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Review

Advancing cell-based cancer immunotherapy through stem cell engineering

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SUMMARY

Advances in cell-based therapy, particularly CAR-T cell therapy, have transformed the treatment of hematological malignancies. Although an important step forward for the field, autologous CAR-T therapies are hindered by high costs, manufacturing challenges, and limited efficacy against solid tumors. With ongoing progress in gene editing and culture techniques, engineered stem cells and their application in cell therapy are poised to address some of these challenges. Here, we review stem cell-based immunotherapy approaches, stem cell sources, gene engineering and manufacturing strategies, therapeutic platforms, and clinical trials, as well as challenges and future directions for the field.

INTRODUCTION

Two unique properties of stem cells, their ability to self-renew and differentiate into multiple cell types, make them an attractive source for cell-based therapies. Stem cells and stem cell-derived products have been investigated for diseases such as muscular dystrophy, heart disease, Parkinson's disease, Alzheimer's disease, spinal cord injuries, diabetes, and cancer.[1] Although much work remains to be done, recent advances in stem cell engineering underscore the promise of a new generation of stem cell-based therapies to alter the treatment landscape for several clinical indications. One such area is cancer, where therapies that rely on genetically engineered stem cells are beginning to enter the clinic and show encouraging signs of safety and efficacy.

Cell therapy, in the form of non-genetically modified hematopoietic stem cell transplantation (HSCT), has been a mainstay of blood cancer treatment for decades.[2] In the late 1900s, as our understanding and acceptance of the relationship between the immune system and cancer matured, investigators began utilizing immune cells to fight cancer.[3] Several clinical trials reported the application of *ex vivo*-expanded tumor-infiltrating lymphocytes and lymphokine-activated killer cells to treat end-stage solid tumors, and noteworthy clinical responses were observed.[4,5] Soon after, developments in gene therapy ushered in another form of cell therapy for cancer, centered on tailor-made, genetically modified immune cells.[6] Chimeric antigen receptor (CAR)-T cell therapy has transformed the treatment of hematological malignancies, with six products approved by the FDA so far. Although instrumental in treating liquid cancers and propelling the field of cell therapy forward, the current CAR-T cell therapies face several limitations.[7–11] The products are autologous, obtained from the patient itself, which impedes their scalability, affordability, and accessibility. The vein-to-vein manufacturing process also prevents the administration of CAR-T cell therapy to patients with rapidly progressing disease, and individualized starting material coupled with an *ex vivo* manufacturing process can result in variable and suboptimal final products.[12–17] For example, patient-derived T cells and current expansion protocols can cause the therapeutic cells to enter a latter differentiation state and express inhibitory receptors. A growing body of work contends that T cell fitness impacts clinical activity, with "younger," less differentiated, less exhausted T cells correlating with improved responses.[18–24] In addition to production and quality control challenges, CAR-T cell therapies can cause serious adverse events, namely cytokine release syndrome (CRS) and neurotoxicity.[25–27] Despite outstanding response rates, the majority of the CAR-T cell recipients relapse.[28–30] Finally, CAR-T cell therapies have often failed against solid tumors, except for the anti-glioma activity of GD2-targeting CAR-T cells[31,32] and the success of Claudin-18.2 CAR-T in treating gastric cancers.[33]

Allogeneic cell therapies have the potential to overcome some of the hurdles faced by autologous therapies but are not without challenges.[34,35] Many concerns about safety and potency are not readily addressed and will remain important considerations as allogeneic cell immunotherapies advance further. Healthy donor-derived cells can improve the manufacturability and standardization of products. They provide readily administrable "off-the-shelf" cell therapies and can be pre-screened for the desired

characteristics. However, they also pose the risk of graft-versus-host-disease (GvHD) and are subject to host cell-mediated allorejection. GvHD is mediated by alloreactive donor T cells and can be avoided by T cell receptor (TCR) editing or the use of immune cells with known specificities or a lack of response to peptide-MHC mismatches.[36–38] Examples are virus-specific T cells, tumor-antigen-specific T cells, innate-like T cells, natural killer (NK) cells, and macrophages.[39–42] Through innate-like TCRs and/or NK receptors, gamma delta ($\gamma\delta$) T, invariant NK T (iNKT), mucosal-associated invariant T (MAIT), and NK cells exhibit intrinsic cancer cytotoxicity against many liquid and solid cancer types.[43–46] Equally, macrophages can display innate cancer phagocytosis.[44,47] Multiple tumor-targeting mechanisms are necessary for durable remission to oppose tumor antigen escape and antigen heterogeneity. Although it is possible to engineer dual-targeting CARs and other means of tumor recognition onto conventional T cells, the limits of the current techniques for genomic alterations must be considered. Lentiviral and retroviral vectors remain the most common gene engineering methods. Given their relatively small gene payload capacity, the number of genes introduced in a single production round for mature immune cells is limited.[48] Between CARs, safety switches, immunomodulatory proteins, and other enhancements, selecting the optimal combination for transduction into mature immune cells is challenging, and viral transduction and genomic editing affect cell yield and quality.[49] Potentially desirable immune cell populations, such as iNKT and $\gamma\delta$ T cells, have low frequencies in peripheral blood, requiring extensive expansion for clinical usage. This expansion can introduce variability, limit manufacturability, and produce highly differentiated cell products.

Regardless of the allogeneic cell type, recognition and elimination by the host immune system may limit the persistence and therapeutic efficacy of the allogeneic cells.[50] Interestingly, targeting B cell malignancies is a unique scenario, as B cell aplasia is a common sequela of CD19 CAR-T cell treatment, and thus, humoral-mediated immunorejection of an allogeneic cell therapy will be diminished.[51,52] Cord-blood-derived, HLA-mismatched NK cells transduced to express CD19 CAR and IL-15 were administered to 11 patients with CD19-positive lymphoid tumors and resulted in a 73% response rate.[29] The infused CAR-NK cells expanded and persisted at low levels for at least 12 months.[29] This long-term persistence was attributed to a permissive environment created by the lymphodepleting regimen combined with the ectopic expression of IL-15 by the CAR-NK cells.[29] However, the presence of therapeutic cells was not sufficient to prevent relapse.[29] Particularly for treating solid tumors, the rational design of allogeneic cells resistant to host rejection may be necessary to create a therapeutic window. Immunoevasion can be achieved by HLA "cloaking," in which the HLA genes are inactivated using gene editing techniques.[53] Cells that lack HLA, however, will be subject to NK "missing self-recognition" and will require additional genetic modifications, such as the overexpression of HLA-E, to resist NK killing.[54]

Despite rapid progress in the development of cell therapies, the question remains: can we produce off-the-shelf, safe, scalable cell products that lead to durable responses in cancers, including solid tumors? In this review, we discuss the potential of stem cell-based cell therapies to achieve this lofty goal. We start by reviewing stem cell sources, engineering strategies, and manufacturing details and then examine stem cell engineering challenges, solutions, and optimizations as well as the current stem cell-based therapeutic platforms and clinical trials.

