Currently, there are four registered dose-escalating phase 1 clinical trials ongoing that test CAR-engineered iNK cells in acute myeloid leukemia, multiple myeloma and B cell lymphoma, including two that utilize NK-tailored CAR designs. Patients enrolled in these studies receive a variety of lympho-conditioning regimens, all including cyclophosphamide and fludarabine [18–21, 86]. Interestingly, one study has an arm that does not receive lympho-conditioning, which will provide insight into the benefits of such pretreatment [18]. Three of the tested products combine CAR expression with other engineering strategies, such as the expression of IL15-RF to enhance persistence [18–20, 104]. Two of these studies test iNK cells further engineered to express hnC16 in combination with anti-CD38 (daratumumab) or anti-CD20 (rituximab) antibodies for multiple myeloma and B cell lymphoma [18, 19]. Unlike the clinically applied iT cell products, iNK cells are not thought to require engineering strategies to avoid alloreactivity beyond the on-tumor effect because iNK cells do not express strong, activating receptors with unpredictable specificity (i.e., TCRs) [92]. In fact, a seminal clinical trial testing allogeneic, haploidentical NK cells suggested that a certain level of mismatching

between the NK cells' inhibitory KIR repertoire and the HLA types expressed by the recipient and their tumor cells can contribute to enhanced clinical efficacy [4].

iPSC-Derived CAR$^+$ Myeloid Cells

Myeloid cells, especially macrophages and neutrophils, are appealing cell types to add to the cellular immunotherapy arsenal. Particularly relevant for solid tumors are their abilities to infiltrate the tumor microenvironment (TME) more effectively than T and NK cells and to modulate the tumorigenic nature of the TME [108]. Primary monocyte-derived macrophages have effectively been retargeted to HER2 through the expression of a first generation, CD3ζ-based CAR design (CAR-Macs) and are currently being tested in a first-in-human phase 1 clinical trial [6, 7]. Patients with HER2-overexpressing solid tumors or peritoneal disease receive a single or multi-dosed treatment of a total of 5 billion autologous CAR-Macs, through intravenous or intraperitoneal administration. Initial results have reported grade 1–2 cytokine release syndrome (CRS) but no dose-limiting toxicities or on-target off-tumor toxicity. The best response was stable disease. Additionally, activation of lymphocytes in the TME could be observed and the trial has been expanded to include a combination of intravenous CAR-Mac administration with PD-1 checkpoint inhibition (Pembrolizumab) [7, 109]. However, the broader application of autologous, primary CAR-Macs is challenged by low yields of patient monocytes, insufficient in vitro expansion techniques, and limited efficiency in genetic engineering [110]. In the case of neutrophils, the short half-life of primary neutrophils, in addition to their resistance to genetic engineering along with the neutropenia commonly associated with cancer and its treatment, renders them inaccessible for autologous CAR-Neutrophil generation. However, it had been shown that CAR-engineered UCB-derived HSPCs could be differentiated along the myeloid lineage, without interference of the CAR expression [111]. Additionally, CD34$^+$ cells engineered to express a CD4/CD3ζ or CD4/FcRγ fusion receptor could be differentiated into neutrophils capable to elicit antigen-targeted lysis [112]. These studies suggest that iPSC-derived macrophages (iMac) or neutrophils (iNeuts) could provide an interesting, feasible approach to enhance accessibility and explore the clinical potential of myeloid cells in ACT.

