IPSC-derived NK cells for immunotherapy and therapeutic perspective (Review)

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Abstract. Natural killer (NK) cell-based immunotherapy has emerged as a transformative approach for cancer treatment However, its widespread clinical application faces several challenges, such as donor variability, limited scalability and functional heterogeneity of primary NK cells. Additionally, issues including *in vivo* persistence, resistance to tumor microenvironment and safety concerns related to genomic instability further hinder its clinical application. Induced pluripotent stem cell (iPSC)-derived NK cells offer a promising solution. They provide high homogeneity and quality control, genetic engineering flexibility and inexhaustible cell source. This present review highlighted the unique advantages of iPSC- NK cells, including clonal uniformity, enhanced cytotoxicity and suitability for large-scale production, positioning them as an ideal 'off-the-shelf' therapeutic platform. It discussed the biological properties of iPSC-derived NK cells, advances in differentiation protocols and strategies to augment their anti-tumor efficacy through genetic engineering, such as chimeric antigen receptor integration and cytokine optimization. Despite these advantages, several challenges remain, including the need to optimize differentiation efficiency, ensure the safety of gene editing (such as off-target effects) and improve the *in vivo* migration and infiltration abilities. With technological advances and clinical validation, this present review aimed to guide future research toward overcoming these barriers to clinical implementation. Ultimately, it is expected that iPSC-NK will become a core means of next-generation immunotherapy, promoting the combination of personalized and inclusive cancer treatment.

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Abbreviations: NK, natural killer; IPSC, induced pluripotent stem cell; CAR, chimeric antigen receptor; CR, complete response; CRS, cytokine release syndrome; ADCC, antibody-dependent cellular cytotoxicity; DCs, dendritic cells; IFN, interferon; TNF, tumor necrosis factor; XCL, recombinant chemokine C-motif ligand; CCR, C-C chemokine receptor; MHC, major histocompatibility complex; HLA, human leukocyte antigen; NCR, natural cytotoxicity receptor; KIR, killer cell immunoglobulin-like receptor; EB, spin embryoid body; HPCs, hematopoietic progenitor cells; KIRs, killer immunoglobulin-like receptors; PD-1, anti- programmed death-1; PD-L1, Programmed cell death ligand 1; ADAM17, a disintegrin and metalloproteinase; CD16 non-cleavable, hnCD16; TIGIT, T cell immune receptor with immunoglobulin and ITIM domains; B2M, β-2 macroglobulin; scFv, single-chain variable fragment; MM, Multiple myeloma

Key words: natural killer cells, induced pluripotent stem cell derived natural killer cells, immunotherapy, solid tumors, off-the-shelf

Contents

1. Introduction

As the first line of defense against infections and malignancies, human natural killer (NK) cells are a critical component of innate immune system. Unlike T cells, which require priming, NK cells can eliminate virus-infected cells and transformed

cells through immune mechanisms (1). Various NK cell sources have been investigated in clinical trials for cellular immunotherapy (2). Induced pluripotent stem cel -derived NK cells (IPSC-NK) have emerged as a research hotspot in tumor immunotherapy due to their scalability, standardized manufacturing potential and genetic engineering capabilities. Compared with other immunotherapies, NK cells demonstrate improved cytotoxic activity and are less sensitive to tumor immune escape strategies, making them a promising approach for cancer treatment (3).

NK cells do not require human leukocyte antigen (HLA) compatibility, reducing the risk of complications such as graft-versus-host disease (GVHD) and cytokine release syndrome (CRS), even when administered as allogeneic cells (4). Their favorable safety profile, coupled with notable anti-tumor capabilities, positions NK cells as a compelling cellular candidate for the application of chimeric antigen receptor (CAR) technology (5). This enables the strategic redirection of their cytotoxic capabilities towards precise targets (6,7).

Despite these advantages compared with other immunotherapies, NK cell-based therapies face several challenges, including immunosuppressive tumor microenvironments, limitations in cell manufacturing and insufficient therapeutic persistence. IPSC-NK cells have emerged as an attractive alternative to overcome these limitations (8). IPSC-NK cells facilitate the mass production of homogeneous NK cells, which can be stored for later use. Moreover, both viral and non-viral methods can be used to effectively genetically modify these cells to meet the needs of different cancer treatments (9). Therefore, in biomedical research and clinical applications, iPSCs hold great promise for translational research and clinical applications (10). As the 'off the shelf' product, iPSC-NK cells possess strong drug-forming characteristics and represent a promising strategy in cancer immunotherapy. This present review examined the immunotherapy and therapeutic perspective of NK cells derived from iPSCs.

2. Biological properties of NK cells

Unlike T and B cells, NK cells lack genetically rearranged antigen receptors, allowing them to directly eliminate target cells without prior sensitization (11). NK cells are positioned as promising candidates for cancer adoptive cell therapy because of this unique feature, as well as their 'off-the-shelf' availability and low risk of GVHD (12,13). NK cells are a type of lymphocyte that play a crucial role in the innate immune system. Their development begins in the bone marrow, where hematopoietic stem cells differentiate into common lymphoid progenitors. These progenitors further differentiate into NK cell precursors by downregulating CD34 and upregulating CD56, with IL2RB (CD122) expression marking the entry into the NK cell lineage (14). The precursors migrate to lymph nodes, where cytokines from stromal and dendritic cells facilitate their maturation into functional NK cells. This process is characterized by the differential expression of genes, including CD34, KIT, KLRB1, CD244 and interleukin (IL)-15R (15-17). During this process, NK cells start expressing receptors that enable them to recognize and bind to target cells, such as infected or cancerous cells.

NK cells are broadly categorized into $CD56^{bright}$ and $CD56^{dim}$ NK subsets (Fig. 1). The $CD56^{dim}$ subset constitutes ~90% of peripheral blood NK cells and plays a primary role in direct target cells elimination through the release of perforin and granzymes. These cells exhibit the strongest cytotoxic activity and antibody-dependent cell-mediated cytotoxicity (ADCC) capabilities (18-20).

By contrast, $CD56^{bright}$ cells, which represent 2-10% of peripheral blood NK cells, have less cytotoxic activity but play a crucial role in immunomodulation. They can interact with dendritic cells (DCs) and T cells to maintain the balance and effectiveness of the immune system (21). Activated NK cells secrete cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and granulocyte-macrophage colony-stimulating factor to enhance cytotoxic T cell responses and macrophage antigen presentation. NK cells can be activated by cytokines secreted by DCs, such as IL-12 and IL-18 (22,23).

$CD56^{bright}$ cells also exert anti-proliferative, anti-angiogenic and pro-apoptotic effects on cancer cells while recruiting DCs to the tumor microenvironment via chemokines such as C-C motif chemokine ligand 5, recombinant chemokine C-Motif ligand (XCL) 1 and XCL2, thereby promoting anti-tumor immunity (24). Furthermore, $CD56^{bright}$ cells express chemokine receptors such as C-C chemokine receptor type (CCR) 7, CCR5 and CXCR4, which allow them to migrate and localize to secondary lymphoid organs. They also express CD62L-selectin, which interacts with high endothelial venues to facilitate homing and retention in lymphoid tissues (17,25,26). This characteristic underscores the crucial role of NK cells in immune regulation and cytokine-mediated immune responses (25).

3. Adoptive NK cell therapy

The unique biological properties of NK cells provide a strong rationale for their application in adoptive immunotherapy. NK cell activation and inhibition are governed by a range of activating and inhibitory receptors (Table I) (13). Whether NK cells are activated or inhibited depends on how signals from these receptors interact (27). The capacity of NK cells to detect and eliminate cancer cells through mechanisms such as MHC-I downregulation recognition is a defining characteristic of their activity (28,29). This recognition is facilitated by the presence of killer cell immunoglobulin-like receptors (KIRs) on NK cells, which are sensitive to the absence of major histocompatibility complex (MHC) class I molecules (30). Inhibitory KIRs contain immune receptor tyrosine-based inhibitory motifs, which mediate inhibitory signals, while activating KIRs associate with immunoreceptor tyrosine-based activation motifs, transmitting activating signals (31).

