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Review

Multiplexed iPSC platform for advanced NK cell immunotherapies

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SUMMARY

Human pluripotent stem cell (PSC) derivation advances have revealed enormous potential for improved cancer immunotherapy and clinical-scale blood cell production. PSCs can self-renew indefinitely and be differentiated into specialized cells, making them promising candidates for producing cytotoxic lymphocytes. Deriving natural killer (NK) cells from PSCs unlocks new possibilities for studying developmental hematopoiesis and investigating potential immunotherapy treatments. Cellular therapies, combined with genetic engineering, are potent tools for combating cancer and viral infections. While NK cells directly lyse tumor cells, genetic modifications, such as chimeric antigen receptor (CAR) engineering or the deletion of checkpoint molecules, can enhance their functional capacity. Here, we discuss recent advances in induced PSC (iPSC) editing and guided differentiation, focusing on developing NK cell immunotherapeutic products and optimizing iPSCs as an NK cell source to broaden therapeutic options and address diverse patient needs. This comprehensive review evaluates iPSC-derived NK cell-based therapies, recent advances, and future genome-editing strategies.

INTRODUCTION

Progress in cellular immunotherapy has transformed the therapeutic landscape for cancer and other ailments. Over the last decade, numerous cellular therapies have advanced, from preclinical studies to Food and Drug Administration (FDA)-approved treatments. Recent discoveries in immunology, genetic engineering, and cell production have facilitated novel therapeutic strategies. Live immune cell interventions present the possibility of curative results in cancers and chronic infections that are unresponsive to conventional clinically approved treatments. Living cells offer significant advantages due to their ability to adapt to environmental changes and participate in complex signaling pathways that traditional drugs cannot.[1] Among these, chimeric antigen receptor (CAR)-T cell therapy has garnered attention as a groundbreaking approach in treating certain hematological cancers, with several showing remarkable success in clinical trials and receiving FDA approval.[2] Despite these promising outcomes, CAR-T cell therapy faces significant challenges. Current clinically used treatments are expensive, require complex manufacturing, and can cause serious side effects such as cytokine release syndrome (CRS) and neurotoxicity. For conditions like B cell acute lymphoblastic leukemia, B cell lymphomas, and multiple myeloma (MM), addressing these issues remains critical to expanding the potential of CAR-T cell treatments.[2,3] These hurdles have led researchers to explore alternative immune cell therapies. Natural killer (NK) cells are part of the innate immune system and can employ various killing modalities without antigen-specific recognition. This allows them to target a broad range of cancer cells without complex engineering or challenging engraftment requirements, making them a relatively safer therapeutic approach than their T cell counterparts.[4,5]

With recent advancements in reprogramming technologies, induced pluripotent stem cells (iPSCs) have revolutionized the field of cellular immunotherapy.[6–8] These developments, built over decades, are now being applied to treat several diseases affecting various tissues, including skin, heart, muscle, and blood.[9,10] iPSCs provide a renewable and universal source for immune cell generation with consistent quality and scalability, making them "off-the-shelf" (OTS) cellular drugs. Targeted immunotherapy has advanced significantly with the introduction of NK cells derived from iPSCs, particularly with the use of CAR technology. This innovation substantially broadens the therapeutic landscape for NK cells. CAR-NK cells have the potential to revolutionize cancer treatment, but their potential applications in fibrotic diseases, endometriosis, and neurological disorders are just beginning to be explored. In this review, we discuss the current advancements and challenges faced in the *de novo* generation of NK cells from iPSCs (iPSC-NK), as well as how iPSC technologies can assist in overcoming major obstacles that presently hinder cellular immunotherapies.

NK CELLS: A SYMPHONY OF IMMUNITY

NK cells originate from common lymphoid progenitors (CLPs) that are derived from hematopoietic stem cells (HSCs) in the bone marrow (BM) and are integral to innate immunity.[11,12] NK cells are distinguished from other lymphoid cells by the absence

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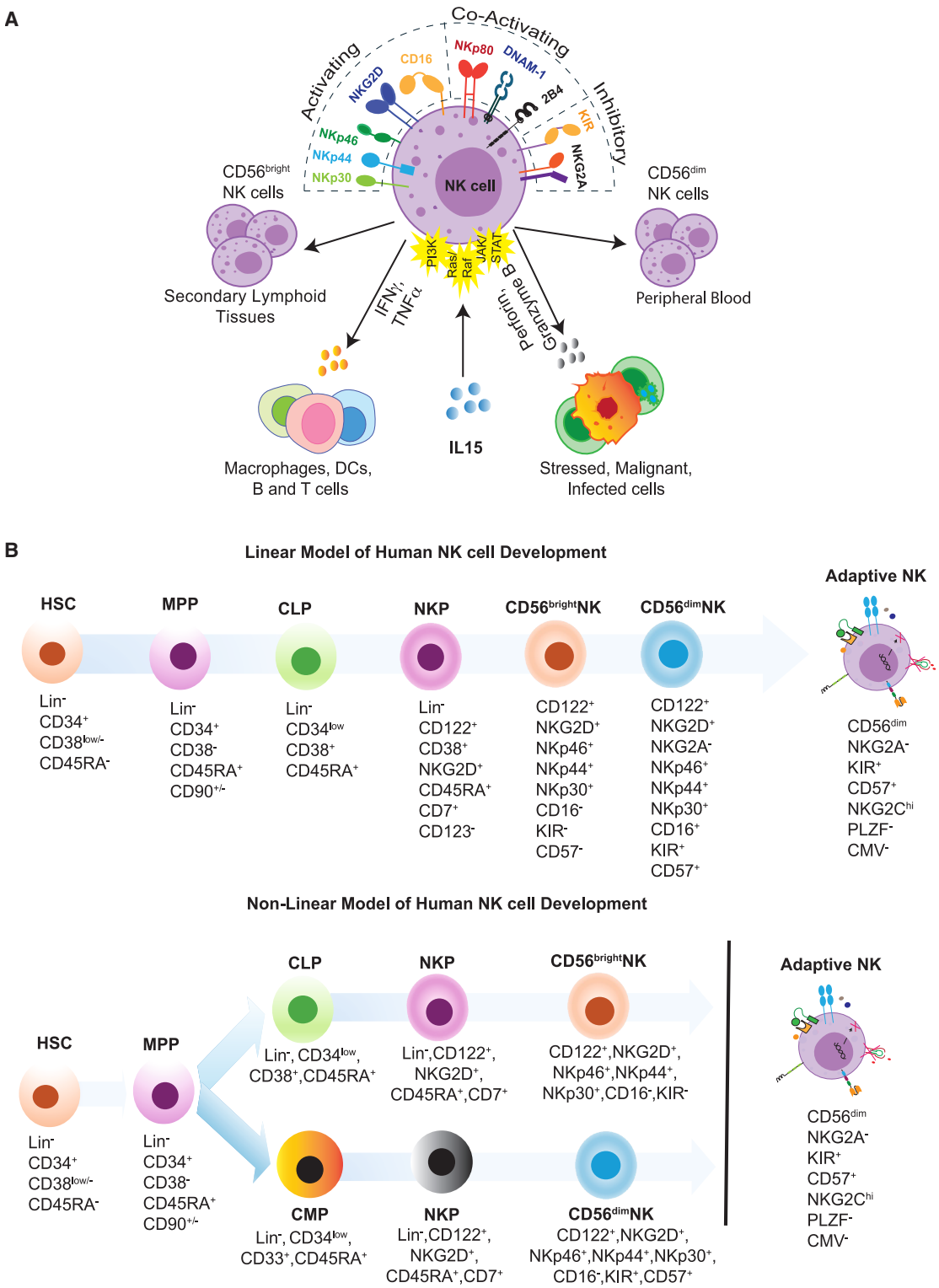