Stem cell sources

The challenges faced by autologous therapy led us to consider alternative master stocks as therapeutic cell sources. Two major stem cell resources, HSCs and pluripotent stem cells (PSCs), have been used to develop therapeutic immune cells and could provide a sustained supply and bypass the unavailability of autologous cell materials.[55,56] Compared with HSCs, PSCs could be utilized as an "unlimited" cell source to derive immune cells for the generation of therapeutic products. However, generating cells from human PSCs has typically been less efficient.[57] Several strategies, such as co-culturing PSCs with stromal cells (e.g., S17 or OP9 cells) and producing embryoid bodies, can improve PSC differentiation and immune cell production.[58–61] In addition, healthy donor periphery blood mononuclear cell (PBMC)-derived immune cells, such as conventional $\alpha\beta$ T, iNKT, MAIT, $\gamma\delta$ T, and NK cells, could be reprogrammed to pluripotency and then re-differentiated into rejuvenated immune cells.[62–67] These PBMC-derived induced PSCs (iPSCs) can be further engineered with CARs for enhanced antitumor efficiency. However, it is necessary to explore the usage of the endogenous promoter to drive transgene expression in PBMC-derived iPSCs, as the endogenous regulatory elements could still be activated in iPSCs, thereby affecting differentiation. Inducible expression systems could be incorporated to address this concern.[57]

Stem cell-derived therapeutic immune cells

The transformative success of immune checkpoint inhibitors and CAR-T cells has reinforced the importance of T cells in cancer immunotherapy. It has further made the creation of stem cell-derived T cells a primary focus in the field of stem cell engineering. Genetic engineering of autologous conventional $\alpha\beta$ T cells to generate tumor-targeting T cells has been pursued for over two decades. Two categories of tumor antigen-specific receptors are applied to grant T cell specificity: physiological TCRs and synthetic CARs. CD19 CAR- and B cell maturation antigen (BCMA) CAR-engineered T cells have been approved by the FDA to treat B cell malignancies and multiple myeloma, respectively.[15,68–72] TCR-engineered T cells have shown promise in the treatment of melanoma, lung cancer, sarcoma, and multiple myeloma.[73,74] Nevertheless, several drawbacks exist for these approaches that may be addressed by stem cell-derived products. Stem cell-derived T cells have alternate cytokine profiles, which may reduce CRS and other safety risks.[75] Additionally, the composition of autologous T cells, such as the $CD4^+$:$CD8^+$ ratio, vary greatly between patients and may affect therapeutic outcomes.[15,24] The heterogeneity of starting materials for CAR-T cell manufacturing may impede therapy efficacy. This issue could be resolved by adjusting a specific ratio/dose of $CD4^+$:$CD8^+$ cells[76,77] or by using allogeneic sources such as HSC- or iPSC-derived off-the-shelf T cells. In these sources, theoretically, $CD4^+$:$CD8^+$ T cells can be tuned and additional genetic engineering performed on the small numbers of starting stem cells (Figure 1A). Notably, it is challenging to derive $CD4^+$ cells from the current protocols that differentiate stem cells

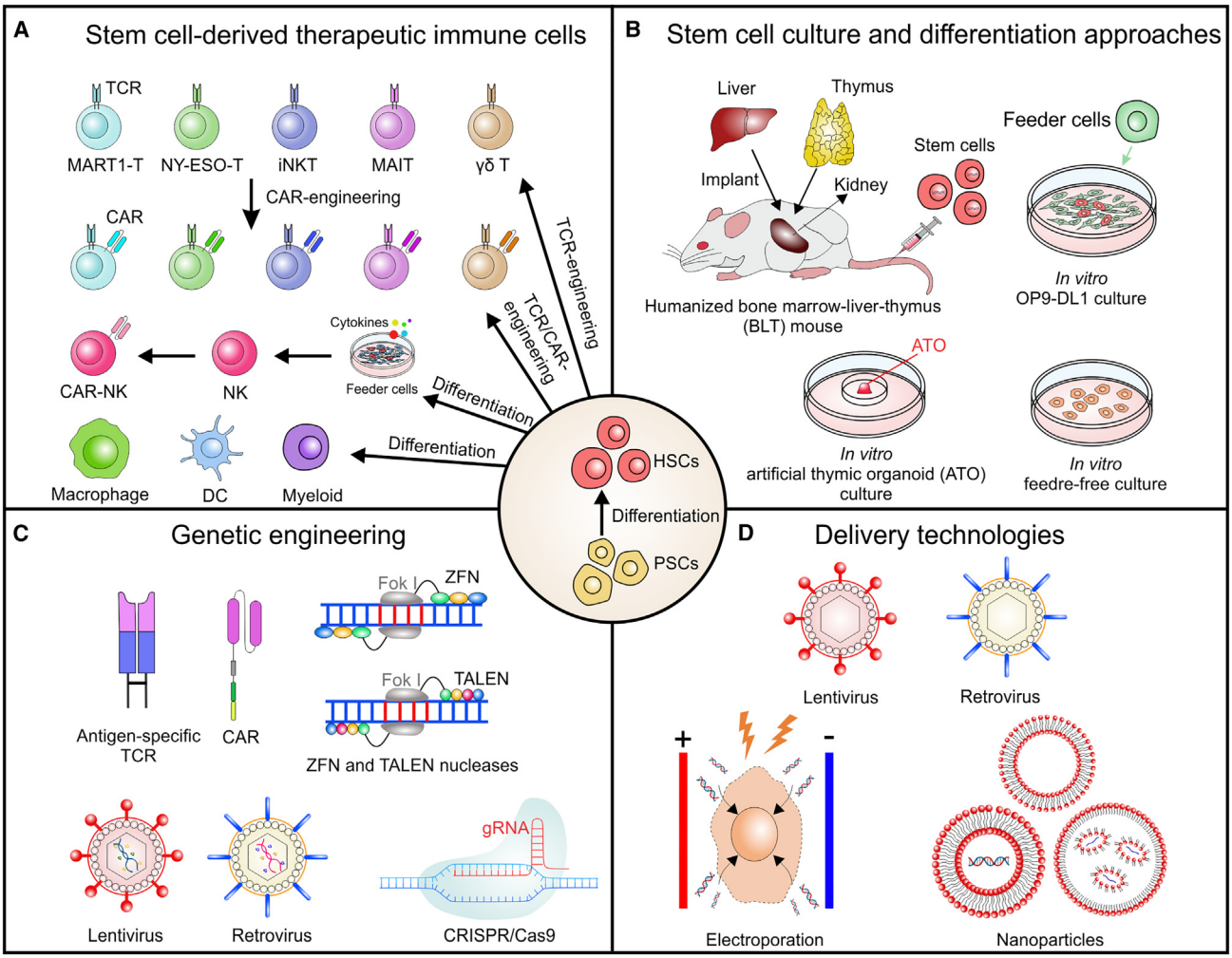

Figure 1. Stem cell engineering technologies, approaches, and therapeutic immune cells

(A) HSCs and PSCs can be engineered and differentiated into a variety of immune cells, such as conventional αβ T cells, innate T (i.e., iNKT, MAIT, and γδ T) cells, NK cells, macrophages, dendritic cells, and myeloid cells. These immune cells could be further engineered with CARs to enhance their tumor targeting capacity.

(B) Various stem cell differentiation culture systems have been developed, such as a humanized bone marrow-liver-thymus (BLT) mouse model, *in vitro* feeder-dependent OP9-DL and artificial thymic organoid (ATO) cultures, and *in vitro* feeder-free cultures.

(C) Genetic engineering strategies have been explored in stem cells and immune cells for antitumor applications, including CAR and TCR engineering, via gene editing using CRISPR-Cas9, designer nucleases such as ZFN and TALEN, and viral vectors.