When KIRs on NK cells encounter and bind to MHC class I molecules on the surface of healthy cells, inhibitory signals are transmitted, preventing the NK cells from attacking these healthy cells (32,33). However, in the case of aberrant expression of MHC class I molecules, the inhibitory receptors on NK cells are unable to recognize the altered MHC class I molecules, resulting in an inability to transmit inhibitory signals (Fig. 2) (34). As a result, when the activating signal outweigh inhibitory signals, NK cells receive an activating signal and proceed to recognize and eliminate the tumor cells. For example,

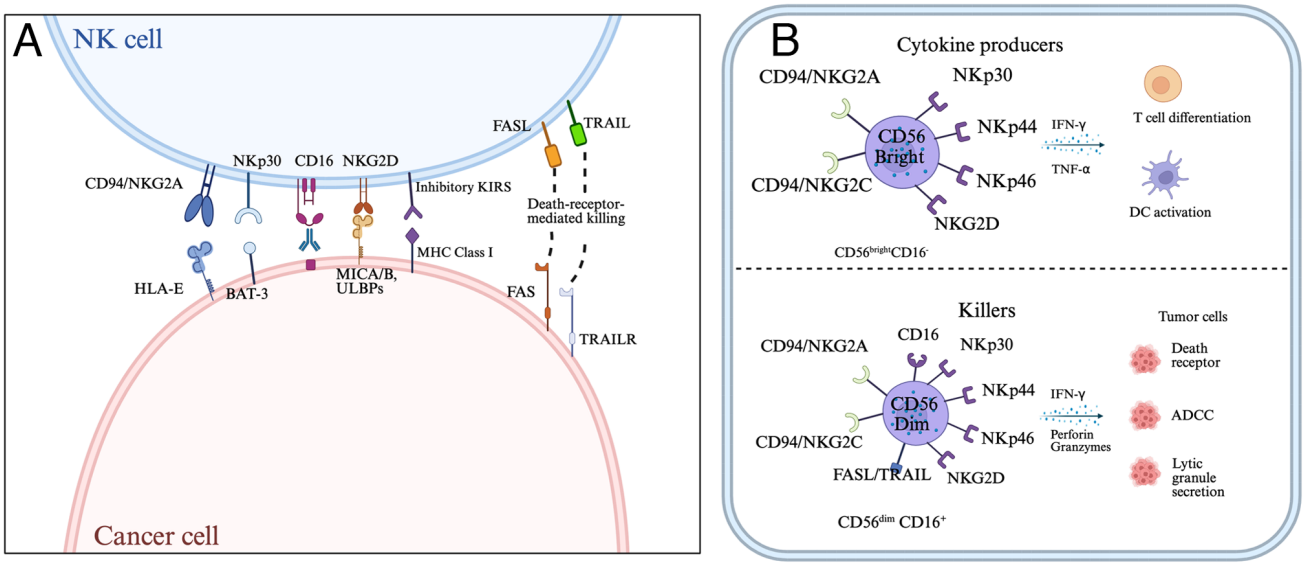

Figure 1. The function of NK cells is governed by a range of activating and inhibitory receptors. (A) The major NK cell receptors and their respective ligands are depicted on the left. (B) CD56^{bright} cells function as cytokine producers, while cytolytic activity was associated with the CD56^{dim} subset. NK, natural killer; HLA-E, major histocompatibility complex, class I, E; FASL, fas ligand; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; TRAILR, tumor necrosis factor related apoptosis-inducing ligand receptor ; MICA/B, MHC class I chain-related protein A/B; KIRS, killer cell Inhibitory receptors; DC, dendritic cells ;ADCC, antibody-dependent cell-mediated cytotoxicity.

when tumor cells downregulate MHC class I molecule expression, inhibitory signals on NK cells are lifted, releasing the 'missing-self' restraint and activating their cytotoxic functions. Simultaneously, MHC-I-deficient tumor cells may upregulate stress-induced molecules, which bind to activating receptors on NK cells, further amplifying cytotoxic signaling (35). This mechanism enables NK cells to target tumors that evade T cell-mediated immune surveillance, forming a complementary anti-tumor immune defense (36). human leukocyte antigen (HLA)-E-related peptides transmit inhibitory signals by interacting with NK inhibitory receptors (37).

For instance, NKG2A dimerizes with CD94 on the cell surface and binds to HLA-E, which is crucial for tumors to resist immune cell activity. In hepatocellular carcinoma (HCC) tissues, the surface expression of NKG2A is increased in NK cells, together with the expression of its corresponding ligand, HLA-E (38,39). Analysis of gene expression data in tumor samples reveals a significant correlation between HLA-E expression levels within tumor tissues and expression levels of NKG2A and CD94 on the surface of NK cells (40). Unlike T cell activation, which requires dual signaling, the activation of NK cells is determined solely by the regulation of surface receptors and is not constrained by MHC. As the tumor progresses, NK cells are rapidly activated, executing their functions ahead of T cells (41,42).

Additionally, NK cells can eliminate tumor cells through ADCC, mediated by Fc γ receptors, which engage antibodies to induce target cell death (43-45). The most important activation receptor in this process is CD16, an antibody receptor that binds immunoglobulin Fc, working alongside other key activation receptors such as NKG2D and natural cytotoxicity receptor (NCR) (46,47). A regulatory mechanism for NK cell cytotoxicity is also provided by the binding of ligands on target cell surfaces to NCR family receptors, including NKp30, NKp44, NKp46 and NKG2D (48,49).

For subsequent gene editing of iPSC-NK cells, it is crucial to understand the characteristics and functions of activating and inhibitory receptors on the surface of NK cells. By exploiting gene editing technology, the expression of receptors on the surface of NK cells can be precisely regulated, thereby optimizing the balance between their activating and inhibitory signals. This approach can enhance the anti-tumor and anti-viral capabilities of NK cells while reducing the risk of potential immune overreaction.

4. Sourcing NK cells

Due to the limited antitumor effect of autologous NK cells, researchers have shifted their focus from autologous to allogeneic NK cell therapy (50). Over the past decade, various NK cell sources have been investigated in clinical trials for cellular immunotherapy, including peripheral blood, umbilical cord blood and NK-92 (Fig. 3). NK cells sourced from peripheral blood are easy to acquire and handle. Each of these sources has distinct advantages. Umbilical cord blood provides a readily available source with weaker allogenic reactions and a low risk of viral transmission (51). Meanwhile, NK-92 cells, an infinitely proliferating cell line, exhibit high cytotoxicity and stable immune properties (52). However, each of these sources has drawbacks. Peripheral blood and umbilical cord blood-derived NK cells are inconvenient to store and subject to donor variability. NK-92 cells, while highly cytotoxic, pose potential carcinogenic risks, lack CD16 and NKp44 and require irradiation for inactivation, which markedly reduces their proliferative capacity and cytotoxicity. The absence of CD16 also hampers their ability to perform ADCC (53,54).

To address these limitations, IPSC-NK cells have emerged as a promising alternative, which can be used as a standardized, 'off-the-shelf' allogeneic cell therapy for treating diverse malignancies and amenable to genome editing (55).

Table I. Human NK cell receptors and function.

