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BANKED ALLOGENEIC IMMUNE EFFECTOR CELLS

# Engineered and banked iPSCs for advanced NK- and T-cell immunotherapies

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**The development of methods to derive induced pluripotent stem cells (iPSCs) has propelled stem cell research, and has the potential to revolutionize many areas of medicine, including cancer immunotherapy. These cells can be propagated limitlessly and can differentiate into nearly any specialized cell type. The ability to perform precise multigene engineering at the iPSC stage, generate master cell lines after clonal selection, and faithfully promote differentiation along natural killer (NK) cells and T-cell lineages is now leading to new opportunities for the administration of off-the-shelf cytotoxic lymphocytes with direct antigen targeting to treat patients with relapsed/refractory cancer. In this review, we highlight the recent progress in iPSC editing and guided differentiation in the development of NK- and T-cell products for immunotherapy. We also discuss some of the potential barriers that remain in unleashing the full potential of iPSC-derived cytotoxic effector cells in the adoptive transfer setting, and how some of these limitations may be overcome through gene editing.**

## Introduction

Cell differentiation was once thought to be a unidirectional process involving transition through a sequence of checkpoints along a pathway of restricted potential and increased specialization. This has been challenged by a series of fundamental discoveries leading to the reprogramming of cell fate.[1-6] Advances in cellular reprogramming made over the past several decades are now being translated for the treatment of diseases affecting numerous tissues including skin, heart, eye, skeletal muscle, nerve, pancreas, and blood.[7] Here, we review recent advances in induced pluripotent stem cell (iPSC) gene editing and differentiation to generate cytotoxic lymphocytes tailored for cancer therapy.

## Adoptive cellular immunotherapy

The adoptive transfer of natural killer (NK) cells and T cells has been tested in clinical trials for the treatment of patients with various types of advanced cancer over the past 35 years. Early clinical studies tested the safety and efficacy of autologous lymphokine-activated killer cell adoptive transfer. The clinical experience of treating patients with lymphokine-activated killer cells and interleukin-2 (IL-2) demonstrated that adoptive cellular immunotherapy could result in significant tumor regression in some patients for whom no other effective therapy is available. However, relatively low overall response rates, short remissions, and IL-2 toxicity were limitations to this approach.[8,9] To take advantage of mismatches between NK-cell inhibitory receptors and HLA, allogeneic NK-cell adoptive transfer emerged as an attractive immunotherapy strategy. In a landmark clinical study, NK cells sourced from HLA haploidentical donors were adoptively transferred to patients with relapsed/refractory acute myeloid leukemia (AML), along with low-dose IL-2. Among patients who received higher intensity chemotherapy regimens before adoptive transfer, complete responses (CRs) were observed in 5 of 19 patients, and treatment-related side effects were mild.[10] However, this approach has several limitations. NK cells comprise a small fraction (~5%) of the lymphocyte pool in the peripheral blood, necessitating large donor apheresis and considerable sample processing at a high cost to obtain sufficient cells for a single therapeutic dose. There is also intra-individual variability in NK cell cytotoxicity, inflammatory cytokine production, and proliferative potential. Furthermore, limited clinical efficacy has been observed in peripheral blood NK-cell adoptive therapy trials outside AML.

Adoptive T-cell therapy aims to use the antigen-specific nature of the T-cell receptor (TCR) to target tumor cells. To enhance the proportion of tumor-specific T cells and overcome tolerance, T cells can be redirected toward tumor-associated antigens through the introduction of an antigen-specific TCR into a polyclonal T cell population.[11-18] However, this approach is HLA-dependent and restricts its applicability. Furthermore, cross-pairing with endogenous TCR chains could result in autoimmunity.[19] An alternative method to re-direct T cells toward tumor-associated antigen involves the use of chimeric antigen receptors (CARs), which consist of an immunoglobulin-derived single chain variable fragment coupled to costimulatory domains for HLA-independent antigen recognition and subsequent T cell activation and proliferation.[20,21] CARs have been designed targeting an extensive number of tumor-associated

antigens. Most notably, autologous CD19-targeting CAR T-cells have shown dramatic outcomes in patients with relapsed/refractory hematologic malignancies,[22] resulting in the approval the of first autologous CAR T-cell therapy by the Food and Drug Administration (FDA) in 2017. Despite the clinical success of CD19-targeting CAR T-cell therapy, its widespread application remains a challenge. CAR T-cell manufacturing requires highly specialized facilities for isolation, engineering, and cell expansion. Patients also need to be able to provide sufficient healthy T cells and undergo bridging therapy to prevent rapid disease progression before CAR T-cell infusion.[23] For wider application of CAR T-cell therapy, allogeneic CAR T-cell sources, which can be generated in large quantities and provided on short notice, are required. iPSCs can now be routinely derived from a variety of sources and, once reprogrammed, have essentially unlimited expansion potential in vitro while maintaining pluripotency.[24] Thus, iPSCs represent a valuable resource for producing large numbers of allogeneic NK and T cells for off-the-shelf immunotherapy.