(D) In addition to lentiviral or retroviral transduction, delivery systems such as electroporation and nanoparticles achieve stable and efficient gene delivery to stem cells and their derivative immune cells.

into T cell lineages.[78–81] One possible reason is that the current stem cell differentiation systems utilize Notch signaling to induce T lineage development. Notch biases the CD4/CD8 T lineage decision, favoring CD8 over CD4 T cells.[82–84]

NK cells, potent innate cytotoxic lymphocytes, effectively target virus-infected cells and cancer cells.[45] NK cell-based clinical trials attempt to treat cancers using a variety of NK cell sources, including NK cell line NK92, autologous patient-derived NK, allogeneic healthy donor-derived NK, and HSC- and iPSC-derived NK cells (Figure 1A).[29,40,85–95] Unlike conventional αβ T cells expressing rearranged TCRs, NK cells recognize target cells through the integration of signals from activating and inhibitory receptors.[88,91,96,97] NK cell recognition is independent of MHC restriction and prior sensitization, thereby free of GvHD risk.[29,91,98] The efficacy of NK cell-based immunotherapy could be potentially improved via multiple strategies, such as arming CARs on NK cells,[45,85,88,99] engineering IL-2 or IL-15 to enhance NK cell antitumor activity and persistence *in vivo*,[40,100] directing antibody-dependent cellular cytotoxicity (ADCC) through NK Fc receptor CD16,[101] and blocking NK inhibitory receptors such as NKG2A or killer cell immunoglobulin-like receptor (KIR).[102]

Unconventional T cells, such as iNKT, MAIT, and γδ T cells, recognize tumor cells via TCRs and NK activating receptors, independent of MHC restriction. These unique features allow innate T cells to target tumor cells without inducing GvHD. Generation of iNKT, MAIT, and γδ cells through genetic engineering and differentiation of HSCs or iPSCs has been successful (Figure 1A). The resulting innate T cells respond to their agonist stimulation and display potent tumor-killing abilities in leukemia, multiple myeloma, and solid tumors.[56,62,103–109] These

pre-clinical studies support the potential of developing off-the-shelf innate T cell-based cancer immunotherapies. Other innate immune cells, such as macrophages, dendritic cells (DCs), and myeloid cells, can also be generated from iPSCs (Figure 1A).[110–113] These iPSC-derived innate immune cells display immunostimulatory function and may facilitate vaccination-based immunotherapy.[111]

Stem cell engineering: Technologies and manufacturing

Stem cell culture and differentiation

Various stem cell culture and differentiation systems have been developed in the past decades, as comprehensively described in earlier reviews (Figure 1B).[55,56] These systems include humanized mouse models (e.g., bone marrow-liver-thymus [BLT] mouse model),[105,106,114–116] feeder-dependent cultures (e.g., OP9-DL and artificial thymic organoid [ATO]),[78–80] and feeder-free cultures (e.g., *ex vivo* HSC-iNKT culture).[104]

Considering the off-the-shelf purpose and safety profile of cellular products, the mouse origins of humanized mouse models severely limit their clinical application. Feeder-dependent cultures containing mouse stromal cells have safety concerns that need to be addressed. One study reduced contamination by mouse feeder cells using a porous membrane, with feeder cells seeded at the bottom of the membrane and stem cells cultured on the other side of the membrane.[117] In addition, human-derived feeder cells such as foreskin fibroblasts, mesenchymal stem cells, and adipose-derived stromal cells were utilized as feeders for human embryonic stem cell (ESC) and iPSC cultures.[118–122] Alternatively, feeder-free systems were developed to meet the clinically applicable and scalable requirements. For instance, an *ex vivo* feeder-free HSC-iNKT culture allowed to generate allogeneic iNKT cells with high yield and purity.[104] The feeder-free culture adopted a system of plate-bound DLL4 and VCAM-1 to induce T cell commitment from stem cells.[123,124] The generated allogeneic HSC-engineered iNKT cells displayed potent antitumor efficacy and antiviral capacity.[104] They could be further engineered with CARs to enhance their tumor targeting and gene-edited with CRISPR-Cas9 to ablate surface MHC molecules.[78] Another feeder-free differentiation culture system spanned from iPSC maintenance to T cell proliferation stages, enabling large-scale iPSC-derived T cell generation for cancer immunotherapy.[125] Further improvements on the feeder-free cultures will be necessary to achieve a more stable and efficient immune cell production.

Stem cell gene editing

The common premise of engineering immune cells for cancer immunotherapy is to grant immune cells the ability to specifically target tumor cells. TCRs and CARs are widely used in stem cell engineering to enhance immune cell specificity, given their validation in mature PBMC T cells (Figure 1C).[15] Tumor antigen-specific TCRs are typically obtained from patient-derived, tumor-responsive T cell clones,[15,126] humanized mouse models,[127,128] or using phage display technology.[129,130] Unlike TCRs that require CD3 co-expression and are MHC-restricted, synthetic CARs function independently of MHC restriction and can be applied to other cells such as NK cells, macrophages, and myeloid cells,[45,113] enhancing tumor targeting capability.[131] In addition to directly engineering mature PBMC-derived or stem cell-derived immune cells, retroviral or lentiviral vectors are used to stably introduce TCRs and CARs into stem cells (Figure 1D).[132] These vectors represent a promising approach to generate long-lasting immune cells with defined antigen specificity.[133,134]

Gene-editing technologies, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas9 have been utilized in cancer immunotherapy (Figure 1C).[36,38,135–138] These technologies enable efficient gene knockout, site-specific knockin, and genome-wide screen in target cells, including immune cells and stem cells. Knockout of TCR genes (e.g., *TRAC* and *TRBC*) avoids T cell-triggered GvHD, knockout of MHC-related genes (e.g., *B2M* and *CIITA*) reduces host T cell-mediated allorejection, and knockout of immune checkpoint genes (e.g., *PDCD1*, *LAG3*, *CTLA4*, and *DGK*α) improves immune cell antitumor efficacy (Figure 2).[38,136,139–149] Knockin of a CAR gene into the *TRAC* locus via CRISPR-Cas9 results in a uniform CAR expression on T cells, lack of endogenous TCR expression, and enhanced T cell potency.[150,151] In addition, CRISPR-Cas9-mediated genome-wide screening of immune and stem cells can help to identify gene targets for cell-based therapies.[152–157] Compared with engineering CAR/TCRs or gene editing in mature immune cells, gene editing in stem cells could mean a reduction of required materials, such as lentivirus and CRISPR-Cas9/gRNA. It might also lead to higher gene editing efficiency.[78,158]

Gene knockouts or knockins that enhance the antitumor capacity of immune cells are being widely explored. For example, incorporating IL-15 into NK or iNKT cells improves their *in vivo* persistence and tumor killing.[159–162] Introducing HLA-E or HLA-G into MHC knockout cells grants resistance to host NK cell-mediated allorejection.[78,163,164] Disrupting CD52 makes these cells resistant to lymphodepleting drugs such as alemtuzumab[26,165] (Figure 2).

Stem cell clinical manufacturing

Cell-based therapy has revolutionized cancer treatment. Unique features of cellular products, such as high variability, a long manufacturing process, low stability, complicated storage and transportation, insufficient characterization, and an unclear mechanism of action, make these cells different from other chemical drugs or antibody products.[166] Therefore, a GMP-grade regulation is especially necessary for cell therapy products. In compliance with official standards, such as the United States Pharmacopoeia (USP) or the European Pharmacopoeia (EurPh), the complete characterization of cellular products should be tested, including identity, yield, purity, viability, and potency. In addition, tumorigenicity and biocompatibility testing should be performed, if necessary. Multiple aspects should be taken into consideration, such as cell origin (autologous versus allogeneic), safety, immunogenicity, *in vivo* efficacy, persistence, administration route, exposure duration, use of combination products, and others.[167]

One concern of PSC-derived cell products is the presence of residual undifferentiated PSCs, which could develop into teratomas in the recipient patients.[168] Several preclinical studies have reported that once PSCs are differentiated into immune cells, a few residual PSCs persist and form teratomas in animal studies. These results have been considered sufficient to demonstrate the safety of PSC-derived cellular products, and therefore, these products have entered phase I clinical testing.[169] Another concern of PSC-derived cell products is immune matching and

