Title

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Journal

Trends in Biotechnology, 41(7)

ISSN

0167-7799

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Publication Date

2023-07-01

DOI

10.1016/j.tibtech.2023.02.003

Trends in Biotechnology

CellPress

Review

Induced pluripotent stem cell-derived engineered T cells, natural killer cells, macrophages, and dendritic cells in immunotherapy

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T cells, natural killer (NK) cells, macrophages (Macs), and dendritic cells (DCs) are among the most common sources for immune-cell-based therapies for cancer. Antitumor activity can be enhanced in induced pluripotent stem cell (iPSC)-derived immune cells by using iPSCs as a platform for stable genetic modifications that impact immuno-activating or -suppressive signaling pathways, such as transducing a chimeric antigen receptor (CAR) or deletion of immunosuppressive checkpoint molecules. This review outlines the utility of four iPSC-derived immune-cell-based therapies, highlight the latest progress and future trends in the genome-editing strategies designed to improve efficacy, safety, and universality, and provides perspectives that compare different contexts in which each of these iPSC-derived immune cell types can be most effectively used.

Highlights

Induced pluripotent stem cells (iPSCs) provide a platform to generate clonal engineered immune cells on a large scale and have been leveraged and highlighted as one of the most immediate and exciting applications in the development of cancer immune cell therapies.

Immunoenhancing engineering strategies for iPSC-derived immune cells include antigen recognition, effect cell function activation, expansion and persistence, trafficking, immunomodulation, and metabolic fitness, and have greatly enhanced iPSC applications in cancer immunotherapy.

Hypoimmunogenicity engineering strategies for iPSC-derived immune cells focus on the avoidance of graft-versus-host disease and allogeneic cell rejection by the recipient's immune system.

Careful consideration is warranted for how to use iPSC-derived immune cell types such as T cells, natural killer cells, macrophages, and dendritic cells most advantageously in different biological and clinical contexts.

iPSC-derived immune cells herald a new era in immunotherapy

Adoptive cell therapies that infuse therapeutic immune cells to patients have shown efficacy in treating malignant tumors that are refractory to other treatments. Various immune cells in the hematopoietic system are worth exploring, but here, we are most interested in the important players in cancer immunology, namely, T cells, NK cells, Macs, and DCs. Their basic roles in immunosurveillance and tumor elimination are summarized in Box 1.

However, the use of these primary immune cells has demonstrated limitations in preclinical assessments and clinical trials. For example, autologous peripheral blood (PB)-derived CAR-T cells have demonstrated powerful cytotoxicity against B cell malignancies, but they may cause **cytokine release syndrome (CRS)** (see Glossary) and **immune effector cell-associated neurotoxicity syndrome (ICANS)** [1]. Moreover, high cost from autologous preparation poses a barrier to wider clinical application. Allogeneic T cells offer a wider range of cell sources and can be a lower cost 'off-the-shelf' product. However, they can lead to life-threatening **graft-versus-host disease (GVHD)** and may be quickly removed from the recipient body without depleting **T cell receptor (TCR)** and human leukocyte antigens (HLAs) [2]. NK cells have a lower risk of CRS and ICANS due to a shorter lifespan and distinct cytokine release spectrum upon immune activation [3] and a lower risk of GVHD due to the absence of TCRs that mediate the attack on recipient cells. However, PB- and cord-blood-derived NK cells are donor dependent and nonrenewable. Genetic modification of primary NK cells also has limited efficiency, causing a heterogeneous population of the product cells. The cell line NK-92 can proliferate indefinitely and the cells are easier to manipulate. However, they do not express Fc receptor

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Box 1. An introduction to the roles of T cells, NK cells, Macs, and DCs in immune defense

T cells, especially typically cytotoxic αβTCR$^+$CD8αβ$^+$ cells, are key effectors in immune surveillance. They directly attack cancerous and/or pathogen-harbored cells by releasing inflammatory cytokines and cytolytic molecules (e.g., granzyme B and perforin). T cells proliferate and perform effector functions when αβTCRs recognize specific antigens in an HLA-restricted manner [61].

NK cells, whose cytotoxic activity is functionally similar to CD8$^+$ T cells, belong to innate lymphoid cells, and treat target cells depending on the net balance between activating signals and inhibitory signals transmitted on the surface of NK cells [62].

Macs are classically considered as important phagocytic effector cells during immune defense, as these cells have the capacities to directly phagocytose tumor cells, infiltrate tissues, and enhance endogenous T cell functions through antigen presentation [63]. They may exert protumor or antitumor effects in response to environmental cues [64].

DCs, though constituting a rare population of immune cells within tumors and lymphoid tissues, are professional APCs that directly present or cross-present TAAs to T cells to initiate immune responses [65].

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CD16a (FcγRIIIA) [4], an essential molecule for **antibody-dependent cell-mediated cytotoxicity (ADCC)**, and they need to be irradiated prior to clinical use, which limits their overall survival and expansion *in vivo* [4]. Primary Macs and primary DCs are mainly derived from PB mononuclear cells (PBMCs) and they are particularly resistant to gene modification approaches, due to host restriction factors, such as SAMHD1 [5]. Although the use of unmodified autologous DCs as tumor vaccines such as sipuleucel-T has been approved by the FDA for the treatment of prostate cancer, only a 4.1-month improvement in median survival has been obtained [6]. Thus, new sources with unlimited capacity to generate editable and off-the-shelf therapeutic cell products are more desirable.

iPSCs are derived from somatic cells by expression of reprogramming factors, including OCT3/4, SOX2, KLF4, MYC, NANOG, and LIN28 [7,8]. Since the first report in 2006, iPSCs have been developed as a new avenue to generate useful transplantable antitumor immune cells from genome-edited clonal seed cell lines (Figure 1), due to their essentially infinite proliferative capacity, ease of editing, and potential to differentiate into various immune cell types. The novel source showed promising signs in overcoming previous challenges of primary cells such as heterogeneity in genome editing efficiency, and incompatibility of standardization of cell product manufacturing. Furthermore, the past few years have witnessed much improvement in our understanding of immune cell differentiation dynamics, and various genome editing strategies to enhance the immune functions and to reduce the immunogenicity in iPSC-derived immune cells. Thus, we highlight the latest progress in genome editing strategies designed to improve efficacy and universality, and compare different contexts in immuno-oncology in which each of these iPSC-derived immune cell types can be most effectively used.

Engineering strategies for iPSC-derived T cells (iT cells)

Engineering TCRs in iT cells

Typically, T cell activation requires the recognition and binding of the cognate antigenic peptide presented by **MHC** molecules by TCRs, the engagement of coreceptors, and the initiation of costimulatory signaling. To redirect the specificity of T cells against a particular antigen, one approach is to engineer T cells with a TCR construct specific for that antigen. TCR-engineered primary T cells targeting melanoma antigen MART1 [9] and germline antigen NY-ESO1 [10] have demonstrated efficacy in clinical trials. Studies to engineer TCRs to generate potent T cells from iPSCs have produced exciting results. For example, NY-ESO-1 TCR-engineered naive T cells showed significant antigen-specific cytotoxicity against NY-ESO-1-expressing K562 leukemia cells at effector-to-target ratios as low as 1:32 [11]. These cells were also capable of slowing the growth of subcutaneously implanted NY-ESO-1 expressing K562 tumors in **NSG**

mouse models [11]. TCR-mediated therapies from iT cells have been performed either by inheriting the endogenous TCR genes of the parental antigen-specific T cells [12–14] or by genetically loading exogenous TCRs [11,15]. Of note, the original antigen specificity of **T-iPSC**-derived T cells may be compromised as they are frequently subjected to additional TCRα assembly during differentiation and become polyclonal TCR-expressing T cells [12–15]. For example, up to 40% of GPC3-T-iPSC-derived CD8αβ T cells lost their antigen specificity due to rearranged TCRs [15]. The first possible solution is to stimulate TCR signaling, which blocks the expression of ***RAG*** that mediates TCR rearrangement, during differentiation [13]; the second solution is to knock out *RAG2* via **CRISPR/Cas9 systems** in T-iPSCs (Figure 2A) [15,16], which prevents undesired TCR rearrangement entirely. Alternatively, iPSCs can be transduced with any exogenous TCR of interest (Figure 2B) [15,16]. One advantage of this approach is that it facilitated TCRαβ T cell generation, compared with nontransduced iPSCs [16]. Another advantage is improved TCR stability, as monoclonal T cells derived from TCR-transduced iPSCs tend to solely express the fully rearranged exogenous TCR through the mechanism of **allelic exclusion**, and without TCR mispairing that usually occurs in TCR-transduced primary T cells [15]. The authors argued that the TCR stability likely made WT1-TCR engineered non-T-cell-derived iT cells to have comparable or even better efficacy than WT1-TCR-engineered PB-T cells for the treatment in a WT1-overexpressing PC9 xenograft model [15]. However, other factors should also be considered, such as the rejuvenated features of iT cells obtained during the reprogramming process, versus the loss of stem-cell-like memory T cell phenotype of the primary PB-T cells caused by prolonged *ex vivo* expansion and culturing.