## The development of methods to generate iPSC-derived NK cells

In 2006, Takahashi and Yamanaka described methods for reprogramming mouse fibroblasts into pluripotent stem cells by introducing genes encoding 4 transcription factors. The resultant iPSCs were similar in nature to embryonic stem cells (ESCs).[6] A year later, the same method was used to reprogram human fibroblasts to generate iPSCs.[25] ESCs and iPSCs with similar morphology, developmental potential, and proliferative capabilities. However, global transcriptional and DNA methylation analyses have revealed differences in gene expression and epigenetic imprinting.[26,27] Although ESCs can serve as a potential source for the generation of off-the-shelf NK cells for immunotherapy,[28] iPSCs are easier to obtain and have the potential for greater donor diversity. The protocols for generating NK cells from pluripotent stem cells have evolved over time. In initial studies by the Kaufman group, human ESCs were cocultured with murine bone marrow stomal cells and then sorted and co-cultured with a second stromal line in media supplemented with cytokines that support NK-cell differentiation.[29] This protocol was then modified to adopt a "spin-embryoid body" method to cultivate hematopoietic progenitor cells in stroma-free, serum-free conditions before differentiation toward the NK-cell lineage.[30] NK cells generated from ESCs exhibited antitumor and antiviral activity both in vitro and in vivo.[31,32] Updated methods to reduce the amount of time needed to derive ESC- or iPSC-derived $CD34^+$ progenitor cells have recently been published.[33]

Early efforts aimed at human iPSC clone selection and characterization were low throughput due to suboptimal culture systems and were not scalable for industrial or clinical use because of the use of mouse embryonic fibroblast feeder cells and nondefined media containing serum.[34-36] Moreover, nonrandom genetic changes, including copy number variants, point mutations, and chromosomal alterations that could potentially lead to malignant transformation, can arise in iPSC lines.[37-40] Valamehr et al[41] developed a system that allows for rapid selection, characterization, and expansion of human iPSC clones under feeder-free culture conditions that maintain pluripotency and genomic stability. The key ingredient of this system was a small molecule cocktail of signaling pathway inhibitors added as a media supplement that improved clonality and maintained iPSC genome stability. The media formulation for this iPSC culture system was further refined with the derivation of a maintenance medium containing small molecule inhibitors, basic fibroblast growth factor, and leukemia inhibitory factor. iPSCs cultured with this maintenance media exhibited far less spontaneous differentiation and had gene expression profiles resembling the ground state of pluripotency.[24] These high-quality iPSCs were used as source material to generate large numbers of NK cells that were broadly cytotoxic toward both hematologic and solid tumor cells in vitro and cooperated with T cells and programmed death 1 blockade for durable tumor control in vivo.[42] A broad overview of the steps required to generate iPSC-derived NK and T cells is provided in Figure 1.

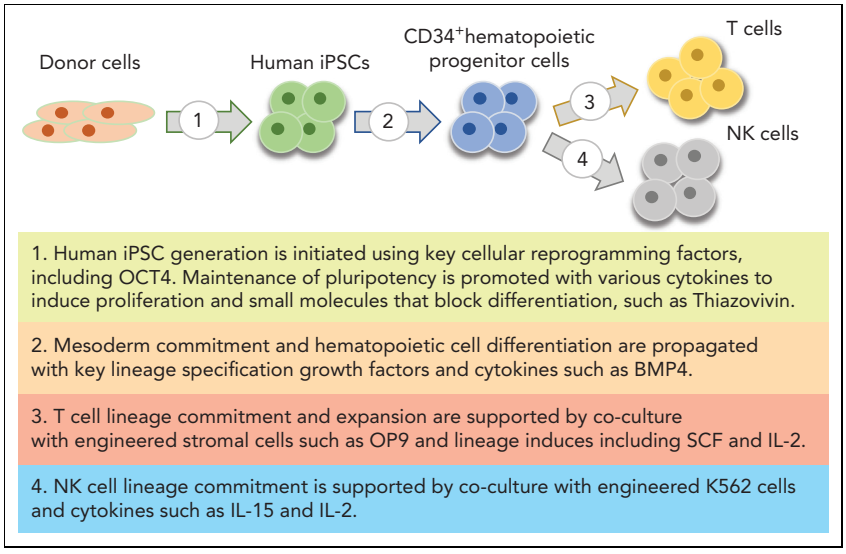


**Figure 1. Off-the-shelf hematopoietic cell products derived from renewable engineered pluripotent cell lines.** Schematic of the overall approach to generate iNK and iT cells from engineered iPSCs. Figure generated using BioRender.