CAR-Engineered iMacrophages

Multiple reports have been published on the generation of iPSC-derived CAR$^+$ macrophages (CAR-iMacs). CAR-iMacs have been successfully differentiated from human embryonic stem cells (hES) and [25, 113], PBMC- [114, 115], or CD34$^+$- [27] derived iPSCs (Fig. 1), against a variety of TAAs, including CD19 [114, 116], mesothelin [117–119], chlorotoxin [113], EGFRvIII, and Glypican-3 [115]. CAR designs utilized either "conventional" T cell-derived first- or second-generation CAR designs or macrophage-optimized CAR designs [113, 114, 116, 117]. Macrophage-specific CARs included signaling domains derived from phagocytic receptors, such as the FcRγ combined with the p85-recruitment domain of CD19 [116], DAP12 [111], toll-like receptor 4 intracellular toll/IL-1R (TIR) domain in combination with CD3ζ [115] or Bai1, MegF10, or MerTK as signaling domains [117]. The best-performing CAR design was generally selected utilizing THP-1 derived macrophages [114, 117] or in primary CD34$^+$ cells which were subsequently differentiated toward macrophages [116] prior to CAR-iPSC engineering. Interestingly, CAR designs containing Macrophage-specific signaling domains did not always have the highest therapeutic potential [114, 116, 117]. Both engineering strategies utilizing random integration as well as targeted integration into the AAVS1 genomic safe-harbor have successfully been applied for CAR-iMac generation [113, 117]. There has been only one report of random integration resulting in downregulation of CAR expression during differentiation [117]. Similar to the findings in UCB-derived HSPCs, constitutive CAR expression in iPSCs does not interfere with myeloid differentiation in vitro [114]. However, the CAR design might affect the properties iMacs acquire. Macrophages have an inherent ability to alter their properties, so called plasticity. Macrophages can acquire anti- (M1) as well as pro-tumorigenic (M2) characteristics in response to environmental cues. Although macrophage plasticity has been established to be far more complex than just a gradient between the M1 and M2 phenotypes, studies still use the associated phenotypical markers due to the extensive experience of correlating those phenotypes with tumor prognosis [120]. iMacs targeted toward CD19 or Mesothelin utilizing a conventional T cell-tailored 4-1BB/CD3ζ design expressed conventional myeloid markers including CD45, CD11b, and CD14 but had a transcriptional signature more akin to a M2 phenotype, while functionally showing increased production of pro-inflammatory cytokines, increased phagocytosis and the ability to control tumor growth in vivo compared to untreated mice [114]. The pro-inflammatory nature of these CAR-iMacs and their in vivo antitumor capacity could be further improved through a knockout of ACOD1, promoting a stronger M1-like, glycolytic, phenotype and an increase in CD80 and CD86 cell-surface expression [119]. Alternatively, constitutive expression of a Macrophage-optimized CAR structure (containing the FcRγ domains and p85-recruitment domain from CD19) did result in CAR-iMacs with a M1-like phenotype, showing increased activation of antiviral and cytokine-mediated responses as well as pro-inflammatory genes (including CD80, CD40, CCL5, and CCL2) compared to GFP-transduced counterparts, as well

as increased phagocytosis and cytokine secretion (IL-6 and TNFα) [116]. Similarly, the use of a TIR-CD3ζ CAR design resulted in CAR-iMac marked by an increased transcription of CD80, CD86, and CD83, and a decrease in CD206 and CD163 compared to iMacs expressing truncated CARs (CARs devoid of an intracellular signaling domain). Besides increased phagocytosis [115], the addition of checkpoint blockade, such as anti-CD47 [115, 116, 121], and the reduction of sialoglycans on tumor cells, can further increase the antitumor potential of iMacs [118]. In addition to their direct antitumorigenic function, iMacs can support T and NK cell responses and thus have potential in combinatorial treatment. iMacs have been reported to be able to increase T cell activation [116], and improve AML tumor cell killing in vitro when combined with iNK cells [121]. Furthermore, iPSCs engineered to ectopically express IL-12 give rise to iMacs that provide enhanced support to T cell responses in preclinical models [122]. Although direct, functional comparisons between CAR-Macs and -iMacs have not been reported, the results suggest that iMacs behave in a manner similar to their primary counterparts. Therefore, considering the pivotal role that macrophages play in CRS [123, 124], caution is required with large-scale infusion, especially when applied in combination with treatment modalities involving the activation of T cells.

CAR-Engineered iNeutrophils

Although the first CAR^+ neutrophils were generated in vitro in 1998 [112], reports of PSC-derived CAR-iNeuts for ACT have only started to gain traction more recently. Besides the early report on CAR-iNeut differentiation from $CD34^+$ cells [112], CAR-iNeuts have been differentiated from hES [26, 125] and FiPSCs [26], targeting either CD4 [112], PSMA [125], chlorotoxin, and IL-13Ra2 [26]. Different CAR signaling modalities have been tested in iNeuts. A first-generation design utilizing a CD4 transmembrane and a CD3ζ intracellular signaling domain was consistently the most functional design [26, 112, 126], compared to designs incorporating FcRγ [112], NKG2D and 2B4 [26] or CD32a [126] domains. Although constitutive CAR expression did not interfere with neutrophil differentiation from $CD34^+$ cells, downregulation of CAR expression has been reported, possibly due to (partial) transgene silencing [112]. Consecutive studies targeting the CAR transgene into the AAVS1 locus did not report such downregulation [26, 125]. CAR expression from the AAVS1 locus did not affect neutrophil differentiation, resulting in phenotypes (based on CD16, CD11b, CD15, CD66b, CD18, and MPO expression) similar to cells derived from unedited PSC and primary neutrophils [26]. However, RNA analysis suggests that CAR-iNeuts are potentially more immature in their phenotype and function, and have reduced sensitivity to chemoattractants [26, 125]. In vitro, CAR-iNeuts showed enhanced killing of glioblastoma multiforme and prostate cancer targets compared to CAR^- iNeuts or primary neutrophils [26, 125]. Target cell killing was mediated through reactive oxygen species production, phagocytosis, and neutrophil extracellular trap formation [26]. In an orthotopic in vivo model of glioblastoma multiforme, weekly administration of 5 million CAR-iNeuts intravenously showed that CAR-iNeuts were able to effectively cross the blood-brain barrier, control the tumor burden and provide a marginal increase in survival [26]. The anti-tumoricidal function of CAR-iNeuts could be improved in vivo by harnessing their ability to carry and deliver chemotherapy, increasing the survival over CAR-iNeuts only [26].