Figure 1. Major roles and development of NK cells in humans

(A) NK cells release cytotoxic proteins in response to interaction with stressed, malignant, or infected cells. NK cells can modulate adaptive immune cell abundance, maturity, and activity by secretion of IFN-γ and TNF-α. CD56^{dim} NK cells predominate peripheral blood NK cell populations and primarily perform cytolytic activity and immune surveillance. IL-15 secreted by multiple cell types can impact NK cell activity through Ras/Raf, PI3K, and JAK/STAT pathways. CD56^{bright} NK cells make up most NK cells residing in secondary lymphoid tissues, where they perform primarily immune-modulating functions.

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of CD3 and high levels of CD56 expression, and their survival and proliferation are driven by interleukin (IL)-15 signaling through the JAK/STAT, phosphatidylinositol 3-kinase (PI3K), and Ras/Raf pathways.[13] Upon maturation, NK cells can migrate from the BM to the bloodstream and establish themselves in numerous tissues due to their ability to move between lymphatic and non-lymphoid organs.[12,14,15] Fully mature NK cells acquire effector roles, including natural cytotoxicity against stressed, malignant, and virally infected cells, as well as the modulation of adaptive immune responses through the secretion of cytokines, chemokines, and growth factors[16,17] (Figure 1). In contrast to T cells, NK cells can rapidly recognize and kill target cells without prior sensitization.[11]

Developmental insight

Understanding the development of human NK cells and other innate lymphoid cell (ILC) lineages is key to the development of cellular products. However, canonical NK cell development is insufficiently understood, creating challenges for *in vitro* replication. The current predominant model proposes a linear, gradual transition where CLPs decrease CD34 expression and increase CD56 expression.[18,19] The earliest stages of NK cell development in the tonsil begin with $CD34^+CD38^-CD45RA^+CD10^+CD7^+CD117^-CD94^-CD16^-$ CLPs possessing Pro-B, Pre-T, and NK progenitor (NKP) potential, as well as potential for other ILC lineages.[18,20,21] The acquisition of IL-1R1 marks the beginning of NKP ($Lin^-IL1R1^+CD122^+CD38^+CD123^-CD45RA^+CD7^+$) commitment. NK cell lineage specification from CLPs, which can also be found in circulation, implies that $CD122^+$ NKP cells are a developmental intermediate capable of seeding peripheral tissues before further differentiation.[12,14] NKPs then differentiate into more restricted immature NK (iNK) precursor cells, characterized by higher expression of IL-1R1 and the appearance of CD314 (NKG2D), CD335 (NKp46), CD337 (NKp30), and CD161 (KLRB1). The next transition is marked by the emergence of $CD117^{+/-}CD94^+CD16^-CD56^{bright}$ NK cells, characterized by elevated CD56 levels alongside maximal expression of NKG2D, NKp46, NKp30, and KLRB1. Subsequently, these $CD56^{bright}$ NK cells further differentiate into $CD117^-CD94^{+/-}CD16^+$ $CD56^{dim}$ NK cells ($CD56^{dim}$ NK), which are distinguished by reduced CD56 levels, heightened CD16 expression, and the acquisition of killer immunoglobulin-like receptors (KIRs)[12,18,20] (Figure 1A). In support of a linear development model, $CD56^{bright}$ NK cells display longer telomeres than $CD56^{dim}$ NK cells and can acquire CD16 and KIR upon activation. Furthermore, studies using adoptive transfer into mice demonstrated the apparent maturation of $CD56^{bright}$ cells.[22–24]

Recently, a non-linear model of human NK cell development was proposed, asserting that common myeloid progenitors and granulocyte-monocyte progenitors can differentiate into NK cells when exposed to NK-supporting cytokines and stromal cells[20,25](Figure 1B). Furthermore, research suggests that $CD56^{bright}$ and $CD56^{dim}$ NK cells may have different ontogenies.[20,26] $CD56^{bright}$ NK cells in peripheral blood exhibit low cytotoxic capacity but display strong cytokine secretion when stimulated. Conversely, $CD56^{dim}$ NK cells have robust natural cytotoxicity, can execute antibody-dependent cellular cytotoxicity (ADCC), and display an abundance of cytolytic granules.[22,23,27] Evidence shows that the $CD56^{dim}$ NK cell population can also serve as a reservoir for NK cells possessing immunological memory for encounters with haptens, cytomegalovirus, or inflammatory cytokine stimulation. Memory (or memory-like) NK cells are phenotypically distinct subsets characterized by enhanced persistence, metabolic fitness, and antitumor activity. They also exhibit specific and amplified responses to secondary exposure events.[19,25,28]

Contrasting hallmarks of NK cells vs. T cells

Considering the pivotal role of NK cell responses in tumor immunity, promising avenues exist for harnessing this activity in immunotherapy. NK cells possess a broad distribution of activating receptors for recognizing distressed and transformed cells and can quickly distinguish and lyse tumor cells without antigen priming.[29,30] This natural cytotoxicity can complement antigen-specific targeting approaches for NK cell therapy. Even when ideal tumor targets are available, antigen escape mechanisms present significant hurdles to successful T cell immunotherapy.[30,31] For instance, human leukocyte antigen (HLA)-I downregulation is a common tactic exploited by malignant cells to evade T cell receptor (TCR)-mediated immunity, impacting more than 90% of patients in some cancer types. NK cells readily identify and destroy infected or transformed cells lacking HLA-I through a phenomenon known as "missing self."[32,33] While T cell treatments have key advantages, such as enhanced memory response and prolonged *in vivo* survival, the unique attributes of NK cell biology render them promising candidates in diseases where T cell antitumor action is insufficient or intolerable. Although primarily active in early tumor control, NK cells also play a role in late-stage disease, demonstrating the capacity to limit metastasis through interferon (IFN)-γ production or direct cytotoxicity.[34,35]

Antigen escape often accompanies metastasis as a function of tumor evolution, contributing to poor T cell activity against residual and metastatic disease. A "cold" tumor possessing an immunosuppressive tumor microenvironment (TME) is an additional negative prognostic marker of T cell-based therapies.[36,37] Through their modulation of adaptive immunity, NK cells may be capable of turning cold tumors "hot" by enhancing macrophage and dendritic cell antigen presentation and stimulating T and B

(B) Linear and non-linear models of human NK cell development. In the linear model, hematopoietic stem cells (HSCs) generate lymphoid-primed multipotent progenitors (MPPs), which differentiate into common lymphoid progenitors (CLPs) and then NK cell precursors (NKPs). These precursors mature sequentially into $CD56^{bright}$ and $CD56^{dim}$ NK cells, with adaptive NK cells emerging later in response to viral infection. The non-linear model suggests a more flexible developmental trajectory. HSCs still generate MPPs, but these can differentiate into either CLPs or common myeloid progenitors (CMPs), both capable of producing NKPs. CLPs predominantly give rise to $CD56^{bright}$ NK cells, while CMPs tend to generate $CD56^{dim}$ NK cells, both of which can ultimately differentiate into adaptive NK cells. This model underscores the growing recognition of NK cell heterogeneity and developmental plasticity in different physiological and disease settings.