The platforms for generating NK cells discussed above use fibroblasts as the source material for iPSC creation. iPSC-derived NK cells express a broad array of activating and inhibitory receptors, have a high cytotoxic granule content, and share a core gene expression signature with peripheral blood NK cells.[28,29,32,42] The precise relationship between iPSC-derived NK cells and NK cells circulating in the peripheral blood of humans has not been explored in depth. NK cells, which are innate lymphocytes, were long considered to comprise a relatively homogeneous population. However, a more recent study of NK-cell receptor repertoire diversity using mass cytometry revealed a surprising degree of diversity, with an estimated 6000 to 30 000 phenotypic populations within an individual and more than 100 000 phenotypes across a donor panel.[43] Using phenotypically defined NK cells from healthy individuals or tumor-infiltrating NK cells from the tissues of patients with cancer as source material for reprogramming to generate iPSCs that could then be differentiated along the NK-cell lineage could provide valuable insights into ontological relationships, particularly if epigenetic "memory" is preserved. Technical hurdles must be overcome for such experiments to be successful, given the inefficiency of NK-cell transduction and transfection protocols. One recent study describes reprogramming of human umbilical cord blood NK cells to the iPSC state, but only a limited characterization of these cells was reported.[44]

## Engineered iPSC-derived NK cells for immunotherapy

A clear advantage of the iPSC platform is its ability to perform efficient genetic engineering to modify NK and T cell effector responses for enhanced immunotherapy. A recent example is the deletion of cytokine-inducible SH2-containing protein (*CISH*) in iPSC-derived NK cells.[45] CIS (the protein encoded by *CISH*) is a member of the suppressor of cytokine signaling family and negatively regulates cytokine signal transduction.[46] NK cells from *Cish*$^{-/-}$ mice were hypersensitive to IL-15 signaling and exhibited superior tumor control in models of experimental lung metastasis.[47] Knockout of *CISH* was achieved in iPSC-derived NK cells using CRISPR-Cas9. *CISH*$^{-/-}$ iPSC-derived NK cells displayed elevated phosphorylation of JAK1, STAT5, and STAT3 in response to IL-15 stimulation, and exhibited increases in both glycolysis and mitochondrial oxidative phosphorylation, which correlated with increased mTOR signaling. Additionally, *CISH*$^{-/-}$ iPSC-derived NK cells persisted at higher frequencies and mediated superior anti-tumor activity compared to control iPSC-derived NK cells after adoptive transfer in a xenogeneic mouse model of AML.[45]

Another example of an engineered iPSC-derived NK-cell product generated for cancer immunotherapy is FT596, which contains 3 gene edits designed to enhance the effector function and persistence. The first modification involved the introduction of a high-affinity, noncleavable variant of CD16 (hnCD16), using a single-cell iPSC engineering platform. CD16 is a low affinity Fc-binding receptor that mediates NK-cell antibody-dependent cellular cytotoxicity (ADCC). As a normal consequence of NK-cell activation, CD16 is cleaved by metalloprotease ADAM17.[48] Presumably, this limits the hyperactive immune response. Substitution of the serine at position 197 in the middle of the cleavage region for a proline (S197P) effectively blocks ADAM17-mediated cleavage.[49] Adoptive transfer of iPSC-derived NK cells expressing hnCD16 (FT516) in combination with the therapeutic anti-CD20 monoclonal antibody rituximab yielded strong antitumor activity and long-term survival in a mouse xenograft model of lymphoma.[50] The second gene edit was the introduction of a membrane-bound IL-15/IL15R fusion (IL-15RF) protein to support in vivo persistence, and the third edit was the addition of an NK cell-optimized anti-CD19 CAR for direct tumor targeting.[51] The rationale for a dual targeting approach to treat B-cell leukemia and lymphoma by combining hnCD16 and an anti-CD19 CAR came from clinical experience with rituximab and anti-CD19 CAR-T cell therapy where loss of CD20 during rituximab has been reported,[52,53] and CD19 loss has been observed frequently in patients that relapse after anti-CD19 CAR treatment.[54-56] In preclinical studies, FT596 cells effectively eliminated both CD19$^{+}$ and CD19$^{-}$ lymphoma cells and had a unique propensity for targeting malignant cells over healthy B cells. Additionally, FT596 cells showed enhanced natural cytotoxicity and persisted at high levels for at least 4 weeks in the peripheral blood, spleen, and bone marrow in the absence of exogenous cytokines after adoptive transfer into immunodeficient mice.[51] It should be noted that there is still some debate in the field regarding the importance of activation-induced clipping of CD16 to NK-cell cytotoxicity. In vitro experiments demonstrated that repeated activation of CD16 decreases the amount of perforin secreted by NK cells. The inhibition of CD16 clipping results in extended contact times with opsonized target cells and greater activation-induced death of NK cells. CD16 clipping may promote the timely detachment of NK cells from targets and increase the serial engagement of target cells.[57] However, in our iPSC-derived NK cells, we did not observe defective ADCC responses for FT516 or FT596 cells in vitro or in vivo.