Although the studies into the application of CAR-iNeuts are still very limited, initial results look promising. Due to the current inability to generate primary CAR^+ neutrophils, direct phenotypic and functional comparisons to the PBMC-derived counterparts are not feasible. However, this underscores the potential of iPSC-derived ACT, rendering cell types accessible which cannot be derived from peripheral blood. The short half-life of neutrophils provides an additional challenge for the effective application of CAR-iNeuts in ACT. Further (pre) clinical studies will be required to assess the best treatment design, balancing their antitumor function, capacity to modulate the TME and possible role in CRS [127].

Discussion

iPSCs provide a source of allogeneic, homogeneously engineered, "off-the-shelf" immune effectors for adoptive cell therapy. As we described here, diverse differentiation protocols and engineering strategies have been developed to generate iPSC-derived T cells, NK cells, macrophages, and neutrophils for their application in oncology. These engineering strategies address tumor targeting (CAR, hnCD16), alloreactivity (*TRAC* or *RAG2* knockouts), and histocompatibility (*B2M* knockout) (Fig. 2). For allogeneic therapy, the efficiency of these engineering strategies is of vital importance to limit toxicities. Even small fractions of allogeneic, PBMC-derived $\alpha\beta TCR^+$ T cells can induce graft-versus-host responses [128] and thus homogeneously edited iPSC-derived T cells could reduce this risk. Histocompatibility is required to avoid immune-rejection of the effector cell population. Strategies to design "hypoimmunogenic" iPSCs have been described [129, 130]. Most of the iT and iNK cells currently in clinical trials are not edited to achieve hypoimmunogenicity, but one iNK-based clinical trial incorporates the expression of an "Alloimmune Defense Receptor" (ADR, Fig. 2) to avoid T- and NK-mediated immune-rejection [131, 132]. Combined, these clinical trials will provide insight