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Table 1. A comparative analysis of NK cells and T cells through defining features of tumor immunity

Characteristics	NK cells	T cells
Killing modalities	• contact-dependent death ligand-mediated lysis • ADCC • recognition of "lack of self" • recognition of stress ligands	• TCR-mediated lysis, highly specific • contact-dependent death ligand-mediated lysis
Memory potential	• minimal, contained within small subpopulations (CIML and adapt-NK)	• high and robust, but small fraction of total populations
Immune modulation	• extensive, key regulators of adaptive immunity	• moderate, release immune-stimulating cytokines upon killing
Immunosuppression	• activated by MHC downregulation • inhibited by Treg cells • inhibited by TGF-β • inhibited by immune checkpoint proteins	• heavily impacted by MHC downregulation • inhibited by Treg cells • inhibited by TGF-β • inhibited by immune checkpoint proteins
Research feasibility	• low yields from donor blood • moderately expandable • low viral and plasmid-based gene transfer efficiency • sensitive culture requirements (both primary NK cells and iPSC-derived NK cells)	• high yields from donor blood • highly expandable • high feasibility of gene transfer • relatively insensitive culture
Clinical use	• positive safety profile • few engraftments' requirements • potential benefits outside of cancer: HIV and malaria • few FDA-approved therapies	• moderate safety profile; common off-tumor toxicities • numerous engraftment requirements to avoid toxicities • multiple direct cell therapies and auxiliary drugs are FDA approved

cell antitumor activity.[38,39] T cell therapies can result in high incidences of adverse events such as CRS, neurotoxicity, and graft-vs.-host disease due to off-tumor effects.[40,41] In contrast, NK cell infusions pose minimal risks of these complications, even with allogeneic grafts.[42,43] Together, these positive impacts of NK cells on immune regulation and disease control support their continued use as independent cellular therapies or in conjunction with other cancer treatments (Table 1).

Guardians of health and fighters of disease

As part of the innate immune system, NK cells are crucial in combating cancer, infectious diseases, autoimmune disorders, and chronic inflammation.[30,44] NK cells act as a first line of defense against infected and abnormal cells by monitoring cell stress and actively detecting hematological and solid cancers, circulating tumor cells, and metastatic progression.[17,45,46] Notably, cytotoxic NK cell infiltration into tumors is a favorable prognostic indicator for melanoma, renal cell carcinoma, and liver, lung, and breast cancer.[47–49] NK cell killing is initiated when the balance of inhibitory and activating signals received by the NK cell is tipped by pro-inflammatory cytokines, engagement of activating receptors is increased, or there is a loss of engagement through inhibitory receptors.[16] NK cell killing encourages further inflammation through cytokine secretion, which stimulates effector immune populations to enhance the immune response. NK cells play a role in both early- and late-stage malignancies. However, immunosuppressive factors in the TME can deter their impact in both, including transforming growth factor β (TGF-β) and regulatory cells such as T_{regs}, anti-inflammatory macrophages, and N2-like neutrophils.[50] NK cells also possess numerous regulatory mechanisms exploitable by tumors, including checkpoint receptors, NK cell localization factors, and NK cell self-tolerance mechanisms. Novel therapeutic strategies must account for these features to avoid limited treatment responses. When this tolerance fails in healthy individuals, NK cells can participate in the emergence of numerous autoimmune diseases, where they have been described as protective and pathogenic.[51] NK cell cytokine production can activate immature dendritic cells, macrophages, and $CD4^+$ T cells via IFN-γ and tumor necrosis factor (TNF) secretion. Reciprocal interactions between NK and other immune cells can modulate or activate NK cell proliferation and cytotoxicity.[16] NK cells are also physically localized to optimize their immune functions, with more cytotoxic $CD56^{dim}$ populations serving as sentry cells in the peripheral blood and more cytokine-producing $CD56^{bright}$ populations localizing to lymphoid tissue where they can influence other immune cells.[15,16,52] The functions of NK cells in healthy tissues, particularly in secondary lymphoid tissues, are becoming a topic of increasing investigation, and efforts are ongoing to translate NK cell functions in healthy tissues into therapeutic advances.[53]

HARNESSING NK CELLS: THE PROMISE OF ADOPTIVE IMMUNOTHERAPY

Immunotherapy has rapidly transformed clinical oncology, cementing itself as the fourth arm of cancer therapy in addition to

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surgery, radiotherapy, and chemotherapy. In a field dominated by T cell studies, NK cells are gaining traction due to their beneficial safety profiles, immunoregulatory functions, and capacity for quick, effective targeting and killing of tumor cells.[54,55] Therapeutic NK cells can be gathered from multiple primary and synthetic sources, each with unique implications for their clinical use. Many approaches use NK cells collected from healthy donor peripheral blood. Despite comprising a small fraction of total lymphocytes (~5%), peripheral blood NK cells can be enriched and expanded with engineered feeder cells to obtain large numbers for immunotherapy.[56,57] Alternatively, NK cells can be harvested from umbilical cord blood units and expanded to clinically relevant levels.[56] Despite originating from different tissues, peripheral and cord blood NK cells demonstrate similar relative cytotoxicity against tumor cells.[58,59] While efficacious, primary-sourced NK cell yields are highly inconsistent between donors and are heavily influenced by batch purification methods.[30,60] Naive cord blood NK cells also exhibit a relatively immature phenotype, with low expression of CD16, KIRs, and key NK cell adhesion molecules such as CD2.[61] However, this limitation can be overcome through *ex vivo* expansion into functionally mature NK cells, as demonstrated by Luevano et al., using cytokine-based differentiation protocols.[62] One group recently described a potential third source of donor-derived NK cells, isolated and expanded from full-term human placentas, offering an additional promising alternative for NK cell-based immunotherapies.[63]

NK cell lines have been established for indefinite culture to avoid donor variation and facilitate consistent yield, purification, and cell characteristics,[64] but there are challenges. One clinically tested NK cell line, NK-92, lacks expression of several key receptors associated with NK cell maturation, including CD16.[64] NK-92 cells also pose some safety concerns. As a neoplastic line, NK-92 cells require irradiation before administration to avoid proliferation and establishment of leukemia in the host.[64] In preclinical and clinical investigations of CAR-T cell therapy, extended *in vivo* longevity is associated with durable tumor clearance. Similar findings have been reported in some NK cell studies.[65,66] Irradiation prevents NK-92 *in vivo* proliferation, which may explain the limited effectiveness of NK-92 cells in various clinical trials.[67]