The testing of hnCD16 iNK cells (FT516) and hnCD16/CD19 CAR/IL-15RF (FT596) NK cells in combination with rituximab for the treatment of relapsed/refractory B-cell leukemia and lymphoma is currently ongoing in phase 1 clinical trials. As of 07 July 2021, 13 patients (2 at 30 million cells per dose, 4 at 90 million cells per dose, and 7 at 300 million cells per dose) were enrolled in the FT516 trial and had at least 3 months of follow-up. Patients received 3 weekly doses of cells, each with IL-2 support, and were eligible for up to 2 rounds of treatment. No dose-limiting toxicities or FT516-related serious adverse events were observed. Eight of 11 patients treated with at least 90 million FT516 cells achieved an objective response. Seven patients achieved CRs, including 2 patients who had disease progression following anti-CD19 CAR-T cell therapy. The 2 patients treated with the lowest dose of 30 million FT516 cells had progressive disease. Of the 8 responders, 5 were still in remission between 4.6 and 9.5 months.[58] As of 25 June 2021, 20 patients were treated in dose escalation with FT596, including 10 patients treated with a single dose of FT596 cells as a monotherapy (Regimen A) and 10 patients treated with FT596 cells and rituximab (Regimen B). No dose-limiting toxicities were reported for either of the treatment regimens. Of the 17 patients evaluable for efficacy, 5 of 8 in Regimen A and 4 of 9 in Regimen B achieved an objective response after the first treatment cycle. At single-dose levels of at least 90 million FT596 cells, 8 of 11 evaluable patients achieved an objective response, including 7 CRs. Among 4 patients with prior CAR-T cell therapy treated with at least 90 million FT596 cells, 2

**Table 1. Clinical trials testing iPSC-derived NK and T cells**

| Trial # | Phase | Product | Disease settings | Stage |
|---|---|---|---|---|
| NCT03841110 | 1 | Nontransduced iPSC-derived NK cells (FT500) with checkpoint blockade | Advanced solid tumors | Active, not recruiting |
| NCT04363346 | 1 | FT516 (hnCD16) iPSC-derived NK cells | COVID-19 | Completed |
| NCT04023071 | 1 | FT516 (hnCD16) iPSC-derived NK cells with obinutuzumab | AML, B-cell lymphoma | Recruiting |
| NCT04630769 | 1 | FT516 (hnCD16) iPSC-derived NK cells with enoblituzumab and IL-2 | Ovarian cancer | Completed |
| NCT04551885 | 1 | FT516 (hnCD16) iPSC-derived NK cells with avelumab | Advanced solid tumors | Active, not recruiting |
| NCT04714372 | 1 | FT538 (hnCD16/*CD38*KO/IL-15RF) iPSC-derived NK cells with daratumumab | AML | Recruiting |
| NCT05069935 | 1 | FT538 (hnCD16/*CD38*KO/IL-15RF) iPSC-derived NK cells with monoclonal antibodies | Advanced solid tumors | Recruiting |
| NCT04614636 | 1 | FT538 (hnCD16/*CD38*KO/IL-15RF) iPSC-derived NK cells with daratumumab or elotuzumab | AML, multiple myeloma | Recruiting |
| NCT04555811 | 1 | FT596 (hnCD16/anti-CD19 CAR/IL-15RF) iPSC-derived NK cells with rituximab | NHL, diffuse large B-cell lymphoma, high-grade B-cell lymphoma | Recruiting |
| NCT04245722 | 1 | FT596 (hnCD16/anti-CD19 CAR/IL-15RF) iPSC-derived NK cells with rituximab or obinutuzumab | B-cell lymphoma, CLL | Recruiting |
| NCT05395052 | 1 | FT536 (hnCD16/*CD38*KO/anti-MICA/B CAR/IL-15RF) iPSC-derived NK cells with monoclonal antibodies | Advanced solid tumors | Recruiting |
| NCT05182073 | 1 | FT576 (IL-15RF/*CD38*KO/anti-BCMA CAR) iPSC-derived NK cells with daratumumab | Multiple myeloma | Recruiting |
| NCT05336409 | 1 | CNTY-101 (sIL-15/EGFRt/anti-CD19 CAR) iPSC-derived T cells with IL-2 | $CD19^+$ B-cell malignancies NHL | Not yet recruiting |
| NCT04629729 | 1 | FT819 (CD8αβ anti-CD19 CAR) iPSC-derived T cells with IL-2 | Lymphoma, CLL, precursor B-cell ALL | Recruiting |

ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin lymphoma.

achieved a CR.[59] These interim clinical results demonstrate the safety of these NK-cell products and are providing early indications of efficacy. A list of the recently completed and ongoing clinical trials testing iPSC-derived NK and T cells is presented in Table 1. The advantages and disadvantages of different cell sources for NK-cell immunotherapy are summarized in Table 2.

## Engineered iPSC-derived T cells for immunotherapy

In vitro T cell differentiation was first established using murine ESCs,[60] and later with human cord blood-derived $CD34^+$ cells,[61] through the coculture of $CD34^+$ progenitors with Notch ligand-expressing feeder cells (OP9 or MS5) to direct hematopoietic progenitors to the T cell lineage. Although these protocols effectively differentiated cells, random rearrangement of the TCR α and β chains resulted in a heterogeneous population of T cells with random TCR specificity. TCR specificity is of great importance not only to ensure the targeting of a tumor-associated antigen but also to mitigate the allo-reactive potential. To generate T cells with a known specificity, iPSCs can be generated from TCR-selected T cells,[62-64] the transduction of specific TCRs into iPSCs derived from other cell sources,[65-67] or through expression of a CAR.[68-73]

The reprogramming of T cells with known TCR specificity and subsequent re-differentiation to iPSC-derived T cells has been successfully achieved for multiple antigens, including MART-1,[63] Nef,[62] MR-1,[64] LMP1/2,[74,75] and WT-1.[66,75] The resulting iPSC-derived T cells generally retain their TCR specificity, although additional TCR α-chain rearrangement have been reported. This can be prevented through *RAG* knockout.[65] TCR-specific iPSC-derived T cells have shown efficacy in preclinical in vivo models of hematologic malignancy[74,75] and solid tumors.[65,66,75] Retargeting of iPSC-derived T cells to tumor antigens with a CAR has been described for CD19,[67,68,72,73] CD20,[71] GPC3,[69] and LMP-1,[70] either through editing at the iPSC level[68-70,73] or transduction at later stages of differentiation.[67-73] The resulting CAR iT cells have effective antitumor efficacy in in vivo mouse models.[67,71,73] However, iPSC-derived T cells require higher doses and more exogenous cytokine support to achieve tumor control comparable to peripheral blood-derived T cells. This disparity in function could be attributed to the

**Table 2. Advantages and disadvantages of different sources to produce NK cells for immunotherapy**

| Source | Advantages | Disadvantages |
|---|---|---|
| Donor peripheral blood | Cells are mature; therefore, there is no need for lengthy differentiation protocols. Heterogeneity within the donor population allows for the selection of cells with favorable attributes such as KIR mismatch. Demonstrated clinical efficacy in treatment of AML. | Low frequency of NK cells in the peripheral blood limits the options for multiple dosing. Low efficiencies of transduction and transfection limits opportunities for genetic engineering. Variability between donors with respect to NK-cell function and proliferation. |
| Autologous peripheral blood | Lack of allo-reactivity could allow for longer persistence of cells after infusion. No need to go through the complex donor selection process. | No clinical benefit has been observed for autologous NK-cell adoptive transfer. |
| Cord blood $CD34^+$ cells | Easier to engineer compared to peripheral blood NK cells. Large numbers of NK cells can be generated using in vitro differentiation protocols. Anti-CD19 CAR NK cells derived from cord blood $CD34^+$ cells demonstrated clinical efficacy in the treatment of CLL. | Nonrenewable cell source with donor heterogeneity. |
| iPSCs | Unlimited renewal capability from well-characterized master cell banks. Precise multiplex engineering that only needs to be performed once per product derivation. Cells are generated from a single clone resulting in product homogeneity and consistency. Ability to generate large numbers of cells off-the-shelf for multiple patient doses. | Labor-intensive manufacturing process requires genetic engineering and cell culture expertise. Clinical testing is still in early stages. |