iPSC-derived CAR-T cells (iCAR-T cells)

TCR-mediated therapies have achieved favorable outcomes, but their effectiveness depends on the expression of ideal target antigens and HLA molecules on tumor cells. Moreover, TCR insertion alone without costimulation may not be sufficient to trigger an antitumor response [17]. It is an alternative solution to introduce a CAR, whose structure and design follow certain principles (Box 2). Adding a CAR to iPSCs or directly to iT cells to derive antigen-specific T cells (Figure 2C) has demonstrated good outcomes [16,18–21]. CD19 is a widely targeted antigen in treating B cell malignancies. CD19-CAR-T-iPSC-derived T cells delayed $CD19^+$ Raji lymphoma progression as effectively as PB CD19-CAR **γδT cells** [18]. CD19-CAR iT cells armed with a membrane-bound chimeric IL-15 showed antigen-dependent cytotoxicity against $CD19^+$ NALM-6 (a B cell precursor leukemia cell line) *in vitro* and enhanced the overall survival rate to an extent similar to CD19-CAR PB-T cells [16]. However, these CD19-CAR iT cells lacked the $CD4^+$ single-positive cell population, which may account for the inferior effect compared with primary CAR-T cells, suggesting that further differentiation optimization may be needed (discussed later). To further enhance functions of iCAR-T cells, additional engineering strategies have been employed. For instance, to reduce the possibility of antigen escape, dual-antigen targeted iCAR-T cells were designed to simultaneously target **LMP1** and **LMP2** antigens, which contributed to enhanced cytotoxicity against Epstein–Barr virus (EBV)-associated lymphomas [19].

The field has also been moved forward by clinical studies, such as studies related to the development of FT819 (Table 1), one of the first off-the-shelf iCAR-T-cell therapies, which was engineered by knocking the CD19-CAR gene into the *TRAC* locus to eliminate TCR expression in the final product. FT819 also included CD16 in its design so that the addition of rituximab [anti-CD20 monoclonal antibody (mAb)] could result in ADCC against CD20-expressing cancer cells to obtain enhanced therapeutic efficacy. The interim Phase 1 clinical data of FT819 were published in the American Association for Cancer Research Annual Meeting, which assessed the safety, antitumor activity and pharmacokinetics of FT819 in patients with relapsed/refractory B cell lymphoma and leukemia (Table 1) [22].

Glossary

Allelic exclusion: a characteristic of immunoglobulin gene in that the expression of an allele is from one chromosome of a pair, which guarantees TCR monoclonal expression in T cells.
Antibody-dependent cell-mediated cytotoxicity (ADCC): an immunoactivation process in which the Fab segment of an antibody binds to the antigenic epitope of virus-infected cells or tumor cells, and its Fc segment binds to the FcR on the surface of killer cells, mediating direct killing of target cells.
Antigen cross-presentation: generally, endogenous antigens are presented on MHCI molecules to activate $CD8^+$ T cells, and exogenous antigen is presented on MHCII molecules by APCs to activate $CD4^+$ T cells; antigen cross-presentation means exogenous antigen is presented on MHC I molecules by APCs to activate $CD8^+$ T cells.
***B2M*:** an HLA-I-related gene encoding a critical subunit β2M of HLA-I; HLA-I is responsible for $CD8^+$ T cell activation.
***CIITA*:** an HLA-II-related gene encoding a trans-activator protein involved in HLA-II expression
CRISPR/Cas9 system: clustered regularly interspaced short palindromic repeat/CRISPR associated protein9 system; the system can edit genome by using a Cas9 nuclease and a single-guide RNA.
Cytokine release syndrome (CRS): a life-threatening syndrome with excessive production of various proinflammatory cytokines in body fluids, including IL-6 and IL-2, mainly caused by severe stimuli (such as infection or CAR-T therapy).
Cytotoxic T lymphocytes (CTLs): most CTLs are $CD4^-CD8\alpha^+CD8\beta^+$ αβ T cells, often with a TCR targeting a specific antigen in an HLA-restricted manner and have the ability to kill target cells directly.
γδT cells: a relatively small subpopulation of T cells whose TCRs are composed of γ and δ chains that recognize antigens through γδTCRs in an HLA-unrestricted manner.
Graft-versus-host disease (GVHD): a condition in which transplanted immunocompetent T cells attack the host; usually occurs after allogeneic transplantation.
***HLA-E*:** a gene encoding HLA class I molecule that interacts with the inhibitory CD94/NKG2A complex on NK cells to prevent NK activation.
HLA-null-mediated NK-cell activation: abnormal cells that fail to

Issues of T cell lineage specification of iT/iCAR-T cells

Engineering strategies not only affect immune functions of T cells, but also impact T cell lineage commitment during differentiation. Various differentiation culture systems can impact both T cell lineage commitment and immune functions. The differentiation methods to generate human iT cells include the 2D delta-like ligand (DLL)1/DLL4-expressing stroma system [12–15,18], 3D artificial thymus organoid (**PSC-ATO**) system [11,21], and 2D stroma-free system [16,20]. However, T-iPSC/CAR-T-iPSC-derived T-lineage-committed cells from the 2D DLL1/DLL4-stroma system exhibited undesirable innate/γδTCR-like features, including $CD4^{-}CD8\alpha\beta^{-}$ and $CD8\alpha\alpha^{+}$ phenotype [14,18] and expression of NK cell surface markers [14,15,18]. One explanation is that the formation of $CD4^{+}CD8\alpha\beta^{+}$ double-positive cells was disturbed by the double-negative cells during differentiation [14]. Premature αβTCR signaling from T-iPSCs or constitutively expressed CARs can interfere with double-positive cell formation as well [23]. It was shown that delayed expression and downregulated signal strength of CAR allowed TCR^{-} iCAR-T cells to divert from the default innate-like fate and acquire the conventional $CD8\alpha\beta^{+}$ phenotype [23]. The 3D PSC-ATO system could address the issue of T cell differentiation interference by CAR introduction, as it allowed generation of the conventional $CD8\alpha\beta^{+}$ iCAR-T cells with comparable antitumor effects and cytokine secretion pattern upon activation compared with PB-CAR-T cells [11,21]. It has been reported that the 3D PSC-ATO system reduced the CAR expression and T cell differentiation by inducing hypermethylation of the CAR transgene promoter [21]. To avoid the use of xenogeneic feeder cells, a stroma-free system based on immobilized DLL4 was developed and it allowed faithful T cell development [20]. Coupling this system to knocking down EZH1, a suppressor of lymphoid commitment, favored differentiation into αβTCR iT cells with mature molecular signatures rather than innate γδTCR iT cells [20].

Supportive molecules such as the cytokine interleukin (IL)-15 can enhance iT cell or iCAR-T cell therapeutic effects *in vivo* [21]. However, the choice of cytokines and the supplementation window are worth noting because some molecules interfere with cell fate. For example, as an NK-promoting cytokine, IL-15 induced more innate-like iT cells that lost the capacity of expansion at the maturation stage [24]. Of note, massive expansion by antigen stimulation may not mean good quality either, because it may lead to more differentiated T cell phenotype and compromised *in vivo* persistence and antitumor effects [24].