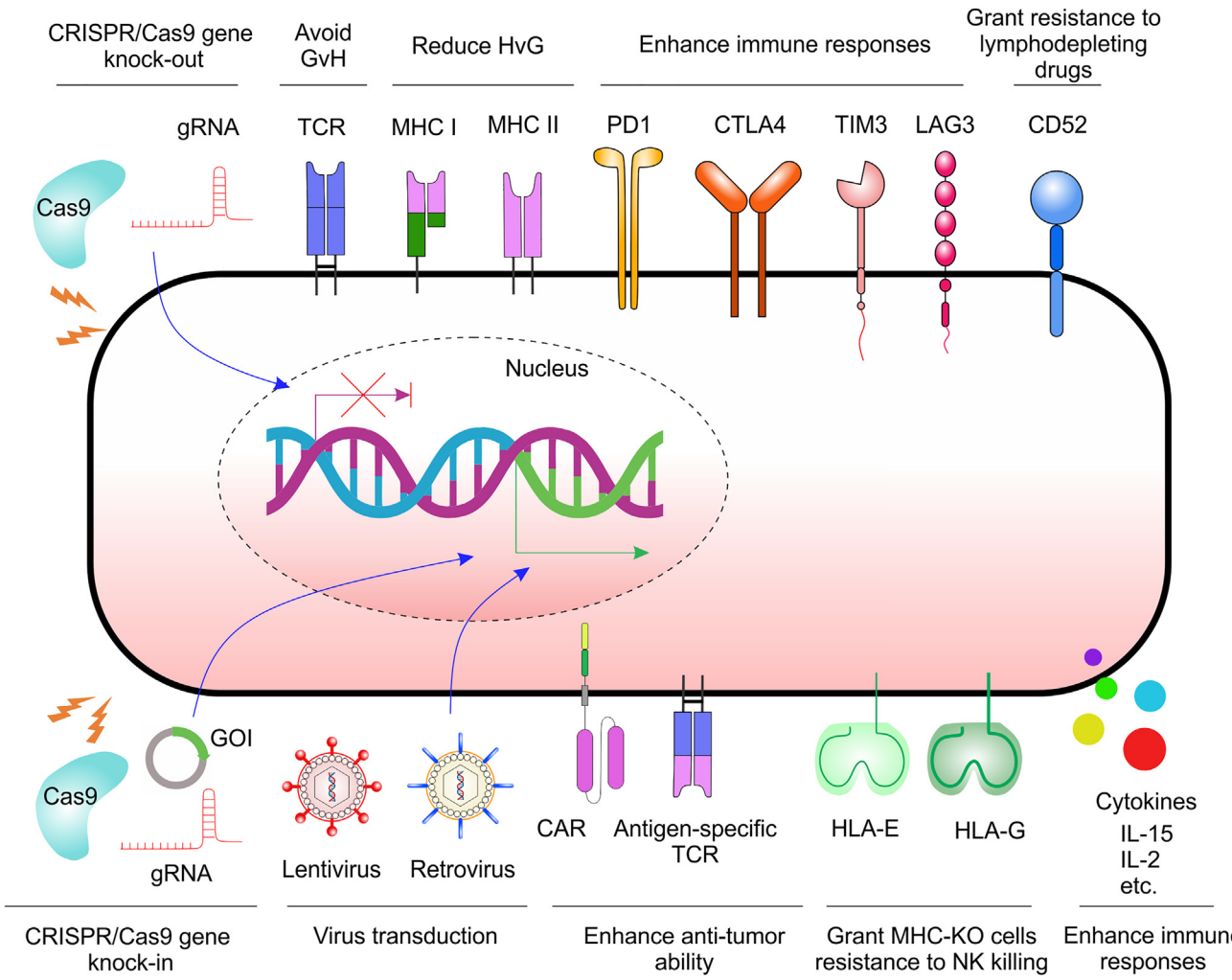

Figure 2. Applications of viral vector transduction and CRISPR-Cas9 gene editing in stem cell-based immune engineering

Lentiviral or retroviral-vector-mediated delivery of CARs, antigen-specific TCRs, and immune-enhanced genes (e.g., IL-2 and IL-15) enhance the antitumor response of immune cells. CRISPR-Cas9 gene editing enables multiple gene knockouts to avoid graft-versus-host-disease (GvHD) (e.g., knockout of TCR), reduce allorejection (e.g., knockout of MHC I and MHC II), and enhance the immune response (e.g., knockout of immune checkpoint proteins such as PD-1, CTLA-4, TIM-3, and LAG-3). In addition, CRISPR-Cas9 allows the site-specific knockin of genes of interest in target cells.

tolerance.[168] Different strategies are being pursued to resolve the issue of allorejection, and these are discussed below. Additionally, high costs and a time-consuming production process are other problems in manufacturing. Different from a vaccine or an antibody, generating human immune cells requires longer periods in a GMP environment, multiple culture and engineering steps, and specific sets of reagents and final formulations. This issue is further complicated, as each patient and condition might require a different number of cells for treatment.[170–172] Nevertheless, the rapid development of PSC-related knowledge and technology will provide more and less expensive alternatives, bypassing the current limitations in iPSC protocols.

The engineered stem cell product: Challenges and optimizations

Safety

In addition to the traditional CAR-T cell adverse events, such as CRS and neurotoxicity,[173] stem cell-derived therapies have other safety concerns that must be addressed.[174] The self-renewal property of stem cells, in particular PSCs, raises concerns about tumorigenicity.[175] It is also possible that undifferentiated and/or immature cells are retained in the final cell product. Even a few residual PSCs could result in teratoma formation.[176] In addition, tumorigenic mutations can arise during *in vitro* culture, which is prone to cause genetic alterations, such as chromosomal abnormality, copy-number variation, and single nucleotide mutations.[177] Allogeneic stem cell-based products are subject to several *in vitro* and *in vivo* tumorigenicity-associated tests.[178] Karyotyping is traditionally used to monitor chromosomal abnormalities,[179] such as chromosomal deletion, duplication, or rearrangement. Clones with such alterations are discarded. Next-generation sequencing can identify single nucleotides and other genomic alterations. The ultimate effect of these smaller genetic events on the final cell product remains controversial, and further research will indicate whether these changes should preclude utilizing specific cell products.[176]

Flow cytometry and quantitative reverse-transcriptase PCR (qRT-PCR)/droplet digital PCR are also employed to detect stem cells in the final product.[180] In products that rely on the use of iPSCs, reprogramming factors are at risk to cause tumorigenesis.[181] The Yamanaka factors, especially c-Myc, are often overexpressed and known as driver mutations of human cancers. Although current practice utilizes Sendai virus transduction of reprogramming factors for episomal expression,[182] care must be taken to ensure that the reprogramming factors are not integrated as transgenes prior to clinical development.[180,182,183] Finally, suicide switches can be included in stem cell-derived therapies to eliminate the cells in case of tumor formation.[184]

Although iPSC technology may ultimately unlock the door to autologous cell therapies of any cell type, the cumbersome and costly process of iPSC reprogramming and differentiation currently prohibits the widespread application of individualized iPSC precision medicine. Thus, stem cell-derived therapies will likely be allogeneic. An important safety concern for allogeneic cell therapies, particularly T cell-based therapies, is GvHD.[185] Our understanding of GvHD stems largely from the longstanding use of allogeneic HSCTs to treat hematological diseases.[186] It remains a major cause of patient morbidity and mortality.[187–189] Therefore, conventional $\alpha\beta$ T cell-based allogeneic therapies require genetic engineering to remove or alter the endogenous TCR. TCR KO is a common approach for creating universal allogeneic T cell therapies (such as UCART). Other methods, such as gene insertion into the T cell *TRAC* locus, can be used to eliminate the risk of GvHD.[138,150] Importantly, several alternative immune cell populations obviate the need for genetic manipulation to avoid GvHD. Germ line-encoded NK receptors and the TCRs on innate T cells do not respond to peptide-MHC mismatches.[55] A growing number of clinical trials validate the safety of these allogenic cell types for cancer immunotherapy.[190] Further support for the safety of innate and innate-like immune cells comes from extensive research identifying cellular components of allo-HSCT grafts that reduce the risk and severity of GvHD without diminishing normal immunological functions, including NK and iNKT cells.[191,192]