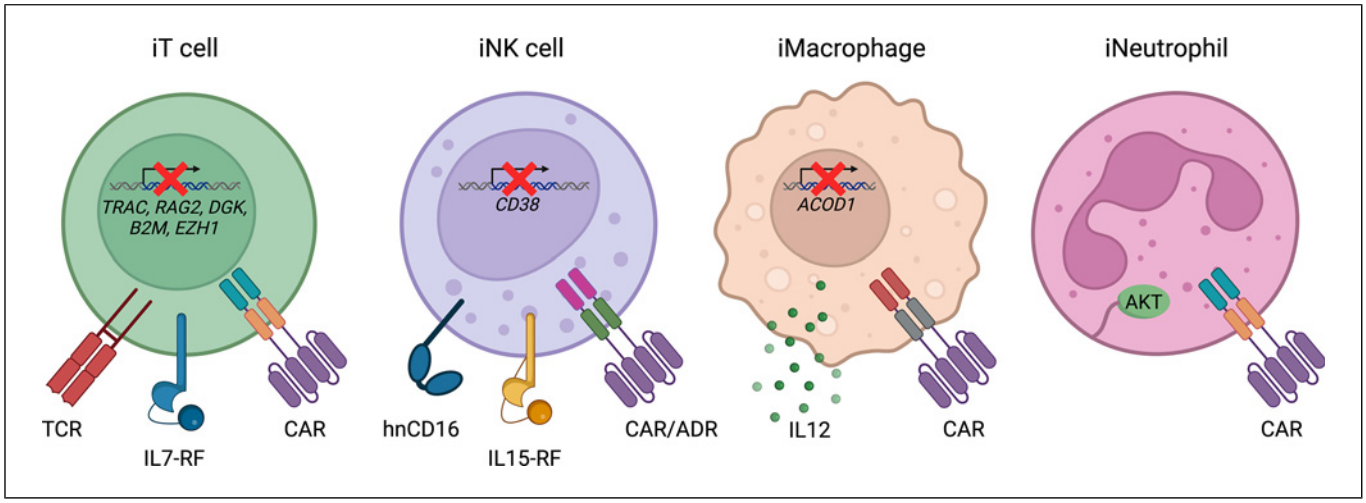

Fig. 2. Engineering strategies applied in iPSC-derived immune effectors. Shown is a representative selection of iPSC level engineering strategies currently applied to redirect iPSC-derived immune effector specificity (TCR, CAR, hnCD16), to prevent the expression of unknown antigen-receptors (*TRAC* and *RAG2* knockouts), to avoid elimination by immune-rejection (*B2M* knockout, ADR) or therapeutic anti-CD38 antibodies (*CD38* knockout), to optimize redifferentiation (*EZH1* knockdown) and to enhance antitumor function (*DGK* and *ACOD1* knockouts, IL7-RF, IL15-RF, IL-12, AKT).

into the in vivo persistence of iNK and iT cells and the requirement of hypoimmunogenicity to achieve therapeutic efficacy. An alternative to engineering strategies to avoid rejection is the generation of HLA-homozygous haplobanks [133, 134]. While such banks are already being established, this would require the generation of parallel iPSC master cell banks for each haplotype, whereas engineering strategies could allow one bank to accommodate all haplotypes.

The current generation of iPSC-derived immune effectors largely resembles their PBMC-derived counterparts in terms of their cell-surface marker expression, effector functions, and transcriptome. iT and iNK cells show remarkable tumor control in vivo. However, the current body of work reviewed here has revealed that notable differences between iPSC- and PBMC-derived immune effectors do remain (Tables 1–2). iT cells produce less cytokines, do not contain a robust $CD4^+$ helper subset and consistently require higher doses and/or cytokine supplementation to match the antitumor efficacy of PBMC-derived products. iNK cells' lower levels of KIR expression suggest that they are not fully maturated, whilst their lower CD16 levels limit their ADCC-potential. Future work should continue to benchmark properties of iT cells (in vitro proliferation and in vivo persistence), iNK cells (cytokine production) and myeloid cells (where feasible) against their PBMC-derived counterparts equipped with the same genetic edits.

Because iPSCs are a virtually unlimited source of immune effectors, they allow for the production of large numbers of cells and therefore higher or repeated dosing could compensate for lesser effector functions. However, one advantage of ACT is the requirement of only one treatment dose. Therefore, strategies to increase the potential of iPSC-derived immune effectors are being pursued. Single-cell transcriptomics can provide insights into the difference between in vitro and in situ differentiation. These comparisons as well as genetic screens can guide optimization of iPSC-differentiation protocols to generate immune effectors that more closely resemble their PBMC-derived counterparts. The potential of this approach was recently exemplified by the enhancement of $\alpha\beta TCR^+$ iT cell differentiation through the knockdown of histone methyltransferase EZH1, directing more cells to a $CD8\alpha\beta^+$ phenotype, at the expense of an innate-like $CD8\alpha\alpha^+$ phenotype [39]. Alternatively, iPSCs can be engineered to overexpress molecular signals and receptors their derivatives lack, such as the aforementioned hnCD16 or IL7-RF. However, such engineering strategies need to be carefully designed and evaluated, since they can disrupt the delicate signaling balance required for immune effector differentiation. This includes both the selection of genes to be knocked out or in, as well as the engineering methodology used. As discussed here, transgene silencing and promotor choice can affect its expression and thereby the lineage commitment of the differentiating immune effector [49, 112], which can be mitigated by targeted integration into a locus with an expression pattern that allows for appropriately timed and stable expression [26, 49]. Augmenting in vitro differentiation to generate bona-fide immune effectors combined with engineering strategies to enhance their effector functions, could potentiate iPSC-derived adoptive cell therapy to reshape the current therapeutic landscape of oncology.

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Conflict of Interest Statement

P.L.L. and S.J.C.v.d.S. are members of the Michel Sadelain laboratory, which receives research funds from Fate Therapeutics. This conflict has been disclosed to the Memorial Sloan Kettering Cancer Center who manages this conflict according to institutional policy.

Author Contributions

Both authors contributed to the design of the review, the interpretation of primary and secondary sources, and writing and editing of the manuscript.

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