Engineering strategies for iPSC-derived NK cells (iNK cells)

Enhanced ADCC of NK cells

mAb-mediated ADCC is a key effector mechanism of NK cells to target tumor antigens and it is mediated by the Fc receptor CD16a (FcγRIIIA) on human NK cells. CD16a is susceptible to removal by ADAM17 upon immune activation [25] and its variants have different binding affinity to IgG [26]. Thus, a promising approach to enhance the ADCC of NK cells was to identify and mutate the cleavage site and screen for high-affinity variants (Figure 3A). The S197P amino acid substitution confers resistance to ADAM17 cleavage to CD16a [27]. Additionally, ADAM17 cleavage-resistant CD16a is further modified into a high-affinity noncleavable form (hnCD16) through single amino acid substitution (F158V). In combination with rituximab (anti-CD20 mAb), hnCD16 iNK cells were demonstrated to have more potent antitumor activity with a significantly higher survival rate in a $CD20^{+}$ human B cell lymphoma tumor model compared with PB-NK cells and unedited iNK cells that were also combined with rituximab [27]. CD64 (FcγRI) is a Fc receptor that binds to the same IgG isotypes as CD16a, but with a 30-fold higher affinity. Therefore, a high-affinity recombinant receptor CD64/16a was created by replacing the extracellular domain of CD16a with CD64 that does not contain an ADAM17 cleavage site [28]. CD64/16a iNK cells in combination with trastuzumab (anti-HER2 mAb) or cetuximab (anti-EGFR1 mAb) demonstrated strong cytotoxicity against $EGFR^{+}/HER2^{+}$ SKOV3 cells [29]. The great advantage of ADCC-enhanced

express MHCI molecules may incur NK cell attack, as MHCI ligands can interact with inhibitory receptors on NK cells.

Immune effector cell-associated neurotoxicity syndrome (ICANS): a neuropsychiatric and syndrome that can develop after receiving certain immunotherapies, such as CAR-T therapies; some cases of ICANS can be fatal.

LMP1 and LMP2: latent membrane proteins 1 and 2, expressed in most EBV-associated lymphoproliferative diseases.

MHC: MHC is a cluster of genes encoding major histocompatibility antigens in animals, whose products are closely related to the occurrence of a specific immune response. Human MHC is called HLA (human leukocyte antigen).

NSG mouse models: immunocompromised mouse models ideal for human tissue transplantation, which lack T cells, B cells, and NK cells.

On-target, off-tumor toxicity: antigen-target therapy is based on tumor expression of specific antigens, but it often causes injury to normal tissue expressing the same antigen.

PSC-ATO: an artificial thymic organoid culture system in which human PSCs form embryonic mesodermal organoids before undergoing hematopoietic differentiation.

***PVR*:** an NK cell ligand gene encoding a signal ligand that interacts with $DNAM\text{-}1^{+}$ NK cells and subsequently results in T cell death.

***RAG*:** recombination activating genes, including *RAG1* and *RAG2*. RAG1 and RAG2 form a recombinant enzyme necessary for TCR rearrangement.

Regulatory T cells (Tregs): a specialized subset of T cells that maintain self-tolerance and homeostasis by suppressing the immune response.

Single-chain variable fragment (scFv): a genetically engineered fusion protein composed of the variable region of the antibody heavy chain and the variable region of the light chain connected by a peptide chain.

T cell receptor (TCR): TCRs are heterodimers that are composed of either an α-chain and a β-chain, or a γ-chain and a δ-chain noncovalently associated with the CD3 complex on the cell surface, whose role is to specifically recognize antigens.

T-iPSCs: iPSCs that are reprogrammed from T cells, usually inherit the TCR from the parental T cells.

iNK cells is that there is no need to develop CAR-expressing cell lines for each type of tumor or target, as iNK cells can be combined with off-the-shelf antibodies, which can provide a wider application for different types of tumors. The corresponding product FT516 (hnCD16 iNK cells) has been assessed in clinical trials to treat COVID-19, hematological malignancies, and solid tumors (Table 1).

Type I interferons (IFNs): polypeptides that affect innate and adaptive immune responses; typical of these are IFN-α and IFN-β.

iPSC-derived CAR-NK cells (iCAR-NK cells)

CAR-T cell therapy has been approved by the FDA, but challenges arising from high costs for autologous cell manufacturing and the toxicities such as CRS have led investigators to seek other cell therapeutic modalities with reduced cost and less risk of toxicity, while maintaining sufficient efficacy. CAR-expressing NK cells have been developed as a promising approach in these

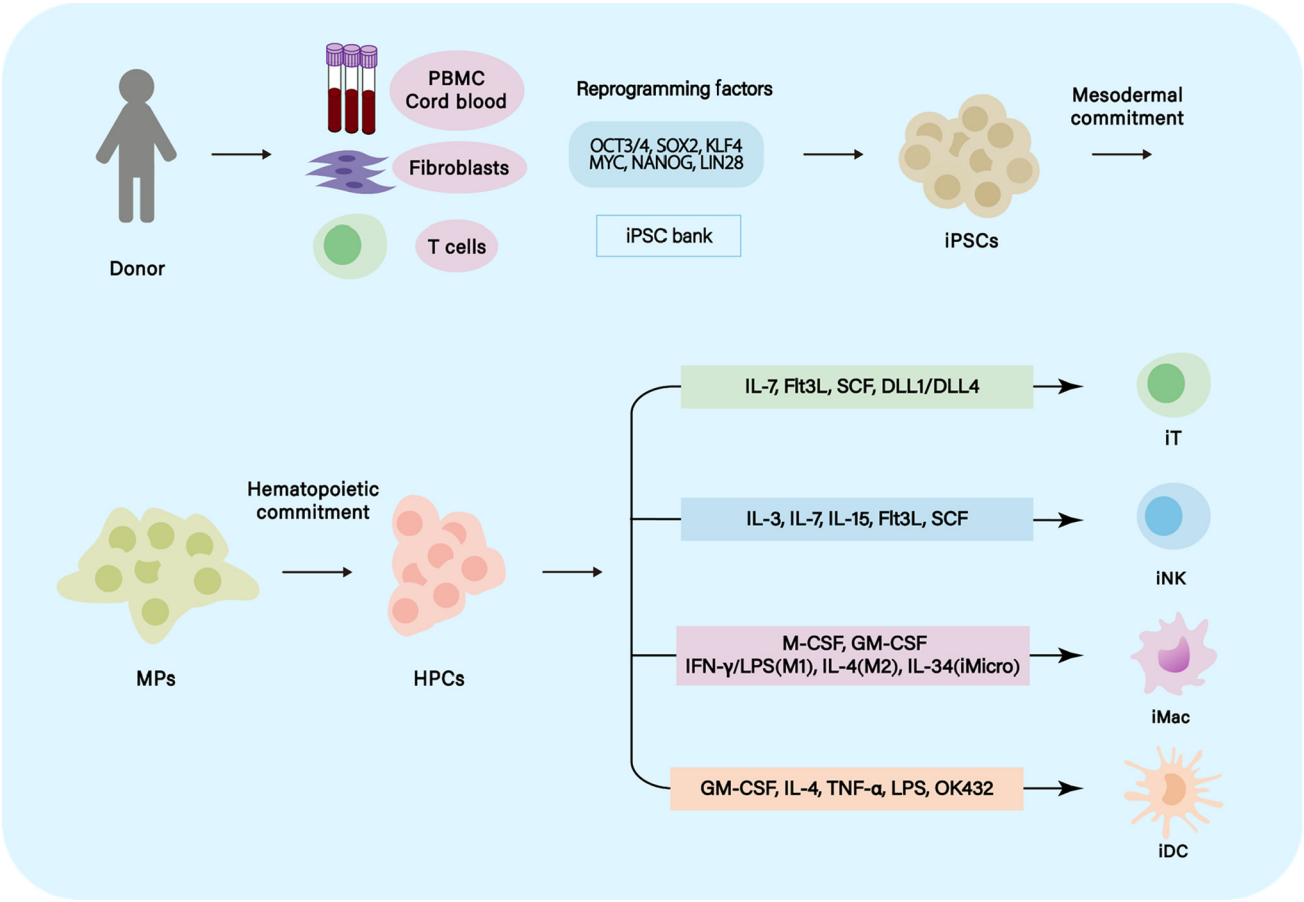

Figure 1. Overview of iPSC-derived immune cell production. iPSCs are derived from donor somatic cells by the introduction of reprogramming factors, including OCT3/4, SOX2, KLF4, MYC, NANOG, and LIN28. First, iPSCs undergo mesoderm induction upon the loss of pluripotency-related gene expression as well as increased expression of mesodermal genes to form multipotent MPs. For driving different lineages of differentiation, such as T cells, $CD56^{+}CD326^{-}$ induced MPs are used as an intermediate population to drive further differentiation [11,21]. Second, HPCs are induced [16,18,20]. The hematopoietic process gradually reduces the self-renewal potential of progenitor cells, and produces a population of cells capable of committing to various cell lineages. Finally, according to the immune cell types and their developmental niches, cell-specific cytokines are applied to specify the progenitor cells to target immune cell types. Abbreviations: DLL, delta-like ligand; Flt3L, FMS-like tyrosine kinase 3 ligand; GM-CSF, granulocyte–macrophage colony-stimulating factor; HPCs, hematopoietic stem and progenitor cells; iDC, induced pluripotent stem cell-derived dendritic cell; IFN, interferon; IL, interleukin; iMac, iPSC-derived macrophage; iMicro, induced pluripotent stem cell-derived microglia; iNK, induced pluripotent stem cell-derived natural killer cell; iPSC, induced pluripotent stem cell; iT, induced pluripotent stem cell-derived T cell; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MP, mesodermal progenitor; PBMC, peripheral blood mononuclear cell; SCF, stem cell factor; TNF, tumor necrosis factor.