Immunogenicity

Immune rejection is a substantial hurdle to the successful application of allogeneic cell therapies. Immunosuppressants are traditionally used to prevent allograft rejection, for instance after organ transplantations. However, severe side effects, including infections, can result from continued immunosuppression.[193,194] When transplanted into immune-privileged tissues, such as ocular or neurological tissues, allogeneic stem cell-derived cells persist for years.[176] For non-immune-privileged areas and to avoid continuous immunosuppression, two strategies can help to prevent immune rejection in the patient: HLA haplotype banks that store human stem cells of different haplotypes and the engineering of hypoimmunogenic immune cell products.

HLA haplotyping is performed before solid organ transplantation and widely used in allo-HSCT, with millions of donors registered in worldwide bone marrow banks.[195] With over 26,000 known HLA alleles reported for humans,[196] creating stem cell biobanks that cover thousands of unique haplotypes is not feasible. However, being able to select specific HLA pairs can maximize population coverage with a minimal number of HLA haplotypes.[197] The key advantage of a haplobanking strategy is that it does not require genetic engineering. Some of the limitations are the need for unique banks for different ethnicities, a benefit for only part of the population, and incomplete immune tolerance, even with HLA matching.[176]

Notably, cells can be engineered to evade the immune system. Genetic deletion of beta 2-microglobulin (B2M) and class II major histocompatibility complex transactivator (CIITA) prevent the expression of MHC class I and II molecules.[198] Cells with genetic B2M and CIITA knockouts resist $CD8^+$ and $CD4^+$ host T cell allorejection, respectively.[198] Cells lacking MHC class I expression are subject to NK "non-self" elimination, and animal and clinical studies have reported a role for monocytes and macrophages in graft rejection.[199] Moreover, HLA class I and II knockouts were paired with CD47 overexpression in iPSCs.[200] CD47 is the canonical "don't eat me" signal and effectively reduces NK and macrophage-mediated elimination. In fully immunocompetent preclinical mouse allogeneic recipients, endothelial cells, smooth muscle cells, and cardiomyocytes derived from mouse hypoimmunogenic iPSCs were well tolerated. Human $B2M^{-/-}$ $CIITA^{-/-}$ CD47 iPSC did not incite any detectable cellular IFN-γ response or antibody response in NSG-SGM3 mice and showed long-term engraftment, whereas non-CD47-engineered hiPSCs were rejected. Alternative hypoimmunogenic cell therapies, in which iPSCs lack B2M-, CIITA-, and NK-activating ligand CD155 and express HLA-E were also tested.[201] T cells differentiated from the hypoimmunogenic iPSC lines showed longer survival than unmodified iPSC-derived T cells in the presence of allogeneic immunity and importantly were more resistant to NK killing than HLA-edited iPSC-derived T cells. Hypoimmunogenic iPSC-derived T cells engineered to express CAR displayed potent antitumor efficacy in mouse models of CD20-expressing leukemia or lymphoma. These studies indicate that hypoimmunogenic stem cell-derived therapies have the potential to produce off-the-shelf therapies that exert therapeutic benefits within reasonable dosing regimens.

Antitumor efficacy

The increasing body of clinical experience confirming the safety of cell-based cancer therapies encourages a focus on enhancing antitumor efficacy. Although these efficacy improvements are primarily developed for mature cells, progress in stem cell research will allow concurrent investigation of such innovations in stem cell-derived products.

A critical barrier to the success of engineered cell therapies is tumor recognition. In hematological malignancies, ubiquitous lineage-specific markers have engendered the striking success of CAR-T cells targeting CD19 or BCMA.[202] Importantly, the elimination of all B cells, cancerous and healthy, can be treated with immunoglobin infusions, providing an advantage over strategies that eliminate cells expressing solid tumor-associated antigens. Other hematological markers, such as CD20, CD22, CD30, CD33, and CD7, are being investigated as CAR-T cell targets.[203] Although CD19 and BCMA CAR-T cells achieve response rates upward of 90% for certain cancers, relapse is common.[140] One possible explanation for relapse after CAR19 CAR T cell therapy is modulation of the CD19 antigen on cancerous cells. For instance, genetic modification leads to partial or complete downregulation of the CD19 receptor or truncation of the protein, which then prevents binding by CD19 CAR-T cells. A lineage switching that leads to the

development of a CD19-negative phenotype can also result in relapse.[204]

Identifying tumor-specific antigens for solid tumors is more challenging. Solid tumors are highly heterogeneous, and tumor-overexpressed antigens are often present on non-disposable healthy tissues. The first wave of CAR-T cells for solid tumors has targeted HER2, EGFRvIII, mesothelin, CAIX, PSMA, and GPC3.[205] A fatal case of HER2-targeting CAR-T cells, potentially due to CAR-T cells attacking lung epithelial cells expressing low levels of HER2, tempered solid tumor CAR-T cell enthusiasm, but many clinical studies have reported tolerable safety profiles,[206] and HER2 has since been targeted safely.[207] Although antigen escape and antigen heterogeneity are two critical immunotherapy evasion mechanisms of cancer cells, several cell-engineering strategies are being implemented to create therapies that target cancer cells through multiple antigens/pathways.[208] Stem cell-derived products are uniquely positioned to provide improved tumor recognition, given their genetic pliability. Importantly, research into efficacy optimization, such as CAR design and the overexpression of immunomodulatory proteins, was first performed using mature immune cells. As such, in most cases, engineered stem cell-derived products would incorporate enhancements originally designed for mature cells. Considerations that are particularly relevant for stem cell engineering remain to be fully elucidated. Among them are, for instance, the optimal time frame to introduce the CAR and other molecules (i.e., pre- or post-differentiation), the influence of genetic alterations on stem cell differentiation, and how to design stem cell-optimized CARs.

One way to prevent antigen escape is to target multiple tumor antigens. Dual-targeting CARs have been reported, and clinical trials, predominantly for liquid cancers using CD19 × CD22 or CD19 × CD20 CARs, are currently ongoing.[25,209,210] Importantly, the evaluation of costimulatory domains and the orientation of antigen recognition modalities are necessary to produce optimized dual-targeting synthetic constructs.[211,212] An example is the synthetic Notch (synNotch) receptor-engineered cell therapy for the treatment of cancer.[213,214] synNotch receptors induce transcriptional activation after recognizing user-specified antigens. They can be used in a highly modular fashion to customize cytokine secretion profiles, differentiation, and local delivery of non-native therapeutic payloads, such as antibodies. SynNotch cellular programming has managed effective and controlled tumor cell killing by targeting antigens that are homogeneous but not fully tumor-specific in glioblastoma.[215]

The antigen sensitivity of engineered receptors can influence the control of tumor cells expressing low levels of antigen. Native TCRs initiate T cell activation after recognizing only a few peptide-MHC complexes, whereas CARs require a higher antigen load.[216] HLA-independent T (HIT) cell receptors have been specifically developed to target tumors with low antigen densities.[217] In this study, the authors edited the heavy and light chains of a traditional CAR scFv into the TRAC locus in human peripheral blood T cells in place of TCRα and β variable chains, reconfiguring the TCR, which maintained its natural CD3 engagement, to the CAR scFv target. HIT receptors consistently afforded superior antigen sensitivity and tumor recognition than CD28-based CARs, the most sensitive CAR design to date. Whether the HIT receptor is applicable to stem cell-derived T cells remains to be demonstrated.