Due to the heterogeneity and adaptability of cancer, multiple studies have explored genetic engineering strategies to enhance NK cell antitumor activity and circumvent tumor-mediated immunosuppression. Transgene delivery technology has improved significantly over the last decade, facilitating the investigation of edits at a high-throughput scale and the delivery of multiple edits to a single cell.[68] Gene edits in NK cells can generally be classified as "improving positive traits" or "disrupting negative traits." Positive edits include the overexpression of chemokine receptors to promote localization to tumor sites, the expression of cytokine receptors for enhanced survival, the introduction of modified CD16α for stronger ADCC, and the induced expression of death receptors, such as TRAILR, to heighten cytotoxicity.[69,70] One innovative approach demonstrated the use of tumor-secreted chemokines to redirect NK cells engineered to express CCR4 and CCR2B to the tumor site. This led to tumor reduction and simultaneous depletion of immunosuppressive cells from the TME.[70] Negative edits are gaining popularity due to their increased ease of implementation via CRISPR-mediated knockout. These include the deletion of *CISH* and *GSK3* to improve NK cell metabolism and the disruption of *TGFRBII* to alleviate tumor-mediated immunosuppression.[20,65,71,72] Recent advancements in CRISPR-mediated knockin, which leverages the cell's endogenous homology-directed repair (HDR) mechanisms, enable simultaneous positive and negative editing by inserting a "positive" gene of interest into the locus of a "negative" gene. HDR knockin also avoids unwanted genomic alterations, such as those caused by lentiviral transfer, by precisely choosing the insertion site to provide stable and long-term expression.[73,74]

There are other methods, outside of genetic engineering, to enhance the antitumor activity of NK cells. As mentioned previously, stimulation of NK cells with IL-12, IL-15, and IL-18 has been used to generate cytokine-induced memory-like (CIML) NK cells.[75,76] These cells secrete high levels of IFN-γ, persist long-term, have potent antitumor activity, and have demonstrated excellent feasibility in manufacturing and replicability.[76,77] Further development of immunotherapy strategies that harness aspects of NK cell memory may broadly strengthen NK cell therapies.

iPSC-DERIVED NK CELLS

In recent years, iPSCs have garnered attention as a promising approach to circumvent the limitations associated with using primary cells and cell lines for immunotherapy. Takahashi and Yamanaka revolutionized regenerative medicine by developing a method for reprogramming mouse fibroblasts into iPSCs using transcription factors (OCT3/4, SOX2, KLF4, and c-Myc), known as "Yamanaka factors."[8] Soon after, this technique was successfully validated in human fibroblasts. Thomson and colleagues then used different factors (OCT4, SOX2, NANOG, and LIN28) to reprogram human somatic cells into iPSCs in 2007.[7] iPSCs share multiple traits with embryonic stem cells (ESCs), such as pluripotency, morphology, proliferation, gene expression, teratoma formation, and the ability to differentiate into multiple cell types.[6,7] While characteristically similar, studies have highlighted global transcriptions and DNA methylation differences between iPSCs and ESCs.[78,79] These studies revealed key variations in iPSC gene expression and epigenetic imprinting that negatively impact their stemness and genomic stability. Therefore, a platform using the reprogramming factors OCT4, SOX2, and SV40LT was designed for the induction and maintenance of transgene-free human iPSCs, allowing for rapid and high-throughput amplification in a feeder-free system.[80,81] This method preserved the pluripotency and genomic integrity of iPSCs by incorporating a small-molecule cocktail of signaling pathway inhibitors into the culture medium.[80,81] iPSCs cultured in this manner exhibited reduced spontaneous differentiation and displayed gene expression profiles that resembled the pluripotent ground state, rendering them more suitable for disease modeling, drug development, and transplantation medicine.

Following preclinical successes with ESC-derived NK cells, iPSC-derived NK cells have emerged as a promising

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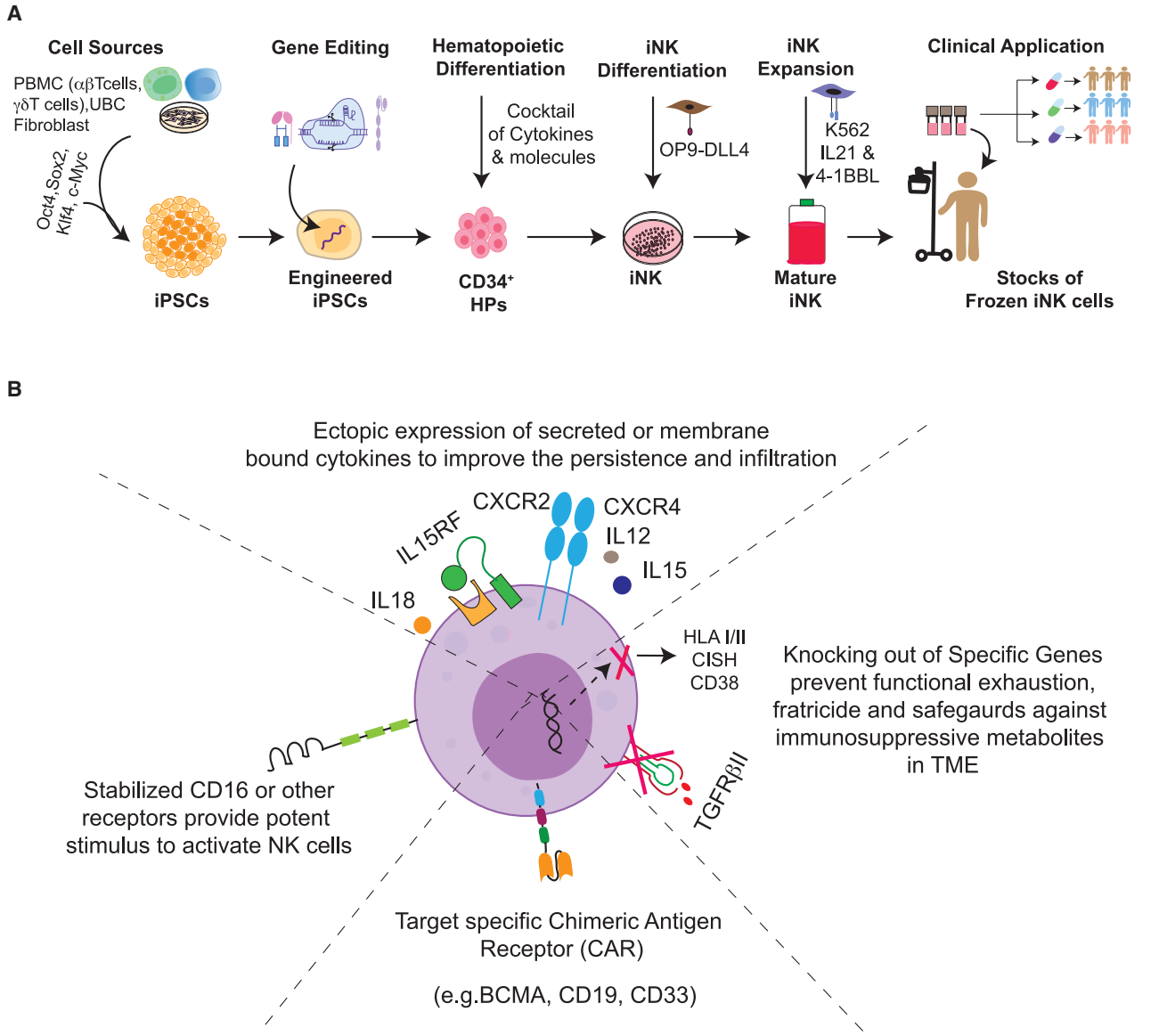

Figure 2. Multiplexed iPSC platform for engineered iNK cell production

(A) Schematic of the hematopoietic progenitors and iNK differentiation from engineered iPSCs.