Receptor	Function	Ligands on tumor
NKG2D	Activation	MIC-A/B, Rael, H60, ULBP
CD94/,NKG2C	Activation	HLA-E
NKP30	Activation	HLA-EB7-H6, BAT3, CMVPP65
NKP44	Activation	Viral HA
NKP46	Activation	Viral HA
CD160	Activation	HLA-C
KIR2DS1	Activation	HLA-C
CD94/NKG2A	Inhibition	HLA-E
CD96	Inhibition	CD155
LAIR1	Inhibition	Collagen
KLRG1	Inhibition	E-Cad
PD-1	Inhibition	PD-L1/2
SIGLEC-3/7/9	Inhibition	Sialic acid
NKR-PIA	Inhibition	CLEC2D
TIGIT	Inhibition	CD155
iKIRs	Inhibition	HLA-I
ILT2	Inhibition	HLA-C
CD96	Inhibition	CD155

NK, natural killer; MIC-A/B, MHC class I polypeptide-related sequence A/B; HLA-E, major histocompatibility complex; BAT3, HLA-B associated transcript 3; CMVPP65, cytomegalovirus phosphoprotein 65; HA, hemagglutinin; CLEC2D, C-Type lectin domain family 2 member D; PD-L1/2, programmed death-1; KIR2DS1, killer cell immunoglobulin-like receptor, two Ig domains and short cytoplasmic tail 1; LAIR1, leukocyte-associated immunoglobulin-like receptor 1; KLRG1, killer cell lectin-like receptor subfamily G member 1; SIGLEC-3/7/9, sialic acid-binding immunoglobulin-like lectin 3/7/9; NKR-PIA, killer cell lectin-like receptor subfamily B member 1; TIGIT, T cell immunoglobulin and ITIM domain; iKIR, inhibitory killer-cell immunoglobulin-like receptors; ILT2, immunoglobulin-like transcript 2.

Furthermore, modified iPSC-NK cells have shown high target specificity, persistence and immune activation potential in cancer treatment (8,56).

5. Development and differentiation of iPSC-derived NK cells

The development of iPSC technology began in 2006. The approach was pioneered by Shinya Yamanaka, who developed a method to generate iPSCs by introducing four transcription factors (OCT3/4, SOX2, KLF4 and c-Myc) into somatic cells (57-59). IPSCs show characteristics identical to embryonic stem cells (ESCs) in terms of morphology, proliferation, gene expression and the ability to form teratomas.

IPSC-NK cell therapy is a novel cancer immunotherapy approach based on iPSC technology. In 2024, Kiran *et al* (60) developed a method to induce and sustain transgene-free human iPSCs, enabling efficient and uniform amplification using reprogramming factors such as SOX2, OCT4, KLF4, MYC, NANOG, LIN28 and SV40 T antigen in a feeder-free system. This method showed an effective performance against malignant brain rhomboid tumor cells. To effectively mitigate the risk of residual exogenous genes, technologies focus on employing non-integrating vectors and small molecule compounds to achieve gene silencing or post-reprogramming removal in iPSCs (61). The differentiation of iPSC into mature NK is typically divided into three stages. Initially, to promote the differentiation of iPSCs into hematopoietic progenitor cells (HPCs), they are co-cultured with a combination of small molecules and cytokines, or irradiated stromal cell lines. Subsequently, $CD34^+$ HPCs are isolated and enriched before being directed towards NK cell differentiation using specific cytokines (IL-3, IL-7, IL-15, SCF and FLT3L) or through co-culture with a second stromal cell line. In the final stage, iPSC-NK cells co-cultured with irradiated and engineered feeder cells to further expand their population (62,63).

In addition, researchers formulated a technique for spin embryoid body (EB) protocol, which has further enhanced the efficiency of iPSC differentiation (64,65). The differentiation of iPSCs into NK cells occurs through a multi-stage process: First, iPSCs are co-cultured with small molecules and induction media, which promotes their differentiation into HPCs. This stage typically takes 12 days to produce $CD34^+$ HPCs. Second, lymphoid induction media and differentiation factors are used to induce lymphoid progenitor cells. The third stage involves guiding the differentiation of iPSCs into NK cells using specific NK induction media. The fourth and fifth stages involve the maturation process of NK cells (Fig. 3).

Zhang *et al* (66) confirmed the successful differentiation process from iPSC to iPSC-NK cells using EBs and analyzed the temporal changes in the expression of key genes through bulk RNA-seq analysis. Their findings revealed that while iPSC-NK cells share transcriptomic similarities with PBMC-derived NK cells, they maintained unique phenotypes characteristics. Pluripotency genes are highly expressed at the iPSC stage but gradually decreased with differentiation, becoming barely detectable at the EB stage. Hematopoietic-related genes were expressed at the EB stage and gradually increased during the differentiation process. Gene's specific to NK cells, such as GZMB, PRF1 and IL2RB, were gradually expressed from the early stage of differentiation and peaked in the late stage. The expression of some NK cell-specific markers (such as NKG2D, NKp46 and NKp30) began at the early stage of in the process of EB differentiation into iPSC-NK cells. During the middle stage, there was an increase in the expression of GATA2, the HSC regulator and key regulator of NK cell maturation. Simultaneously, the expression of mature NK cell-specific markers (such as natural killer cell cytotoxicity receptors and activation receptors) gradually increased. Compared with traditional 2D culture, EB-based iPSC-NK cells improved imitate the *in vivo* microenvironment. combining Clinical-grade iPSC-NK manufacturing is made possible by the cost reduction that results from combining EB with bioreactor technology (66).

6. Utilization of iPSC-derived NK cell in cancer treatment

In recent years, iPSC-NK cells have emerged as a novel direction in the field of immunotherapy. These cells open new possibilities for treating various diseases, particularly cancer, by