ADCC is mediated by the Fc receptor CD16a on immune cells and is the key effector mechanism of therapeutic monoclonal antibodies.[218] Natural CD16a undergoes activation-induced surface cleavage, and different CD16a alleles have a range of antibody-binding affinities, which can limit the therapeutic benefit of CD16a expression.[219,220] Researchers incorporated high-affinity, non-cleavable CD16a expression into PSC-derived NK cells and observed enhanced antitumor activity in combination with antibody therapy.[95]

In addition to genetically engineered tumor cell recognition, several immune cell-intrinsic tumor-targeting mechanisms can be harnessed for anticancer therapy. Tumor antigen-specific T cells can be reprogrammed into iPSCs, although TCR-mediated tumor targeting of the differentiated final cell therapies will be HLA restricted.[221] The natural TCRs of iNKT, MAIT, and $\gamma\delta$ T cells endow these T cell subpopulations with innate tumor cytotoxicity.[222] Adoptive transfer of engineered and non-engineered iNKT and $\gamma\delta$ T cells for the treatment of cancer is ongoing.[160,223] NK cells and macrophages also possess inherent antitumor activities and are promising cell types for adoptive cancer treatment.[222]

Critical bottlenecks in the widespread clinical application of innate and innate-like immune cells are their scarcity, fecundity, and genetic pliability. Starting at the stem cell state mitigates these issues by exploiting stem cell-engineering techniques and expansion potential. For example, a single cord blood donation can be expanded to generate upward of ten thousand doses of HSC-derived iNKT (HSC-iNKT) cells that retain iNKT TCR functionality and tumor targeting, and furthermore, CAR engineering results in superior antitumor efficacy.[78] In 2019, Zeng et al. used an "NK cell-promoting" protocol to differentiate $\gamma\delta$ T-iPSCs, which produced "$\gamma\delta$ NK T cells" that were cytotoxic to a broad spectrum of cancers through $\gamma\delta$ TCR and NK killing mechanisms.[224] CAR-engineered, PSC-derived macrophages also exhibit potent cancer cytotoxicity *in vitro* and *in vivo*.[113] Therefore, utilizing stem cells to produce unique cell populations can potentially expand the armamentarium for cancer treatment and may be instrumental in combatting tumor antigen heterogeneity and escape mechanisms.

Persistence

Their enhanced ability to expand and persist might endow less-differentiated T cells with better antitumor effects compared with fully differentiated effector T cells.[225,226] Increasing the persistence of innate and innate-like immune cells and their stem cell-derived counterparts may be especially important, given their traditionally short lifespans. Several stem cell engineering strategies have been developed to improve the long-term durability of these cells.

Autonomous cytokine secretion is one way to boost the persistence of engineered cell products. Transducing CAR-T cells to express IL-15, IL-12, IL-2, IL-18, and other cytokines promotes *in vitro* and *in vivo* survival.[227] IL-15 signaling is especially important for the maintenance of NK and innate-like T cells[228] and has been incorporated into NK, iNKT, and $\gamma\delta$ T cell-based therapies.[40,160,229] IL-15 signaling can be potentiated by incorporating IL-15 secretion, membrane-bound IL-15 expression, and/or genetic modifications targeting the IL-15 pathway.[230–232]

Genetic knockout of cytokine-inducible SH2-containing protein (CIS; encoded by the gene CISH), a negative regulator of IL-15 signaling, in iPSC-NK cells increased IL-15-mediated Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling activity. CISH$^{-/-}$ iPSC-NK cells displayed improved *in vivo* persistence and inhibition of tumor progression in a leukemia xenograft model, which coincided with CISH KO-mediated metabolic fitness advantages.[95] Other transcription factors, such c-Jun and basic leucine zipper ATF-like transcription factor (BATF),[233,234] protect CAR-T cells from exhaustion and enhance persistence. Further research will be needed to assess the feasibility of these methods in stem cell products. Modifications to improve cell persistence and survival will have to be optimized for each stem cell product and effects on differentiation must be assessed. Thus far, IL-15 signaling modifications have not been reported to negatively influence the differentiation, production, or phenotype of stem cell-derived NK and iNKT cells.

Heterogeneity

Each PSC line differs from others in gene profiling, epigenetic status, and differentiation propensity.[176,235–237] A comparison of the differentiation potential of 17 human ESC lines found that some lines exhibited >100-fold differences in the propensity to differentiate into specific lineages.[236] Others reported distinct differentiation capacities of multiple PSC lines and indicated that PSC lines with lower differentiation potential exhibit an abnormal epigenetic status and are prone to teratoma formation.[235,238] The heterogenicity of PSC lines may limit the broad application of PSC-derived immune cell therapy. Researchers have developed approaches to convert primed into naive PSCs to eliminate PSC heterogeneity. The induction of naive ESCs was reported through treatment with a combination of five kinase inhibitors, specifically inhibitors of mitogen-activated protein kinase (MEK), glycogen synthase kinase 3 (GSK3), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), Rho-associated protein kinase (ROCK), and proto-oncogene tyrosine-protein kinase Src (SRC).[239] Equally, short-term expressions of NANOG and KLF2 reset the human pluripotent state, and the naive state of PSC cells is maintained in the presence of a protein kinase C (PKC) inhibitor.[240] Although these methods are promising, the potential loss of genetic integrity and imprinting in naive PSCs needs to be carefully considered to avoid problems.[241,242]

Therapeutic platforms and clinical trials

iPSC-derived NK cells

The use of iPSC-derived NK cells is receiving increased interest. iPSCs are a renewable cell source that can be expanded indefinitely to produce homogeneous NK cells, addressing the manufacturing and supply chain bottlenecks associated with primary NK cells. Preclinically, iPSC-NK cells have shown powerful antitumor functions against a variety of cancers in xenograft models.[85,90,95] Hermanson et al. showed that the antitumor activity of iPSC-NK cells against MA148 and A1847 ovarian tumor cells was as effective as primary NK cells.[87] In a representative study, non-KIR expressing NK cells derived from donor peripheral blood-iPSCs had greater cytotoxicity against ovarian cancer SKOV3, colorectal cancer SW480 and HCT-8, breast cancer MCF7, and head and neck cancer SCC-25 cells compared with primary NK cells.[67] Engineered iPSC-NK cells that express CARs targeting CD19, CD33, or GPC3 demonstrated improved anti-tumor efficacies against CD19$^+$, CD33$^+$, or GPC3$^+$ tumor cells.[92,243,244] Furthermore, the antigen-specific NK cell signaling and anti-tumor activity of iPSC-NK cells could be enhanced by utilizing a CAR containing the transmembrane domain of NKG2D.[85] iPSC-NK cells with a deletion of the IL-15 signaling regulatory protein CISH demonstrated an improved metabolic profile, increased expansion and persistence, and enhanced cytotoxicity in human AML xenograft tumor models.[95]

Phase I clinical trials are underway for universal off-the-shelf iPSC-NK cell products, including several of Fate Therapeutics' iPSC-NK cell therapies (Clinicaltrials.gov Identifier NCT03841110, NCT04023071, NCT05182073, and NCT04245722). Fate Therapeutics showed the safety and tolerability of allogeneic iPSC-NK cells in liquid and solid cancer patients[245] and has advanced to clinical investigations with engineered iPSC-NK cell products. iPSC-NK cells engineered with CD19-targeting CAR, high-affinity, non-cleavable CD16 Fc receptor, and IL-15/IL-15 receptor fusion promoting cytokine-autonomous persistence iPSC NK cell therapy, showed promising results for treating B cell lymphoma,[246] with 13 of 19 patients achieving an OR with a single dose of the cell therapy. In multiple myeloma patients, Fate Therapeutics' BCMA-targeting cell product resulted in 10 of 14 patients achieving objective responses. These trials provide clinical support for the high tolerability of allogeneic iPSC-NK cell therapies and show signs of antitumor efficacy.