(B) Strategies for genetic modifications to improve iPSC-NK cell function. Several genetic alternations have been engineered to enhance the biology and function of iPSC-derived NK cells for immune therapeutics. Here, we highlighted key components that have been engineered into iPSCs to enhance the functionality of iNK cells.

immunotherapy approach.[82,83] Recent advances have allowed the efficient differentiation of both human ESCs and iPSCs into highly functional NK cells (iNK cells), which share many phenotypic and functional characteristics with primary peripheral blood NK cells[84–86] (Figure 2). While human ESCs exhibit more consistent and efficient differentiation, iPSCs offer significant logistical advantages, providing a more reliable source of stem cells and the capacity for off-the-shelf therapies. Several protocols for NK cell differentiation originally developed with ESCs have since been refined for iPSCs, promoting efficient, large-scale production of iNK cells for therapeutic applications. Early attempts to generate stem cell-derived NK cells utilized a co-culture system with murine BM stromal cells, followed by further co-culture with cytokine-supplemented stromal lines to promote NK cell differentiation.[66,83] Currently, iNK production begins with the co-culture of iPSCs into lymphocytic progenitors using differentiation-promoting small molecules, cytokines, and stromal feeder cells. Once accomplished, the resulting $CD34^+$ hematopoietic progenitor cells (HPCs) can be enriched and differentiated into NK cells with a blend of NK cell-promoting cytokines, including IL-3, IL-7, IL-15, stem cell factor, and FLT3 ligand, or cultured on a secondary stromal cell line.[86,87] In the final stage,

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the fully differentiated iNK cells are isolated from any remaining stromal cells and expanded by co-culture with irradiated, engineered K562 cells to achieve maximum expansion and functionality.[66,85] This method has since evolved into a more refined "spin-embryoid body" technique that stimulates HPC growth in stroma-free, serum-free conditions, eventually driving cells to the NK lineage.[86,88] Under specific conditions, this protocol facilitates the differentiation of HPCs into phenotypically mature NK cells without requiring cell sorting (Figure 2). Additionally, the aryl hydrocarbon receptor antagonist StemRegenin-1 has been shown to significantly enhance the differentiation of human pluripotent stem cells into $CD34^+CD45^+$ HPCs, thereby promoting the development of conventional NK cells and providing a more efficient approach for iNK cell generation.[89] Recent findings demonstrated that iNK cells derived from peripheral blood cell iPSCs lack inhibitory KIR expression, leading to enhanced antitumor cytotoxicity.[90] Both *in vitro* and *in vivo* studies revealed that these iNK cells had potent antitumor and antiviral properties.[91]

iNK cells share many hallmark surface markers with peripheral blood NK cells, including CD56, DNAM-1, CD69, NKG2A/D, and natural cytotoxicity receptors.[82,92] However, iNK cells generally express lower levels of CD16, reducing their capacity for ADCC.[82,92] Additionally, KIR frequencies on iNK cells vary significantly, complicating their activation potential. In contrast, ESC-derived NK cells possess strong, consistent CD16 and KIR expression and exhibit potent cytotoxic activity against malignant cells via direct cell-mediated cytotoxicity and ADCC.[62,82,83] While iNK cells hold great promise as a solution for developing off-the-shelf immunotherapies, their large-scale manufacturing remains more complex and inconsistent relative to ESC-derived NK cell production, requiring further optimization for clinical use.[86,93,94] Despite recent advancements, generating human iNK cells faces several challenges. Initial efforts were hindered by low-throughput, suboptimal culture systems and a dependence on mouse embryonic fibroblast feeder cells. Additionally, non-defined serum-containing media and the prevalence of genetic instability in iPSC lines have raised concerns about the potential for oncogenic transformation.[95,96] These issues underscore the need for continued research to enhance iPSC-derived NK cell function, safety, and scalability to ensure their suitability for therapeutic applications.

ENHANCING iPSC-NK CELL FUNCTION VIA GENETIC ENGINEERING

Recent studies have explored the genetic modification of iPSCs to create engineered iNK cells with enhanced functionality and tumor-killing capabilities. iPSCs provide a stable platform for consistent genetic modifications that can enhance immunotherapy, and several modification approaches have been used to improve NK cell functions (Figure 2). Due to its central role in stimulating NK cell expansion and cytotoxicity, significant focus has been given to manipulating the IL-15 pathway.[43,97] Studies have established that increased NK cell functionality and antitumor activity accompany increased IL-15 signaling activation.[43,98,99] Stimulating IL-15 signaling also reduces negative factors by mitigating the immunosuppressive effects of TGF-β in the TME.[100] The co-expression of IL-15 with αCD19 CARs has shown promise in increasing cord blood NK cell antitumor activity against $CD19^+$ cell lines and primary leukemic cells.[100] This approach has been used to treat patients with relapsed or refractory (R/R) B cell malignancies in clinical studies.[30] Studies have shown that membrane-bound IL-15 expression in peripheral blood NK cells increases survival and proliferation *in vitro* and *in vivo* without needing exogenous cytokine support and enhances functional responses to hematologic and solid malignancies.[101] An alternative technique combines IL-15 superagonist and receptor α fusions for constitutive signaling. Two groups used fusion constructs to enhance peripheral blood NK and iNK cell antitumor efficacy *in vitro* and *vivo*.[66,102] Due to these favorable characteristics, IL-15 presents a clear target for boosting NK cell-mediated antitumor efficacy across platforms.

Cytokine-inducible SH2-containing protein, encoded by the gene *CISH*, is a negative regulator of IL-15 signaling in NK cells.[103,104] CRISPR-mediated *CISH* knockout ($CISH^{-/-}$) increased IL-15-driven JAK-STAT signaling in iNK cells, resulting in enhanced *in vivo* proliferation, cytotoxicity, and persistence in xenogeneic adoptive transfer models utilizing various tumor cell lines.[65] Another promising target is NKG2C, an NK cell-activating surface receptor that provides powerful activating signals and is linked to establishing immune memory. An αNKG2C/IL-15/αCD33 engager was recently developed to stimulate tumor-targeted responses from $NKG2C^+$ iNK cells, resulting in increased degranulation, IFN-γ production, and strong cytotoxicity against $CD33^+$ tumor cells and primary acute myeloid leukemia (AML) cells.[105] As previously noted, NK cells mediate ADCC through the Fc receptor FcγRIIIa (CD16a).[93,94] To exploit this killing pathway, researchers have generated engineered iNK cells (FT596) bearing three genetic edits to improve iNK effector function and persistence.[106] The first of these, a high-affinity, non-cleavable CD16 variant (hnCD16), was designed to inhibit ADAM17-mediated cleavage of CD16 and increase ADCC.[99,107] This approach, combined with the αCD20 monoclonal antibody (mAb) rituximab, demonstrated strong antitumor activity in mouse lymphomas.[69,108] Next, a membrane-bound IL-15/IL-15R fusion protein (IL-15RF) was added to promote constitutive IL-15 signaling and heightened *in vivo* survival.[106] Following this addition, an αCD19 CAR was incorporated to target B cell leukemias and lymphomas. This dual-targeting strategy aimed to maintain therapeutic efficacy if either CD20 or CD19 antigen expression was downregulated or lost by tumor cells.[108] In preclinical studies, FT596 demonstrated potent cytotoxicity and prolonged longevity without the need for exogenous cytokines, efficiently eliminating both $CD19^+$ and $CD19^-$ lymphoma cells.[106,109]