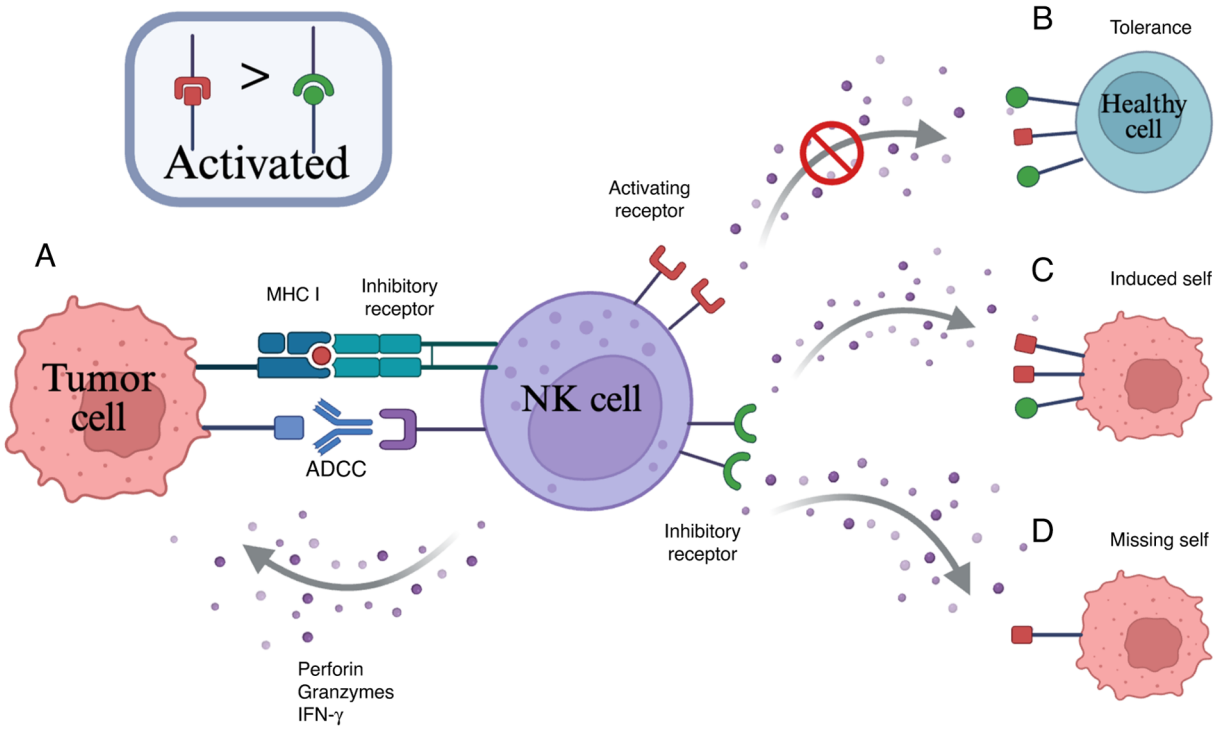

Figure 2. Schematic of NK cell functions. (A) Tumor cell targeting can occur in NK cells via ADCC and MHC-I. (B) In normal cells, MHC class I molecules can engage inhibitory receptors on NK cells, outnumbering and overriding activating receptors, thereby preventing NK-mediated cytotoxicity. (C) In malignant cells, NK cells recognize and respond to target cells through their activating and inhibitory receptors, resulting in NK-mediated cytotoxicity. (D) In tumor cells with downregulation or lack expression of MHC class I molecules, NK cytotoxicity can still be induced through the upregulation of stress-induced activation ligands, which bind to activating receptors on NK cells. NK, natural killer; ADCC, antibody-dependent cell-mediated cytotoxicity; IFN, interferon; MHC-I, major histocompatibility complex class I.

harnessing the advantages of iPSCs and NK cells. They have demonstrated significant clinical benefits in cancer treatment, as well as in autologous and allogeneic transplantation, infectious diseases, antiviral therapy and autoimmune diseases (66,67).

Currently, antibodies are extensively utilized in numerous cancer treatment modalities. However, treatment with antibodies alone might not be enough to increase the immune response because certain tumor patients (both treated and untreated) have severe lymphopenia (68). As a result, the infusion of a substantial quantity of NK cells is often required in the most clinical trials involving NK cells, ranging from $5x10^6$-$1x10^8$ cells/kg (8). One major advantage of iPSC-NK cells is their ability to undergo genetic modification and cryopreservation after differentiation, facilitating the production of homogeneous functional NK cells at a clinical scale (69). A study showed that differentiated iPSC-NK cells exhibit remarkable 3,000-fold expansion when Compared with primary NK cells Over 200 doses, each containing $>1x10^9$ cells, can be produced by utilizing this cell production scale (70). Markedly, iPSC-NK cells retain the characteristic NK cell phenotype, including the presence of activating receptors such as NKG2D, NKp44, NKp46 and DNAM-1 (66).

Furthermore, a study analyzing NK cell populations from peripheral blood, umbilical cord blood and iPSC-NK cells found that these different sources exhibit relatively similar expression patterns of cell surface antigens, as well as activation and inhibitory receptors. Notably, unlike CB-NK and PB-NK cells, iPSC-NK cells show variable expression of killer immunoglobulin-like receptors (KIRs). However, their cytotoxic activity against tumor targets is not markedly impaired by this mechanism. In fact, Compared with PB-NK cells, iPSC-NK cells produced more IFN-γ and TNF-α, thereby enhancing their function (65).

In November 2018, the FDA approved FT500 , the first clinical trial for iPSC-NK cell immunotherapy (56). Recent clinical trials have shown that iPSC-NK cells boost anti-tumor cytotoxicity and promote T cell activation and homing (71). Higher cytotoxicity against lung carcinoma cells, hepatocyte carcinoma cells, ovarian adenocarcinoma cells, melanoma cells and myeloid leukemia cells is demonstrated by their ability to recognize and lyse HLA-I downregulated tumor cells. This is particularly beneficial for solid tumor patients receiving anti-programmed death-1 (PD-1) or anti- programmed death ligand 1 (PD-L1) antibody therapy (72). Moreover, iPSC-NK cells may be cryopreserved and retained for subsequent repeated infusions.

A study comparing fresh, frozen and thawed PB-NK cells showed that among patients who received cell therapy, frozen cells did not proliferate and exhibited reduced cytotoxicity after thawing. By contrast, iPSC-NK cells can be cryopreserved at high densities. For example, both the FT596 and FT396 clinical trials used a density of $1.1x10^8$ for cryopreservation and the thawed cells still demonstrated high recovery rates and cytotoxicity (73). The method of producing cGMP-grade NK cells from iPSC lines is a significant advance. This approach not only improves NK cell production efficiency but also preserves their functional integrity. Additionally, iPSC-NK cells serve as a powerful tool for cancer treatment, serving as