Besides Fate Therapeutics, several companies are developing next-generation iPSC-NK cells for cancer treatment. Shoreline Biosciences generate CISH knockout iPSC-NK cell products with increased durability and activity for use in hematologic and solid tumor contexts.[95] Century Therapeutics develop iPSC-NK cells with multiple targets such as CD19, CD19 × CD79b, CD133 × EGFR, and Nectin-4 to treat B cell malignancies, glioblastoma, acute myeloid leukemia, and other solid tumors.[247] Overall, by being easily engineered, cultivated on a large scale, and adapted to diverse cancers with high safety, iPSC-NK cells have become a viable alternative to conventional CAR-T cells for cancer immunotherapy.[29,97,113,248–250] Nevertheless, *in vivo* persistence and viability of iPSC-NK cells, as well as their efficiency in conjunction with other immune checkpoint inhibitors, are still unclear and must be elucidated before iPSC-NK cell therapies can be widely used in the clinic.

iPSC-derived immune cells

In 2013, two Japanese research groups generated rejuvenated iPSC-derived, antigen-specific T cells.[64,65] Human HIV-1 or MART-1-specific CD8$^+$ T cells were reprogrammed to pluripotency by transducing retroviral vectors encoding OCT3/4, SOX2, KLF4, and c-MYC. The T-iPSCs were then redifferentiated into CD8$^+$ T cells, which displayed the same antigen-specific killing activity and TCR rearrangement pattern as the original CD8$^+$ T cell clone from the patient.[63–65] A safeguard system, inducible caspase-9, was introduced into the iPSCs to ameliorate the tumorigenic potential of undifferentiated iPSCs.[251] Using a similar technology, Wakao et al. reprogrammed human MAIT cells into iPSCs and redifferentiated the iPSCs to MAIT cells with antimycobacterial activity.[66] In the same year, Themeli et al. combined T-iPSC and CAR technologies to develop CD19 CAR-T to treat B cell malignancies.[75] These CD19

CAR-T cells displayed a phenotype closely resembling that of γδ T cells.[75] Following the T-iPSC technology, several groups generated rejuvenated iNKT,[62] NK cells,[67] and DCs[111] from reprogrammed iPSCs. Owing to the unlimited availability of iPSCs, these technologies provide a valuable source of off-the-shelf, allogeneic cell products. Further research also confirmed the translational relevance of iPSC-based strategies. In 2018, Minagawa et al. utilized CRISPR-Cas9 to delete *RAG2* in T-iPSCs and prevent an unwanted TCR rearrangement and then transduced the T-iPSCs with an antigen-specific TCR to endow T cells with tumor-targeting capacity.[252] The resulting TCR-stabilized, regenerated cytotoxic T cells displayed an effective antitumor ability in xenograft cancer models.[252] In 2021, the same group reported a clinically applicable and scalable technology to regenerate T cells from iPSCs derived from an antigen-specific cytotoxic T cell clone or from TCR-transduced iPSCs, as starting materials.[125] A feeder-free, serum-free differentiation culture protocol was also developed to achieve an efficient iPSC differentiation procedure that can be adapted toward clinical application.[125] Overall, these are promising approaches to generate large numbers of tumor-targeting immune cells for the study of T cell differentiation and potential clinical application.

iPSC-derived CAR-T cells

iPSC-derived CAR-T cells have the potential to be an infinite source of phenotypically defined, expandable, and functional CAR-T cells for off-the-shelf cancer therapy.[253] However, compared with iPSC-NK cells, the generation of iPSC-derived CAR-T cells has been challenging and typically requires a preexisting TCR that directs *in vitro* T cell differentiation.[61,64,65,80,252] In addition, T cell differentiation requires notch ligand engagement and can be impaired by CAR expression.[75]

Recently, highly functional CAR T cells were generated through iPSC reprogramming from $CD62L^+$ naive and memory T cells, followed by CD19-CAR engineering and MS5-DLL4 stromal cell-dependent 3D-organoid system differentiation.[254] The primary $CD62L^+$ T cells have superior persistence and therapeutic potential in CAR-T cell treatment, and the pre-existing TCR induces T cell differentiation in a directed manner.[226] The resulting iPSC-derived CD19 CAR-T cells demonstrated conventional αβ T cell phenotypes, homogeneous TCR repertoire, and strong antitumor reactivity.[254] Another iPSC-derived CAR-T cell product was generated by combining histone methyltransferase EZH1 repression, stromal-free T cell differentiation from iPSCs, and CAR engineering.[255] Repression of EZH1 promotes *in vitro* differentiation and maturation of T cells derived from iPSCs. The mature iPSC-T cells are similar to peripheral blood αβ T cells in phenotype and functionality.[255] In addition, CAR-engineered iPSC-T cells showed enhanced cytokine production, potent cytotoxicity, and superior persistence in preclinical mouse models.[255] Overall, these iPSC-derived CAR-T cell platforms lay the groundwork for future efforts targeted at creating an infinite number of potent, allogeneic off-the-shelf CAR-T cells.

HSC-derived iNKT cells

iNKT cells are another potentially promising cell population for cancer immunotherapy. However, the low frequency and high variability of iNKT cells in humans limit their clinical applications.[256] To overcome these challenges, an HSC-iNKT cell platform was developed (Figure 3A). The first generation was based on autologous HSC genetically engineered to express the iNKT TCR that were transferred back into the patient, potentially providing therapeutic levels of iNKT cells for a lifetime (Figure 3B).[105] However, this approach is expensive and difficult to deliver to all patients in need.[37] Subsequent development of the HSC-iNKT cell platform has centered on producing off-the-shelf HSC-iNKT cells, and it was recently shown that HSC engineering followed by *in vitro* differentiation resulted in allogeneic HSC-iNKT cells with high yield and purity (Figures 3C and 3D).[78,257] Allogeneic HSC-iNKT cells have innate cancer-killing capacity and a low risk of GvHD, and to enhance their therapeutic potential, these cells can be engineered to express CARs and undergo ablations of HLA classes I and II.[78] Furthermore, HSC-iNKT cells can modulate the tumor microenvironment, as they can effectively target and eliminate tumor-associated macrophages and other immunosuppressive cells.[104,258,259]

Conclusions

The remarkable success of CAR-T cells in treating hematological malignancies has ignited the field of cell therapy. In 2012, at the age of 6 years old, Emily Whitehead was the first patient to receive CAR-T cells for acute lymphoblastic leukemia and recently celebrated 10 years of cancer-free survival. By all evidence, she is cured.[260] Our goal is to make Emily's story a reality for all cancer patients. However, CAR-T cell therapies are limited by their autologous nature, suboptimal long-term efficacy for many patients, and lack of potency in solid tumors. Off-the-shelf cell therapies that overcome tumor antigen plasticity, heterogeneity, and the immunosuppressive tumor microenvironment can advance the current cell therapy paradigm. Work to date suggests that many immune cell subtypes, such as innate-like T cells, NK cells, and macrophages, might be useful in cancer therapy. The benefits of applying unconventional T and innate immune cells are 2-fold: they have intrinsic antitumor capabilities and pose little risk of causing GvHD. Unfortunately, isolation, gene engineering, and expansion of mature immune cells still represent several bottlenecks in the development of these therapies. Maintaining the desired cell phenotype during expansion, generating sufficient cell numbers for multiple doses, and performing extensive genetic manipulation are all remaining challenges.