Further improvements have been made to iPSC engineering for the large-scale production of iNK cells and dual targeting against MM. Following the promising results of FT596, researchers created an engineered iNK (FT576) harboring four gene edits: hnCD16α, IL-15RF, an NK cell-optimized B cell maturation antigen (BCMA) CAR, and *CD38* knockout to enhance metabolic fitness and prevent NK-mediated fratricide when combined with αCD38 mAb therapy.[110] CD38, an ectoenzyme, has received notice for therapeutic targeting due to its involvement in immunotherapy resistance and NAD^+ metabolism.[24,110]

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Quadruple-edited FT576 cells have demonstrated superior immune function, ADCC, persistence, and adaptive NK cell metabolic and transcriptional characteristics. Combined with the αCD38 mAb daratumumab, these engineered iNK cells demonstrated potent antitumor activity against MM and AML, with no evidence of daratumumab-mediated fratricide occurring.[110] Recently, Thangaraj and colleagues created engineered iNK cells for improved functional activity against hepatocellular carcinoma (HCC).[72] The researchers modified these cells by either knocking out the TGF-β receptor 2 (*TGFBR2*-KO) or introducing a dominant-negative variant (*TGFBR2*-DN) and CARs targeting glypican 3 (GPC3) or alpha-fetoprotein (AFP).[72] *TGFBR2*-KO and *TGFBR2*-DN resisted TGF-β-mediated NK cell inhibition, a key suppressor in the liver TME.[111,112] GPC3 is a heparin sulfate proteoglycan overexpressed in HCC,[113,114] whereas AFP is a glycoprotein that is highly expressed during development but not in healthy adults.[115] Combining TGF-β signaling inhibition with CARs against these HCC antigens improved antitumor efficacy in modified iNK cells. These findings suggest that blocking TGF-β signaling is essential for effective iNK cell-mediated antitumor responses, with or without CARs, especially in tumors with high TGF-β levels in their microenvironment.[72] These advances highlight the potential of genetically modified iNK cells as powerful cancer immunotherapy agents, with ongoing clinical trials providing promising evidence of efficacy and safety. Further research is required to refine these approaches and fully realize the therapeutic potential of iPSC-derived NK cells.

ADVANCING iPSC-NK CELL TUMOR TARGETING WITH CAR ENGINEERING

Following the clinical success of CARs in T cell therapies, they have been repurposed to endow antigen specificity to NK cells, resulting in favorable antitumor efficacy.[116,117] CAR structures comprise an extracellular domain (ECD) for antigen binding, a hinge domain that provides stability, a transmembrane domain (TMD) that facilitates CAR integration into the cell membrane, and an intracellular domain (ICD) that triggers functional signaling cascades. Early CAR-NK cell designs closely followed those established in T cell models, with moderate success. However, recent updates utilizing NK cell-specific domains have shown improved efficacy.[84,118,119] Despite this progress, the optimal CAR structures and component combinations for NK cells remain undefined. For example, while DAP10 plays a key role in transmitting activating signals when paired with NKG2D, one study found that the DAP10 ICD only enhances function when combined with the NKG2D ECD.[118,120] In contrast, when NKG2D was used as a TMD, the presence of the DAP10 ICD reduced CAR-NK cell function, highlighting the complexity of designing effective CAR-NK constructs.[118,120] Conversely, another study found that using an NKG2D TMD followed by 2B4 and $CD3_{\zeta}$ in CAR-iPSC-NK cells highly improved antitumor activity.[84] Other studies suggest that DAP12 and DNAM1 are more effective ICD options than DAP10 and $CD3_{\zeta}$, though these effects may be context dependent.[119,121,122] Various preclinical studies have demonstrated encouraging antitumor activity of CAR-NK cells against ovarian cancer and lymphomas.[123,124] As previously mentioned, solid tumors often respond to therapeutic pressure by selectively downregulating targeted antigens, frequently leading to reduced CAR-T cell efficacy and patient relapse. While combinational targeting can mitigate antigen escape, CAR-NK cells may have extra advantages due to their expression of an array of germline-encoded activation receptors, as evidenced in preclinical data.[125,126] This underscores the endogenous therapeutic advantage of NK cells, even in the absence of potential further improvements and modifications.

Several CAR strategies that have been effective in T cell studies, such as tandem-CARs, dual-CARs, and adapter-CARs for multi-antigen recognition, remain unexplored in CAR-NK cells.[127,128] Dimeric antigen receptors (DARs) are chimeric receptors, similar to CARs, but with an antigen-binding component composed of a complete F_{ab} to enhance binding stability and targeting specificity.[129] Replacement of CARs with DARs could enhance the antigen-specific cytotoxicity of NK cells. Perhaps more potential exists for chimeric switch receptors (CSRs), a derivative of the CAR design that contains an ECD for an inhibitory ligand or receptor such as programmed cell death protein 1 (PD-1) or TIGIT but replaces native ICDs with activating signaling components, thereby switching what would typically be an "off" switch to "on."[30] NK-92 cells transduced with an αPD-1 NK cell-specific CSR demonstrated rapid clearance of PD-1-expressing tumor cells *in vitro*, and similar results were recently reported for peripheral blood NK cells against MM.[130] Due to the critical role TGF-β plays in suppressing NK cell activity, it has become a strong candidate for CSR design. While unmodified NK cells are primarily antigen independent, antigen-targeting modifications in NK cell therapies have necessitated greater attention to antigen availability. Recently, a study reported high tumor killing by CAR-modified NK-92 and peripheral blood NK cells transduced with a synthetic TCR, facilitating multi-epitope binding and, more importantly, recognizing intracellularly derived antigen peptides. However, these cells must lack inhibitory KIRs to bind HLA-bound peptide complexes efficiently and have not been thoroughly evaluated *in vivo*.[131] These studies highlight that while multiple promising synthetic receptor strategies exist beyond CARs, the NK cell field lacks sufficient evaluation of synthetic receptor-based approaches. There is also a need to shift CAR-NK cell models away from NK-92 and peripheral blood NK cells and toward more robust platforms for synthetic receptor testing.