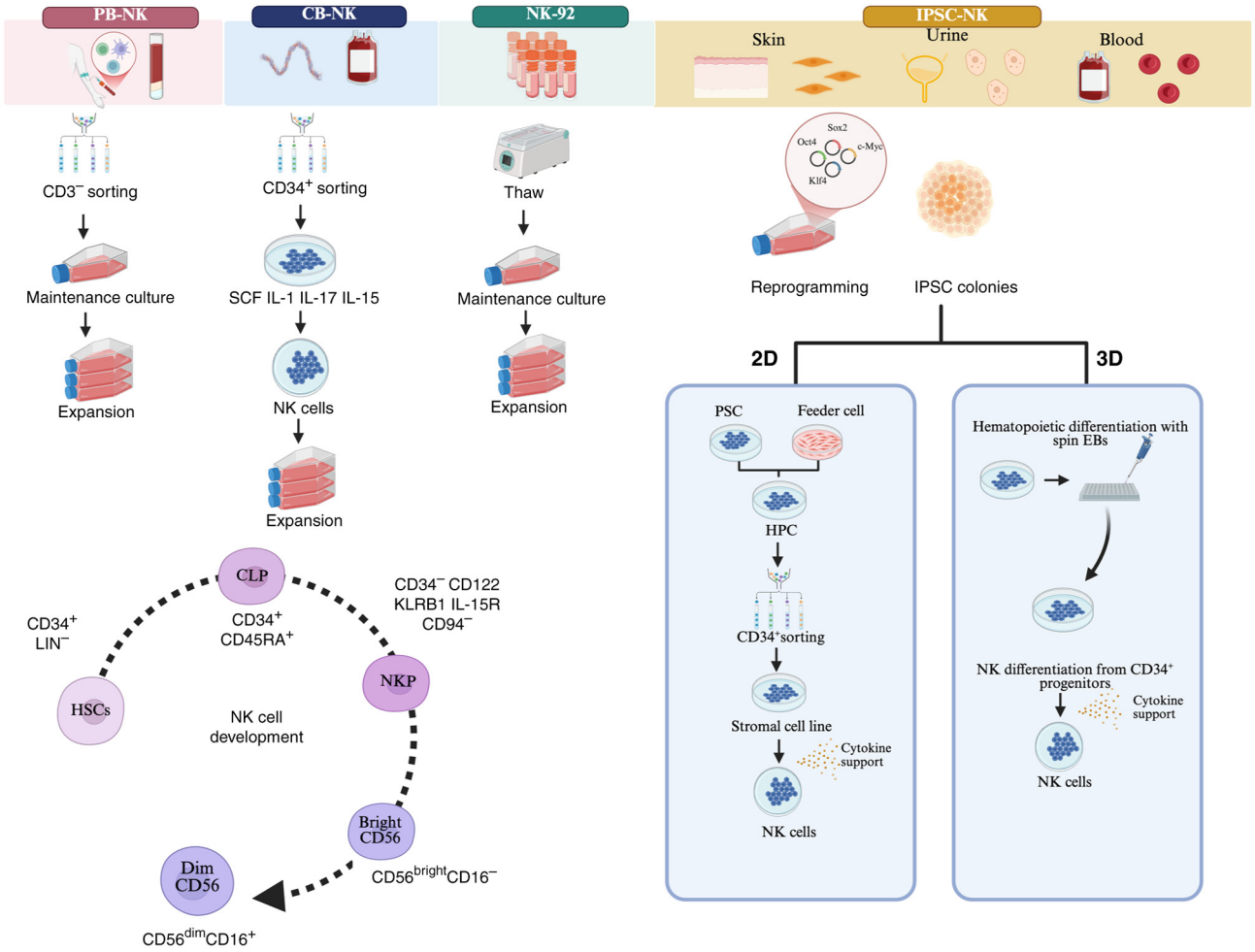

Figure 3. Different sources of NK cells and the development of NK cells. Various NK cell sources have been investigated in clinical trials for cellular immunotherapy, such as peripheral blood, umbilical cord blood and NK-92 cells. In the schematic representation of NK cell differentiation, $CD34^+$ cells differentiate into NK cells through the sequential acquisition of receptors/markers and functional properties. Three main subsets can be identified: NK cell precursors, $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells. The process of iPSC-NK development begins with the introduction of reprogramming factors into somatic cells, primarily fibroblasts, to induce their differentiation into pluripotent stem cells. These pluripotent stem cells are then guided to differentiate into $CD34^+$ HPCs using a combination of small molecules and cytokines, or through co-culture with irradiated stromal feeder cell lines. Next, the HPCs differentiate into iPSC-NK cells following the addition of NK cell initiating cytokines. In the 3D protocol, the differentiation of HPCs occurs in a suspension culture thorough EB formation or a spin-EB approach. The two methods utilize spin EBs to generate $CD34^+$ cells, which are HPCs. Over a period of 3-5 weeks, mature and functional NK cells are developed through this process. NK, natural killer; HPCs, hematopoietic progenitor cells; iPSC-NK, induced pluripotent stem cell-derived NK cells; EB, spin embryoid body. PB, peripheral blood; CB, cord blood.

a cell seed bank (62,74). While NK cells exhibit advantages compared with other immunotherapies, several limitations for solid tumors should also be overcome. The following sections introduce the strategies to enhance the function of iPSC-NK cells (Fig. 4).

7. Effects of cytokines and chemokines on iPSC-derived NK cells

IL-15 plays a pivotal role in the immune system, particularly in the development, maintenance and function of NK cells (75,76). IL-15 induces the proliferation of NK cells through binding to its receptor and activation of downstream signaling pathways, such as JAK-STAT and PI3K/AKT (77,78). Research has shown that culturing NK cells with a combination of IL-15, IL-18 and IL-12 enhances their targeting and killing of tumor cells both *in vivo* and *in vitro* (79). For iPSC-NK cells, Chen *et al* (80) developed a TALEN-based workflow to knock in IL-15, which enhanced the cellular function and persistence of iPSC-NK cells (80). A study by Kim *et al* (45) demonstrate that NK-exosIL-15/21 (natural killer cell-derived exosomes loaded with IL-15 and IL-21) enhances cytotoxicity and apoptotic activity in Hep3B cells. This effect was achieved by activating specific pro-apoptotic proteins, including Bax, cleaved caspase-3, cleaved poly ADP-ribose polymerase, perforin and granzyme B. Additionally, the treatment inhibited the anti-apoptotic protein Bcl-2, which prevents apoptosis and promotes cell survival.

Furthermore, a platform has been developed using CISH knock out in iPSC-NK cells to enhance JAK-STAT signaling through IL-15. Through improved metabolic fitness, which is characterized by mTOR signaling, this alteration directly contributes to enhanced NK function (11). These mechanisms still need to be deeply explored to optimize treatment strategies and translate pre-clinical findings into more effective clinical therapies for cancer patients (81).

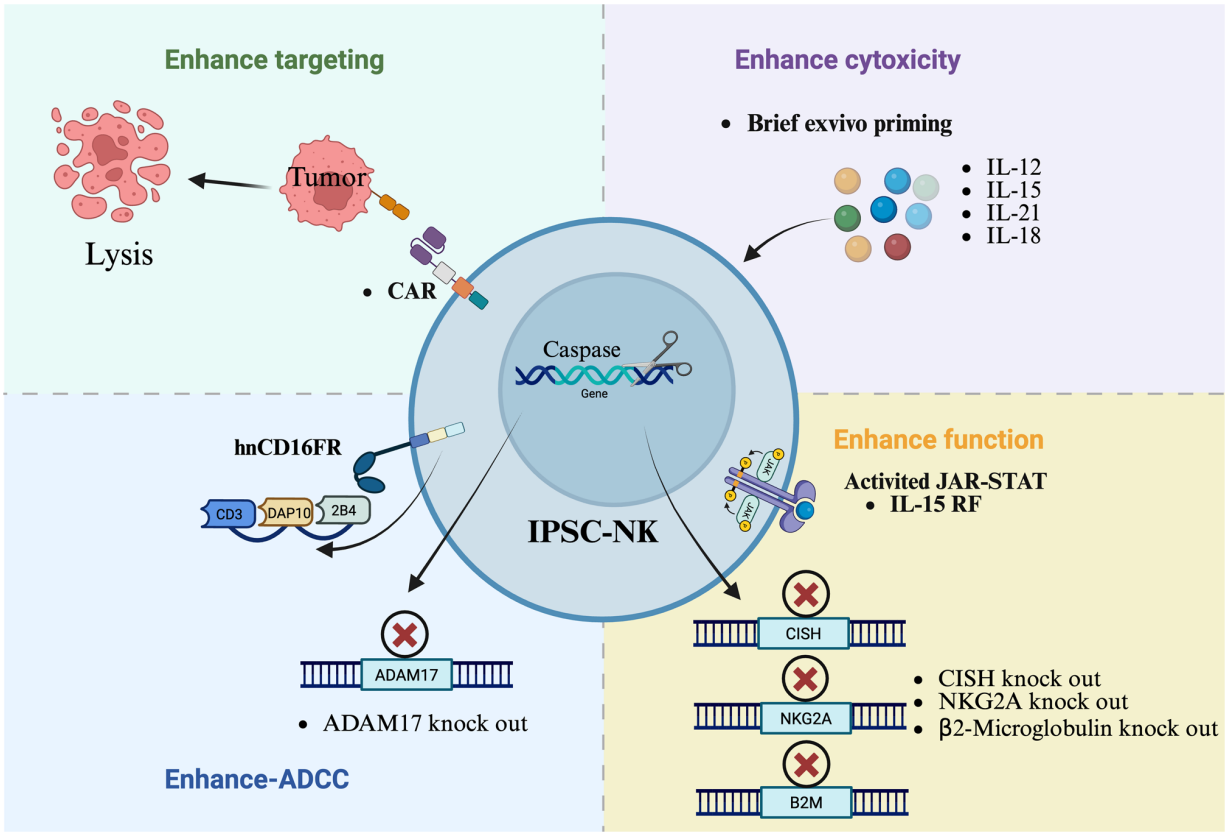

Figure 4. Summary of the various approaches to enhance iPSC-NK cells function. IPSC-NK cells are engineered with CAR to improve the target recognition of the tumor cells. Culturing with cytokines increases the cytotoxicity, while enhancing hnCD16 increases the ADCC. Cytokines increase *in vivo* persistence and activation. iPSC-NK, induced pluripotent stem cell-derived NK cells; CAR, chimeric antigen receptor; ADCC, antibody-dependent cell-mediated cytotoxicity.

8. Increased CD16 receptors on iPSC-derived NK cells

CD16 is a receptor involved in ADCC (82). When CD16a recognizes IgG-coated targets, NK cells release various cytotoxic molecules to mediate the death of the target cells (83,84). The cleavage of CD16a by ADAM17 (a disintegrin and metalloproteinase) is one of the mechanisms underlying its shedding. Activated NK cells exhibit a loss of CD16, which is cleaved by the ADAM17, and the homing receptor CD62L (84,85). Therefore, a strategy to improve cellular ADCC is to target ADAM17 to prevent CD16a shedding (85,86).