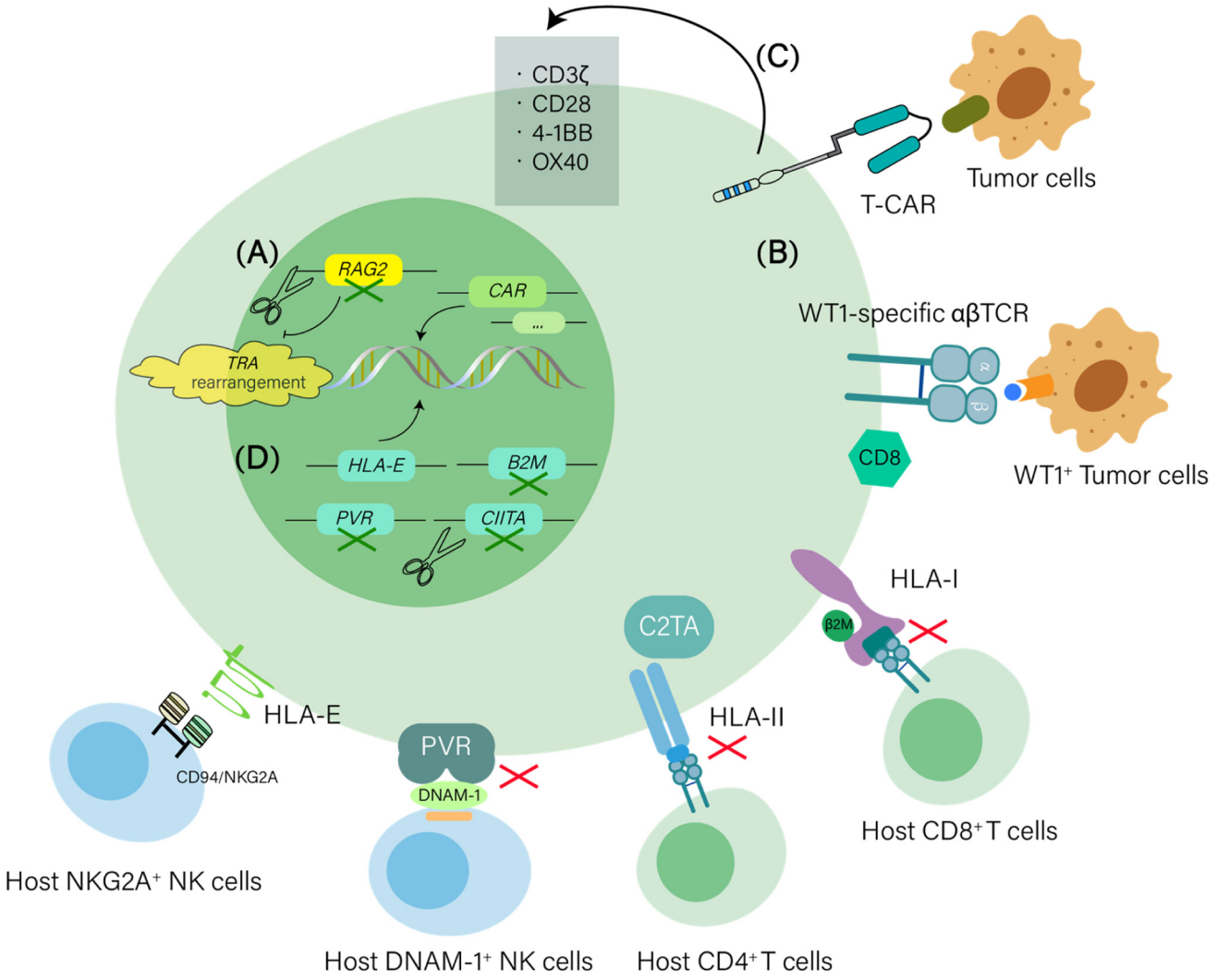

Figure 2. iT cell immune editing strategies. (A) Disruption of the *RAG2* gene prevents *TRA* rearrangement to preserve TCR specificity [15,16]. (B) TCR engineering: CD8αβ$^+$ iT cells derived from WT1 TCR-engineered HLA-homozygous iPSCs demonstrated specific cytotoxicity against the cognate WT1$^+$ mesothelioma cell line NCI-H226 [16]. (C) CAR engineering: iT cells loaded with CAR demonstrated potent antigen-dependent cytotoxicity against tumor cells [16,18,19]. The endodomain of T-CAR is usually composed of one stimulatory molecule (e.g., CD3ζ) and costimulatory molecules (e.g., 4-1BB, CD28, and OX40). (D) Hypoimmunogenic iT cells [57]: disruption of the *B2M* gene blocks HLA-I expression to avoid activation of host CD8$^+$ T cells; disruption of the *CIITA* gene suppresses HLA-II expression to avoid activation of host CD4$^+$ T cells; disruption of the *PVR* gene avoids activation of host DNAM-1$^+$ NK cells; introduction of HLA-E avoids activation of host NKG2A$^+$ NK cells. Abbreviations: B2M: β2 microglobulin; CAR, chimeric antigen receptor; DNAM-1, DNAX accessory molecule 1; iT cell, induced pluripotent stem cell-derived T cell; NKG2A, natural killer group 2A; WT1, Wilms tumor 1.

regards. Early clinical trials using primary NK cells (isolated from PB or cord blood) to produce CAR-NK cells have been launched for treatment of both hematological and solid tumors. For these studies, the CAR constructs used were mostly designed for T cells, and did not involve inherent signaling modules in NK cells. CAR constructs calibrated for NK cells have been proved to be superior to traditional CAR-T constructs for CAR-NK therapy in a study on iCAR-NK cells. Li *et al*. tested a series of specialized CARs containing costimulatory molecule intracellular domains shared by T cells and NK cells (e.g., CD3ζ and 4-1BB) or specific to NK cells (e.g., DAP10, DAP12, and 2B4) and identified NKG2D-2B4-CD3ζ as a potent candidate with enhanced

Box 2. The structure and design principles of CARs

Typically, two signals are necessary for optimal activation of naive T cells: a primary signal triggered by the engagement of TCR-CD3 with an antigen presented on MHC and a second signal triggered by costimulatory molecules such as CD28. In essence, CARs are a simplified form of the TCR-CD3ζ complex, initially designed for T cells, that remove the need for antigen presentation restricted by specific HLA molecules in the process of antigen recognition. First-generation CARs mainly consist of an extracellular **single-chain variable fragment (scFv)** derived from the variable region of an mAb, hinge domain, transmembrane domain for CAR fixation, and an intracellular activation domain (e.g., CD3ζ or FcRγ, which contain critical immunoreceptor tyrosine-based activation motifs so as to activate downstream pathways to implement target-killing functions). Second-generation CARs incorporate a costimulatory domain, such as that of CD28 or 4-1BB, to avoid T cell clonal anergy and improve T cell cytotoxicity, while third-generation CARs include another costimulatory domain. Fourth-generation CARs and fifth-generation CARs were designed based on second-generation CARs. The former is in combination with a constitutively or inducibly expressed chemokine while the latter adds intracellular cytokine receptor fragments [66]. CARs used in NK cells and Macs have the similar extracellular domain, hinge domain, and transmembrane domain as CARs used in T cells.