CLINICAL APPLICATION POTENTIAL OF iPSC-DERIVED NK CELLS

NK cell treatments have made significant headway for the treatment of hematological malignancies, with the most compelling results reported for AML and myeloid malignancies.[76,132–134] Recent efforts to optimize KIR-HLA karyotyping may further strengthen NK adoptive cell therapy (ACT).[135,136] Studies have evaluated harnessing allogeneic peripheral blood NK cells concurrently with mAb products to enhance the impacts of NK cell-mediated ADCC. Allogeneic NK ACT combined with rituximab yielded promising responses in patients with B cell lymphoma with no significant toxicities.[137,138] Additionally, blocking mAbs against inhibitory KIR and NKG2A/CD94 signaling have been developed and studied in phase 1 trials.[139,140] This review

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summarizes key genetic edits being investigated in primary- and stem cell-derived NK cells. Many of these edits have been clinically tested with favorable outcomes observed.[79,80] As these trials progress, results reported may provide better focus to the overwhelming array of edits explored in preclinical settings.[141] While antibody drugs can direct NK cell-mediated ADCC against a chosen tumor antigen, the clinical success of T cell engager therapies such as blinatumomab has inspired multi-targeting engagers with NK cells. Several bi-specific killer engager and tri-specific killer engager designs are undergoing safety and efficacy evaluation in phase 1 and 2 clinical trials (ClinicalTrials.gov: NCT06088654, NCT06594445, and NCT05883449).

CAR-NK cell therapies have largely mirrored their CAR-T cell counterparts in clinical application, primarily focusing on $CD19^+$ hematological malignancies, though solid tumor studies are also underway (ClinicalTrials.gov: NCT05922930 and NCT06066424). One early CAR-NK trial deployed an αCD19 CAR-NK with inducible safety switches against $CD19^+$ chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma (NHL), yielding optimistic results, with a complete response (CR) rate of 63.6% and no significant toxicities at the 13-month follow-up point (ClinicalTrials.gov: NCT03056339).[30,121] CAR-NK products have also shown promising outcomes against R/R AML and R/R NHL with encouraging safety profiles.[30,121] The demand for OTS cellular therapies for improved treatment logistics has generated several related NK cell studies. Among these, ELiPSE (sponsored by Century Therapeutics), FT576, FT522, and FT596 (all from Fate Therapeutics) have entered the clinical stage and may help substantiate the clinical legitimacy of OTS therapies like iNKs. FT576 iPSC-derived CAR-NK cells contain a proprietary BCMA CAR, a novel hnCD16 Fc receptor, an IL-15RF to stimulate NK cells, and CD38 elimination to prevent NK cell-mediated fratricide.[142] In a phase 1 trial (ClinicalTrials.gov: NCT05182073), patients with R/R MM were given FT576 cells alone or with daratumumab. Following two treatment cycles, four patients were stable (stable disease), two were progressive (progressive disease), one had very good partial response (VGPR), one had a minimal response, and one had a partial response.[143,144] Another iNK (FT522) contains five novel synthetic controls of cellular functionality that allow for dual targeting of B cell CD19 and CD20 antigens: hnCD16, IL-15RF, CD38 elimination, and the company's alloimmune defense receptor (ADR) technology, which fosters functional persistence.[145] ELiPSE is a multicenter phase 1 dose-finding study evaluating the efficacy, safety, and pharmacokinetics of CNTY-101, an αCD19 iPSC-derived CAR-NK product, against multiple $CD19^+$ B cell malignancies (ClinicalTrials.gov: NCT05336409). CNTY-101 will follow a similar treatment regime to clinically used CAR-T drugs, applying lymphodepleting chemotherapy followed by single or multi-dose CNTY-101 administration alone or alongside subcutaneous IL-2 administration. Once completed, this study could provide important insights into how CAR-NK and iNK cell products compare to clinically relevant CAR-T counterparts. Recently, FT596 cell therapy has shown clinical promise as an intervention for R/R B cell lymphoma. In a phase 1 study, patients received a single dose of FT596 alone or with rituximab after conditioning chemotherapy.[146] In higher dose cohorts, 75% of patients had objective responses, while 58% had complete responses. The treatment was also well tolerated, with no dose-limiting toxicities or serious adverse events reported, further supporting the safety of CAR-NK cell products over CAR-T cell therapy.[146] Unfortunately, trials examining iNK products remain limited in both number and scope. While current studies have shown substantial responses, it should be noted that in most cases, the majority of patients eventually relapse. Therefore, testing of combinatorial CAR-NK cell strategies that attack the tumor in a multifaceted fashion has become increasingly common. For instance, a recent trial harnessed *ex vivo*-expanded, HLA-mismatched cord blood (CB) NK cells engineered to express an αCD19 CAR and secreted IL-15 to treat R/R B cell malignancies.[30] This innovative method localized IL-15 administration and improved patient response rates.[147] Though still being refined in their implementation, many ongoing CAR-NK cell clinical studies highlight continued optimism for breakthroughs in NK cell immunotherapy. Despite setbacks in solid tumor settings, recent enthusiasm has grown for utilizing NK cells against brain tumors, which are notoriously difficult to treat without damaging sensitive, healthy tissue. Many clinical trials employing various NK cell therapy classes are recruiting and ongoing in this setting (ClinicalTrials.gov: NCT04991870, NCT05588453, and NCT05887882). Multiple studies have experienced early termination, limited recruitment, and other procedural barriers, further emphasizing the need for logistical advancements (ClinicalTrials.gov: NCT04630769, NCT04106167, and NCT03841110). Table 2 depicts a list of clinical trials using iPSC-derived iNK cells.

CAR-NK cells have demonstrated promising efficacy against various cancers. However, recent research indicates that their potential extends beyond oncology into autoimmune diseases. Among the different immune cell types engineered for CAR therapy, CAR-NK cells have received much attention because of their favorable safety profile, including a lower risk of CRS and graft-versus-host disease. Recently, Meng et al. developed a chimeric autoantibody receptor that specifically targets the autoantigen La/SSB associated with various autoimmune diseases. They then incorporated this receptor into NK-92 cells to target autoreactive B cell clones.[148] As of 2025, several clinical trials involving CAR NK cells had been registered, with 12 focusing specifically on autoimmune diseases. Five of these trials are focused on systemic lupus erythematosus (SLE), with CD19 as the target antigen. Notably, one of these SLE trials employs iPSC-derived NK cells, highlighting the growing interest in scalable and renewable NK cell sources for autoimmune therapy (ClinicalTrials.gov: NCT06255028; Table 2). Earlier, the administration of CAR NK-92 cells to SLE mice significantly reduced the number of $CD4^+$ T cells and splenomegaly, as demonstrated by King et al.[149] These findings support the therapeutic potential of CAR-NK cells in modulating autoreactive immune components. With their innate cytotoxicity, allogeneic applicability, and rapid immune engagement, CAR-NK therapies represent a promising, scalable platform for treating a wide range of autoimmune diseases, including SLE, multiple sclerosis, and others.