CLL, chronic lymphocytic leukemia; KIR, killer immunoglobulin-like receptors.

fact that iPSC-derived T cells only generate $CD8^+$ T cells and lack the $CD4^+$ T helper cell compartment, and that, despite expression of the endogenous αβTCR on the cell surface, the iPSC-derived T cell phenotype and function resemble innate-like lymphocytes such as γδT cells[68] or innate lymphocyte cells.[69] However, they are distinct from NK cells.[62,68,72] Recent advances have shown that differentiation toward a more adaptive T cell phenotype, either through regulation of epigenetic modulation,[76] or timed and titrated CAR signaling,[73] results in improved in vivo T persistence and antitumor efficacy.

For widespread allogeneic application of adoptive cell therapy, measures to increase histocompatibility and reduce allo-reactivity are key to mitigating the risks of graft-versus-host-disease or graft rejection. The self-renewing nature of iPSCs makes them an ideal platform for selecting multiplexed, genetically engineered cells for allogeneic applications. To improve histocompatibility, iPSC banks can be generated from HLA-homozygous donors,[77] or pseudo-homozygous iPSC can be engineered through allele-specific HLA knock-outs.[78] Both strategies require the establishment of large iPSC banks to cover the population. 150 HLA-homozygous donors could match 93% of the UK population,[79] and 50 donors would be needed to match 90.7% of the Japanese population.[80] To generate a "generic" iPSC line, full HLA knockout through B2M and CIITA disruption can be achieved.[71] Though full HLA-disruption can protect adoptively transferred cells against host T cell–mediated rejection, it renders the cells sensitive to targeting by host NK cells. One strategy to mitigate this type of rejection is overexpression of HLA-E, which can reduce host NK-cell activation through engagement with the inhibitory receptor NKG2A.[71] Other strategies for engineering T cells to subvert attack from the host immune system include introduction of alloimmune defense receptors[81] or the NK cell-ligand poliovirus receptor CD155.[71] To reduce the risk of allo-reactivity, iPSCs with a known TCR specificity against a common antigen (such as viral antigen) can be used to generate T cells. However, HLA-matching is still required. Figure 2 summarizes the edits that promote antigen-specific cytotoxicity, persistence, and avoidance of rejection by

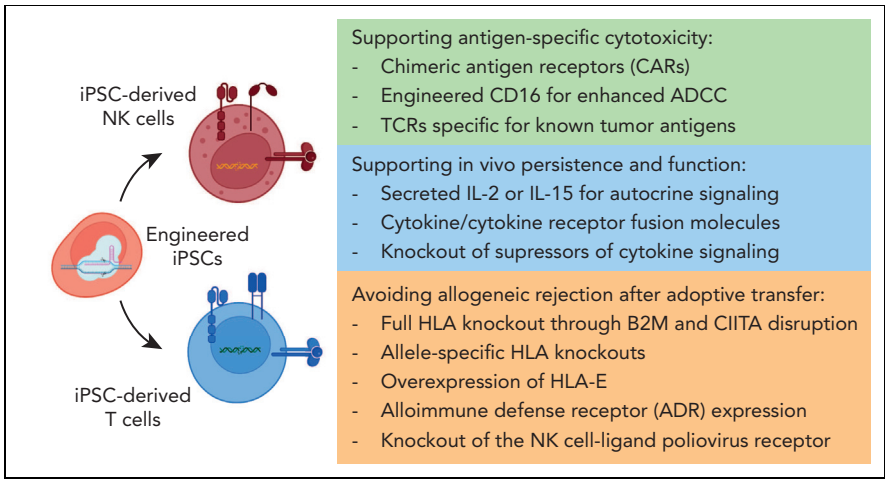


**Figure 2. Gene edits for enhancing iPSC-derived NK and T cell function.** Strategies for enhancing cytotoxic function, supporting persistence, and avoiding alloreactivity through genetic engineering in the generation of iPSC-derived NK and T cells.