Research has found that using CRISPR/Cas9 technology to knock out ADAM17 can lead to improved production of IFN-γ and enhanced NK cell activity both *in vitro* and *in vivo* (87). Clinical trials have modified iPSC-NK cells to express a novel high-affinity 158V, non-cleavable CD16 Fc downregulation and enhance their binding ability with monoclonal antibodies (19). Meng *et al* (86) developed a novel hnCD16 fusion receptor, which consist of the extracellular domain of hnCD16, NK cell-specific co-stimulatory molecules (2B4 and DAP10) and the intracellular domain of CD3ζ. *In vitro*, iPSC-NK cells with hnCD16 fusion receptor demonstrate a marked increase in cytokine secretion against tumor cells compared with the control group. Additionally, it has been demonstrated that the anti-tumor capability of iPSC-NK cells can be markedly enhanced by combing high-affinity, non-cleavable CD16 fusion receptor (hnCD16FR)-iPSC-NK cells with CD20 monoclonal antibodies (88).

9. Increased other receptors on iPSC-derived NK cells to enhance functions

CD38 is a transmembrane protein primarily responsible for catalyzing the activity of molecules such as nicotinamide adenine dinucleotide and nicotinamide mononucleotide (89). Of CD38, ~90% is located on the cell membrane. To enhance the cytotoxic efficacy of NK cells, a strategic approach involves suppressing CD38 activity through genetic ablation (54,90).

Research has shown that knocking out CD38 and introducing the CD16-158V receptor in NK cells results in stronger ADCC effects and improved anti-tumor activity, particularly in multiple myeloma (90). NKG2A is an inhibitory receptor that binds to HLA-E on NK cells. Qin *et al* (68) focus on delating the expression of NKG2A by knocking out the NKG2A receptor gene in iPSC-NK cells, demonstrating excellent killing ability against tumors that highly express HLA-E.

T cell immune receptor with immunoglobulin and ITIM domains (TIGIT) and CD73 are key molecules in immune inhibitory pathways, often highly expressed in the tumor microenvironment, thereby suppressing immune cell function (68). Targeting TIGIT and CD73 alleviates immune suppression, enhancing anti-tumor immune responses. In glioblastoma therapy, inhibiting the TIGIT and CD73 pathways helps restore the activity of NK cells and T cells, leading to more effective tumor cell killing (91,92). Lupo *et al* (93) used the synNotch system to program iPSC-NK cells to express

Table II. CAR-NK cell clinical trials.

Number of NCT	Start year	Stage	Tumors	Target	NK source	Locations
NCT06307054	2024/2/28	Phase 1	Relapsed Adult AML. Refractory AML	CLL-1	PB-NK	Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine
NCT06242249	2024/1/27	Phase1/Phase 2	Multiple Myeloma, Refractory	BCMA	PB-NK	Shahid Beheshti University of Medical Sciences
NCT06201247	2023/12/30	Early Phase 1	Acute Myeloid Leukemia, in Relapse; Acute Myeloid Leukemia Refractory	CD123	PB-NK	Peking University People's Hospital
NCT06182735	2023/12/6	Phase 1	Renal Cell carcinoma	CD70	PB-NK	Fudan University
NCT06066424	2023/9/27	Phase 1	Solid Tumors	TROP2	PB-NK	M.D. Anderson Cancer Center
NCT06045091	2023/9/13	Early Phase 1	Multiple Myeloma; Plasma Cell Leukemia	BCMA	PB-NK	Shanghai Changzheng Hospital
NCT06027853	2023/8/30	Phase 1	Acute Non-Lymphoblastic Leukemia; Myeloid Leukemia; Acute Myeloid Leukemia; Acute Graft Versus Host Disease	CLL1	iPSC-NK	Zhejiang University
NCT06006403	2023/8/17	Phase 1/Phase 2	AML; Blastic Plasmacytoid Dendritic Cell Neoplasm; relapse Leukemia; Refractory Leukemia	CD123	PB-NK	Chongqing Precision Biotech Co., Ltd
NCT05987696	2023/7/24	Phase 1	AML, Adult; Minimal Residual Disease	CLL1/CD33	iPSC-NK	Institute of Hematology & Blood Diseases Hospital, China
NCT05922930	2023/6/20	Phase 1/Phase 2	Pancreatic Cancer; Ovarian cancer; Adenocarcinoma	TROP2	CB-NK	M.D. Anderson Cancer Center
NCT05856643	2023/5/3	Early Phase 1	Ovarian Epithelial Carcinoma	SZ-011	PB-NK	Shantou University Medical College
NCT05845502	2023/4/25	Not Applicable	Advanced Hepatocellular Carcinoma	SZ003		Shantou University Medical College
NCT05842707	2023/3/25	Phase 1/Phase 2	Refractory or Relapsed B-cell Non-Hodgkin lymphoma	CD19/CD70	CB-NK	Shanghai Tongji Hospital, Tongji University School of Medicine
NCT05776355	2023/3/8	Not Applicable	Ovarian Cancer	NKG2D	PB-NK	Hangzhou Cheetah Cell Therapeutics Co., Ltd
NCT05739227	2023/2/11	Early Phase 1	Acute lymphoblastic Leukemia; B-cell Lymphoma; Chronic Lymphocytic Leukemia	CD19	PB-NK	Xuzhou Medical University
NCT05747586	2023/1/30	Not Applicable	Multiple Myeloma in Relapse; Multiple Myeloma, Refractory	BCMA	PB-NK	Zhejiang University
NCT05734898	2023/1/30	Not Applicable	Acute Non-Lymphoblastic leukemia; Acute Myeloid Leukemia;	NKG2D	PB-NK	Zhejiang University
ChiCTR23000766	2023/9/21	Early Phase 1	AML	NKG2D	PB-NK	Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology

CAR, chimeric antigen receptor; NK, natural killer; PB, peripheral blood; CB, cord blood.

molecules capable of disrupting TIGIT and CD73 activity, showing a good performance in disrupting the immunosuppressive network in glioblastoma (93). Advances in gene editing technology have enabled the reduction of immunosuppression by inactivating HLA genes. This is achieved by the deletion of their common component, β-2 microglobulin (B2M), using CRISPR-Cas9 (94).

By employing gene editing techniques, it is possible to generate cell lines with a consistent genetic background. This approach minimizes phenotypic variations arising from genetic disparities, thereby enhancing the precision and reliability of disease research.

10. Increased targeting on iPSC-derived NK cells to enhance functions

Currently, researchers are improving the targeting capabilities of iPSC-NK cells by modifying them with CAR. A classic CAR consists of an extracellular recognition domain, such as a single-chain variable fragment (scFv) that recognizes tumor-specific antigens, along with a transmembrane domain and an intracellular signaling domain (10,95). The remarkable clinical efficacy of CAR-NK cells has sparked significant enthusiasm among researchers worldwide. As a result, clinical trials utilizing CAR-NK cells derived from diverse sources are currently underway at a rapid pace (Table II) (96). However, genetic modification of primary NK cells presents several challenges. The freeze-thaw process further complicates these challenges by compromising both the viability and anti-tumor capabilities of these cells (78). Additionally, the expansion and persistence of CAR-NK cells are limited by metabolic exhaustion and insufficient cytokine support within the tumor microenvironment (79).

By contrast, iPSC-NK cells offer several advantages, including a low requirement for seed cells, scalability, cost-effectiveness and the potential for autologous supply with minimal immunogenicity. Furthermore, these cells can be cryopreserved long-term, ensuring immediate availability for critically patients (9,76,77). Consequently, the development of CAR-iPSC-NK cells has emerged as a promising strategy for generating readily available allogeneic lymphocytes that can specifically target and combat malignant tumors. At present, CAR-iPSC-NK cells have evolved into a promising strategy for producing allogeneic lymphocytes to specifically target and combat certain malignant tumors (97,98). The potential of CAR-iPSC-NK cells was first reported in June 2018, when researchers introduced a CAR construct (scFv-NKG2D-2B4-CD3ζ) into iPSC-NK cells. The results demonstrated that CAR-iPSC-NK cells exhibited antigen-specific cell killing and increased expression of the granule marker CD107a (9).