Table 1. Summary of iPSC-derived immune cells in clinical trials

iPSC-derived agents	ClinicalTrials.gov identifier	Conditions	Start time and (expected) completion time	Status/phase	Sponsors	Refs
FT819: TCR-less, TRAC-targeted allogeneic CD19-CAR-iT cells	NCT04629729	Blood cancers	26 July 2021 – 30 September 2039	Recruiting/1	Fate Therapeutics	[22,68]
FT500: allogeneic iNK cells	NCT03841110	Solid tumors and blood cancers	15 February 2019 – 15 November 2022	Active, not recruiting/1	Fate Therapeutics	[69]
	NCT04106167	Solid tumors and blood cancers	11 June 2019 – December 2037	Recruiting/–	Fate Therapeutics	
FT516: hnCD16-iNK cells	NCT04363346	COVID-19	14 May 2020 – 18 February 2021	Completed/1	Masonic Cancer Center, University of Minnesota	[27,70,71]
	NCT04023071	Blood cancers	4 October 2019 – May 2039	Recruiting/1	Fate Therapeutics	
	NCT04630769	Solid tumors	2 April 2021 – 1 January 2022	Completed/1	Masonic Cancer Center, University of Minnesota	
	NCT04551885	Solid tumors	7 September 2020 – 27 January 2023	Completed/1	Fate Therapeutics	
FT538: CD38 negative, hnCD16, IL15RF iNK cells	NCT05708924	Solid tumors	30 May 2023 – 30 September 2028	Suspended/1	Masonic Cancer Center, University of Minnesota	[72]
	NCT05700630	Persistent low-level HIV viremia	15 March 2023 – 21 August 2023	Not yet recruiting/1	Masonic Cancer Center, University of Minnesota	
	NCT04614636	Blood cancers	17 October 2020 – August 2038	Recruiting/1	Fate Therapeutics	
	NCT05069935	Solid tumors	15 October 2021 – 5 August 2025	Recruiting/1	Fate Therapeutics	
	NCT04714372	Blood cancers	3 November 2021 – 15 December 2025	Recruiting/1	Masonic Cancer Center, University of Minnesota	
FT596: hnCD16, IL15RF, CD19-CAR-iNK cells	NCT04555811	Blood cancers	22 September 2020 – 2 February 2023	Terminated/1	Masonic Cancer Center, University of Minnesota	[73,74]
	NCT04245722	Blood cancers	19 March 2020 – May 2039	Recruiting/1	Fate Therapeutics	
FT536: CD38 negative, hnCD16, IL15RF, MICA/B-CAR-iNK cells	NCT05395052	Solid tumors	31 May 2022 – April 2027	Recruiting/1	Fate Therapeutics	[75]
FT576: hnCD16, CD38 negative, IL15RF, BCMA-CAR-iNK cells	NCT05182073	Blood cancers	24 November 2021 – February 2040	Recruiting/1	Fate Therapeutics	[76]

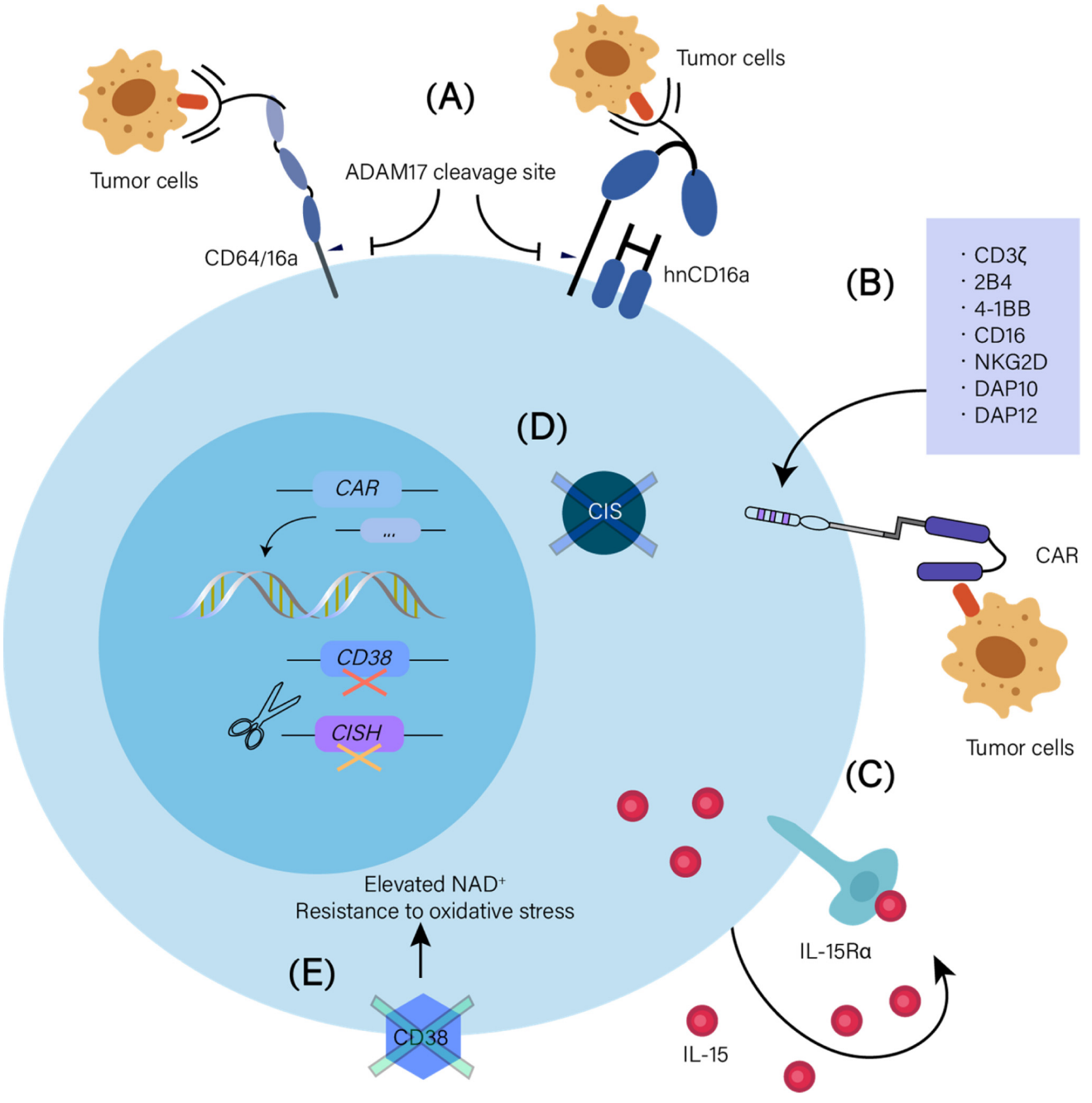

Figure 3. iNK cell immune editing strategies. (A) ADCC enhancement: iNK cells can be engineered with a CD16 variant free of ADAM17 cleavage either through replacement with a CD64 domain (CD64/16a) or through introducing a mutation at the cleavage site (hnCD16) [28]. (B) More specialized CARs for NK cells have been developed based on natural NK cell immune mechanisms (e.g., NKG2D, DAP10, and CD16) [30]. (C) Expression of IL-15/IL-15 receptor fusion protein (IL-15RF) provides signals for iNK cell proliferation and persistence [35]. (D) Biallelic *CISH* knockout redirects the metabolic states of iNK cells by increasing the sensitivity of IL-15 signaling [37]. (E) Adaptive iNK cell engineering: biallelic CD38 knockout gives rise to elevated NAD^+ and resistance to oxidative stress [35]. Abbreviations: ADAM17, a disintegrin and metalloproteinase 17; CAR, chimeric antigen receptor; DAP, DNAX-activating protein; IL, interleukin; iNK cell, induced pluripotent stem cell-derived natural killer cell; NKG2D, Natural Killer Group 2D.

antitumor activity (Figure 3B) [30]. Compared to T-(meso)-CAR-(CD28-41BB-CD3ζ)-iNK cells, NK-(meso)-CAR(NKG2D-2B4-CD3ζ)-iNK cells were more effective *in vitro* or *in vivo*, with the NK-cell-specific CARs demonstrated to better stimulate NK-cell-mediated cytotoxic signaling pathways [30].

Improved NK cell persistence driven by the IL-15 pathway

A key limitation for NK cell therapies may be less persistence *in vivo*. Adoptive NK cell therapies typically use both IL-2 and/or IL-15 to enhance NK cell persistence [31]. Activating the IL-15 signaling pathway is a more preferable choice, as IL-2 concomitantly activates **regulatory T cells (Tregs)** and subsequently suppresses the activity of NK cells [32]. Furthermore, IL-15 is an important cytokine that promotes iNK cell differentiation [33] but needs to be administered frequently due to its short half-life [34]. Ectopic expression of IL-15 constructs such as secreted IL15 or an IL15/IL15-receptor fusion construct (IL-15RF) (Figure 3C) can provide prolonged proliferation signals and enhance antitumor activity of iNK cells both *in vitro* and *in vivo* [35,36].

IL-15 signaling can be suppressed by negative feedback mechanisms, especially the cytokine-inducible SH2-containing protein encoded by the gene *CISH*. To increase the sensitivity of NK cells to IL-15, a genome editing strategy was used to knock out the *CISH* gene at the iPSC stage (Figure 3D), which improved the mTOR signaling-mediated metabolic fitness of iNK cells, and produced iNK cells with improved *in vivo* expansion, persistence, and antitumor effects [37]. $CISH^{-/-}$ iNK cells were phenotypically similar to unmodified iNK cells in terms of the expression of typical NK cell surface markers and the cytotoxic activity.