**Table 3. Advantages and disadvantages of different sources to produce T cells for immunotherapy**

| Source | Advantages | Disadvantages |
|---|---|---|
| Donor peripheral blood | Healthy donors have abundant T cell populations, and genetic engineering and expansion can be performed. Donors with high T cell yields can provide treatment doses for multiple patients. | Careful matching of the donors and recipients is required to avoid GVHD or allo-rejection. 100% purity of genetic engineering required to avoid GVHD or allo-rejection is a challenge. |
| Autologous peripheral blood | Autologous peripheral blood CAR T cell therapy is the gold standard in the field. This approach can lead to long, durable remissions in some patients with B-cell malignancies. | Potential for serious side effects. Complex manufacturing with considerable lag time. Reliant on the quality of patient T cells, which can be poor due to disease and previous treatments. Very high cost. |
| Cord blood T cells | T cells in cord blood are more naïve than those in peripheral blood and could theoretically, exhibit less exhaustion and better persistence after adoptive transfer compared to T cells derived from peripheral blood. | New engineering strategies would be needed to eliminate the potential for GVHD. Few groups have tested this approach and studies have been done in vitro and using immunodeficient mice. |
| iPSCs | All the advantages listed in Table 2 plus disruption of the *TRAC* locus at the iPSC stage can abolish TCRα chain expression, allowing for the generation of CAR T cells without TCR-driven GVHD. | Labor-intensive manufacturing process requires genetic engineering and cell culture expertise. Clinical testing is still in early stages. |

GVHD, graft-versus-host-disease.

iPSC-derived T and NK cells. $CAR^+$ iPSC-derived T cells can control tumors in preclinical models, and a first-in-human clinical trial with iPSC-derived $TCR^-$ $CAR^+$ T cells is ongoing. Table 3 describes the advantages and disadvantages of different cell sources for generating T cells for immunotherapy.

## iPSCs as a source for generating cells from additional lymphoid lineages

Although the focus of this review is iPSC-derived NK and T cells, it is worth mentioning that other immune cell types with potential clinical benefits have been generated from iPSCs. γδ T cells have therapeutic potential with a low risk of graft-versus-host-disease, owing to their highly restricted TCR profile. In humans, 1% to 10% of peripheral blood lymphocytes are γδ T cells, of which Vγ9Vδ2 T cells are the major subset. Vγ9Vδ2 TCRs recognize phosphoantigen on the surface of infected or transformed cells to trigger cellular cytotoxicity.[82] In a two-step process to generate human Vγ9Vδ2 T cells with abundant expression of NK cell–associated activating receptors, primary Vγ9Vδ2 T cells were reprogrammed to iPSCs and then differentiated along the γδ T cell lineage using an "NK cell-promoting protocol." In vitro studies demonstrated direct cytotoxicity and ADCC responses of iPSC-derived Vγ9Vδ2 T cells against an array of cancer cell lines.[83] iNKT cells are a distinct subset of T cells that express a canonical invariant TCR α chain and TCR β chains that use limited Vβ and can regulate innate and adaptive immunity.[84] The antitumor effects of iNKT cells have been demonstrated in several clinical trials.[85-87] Human iNKT cells have been reprogrammed to iPSCs and then re-differentiated and expanded in the presence of IL-15 and TCR stimulation. In vitro studies showed NK cell–like cytotoxicity of iPSC-derived iNKT cells that was dependent on NKG2D and support of polyclonal $CD8^+$ T cell responses.[88] Mucosal-associated invariant T (MAIT) cells are a subset of innate-like T cells that express an invariant TCR α chain, the NK cell–associated receptor KLRB1, and IL-18Rα.[89] MAIT cells are abundant in humans and are important for host defense against a wide range of fungal and bacterial infections.[90] Human MAIT cells have been reprogrammed to iPSCs and re-differentiated to generate MAIT cells that were functional and able to protect mice from *Mycobacterium abscessus* infection.[64] Adoptively transferred iPSC-derived murine MAIT cells contributed to inhibition of tumor growth in a lung metastasis model and prolonged survival through enhanced NK cell–mediated cytotoxic activity.[91] Together, these recent studies demonstrate the expansion of iPSC platforms to generate distinct lymphocyte populations for cancer immunotherapy.