In vivo experiments further show that mice treated with CAR-iPSC-NK cells experienced fewer side effects and much higher survival rates than treated with CAR-T cells. This study provided the first evidence that CAR-targeted NK cell therapy a viable option for treating refractory malignancies when chimeric antigen receptors are combined with the clinical-scale NK cells from iPSCs (9). Karvouni *et al* (99) developed a specific CAR targeting GPRC5D, which is highly expressed in Multiple myeloma (MM). They conducted a series of gene edits on iPSC-NK cells, including the incorporation of IL15/IL15RF fusion protein, hnCD16 and the knockout of CD38. These findings indicated that CAR-iPSC-NK cells a demonstrated strong cytotoxicity and tumor-clearing capabilities in allogeneic MM transplants (99). *In vivo* results indicated that two out of five mice achieved complete tumor clearance by day 80, highlighting the synergistic effect of CAR and hnCD16 (100-102).

Wang *et al* (103) developed 70-CAR-iPSC-NK cells, incorporating four gene edits which exhibited robust cytotoxicity against a wide range of tumors. The efficacy in precisely targeting lymphoma and renal cancer was further substantiated through xenograft models. The study also found that allogeneic reactive T cells expressed high levels of CD70, which 70-CAR-iPSC-NK cells effectively targeted and cleared, enhancing their survival and persistence (103). This finding underscores the importance of selecting an appropriate CAR construct to maximize the anti-tumor efficacy of CAR-iPSC-NK cells. The importance of CAR selection is further emphasized in ovarian cancer xenograft model. Compared with PB-NK cells, iPSC -derived NK cells and T-CAR-expressing iPSC-NK cells (scFv-CD28-CD28-CD137-CD3ζ,), NK-CAR expressing-iPSC-NK (scFv-NKG2D-2B4-CD3ζ) cells demonstrated markedly improved tumor suppression and prolonged survival (9).

Additionally, studies have reported remarkable anti-tumor performance of iPSC-derived NK cells expressing various CAR constructs, such as EGFR-CAR, CD19-CAR and CD33-CAR (104-106). By carefully selecting the CAR construct and employing metabolic engineering and gene-editing strategies, the functionality and targeting capabilities of CAR-iPSC-NK cells can be optimized.

11. Advances in clinical studies of iPSC-derived NK cells

IPSC-NK cells are currently undergoing clinical trials to evaluate their potential in treating various diseases. In order to provide data support for further clinical applications, these trials aim to determine the safety, efficacy and feasibility of iPSC-NK cells (Table III). The fastest-progressing clinical projects for iPSC-NK thus far primarily include those associated with Fate Therapeutics (FT596, FT576) and Century Therapeutics (CNTY-101).

In February 2025, the first clinical trial of an iPSC-derived CAR-NK cells product was completed. FT596 (trial no. NCT04245722) received Investigational New Drug approval from the US FDA (107). This product includes a CD19 CAR, a high-affinity, non-cleavable CD16 Fc receptor and an interleukin-15-interleukin-15 receptor fusion (58,108,109). In the Phase I trial, FT596 demonstrated a complete Remission (CR) rate of 85% (17/20 patients) in relapsed follicular lymphoma, with a median duration of response of ~16.9 months. Among patients previously treated with CD19 CAR-T therapy, the CR rate reached 30%. Furthermore, the study found that the proportion of $PD1^{+}CD8^{+}$ T cells in the tumor was positively associated with efficacy. This suggests that FT596 may exert an auxiliary anti-tumor effect by activating endogenous T cells, achieving a dual combined action of NK cells and T cells (107).

Table III. Ongoing clinical trials with iPSC-NK cells.

Company	iPSC platform	Product	Indications	Phase
Fate Therapeutics	iNK	FT576	Multiple Myeloma	Phase I
	iNK	FT522	B-Cell Lymphoma	Phase I
Century	iNK	CYTN-101	B-Cell Lymphoma	Phase I
	iNK	CYTN-104	Acute Myeloid Leukemia	Preclinical
	iNK	CYTN-106	Multiple Myeloma	Preclinical
Nuwacell	iNK	NCR300	Myelodysplastic syndromes	Phase I
	iNK	NCR301	Myelodysplastic syndrome	Preclinical
	iNK	NCR305	Solid tumor	Preclinical
Cytovia Therapeutics	iNK	CYT-103	Hepatocellular carcinoma	Preclinical
	iNK	CYT-303	Hepatocellular Carcinoma	Preclinical
		CYT-150		
	CAR-iNK	CYT-503	Hepatocellular Carcinoma	Preclinical
	CAR-iNK	CYT-538	Multiple Myeloma	Preclinical
	CAR-iNK	CYT-501	Glioblastoma Multiforme	Preclinical
Hebecell	iNK	HC101	Acute Myeloid Leukemia	Preclinical

iPSC-NK, induced pluripotent stem cell-derived NK cells; CAR, chimeric antigen receptor; NK, natural killer.

FT522 (NCT05950334) is a further optimized version of FT596. In order to expand the range of indications and reduce dependence on preconditioning chemotherapy, it incorporates an allo-defense receptor targeting 4-1BB function. Peripheral B lymphocytes were rapidly and profoundly reduced in patients receiving FT522 therapy in Phase 1 clinical trials. FT522 demonstrated enhanced persistence in comparison to the CAR-iPSC-NK cell product of the preceding generation, FT596 (110).

FT576 (NCT05182073) is an 'off-the-shelf' NK cell therapy derived from iPSC lines. It has been engineered with a CAR targeting B-cell maturation antigen (BCMA) and an IL-15 receptor fusion protein. In an evaluation involving nine patients, the following observations were made: No dose-limiting toxicities, no grade CRS, no immune-related neurotoxicity, no cases of GvHD. Notably, one patient who had previously undergone five lines of treatment achieved a very good partial response after the second administration of FT576 monotherapy, accompanied by a significant decrease in soluble BCMA (102). These findings indicated iPSC-NK cells have promising safety and efficacy for treating solid tumor, offering therapeutic benefits even in patients with extensive prior treatments (100-102).

CNTY-101 represents a pioneering cell therapy product candidate with six precise gene edits (111). These edits include: The incorporation of a CD19-CAR for targeted cell recognition, insertion of transgenes encoding HLA-E protein to disrupt B2M, insertion of transgenes encoding the extracellular and transmembrane domains of EGFR, implementation of Allo-Evasion™ technology (Century Therapeutics) to enhance compatibility across diverse patient populations, abnormal cells can be rapidly eliminated through the integration of suicide genes or drug-inducible switches to rapidly eliminate abnormal cells when toxicity induced by cellular therapies (94). These modifications enhance the cytotoxic effects of CNTY-101, even after over 15 successive rounds of *in vitro* killing. *In vivo* studies have shown significant effects, with fresh cells demonstrated a notable 91% reduction in tumor growth and cryopreserved cells showing a substantial 76% reduction. These results underscore the exceptional potential of CNTY-101, which is attributed to its enhanced properties and efficacy in precisely targeting and suppressing tumor growth in preclinical settings (112-114).

Therefore, CAR-iPSC-NK cells can directly enhance anti-tumor activity by improving ADCC and cytokine secretion. The clinical trials of iPSC-NK cells aim to validate their potential in treating cancer and autoimmune diseases, providing scientific evidence for their widespread application as an innovative cell therapy (3,115).