Metabolic manipulations

Metabolic manipulation refers to engineering the metabolic pathways of NK cells to transform them into their more capable counterparts. The above *CISH* knockout iNK cells are an example of indirect regulation of metabolic pathways. It has been reported that a unique subpopulation of NK cells in cytomegalovirus-seropositive individuals, namely, adaptive NK cells, have elevated metabolic activity [35,38]. Adaptive NK cells exhibit elevated NKG2C and reduced CD38 expression, resistance to cell death induced by oxidative stress, potent IFN-γ production and ADCC, and transcriptional and metabolic features similar to those of $CD8^+$ memory T cells [35,39]. CD38 is an enzyme with NAD^+ glycohydrolase activity, and its expression can lead to NAD^+ depletion and immune cell exhaustion. A strategy to knock out CD38 endowed the iNK cells with metabolic features resembling adaptive NK cells. Biallelic CD38 knockout ($CD38^{-/-}$) iNK cells (Figure 3E) displayed elevated ATP levels, glycolysis and oxidative phosphorylation. $CD38^{-/-}$ iNK cells exhibited not only overall enhanced metabolic fitness but also the capacity to escape 'fratricide' when combined with daratumumab (anti-CD38 mAb) for treatment of multiple myeloma [35]. FT538, a CD38-negative, and hnCD16- and IL15RF-positive iNK-cell product, has been assessed in clinical trials to treat both hematological and solid tumors (Table 1).

Engineering strategies for iPSC-derived Macs (iMacs) and DCs (iDCs)

iMacs

Myeloid cells such as Macs are notorious for their limited expansion capacity. To generate sufficient amounts of iPSC-derived myeloid cells for therapeutic use, Haruta *et al.* transduced genes involved in cell growth or senescence suppression (such as *c-MYC*, together with *BMI1*, *MDM2*, or *EZH2*) to produce human iPSC-derived proliferating myeloid cells (iPS-pMLs), which could be propagated for months and function as primary Macs [40] (Figure 4A) or differentiated into DCs (Figure 5A) in 2–3 days [41]. They also reported that these engineered cell lines had low tumorigenic risks since they proliferate in a cytokine-dependent manner *in vitro*, and the same cytokine-rich condition could not be met in an *in vivo* physiological situation. Thus, iPS-pMLs

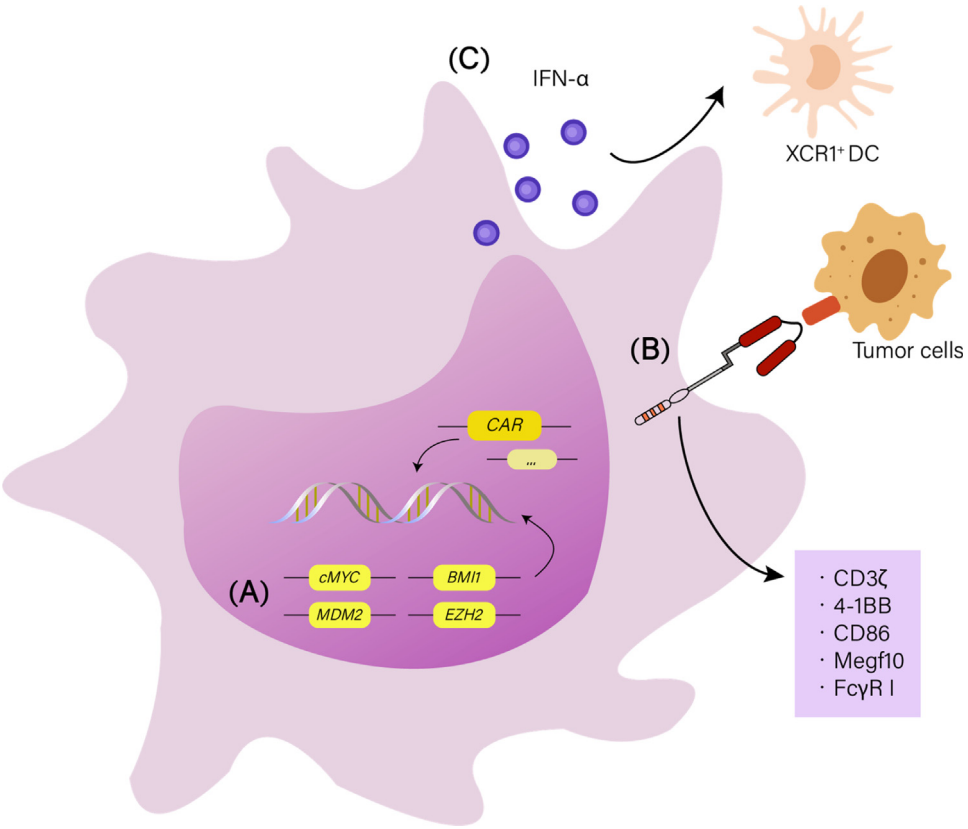

Figure 4. iMac immune editing strategies. (A) To ensure accurate identification of tumor cells, Macs have been engineered with a T-CAR containing 4-1BB and CD3ζ or an M-CAR composed of Megf10 [67], FcγRI [43,67], and CD86 [43]. (B) iMacs manipulated to express type I IFNs (such as IFN-α) can enhance anticancer immunity through XCR1⁺ DCs [44]. (C) Simultaneous introduction of the *cMYC* gene together with *BMI1*, *MDM2*, or *EZH2* can induce the proliferation of iPS-pMLs to obtain a large number of iMacs [40]. Abbreviations: FcγRI, Fcγ receptor I; IFN, interferon; iMac, inducible pluripotent stem cell-derived macrophage; Megf10, multiple EGF-like domains 10.

offered opportunities for treating rapidly progressing tumors with multiple doses. Nevertheless, the oncogenes used to generate iPS-pMLs do not meet clinical safety requirements, and other approaches avoiding using oncogenes to stimulate myeloid cell proliferation and expansion need to be explored.

iPSC-derived CAR-Macs (iCAR-Macs)

In view of the current success of CAR-T cell therapy and the promising potential of CAR-NK cell therapy, other studies have started to explore CAR-expressing Macs. Macs have unmatched phagocytic effector cell functions as well as immunomodulatory regulatory cell functions in the tumor microenvironment (TME). Klichinsky *et al.* engineered human primary Macs with an anti-CD19 CAR containing a CD3ζ intracellular domain. These CAR-Macs exhibited M1-like proinflammatory phenotypes that were resistant to the M2 immunosuppressive polarizing effects of the TME through stimulation by the adenovirus vector [42]. Although the chimeric adenoviral vector Ad5f35 that they used can transduce primary human monocytes and Macs with high efficiency (>75%) [42], the limitation is that the quantity of engineered primary Macs is challenging for large-scale clinical application. Zhang *et al.* established a platform to engineer iPSCs with a CAR and to differentiate them into Macs, which were named CAR-iMacs [43]. The initially