## Potential adverse effects of iPSCs and mitigation strategies

As mentioned above, genetic mutations and chromosomal alterations can occur in iPSCs during cell culturing. An example of this is the amplification of chromosome 20q11.21, which is common in human ESCs and iPSCs. One of the 3 genes within 20q11.21 is *BCL2L1* (an isoform of BCL-XL). Elevated BCL2L1 expression provides a selective advantage to these cells. Amplification of the 20q11.21 region has also been identified in human carcinoma cell lines and some teratocarcinomas, linking this mutation to malignant transformation.[38,39] While less common, *TP53* mutations seen in human cancers have also been identified in human pluripotent stem cell lines.[40] One way to reduce mutation rates in iPSCs is to modify culture conditions using small molecule pathway inhibitors to dampen stimulatory signaling and maintain a more quiescent state.[24,41] Another potential danger is tumorigenicity of residual iPSCs and undifferentiated cells after adoptive transfer through the formation of teratomas, which has been observed in transfer experiments using immune-deficient animals or autologous cells.[92-94] A recent study of allogeneic iPSC transfer into immune-competent Macaques, which more closely model clinical application of iPSC-derived products, demonstrated no tumorigenicity.[95] There are also ways of eliminating residual iPSCs or undifferentiated cells from cellular products should contamination from these populations occur. Lastly, differentiation of iPSC to NK or T cell lineages currently relies heavily on the coculture of murine (OP9) or human (K562, MS5) feeder cells to facilitate

lineage commitment and the risk presence of residual cells in the final clinical product. Irradiation of the feeder cells and extensive quality control testing of the final product minimize the risk of feeder-transfer, and feeder-free protocols are in development to eliminate feeder cells in the near future.[96-98]

## Key factors supporting the design of iPSC-derived lymphocytes

Although cellular reprogramming and gene editing technologies have advanced substantially over the past decade, questions remain regarding how best to engineer cytotoxic lymphocytes to achieve optimal clinical efficacy in the setting of cancer immunotherapy. An ideal cellular product should exhibit functional persistence by homing to the tumor microenvironment (TME) and without suppression of factors within the TME. The precise way to achieve this goal with gene editing technologies remains to be determined and will require basic scientific and translational studies that evolve into human clinical trials, further guided by high resolution immune monitoring of not only the blood but also the TME. To date, most studies have evaluated persistence after adoptive transfer in the blood. However, tumor-infiltrating cells must be highly functional in the TME, and blood persistence may be indicative of inadequate homing or activation. It is also becoming increasingly clear that the function of banked cell products may not represent their actual in vivo function after 1 day, 1 week, 1 month, or 3 months after infusion. Further studies are needed to understand the in vivo functional persistence and tissues that cells traffic to after infusion. It is possible that ex vivo expansion leads to cytokine and growth factor dependency, which may diminish cell function or lead to apoptosis after adoptive transfer. Understanding the in vivo function of cell products in the physiological setting of immune suppression within the TME is also required.

It remains controversial exactly how much persistence and in what compartment is needed for clinical efficacy. In contrast to "one and done" autologous CAR-T cells, allogeneic banked products can be multidosed for primary treatment or maintenance. Lastly, high dose lymphodepletion, as originally described by Rosenberg and colleagues[8] is not tolerable for heavily pretreated patients or those with other age-related comorbidities. Therefore, gene editing in iPSC-derived NK or T cells that enables evasion from allogeneic rejection or safer, more directed lymphodepletion strategies is needed. It is expected that there may be many effective strategies to balance these variables in the future.

## Conclusion

In summary, the development of gene-edited iPSCs and methods for their differentiation into NK and T cells has accelerated rapidly over the past several years. Although these technologies are new, promising results are now being observed in clinical trials for the treatment of patients with relapsed/refractory diseases. We anticipate that these platforms will continue to improve, and clinical trials will begin to advance beyond early stages and toward late stages and commercialization. The promise of banked allogeneic iPSC-derived NK and T cells as next-generation immunotherapies is now being realized.


## Acknowledgment

This work was supported in part by grants from the National Institutes of Health (NIH), National Cancer Institute: CA111412 (J.S.M. and F.C.), CA65493 (J.S.M. and F.C.), CA197292 (J.S.M.); and the NIH, National Heart, Lung, and Blood Institute: HL155150 (F.C.).


## Authorship

Contribution: F.C., S.J.C.v.d.S., and J.S.M conceptualized the review, collected data, and wrote the manuscript.

Conflict-of-interest disclosure: F.C. and J.S.M. are paid consultants for Fate Therapeutics, receive stock options, and research funds from this relationship. S.J.C.v.d.S. receives research funds from Fate Therapeutics. These conflicts have been disclosed to the University of Minnesota and Memorial Sloan Kettering Cancer Center who manage this conflict according to institutional policy.


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## Footnotes


Submitted 22 August 2022; accepted 28 October 2022; prepublished online on *Blood* First Edition 3 November 2022. https://doi.org/10.1182/blood.2022016205.


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