Herein lies the allure of stem cell engineering, which promises scalable, easily modifiable, and homogeneous cell products that are optimized for safety and efficacy. As of yet, hypoimmunogenic, persistent, and potent stem cell-derived therapies are under development. Despite this promise, several outstanding questions still need to be addressed. It was recently shown that iPSC-derived CAR-T cells exhibit a reduced CAR expression compared with PBMC-derived CAR-T cells.[254] In addition, previous work indicated that αβ iPSC-derived CAR-T cells are closely related to γδ T cells based on the gene expression analysis.[75] It is noteworthy that multiple stem cell engineering and differentiation protocols have been documented, which have the potential to yield considerable variability in the resultant cell products, even when such products are classified as being of the same cellular subtype. We also do not know whether cells created *in vitro* are comparable with their naturally occurring counterparts. Stem cell-derived products might be more

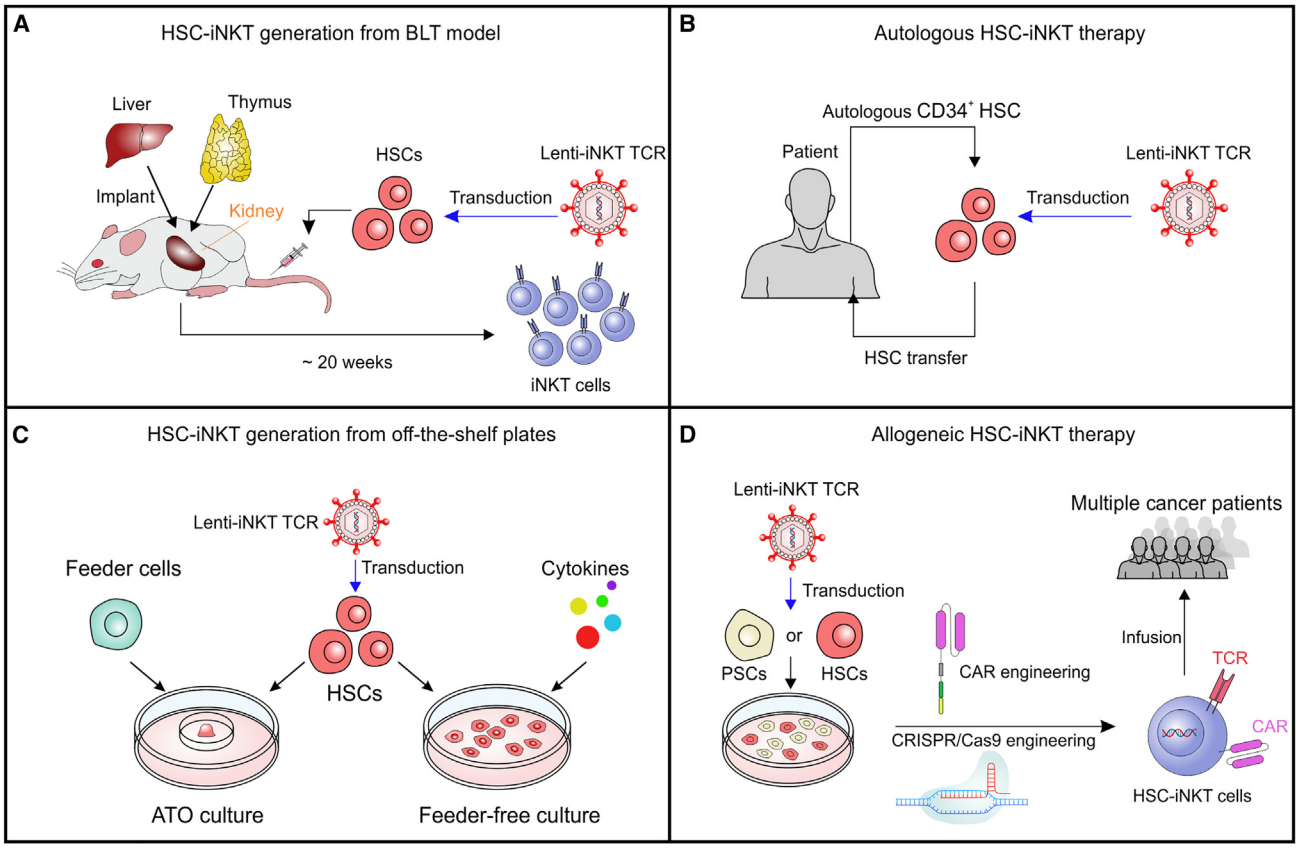

Figure 3. Development of HSC-engineered iNKT (HSC-iNKT) cell therapies for cancer HSC-iNKT cells are presented as an immune cell example

The different stem cell differentiation and culture strategies could be easily applied to generate other TCR-engineered T cells, such as MAIT, γδ, and antigen-specific αβ T cells. The proposed autologous and allogenic cell therapy could also use other TCR-engineered T cells as cell carriers, depending on the tumor types.

(A) Generation of HSC-iNKT cells in a bone marrow-liver-thymus (BLT) humanized mouse model.

(B) Development of an autologous HSC-iNKT cell therapy for cancer.

(C) Generation of allogeneic HSC-iNKT cells in an ATO or a feeder-free culture.

(D) Development of an allogeneic HSC-iNKT cell therapy for cancer. CAR engineering and CRISPR-Cas9 gene editing could be incorporated into HSC-iNKT cells to enhance their immune response and safety profile.

affected by the changes in gene regulation, such as epigenetic silencing. Importantly, most of the engineering and optimization strategies applied to stem cells were developed in mature T cells. Are there any modifications, such as different CAR designs or cytokine secretion patterns, that could enhance specific features of stem cell-derived products? It will also be important to assess whether stem cell engineering influences the differentiation potential, identity, and function of the final product. CARs, for example, can cause tonic signaling, which could impact stem cell differentiation. Leveraging cutting-edge technologies, such as multi-omics, CRISPR screens, and automated culture systems, will enable researchers to better understand the underlying mechanisms of engineered stem cell functionality and perform high throughput screens to optimize the potential of stem cell therapies.[261,262]

Given the complexity of diseases such as cancer, combination therapies will likely be necessary to achieve durable therapeutic benefits.[263] Engineered stem cell products, with their off-the-shelf nature, hold promise as a component of treatment regimens that include established cancer treatments, such as surgery, radiation, chemotherapy, and targeted therapies, as well as emerging cancer immunotherapies, such as immune checkpoint inhibitors, oncolytic viruses, cancer vaccines, and adoptive cellular therapies.[15,56] The versatility of engineered stem cell products and their ability to be tailored and scaled for specific cancer types make them a promising addition to the oncology arsenal, with potential to enhance treatment outcomes and improve patients' quality of life. As the field continues to evolve, it is crucial that we maintain a cautious and meticulous approach to ensure the safe and effective translation of stem cell-based therapies to clinical settings.

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AUTHOR CONTRIBUTIONS

Y.-R.L. and Z.S.D. wrote the manuscript with assistance from Y.Y. and M.L. L.Y. and P.W. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

Y.-R.L., Z.S.D., P.W., and L.Y. are inventors on patents relating to this manuscript. Y.Y. is currently an employee of the Fate Therapeutics. P.W. is a co-founder, stockholder, consultant, and advisory board member of HRain Biotechnology, TCRCure Biopharma, and Appia Bio. L.Y. is a scientific advisor to AlzChem and Amberstone Biosciences and a co-founder, stockholder, and advisory board member of Appia Bio. None of the declared companies contributed to or directed any of the writing of this manuscript.

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