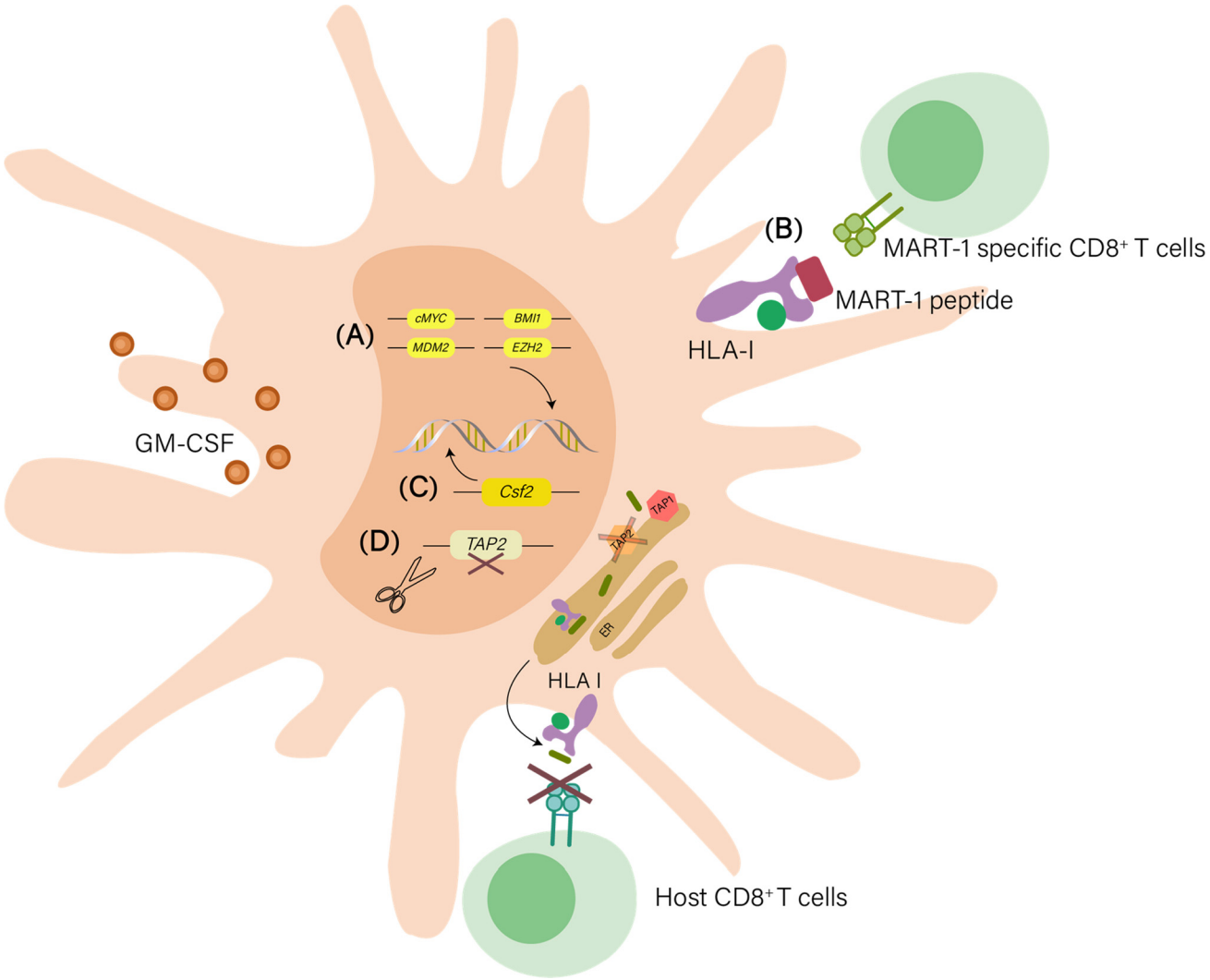

Figure 5. iDC immune editing strategies. (A) DCs derived from MART-1 gene transduced human PSCs can effectively prime MART-1-specific CD8⁺ T cells [50]. (B) Simultaneous introduction of the *cMYC* gene and *BMI1*, *MDM2*, or *EZH2* can induce the proliferation of iPS-ML cells to obtain a larger number of iDCs [40]. (C) GM-SCF (encoded by *Csf2* gene) expressing mouse iDCs have more potent proliferative capacity and augment overall antitumor effect [47]. (D) *TAP2* knockout reduces the response of alloreactive CD8⁺ T cells through blockage of the interaction between HLA I and TCR [41]. Abbreviations: GM-CSF, granulocyte–macrophage colony-stimulating factor; iDC, iPSC-derived dendritic cell; MART1, melanoma antigen recognized by T cells 1; TAP2, transporter 2, ATP binding cassette subfamily B member; TCR, T cell receptor.

screened M-CARs were composed of CD86 and CD64 (FcγRI), which were exclusive to Macs, but the phagocytic ability was not more powerful than that of conventional T-CAR (containing 4-1BB and CD3ζ)-expressing iMacs (Figure 4B). Of note, without antigen exposure, CAR-iMacs derived with the system were not activated, but incubation with CD19-expressing leukemia cells or mesothelin-expressing ovarian cancer cells transformed the corresponding CAR-iMacs toward the direction of an activated state.

Enhancing effector cell trafficking toward tumors

Approaches to enhance trafficking have long been explored, since effector cell (e.g., T cell and NK cell) trafficking to the tumor site plays a crucial role in successful cancer treatment. One way to

deliver effector cells is to inject cells directly into the tumor site; however, different types of tumors are not always accessible. Another way is to enhance infiltration capacity of effector cells. In one study, researchers engineered human iPS-pMLs with **type I interferons (IFNs)**, which were cytokines that remodeled the TME, conferred the M1 phenotype to Macs, and further transformed local tumor-associated Macs into the proinflammatory state [40]. Importantly, the type I IFNs play a crucial role in T cell infiltration into the TME. Direct administration of IFNs is often restricted to tolerable doses since systemic injection of type I IFNs can elicit serious side effects. iPSC-pMLs engineered to express type I IFNs (Figure 4C) could not only persistently produce appropriate doses of type I IFNs locally, but also promote T cell penetration into tumors, and stimulate T cell activity through **antigen cross-presentation** mediated by XCR1$^+$ DCs [44]. IFN-β expressing iPS-pMLs was superior to IFN-α expressing iPS-pMLs in treating peritoneally disseminated SK-MEL28 melanoma in xenograft models due to the difference in the sensitivity of tumors to the specific cytokine or the greater tissue affinity to IFN-β over IFN-α [40], suggesting the importance of selecting specific cytokines to engineer.

iDC-based vaccines

DCs can originate from common lymphoid progenitors and common myeloid progenitors *in vivo*, but most iDCs pass through myeloid lineage during differentiation [45–48]. Thus, this review classifies DCs as myeloid cells generally. DCs are renowned as potent antigen-presenting cells (APCs). DCs loaded with tumor-specific antigens are applied as vaccines for cancer immunotherapy. They boost the patient's immune system in advance, prepare the immune system to combat cancer, and amplify the magnitude of T cell activation to increase overall antitumor activity.

Conventional manipulation of DCs has been focused on antigen loading for efficient antigen presentation to T cells [49], but neither clinical-grade payload production nor laborious primary DC engineering is needed since iPSCs can be genetically modified to permanently express specific antigens (Figure 5B). For example, DCs derived from MART-1-transduced human PSCs prime MART1-specific CD8$^+$ T cells more effectively than MART-1-peptide pulsed human PSC-DCs do [50]. Traditional primary DCs without any genetic modifications have the ability to stimulate naive CD4$^+$ T cells through antigen presentation but are insufficient for cross-presentation to MHCI-restricted CD8$^+$ T cells. However, iDCs have wide antigen cross-presenting potential; for instance, completely mature iDCs stably expressing tumor-associated antigen (TAA) show MHCI-presented TAA-specific peptides and induce **cytotoxic T lymphocyte (CTL)** stimulation in immunized mice [51]. It is believed that the CD141$^+$XCR1$^+$ cDC1 type, rare in PB, is the reason for antigen cross-presentation and more potent immune defense [45,52]. To obtain cDC1-like iDCs in large numbers, Oba *et al*. induced notch signaling and identified it as a substantial requirement to generate antigen cross-presenting iDCs [45]. In certain cases, antitumor vaccination needs to be boosted to combat strong immunosuppressive signals in the TME. *Csf2*-transduced iPS-pMLs generated GM-CSF-producing iDCs (Figure 5C), and they suppressed myeloid-derived suppressor cells and mediated a CTL-mediated antitumor response [47].

Increasing DC migration capacity after immunization is crucial to cancer immunotherapy success. To this end, a recent study found that in a tumor model refractory to immune checkpoint inhibitors (ICIs), in combination with local radiotherapy, *in situ* delivered iDCs showed enhanced ability to migrate to tumor-draining lymph nodes and crosstalk with T cells, promoted CTL infiltration, and sensitized programmed death ligand (PD-L)1 blockade, thereby stimulating systemic immune defense and slowing remote tumor progression [46]. Thus, these findings provide a rationale that the next-generation DC vaccines combined with ICIs and traditional cancer treatment (e.g., chemotherapy and radiotherapy) may be effective for treating various types of

tumors. Such synergistic strategies to boost the overall immune system avert the need to identify antigens specific to a tumor target and to engineer a CAR on the immune cells. Human engineered iDCs have not yet been implemented in the clinic, but the preclinical work is promising.

Engineering strategies for universality in iPSC-derived immune cells

Compared to most current cell therapies, which are confined to autologous settings, the application of universal effector cells in cancer therapy could significantly reduce the time and cost of cell product manufacturing. HLA matching through the construction of HLA-haplotype banks is anticipated to provide off-the-shelf iPSCs [53,54]. However, even if the iPSC-derived products are autologous, or well matched, they might still arouse immune rejection when transplanted to patients. One of the possible mechanisms is that during reprogramming and differentiation procedures, mitochondrial DNA mutations emerge, accumulate, and result in the production of neoantigens [55]. Thus, genetic manipulation of iPSCs to achieve universality is necessary.