There are numerous barriers hindering NK cell immunotherapies in the clinical setting, including poor NK cell persistence,

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Table 2. Clinical trials of iPSC-derived NK cells in cancer immunotherapy

Product	Title	Disease	Phase	Status	Study ID	Country
iPSC-NK (FT500)	FT500-derived NK cells as monotherapy and in combination with immune checkpoint inhibitors	advanced solid tumors, lymphoma	1	completed	NCT03841110	USA
iPSC-NK (FT516)	study of FT516 for the treatment of COVID-19 in hospitalized patients with hypoxia	COVID-19	1	completed	NCT04363346	USA
iPSC-NK (FT516)	FT516 (hnCD16) and IL-2 with enoblituzumab for ovarian cancer	ovarian cancer, adenocarcinoma, and primary peritoneal cavity cancer	1	completed	NCT04630769	USA
iPSC-NK (FT516)	FT516 (hnCD16) in combination with monoclonal antibodies (avelumab)	advanced solid tumors	1	terminated	NCT04551885	USA
iPSC-CAR-NK (FT596)	FT596 (hnCD16/anti-CD19 CAR/IL-15RF) iPSC-derived CAR-NK as a monotherapy and in combination with anti-CD20 monoclonal antibodies (rituximab or obinutuzumab)	B cell lymphoma (BCL), CLL	1	terminated	NCT04245722	USA
iPSC-CAR-NK (FT596)	FT596 (hnCD16/anti-CD19 CAR/IL-15RF) iPSC-derived CAR-NK with rituximab	autologous hematopoietic stem cell transplantation (HSCT) for NHL, B cell lymphoma	1	completed	NCT04555811	USA
iPSC-NK (FT516)	FT516 (hnCD16) in combination with monoclonal antibodies (obinutuzumab)	hematologic malignancies, AML, B cell lymphoma	1	terminated	NCT04023071	USA
iPSC-CAR-NK (FT538)	FT538 (hnCD16/CD38KO/IL-15RF) iPSC-derived NK cells in combination with daratumumab	acute myeloid leukemia (AML)	1	completed	NCT04714372	USA
iPSC-CAR-NK (FT538)	FT538 (hnCD16/CD38KO/IL-15RF) iPSC-derived NK cells in combination with monoclonal antibodies	advanced solid tumors	1	terminated	NCT05069935	USA
iPSC-CAR-NK (FT538)	FT538 (hnCD16/CD38KO/IL-15RF) iPSC-derived NK cells in combination with elotuzumab	hematologic malignancies, AML, multiple myeloma	1	terminated	NCT04614636	USA
iPSC-CAR-NK (FT538)	FT538 (hnCD16/CD38KO/IL-15RF) iPSC-derived NK cells in combination with elotuzumab a3 domain of MICA/B-CAR	ovarian, fallopian tube, and primary peritoneal cancer	1	recruiting	NCT06342986	USA
iPSC-CAR-NK (FT522)	FT538 (hnCD16/CD38KO/IL-15RF) iPSC-derived NK cells in combination with CD19-CAR + ADR	BCL	1	recruiting	NCT05950334	USA
iPSC-CAR-NK (CNTY101)	a study of CNTY-101 in participants with CD19-positive B cell malignancies	R/R $CD19^+$ B cell malignancies, aggressive or indolent NHL	1	active, not recruiting	NCT05336409	USA
iPSC-CAR-NK (FT576)	a phase 1 study of FT576 (IL-15RF/CD38KO/anti-BCMA/CAR) as monotherapy and in combination with daratumumab	MM, myeloma	1	active, not recruiting	NCT05182073	USA
CAR iPSC-NK (FT536)	FT536 iPSC-derived CAR-NK (hnCD16/CD38KO/anti-MICA/B/CAR/IL-15) monotherapy and in combination with monoclonal antibodies	advanced solid tumors	1	terminated	NCT05395052	USA
iPSC-CAR-NK	iPSC-derived NK cells targeting CD33	AML	1	terminated	NCT05601466	China
iPSC-CAR-NK	iPSC-derived NK cells targeting CLL1	AML, chronic myeloid leukemia (CML)	1	recruiting	NCT06027853	China
iPSC-CAR-NK	iPSC-derived NK cells targeting CD19	systemic lupus erythematosus (SLE), lupus nephritis	1	recruiting	NCT06255028	USA

heterogeneous expansion, tumor-mediated immunosuppression, and overreliance on cytokine and feeder cell support. These factors, combined with an incomplete elucidation of canonical NK cell development and biology, contribute to a lack of clinically approved products relative to those achieved in the T cell immunotherapy field. Incidentally, last year, the FDA provided the allogeneic NK cell product IGNK001 (gengleucel) with orphan drug designation for use against AML.[150] Researchers, clinicians, patients, and their families eagerly await similar achievements, and many ongoing CAR-NK cell clinical studies highlight continued optimism for breakthroughs in NK cell immunotherapy.

OUTLOOK

This review describes several promising NK cell therapeutic strategies, technologies, and platform advancements. CAR-NK cells have thus far demonstrated favorable safety profiles and multiplexed immune responses for cancer immunotherapy. iPSCs offer unique advantages in realizing this potential. iPSCs can be derived from diverse sources, reprogrammed, and shown to exhibit virtually unlimited proliferation potential *in vitro*, reducing production costs and increasing the accessibility of NK cells for clinical use. Using iPSCs as a gene-editing platform provides significant opportunities to enhance the functionality of NK cells, particularly CAR-NK products, positioning them as a powerful tool against hematologic and solid malignancies. iPSC-derived CAR-NK cells hold promise as an innovative and effective ACT for patients with cancer, which may overcome the limitations of traditional CAR-NK cells sourced from peripheral or cord blood, though further clinical data are needed.

As observed in T cell immunotherapy, feedback from clinical studies helps focus preclinical efforts and studies and guide combinatorial strategies. However, there is currently a lack of FDA approvals relative to the numerous NK cell immunotherapy studies underway. This is partly due to the recent increase in costs of iPSC clinical translation, caused by various technological and regulatory challenges. For one, a proper clinical study requires a comprehensive sponsor-led risk assessment and testing for adventitious agents in raw materials. To further complicate matters, iPSC product development laws vary by jurisdiction, are inconsistently updated, and many are currently being contested. Clinical successes in cellular therapy stem from understanding core biological and biochemical mechanisms, as well as the ease with which living drugs can be harvested, modified, and manufactured. Thus, prioritizing the elucidation of NK cell lineage commitment, maturation, signaling, and cellular interactions is critical. To strengthen the logistical foundation of NK cell immunotherapy, advancing NK cell sourcing, gene transfer methods, expansion and stimulation protocols, and cryopreservation is imperative. Effective feeder-free culture methods and xeno-free culture material for iPSC genesis and proliferation represent significant breakthroughs in this effort. Unfortunately, the lack of commercially accessible Good Manufacturing Practice (GMP)-grade and xeno-free stem cell reagents has hindered the use of sophisticated and time-consuming GMP-compliant production processes that involve numerous biological components. These setbacks must be resolved so that current iPSC-NK products can fully realize their potential benefits.

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AUTHOR CONTRIBUTIONS

Writing – original draft, A.K.; writing – review and editing, C.F. and F.C.; supervision, J.S.M.

DECLARATION OF INTERESTS

J.S.M. and F.C. consult for, receive research support from, and hold stock options in Fate Therapeutics, an iPSC company. J.S.M. also consults for, receives research support from, and holds stock options in GT Biopharma, an NK cell engager company, and advises for Sanofi and Vycellix. These interests have been reviewed and managed by the University of Minnesota in accordance with its conflict-of-interest policy.

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