12. Challenges of iPSC-derived NK cells for clinical application

The performance of iPSC-NK cells in treating malignancies has generated great interest in their application. However, several obstacles must be tackled before their successful use. First, quality challenges cannot be overlooked. The preparation and differentiation of iPSCs require strict control of various conditions to ensure the final cell products are consistent and of high quality (71).

Heterogeneity in differentiation protocols may yield iPSC-NK cells with divergent phenotypes and functional profiles (116). For example, compared with feeder-free differentiation strategies, a lymphoid-based differentiation strategy using OP9 cells may produce more mature and potent iPSC-NK cells (117). However, due to technical limitations and the complexity of cell biological characteristics, it is difficult to completely avoid issues such as cell variation and contamination in practical operation. Researchers should evaluate the long-term safety of cell therapy and closely monitor patients

for adverse reactions and side effects when applying these cells to cancer patients (118). This will include monitoring immune responses, tumor formation and other potential risks associated with cell therapy. To enhance the efficacy and safety of iPSC-NK cells in the tumor microenvironment, future research needs to further explore how to optimize their preparation and modification methods.

Second, the tumor microenvironment plays a crucial role in enabling tumor cells to evade NK cell immune surveillance (10,119). The migration and infiltration abilities of iPSC-NK cells are crucial for targeting tumors. However, the dense stroma, high interstitial fluid pressure and abnormal vascular structures in the tumor microenvironment can impede these processes (116,120). Lactic acid in the tumor microenvironment suppresses NK cell cytotoxicity by inhibiting mTOR signaling (121-123).

To counteract this effect, strategies such as engineering iPSC-NK cells with lactate dehydrogenase overexpression have been employed (124,125). Enhancing the migration and infiltration abilities of iPSC-NK cells to enable them to reach the tumor site more effectively is an important research direction. Moreover, immunosuppressive factors in the tumor microenvironment also negatively impact the activity of iPSC-NK cells (126,127). The proliferation, activation and effector functions of iPSC-NK cells can be inhibited by these factors, which include regulatory T cells, myeloid-derived suppressor cells and immunosuppressive molecules (127,128). Thus, it is imperative to overcome these immunosuppressive factors to improve the survival rate and antitumor activity of iPSC-NK cells in the tumor microenvironment.

Tumor heterogeneity poses a significant challenge for iPSC-NK cells cell therapy. Genetic and epigenetic heterogeneity among tumor cells drive the emergence of diverse cellular subpopulations within tumors, resulting in varied responses to treatment and uncertainty in therapeutic efficacy. Studies have indicated that NK cells do not persist well *in vivo*, which limits the durability of their response against tumors (129-131). Therefore, enhancing the persistence of iPSC-NK cells is crucial for their therapeutic efficacy *in vivo*.

In summary, although iPSC-NK cells have the potential to recognize and kill tumor cells, several challenges must be addressed for their clinical application: Enhancing *in vivo* persistence, improving tumor targeting, overcoming the effects of the tumor microenvironment, optimizing differentiation strategies, achieving scalable production and quality control and managing immunogenicity and toxicity issues. Improving the specificity and targeting efficiency in the complex tumor microenvironment will aid in advancing the development and application of iPSC-NK cells therapies.

13. Discussion and future perspectives

To date, only Fate Therapeutics has completed a phase I clinical trial using iPSC-NK cells to treat cancer (107). Partial clinical trial data indicate that both genetically modified and unmodified iPSC-NK cells have demonstrated good safety and tolerability, with no severe adverse events related to NK cells (58,73,109). It has been reported that unmodified iPSC-NK cells show therapeutic effects against various types of tumors, especially in solid tumors (62). Meanwhile, genetically modified iPSC-NK cells have displayed favorable overall response rates and complete response rates in treating relapsed or refractory lymphomas (110). The development of CAR-iPSC-NK cells has brought new hope to the field of cancer therapy (6,88,132).

Despite these promising results, potential risks associated with iPSC-NK cells remain. Hypoxia and the accumulation of metabolites are two factors that may affect the metabolic adaptability and function of CAR-NK cells. For example, the activation receptors of CAR-NK cells may be inhibited in hypoxic environments, leading to a reduction in their cytotoxicity. Additionally, NK cells can enhance their utilization of glucose to promote glycolysis and mitochondrial oxidative phosphorylation. This metabolic adaptation provides sufficient energy and biosynthetic precursors, thereby supporting their proliferation and cytotoxic functions. (133,134).

The rapid utilization of glucose by tumor cells may also induce metabolic reprogramming in NK cells, thereby affecting their effector functions (123). The specificity and durability of CAR-iPSC derived NK cells have been further enhanced by recent advances in CRISPR-Cas9-based gene editing technology. For example, Shankar *et al* (135) used non-viral CRISPR gene editing technology on iPSC-NK cells to successfully insert CAR to GD2 while knocking out the KLRC1 gene. This modification markedly enhanced the ability to kill solid tumors. The improvements enhance the NK cells to proliferate by prolonging their survival *in vivo* and alleviating inhibitory signals. This improvement may be partly attributed to modifications in CAR that influence cellular metabolism. Therefore, further in-depth research is needed to explore strategies for overcoming the immunosuppressive effects of metabolites through CAR-iPSC-NK cells.

In the context of combination therapies, integrating iPSC-NK cells with monoclonal antibodies targeting tumor antigens may further enhance tumor recognition and eradication. Existing studies show that iPSC-NK cells and PD-1/PD-L1 inhibitors can synergistically enhance anti-tumor efficacy by overcoming immunosuppression in the tumor microenvironment, ultimately improving anti-tumor immune responses (136,137). Currently, clinical trials employing iPSC-NK cells in combination with monoclonal antibodies mainly include FT596 and CD19t-haNK (NCT06334991). CD19t-haNK is a clinical trial evaluating CAR-NK as a monotherapy or in combination with rituximab for treating relapsed/refractory CD19 and CD20 B-cell non-Hodgkin lymphoma (138). The first patients were successfully dosed in October 2024, with additional data expected in subsequent phases.

As reprogramming factors such as Oct3/4, Sox2, Klf4 and c-Myc are closely associated with tumorigenesis, their use in iPSC generation raises concerns. Notably, c-Myc mutations are frequent in human cancers, further highlighting the need for caution in iPSC-based therapies. To fully unlock the potential of iPSC-NK cells therapies in oncology, rigorous safety measures and technological advancements are critical for addressing these risks (139). Therefore, meticulous control of the iPSC cell amplification process is necessary. This includes selecting iPSC lines that have already demonstrated stability and safety in preclinical studies and employing gene-editing techniques to repair or correct potential mutation-causing sites

to enhance iPSC stability. For example, to replace oncogenic factors, Ding *et al* (140) employed transient mRNA delivery or small molecules.

Meanwhile, single-cell RNA sequencing technology can markedly contribute to resolving the heterogeneity during the differentiation process of iPSCs. By identifying functional subsets of NK cells, it can optimize the differentiation strategy of iPSC-NK cells (42,117). This technology has been used to analyze gene expression differences in iPSC-NK cells within the tumor microenvironment, shedding light on their interaction mechanisms with tumor cells, immunosuppressive cells and stromal cells. Additionally, single-cell sequencing has the potential to identify new therapeutic targets, thereby advancing iPSC-NK cells therapy in cancer treatment and immunotherapy (141). Using single-cell sequencing technology help detect abnormal genomic mutations, thereby reducing potential iPSC-related risks.

IPSC-NK cells are emerging as a novel and promising cell therapy tool in multiple ongoing clinical studies for solid tumor. They have demonstrated safety and potential efficacy in several reported clinical studies. However, some challenges remain in the clinical application of iPSC-NK cells, such as tumor microenvironment, differentiation strategies and quality control. Thus, more preclinical and clinical studies are required to push the iPSC-NK therapy into clinical applications. The establishment of allogeneic iPSC-NK cells as a next-generation immunotherapy, particularly in oncology, will be strongly supported by overcoming these hurdles.

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