Therapeutic cell universality would resolve issues on two main fronts: GVHD and allogeneic cell rejection by the recipient's immune system. Especially for T cells, the former issue can be addressed by disrupting TCR encoded in the *TRAC* locus [56] or choosing iPSC clones of innocuous TCR [21]. For the latter issue, one should consider immune escape from host effector cells, especially CTLs. An early study focusing on the heterodimer TAP, which is critical for HLA I cell surface expression, showed that knockout of one subunit, TAP2, reduces alloreactive $CD8^+$ T cells response (Figure 5D) [41]. Recent comprehensive iPSC genetic engineering studies considering more effector cells (host $CD8^+$ T cells, $CD4^+$ T cells, and NK cells) simultaneously knocked out ***B2M***, ***CIITA*****,** and ***PVR*** and transduced ***HLA-E*** during the iPSC stage (Figure 2D) [57]. This approach blocked the presentation of antigens from iPSC-derived cells and inhibited **HLA-null-mediated NK-cell activation**, prolonging the survival of resulting iT cells both *ex vivo* and *in vivo* compared with that of HLA-intact iT cells, without compromising therapeutic function. Of note, to generate an iDC-based vaccine, HLA genes must be maintained for antigen presentation to stimulate endogenous CTLs, and thus, a balance between depleting and retaining the HLA apparatus needs to be carefully considered.

Comparative analysis of different iPSC-engineered immune cells

Currently, lymphoid immune cells, such as T cells and NK cells, and myeloid immune cells, such as monocytes, Macs, and DCs, can all be efficiently induced through iPSCs, and with the aid of genome editing, a variety of engineered iCell products have come to the horizon and are in different stages of research in preclinical and clinical settings. An understanding of their strengths and limitations and of the contexts in which they may be most useful is highly needed. Theoretically, lymphoid cells are the more potent effector cells in directly killing tumor cells. For instance, traditional genetic engineering strategies were mainly applied to T cells due to their unparalleled role in cytotoxicity against tumor cells. Prevalent engineering strategies, such as introducing a CAR, confer T cells with strong targeting specificity. Among the types of immune cells, one advantage for T cells is that there is a subset of memory cells that can last long in the human body, and the applications of this feature for preventing tumor relapse have been heavily investigated. Another advantage is that T cells are highly proliferative cells, which guarantees their *in vivo* expansion to combat a large number of tumor cells, albeit with stronger risks of toxicity such as CRS and ICANS.

The intrinsic multiple killing mechanisms of NK cells, including ADCC, cytotoxic granules release, upregulation of death ligands, and no need for antigen priming, make them a new alternative for cancer immunotherapy. Once engineered with a CAR, CAR-NK cells can target tumors in a CAR-dependent or -independent manner. Regarding safety issues, early clinical results showed that engineered allogeneic CAR-NK cells do not cause GVHD, CRS, and ICANS [58], while these

syndromes are often seen with CAR-T therapy. Besides, it is believed that NK cells are more suitable for the development of universal off-the-shelf cell products without the extra genome editing of TCRs that is intrinsic to T cells. However, their short lifespan, as well as expression of inhibitory receptors, might restrict the efficacy of iNK cell treatment.

Distinct from cytotoxic lymphoid effector cells (T cells and NK cells), myeloid cells (Macs and DCs) are considered to have both immunomodulatory effects and phagocytic effector cell capacity. Thus, the primary goal of iMac engineering is not only to enhance their phagocytic function but also to control the proinflammatory/antitumor state and allow them to stimulate the endogenous immune system. Macs are notorious for their plasticity when domesticated by the TME, and thus genetically engineering iMacs to make the cells constitutively stay in the M1-like state is critical. By leveraging their infiltrating capacity to enter the tumor, it is rational to generate engineered M1-like Macs expressing certain proinflammatory factors to shift the TME. The feasibility of iPSC-derived myeloid cells delivering IFNs has been confirmed as described before, and recent advances have demonstrated powerful potency in reversing the immunosuppressive TME by genetically engineered IL-12-carrying primary myeloid cells [59], whose antitumor ability might be further enhanced if the iPSC platform is used to overcome the issue of low transduction efficiency. Combination therapy with ICIs, such as anti-PD-1/PD-L1 antibodies [44] or anti-CD47 antibodies, can also strengthen the tumoricidal effects of engineered iMacs. One particularly important application for iMacs or iDCs is their use as tumor vaccines; this application has been illustrated by the case of IL-12-expressing myeloid cells [59] and by other iDC studies using syngeneic mouse models in which engineered mouse myeloid cells can sufficiently remodel the syngeneic TME upon transplantation, and prevent tumor relapse or resist tumor cell rechallenge.

Immune cells are inextricably linked, which provides the rationale for combining different iPSC-derived immune cells. iPSC-derived lymphoid effector cells (iT and iNK cells) shoulder the responsibility for acute immune defense and have shown efficacy in treating hematological malignancies. Engineering strategies such as enhancing their persistence and overcoming immunosuppressive TME are highly expected to compensate for their shortcomings. iPSC-derived myeloid cells (iMacs and iDCs) wear two hats, phagocytosis and environment modulation. Though their direct cytolytic ability is weaker than that of iPSC-derived lymphoid cells, it is anticipated that they not only provide an auxiliary therapeutic modality but also might become essential when the modulation of an immune-unfavorable TME is critically needed, particularly in the treatment of solid tumors where CAR-T and CAR-NK therapies alone still face challenges.

Concluding remarks and future perspectives

iT cells, iNK cells, iMacs, and iDCs have inaugurated a new era in tumor immunotherapy. They have provided an opportunity for producing large amounts of well-controlled off-the-shelf products. An increasing number of studies have focused on approaches to enhance their immune function, to let them reprogram the TME, to overcome antigen heterogeneity, to achieve hypoimmunogenic features, to elicit durable responses, and to combine them with other therapeutic modalities. Despite intense investigations of immunoenhancing engineering strategies, cell fate engineering during iPSC differentiation to precise subsets of the four immune cell types is relatively understudied in the context of immunotherapy (see Outstanding questions). Also, more practical issues related to manufacturing and quality controls need to be carefully addressed, such as cryopreservation of cell drugs without loss of function, especially for fragile NK cells that are notorious for losing viability after thawing [60]. Overall, with the continuous progress of cell manufacturing and genome editing, we are confident that engineering better iPSC-derived immune cells, no matter whether they are focused on cell fate specification, immune functions, or product universality, will greatly impact cancer immunotherapy in the near future.

Outstanding questions

How can heterogeneity be overcome in different iPSC lines, resulting in variation of differentiation deficiency?

How can iPSC-derived immune cells be precisely engineered in terms of cell fate, or is it possible to introduce specific transcription factors to direct differentiation of different iPSC-derived immune cells, especially for lymphoid lineage cells, to capitulate specific subsets of their primary counterpart?

How can CAR-immune cell specificity and cytotoxicity be maximized against tumors without causing safety issues such as CRS and **on-target, off-tumor toxicity**?

Is there a way to design more effective M-CARs or modify the CAR-iMacs to further enhance efficacy and persistency?

Can we design a kind of CAR to develop the immune regulatory function of iCAR-Macs?

Is it possible to deliver blood vessel-penetrating CAR-iPSC-derived monocytes and make them differentiate into iCAR-Macs in the TME?

Can iPSC-derived immune cells preserve considerable therapeutic function after cryopreservation?

Is it possible to enhance the overall fitness of other iPSC-derived immune cells in addition to iNK cells through metabolic reprogramming?

Does it make sense to add a CAR on DCs to develop more effective vaccines?

Is it possible to combine different iPSC-derived immune cells to elicit a more synergistical and comprehensive treatment effect against tumor malignancies?

Acknowledgments

This work was sponsored by the National Key Research and Development Program of China (2018YFA0107103 and 2018YFC1005002), the National Natural Science Foundation of China (31871453 and 91857116), Zhejiang Innovation Team grant (2019R01004), and the Zhejiang Natural Science Foundation (LR19C120001).

Declaration of interests

J.Z. is a cofounder of CellOrigin. D.S.K. is a cofounder and advisor to Shoreline Biosciences and has an equity interest in the company. The terms of these arrangements for D.S.K. have been reviewed and approved by the University of California, San Diego in accordance with its conflict-of-interest policies. The remaining authors have no interests to declare.

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