

REVIEW ARTICLE

iPSC-mediated genetic manipulation
promotes natural killer cell-centered cancer
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Abstract

Natural killer (NK) cells demonstrate potent cytotoxic activities and the capacity to secrete cytokines. Their distinctive capability to trigger cell death, bypassing the need for major histocompatibility complex recognition, opens promising avenues for their use in clinical settings such as allogeneic transplantation and tumor immunotherapy. Although the ability of NK cells to kill hematological tumors has been widely recognized, their effectiveness in treating solid tumors is not as pronounced. The intricate interplay of NK cells with the tumor microenvironment, specifically in the context of solid malignancies, has been noted to attenuate the anti-cancer prowess of NK cells and foster the ability of malignant cells to elude immune surveillance. Successful NK cell-centered immunotherapy hinges on obtaining a substantial quantity of NK cells with potent tumor-killing capabilities. However, the current challenge lies in the limited *ex vivo* expansion of NK cells and the inefficiency of gene introduction methods. Induced pluripotent stem cells (iPSCs) are multipotent stem cells with relatively easier gene transfection capability and theoretically unlimited proliferation potential. NK cells derived from iPSCs circumvent the challenge of difficult genetic modification in NK cells, offering various potential strategies to counteract the immune suppression induced by the tumor microenvironment.

Keywords: Induced pluripotent stem cells; Natural killer cells; Solid cancer; CAR-NK cells; Immunotherapy

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1. Introduction

Innate lymphoid cells (ILCs) are classified into three principal groups: Group 1, Group 2, and Group 3 ILCs. Natural killer (NK) cells are categorized within Group 1 ILCs, along with ILC1.¹ Each of these groups has a corresponding counterpart in adaptive immunity characterized by T helper (Th) cells: Th1 for Group 1, Th2 for Group 2, and Th17 for Group 3, reflecting their roles and functions in the immune response.² NK cells, first identified in the 1970s, derive their name from their innate ability to kill target cells

without prior antigen stimulation, in contrast to T cells, which require such activation.^{3,4} NK cells are primarily involved in directly killing target cells and modulating the activity of other immune cells via cytokine secretion. Their typical targets include virally infected cells and cells that have undergone malignant transformation.⁵ Originating from hematopoietic stem cells (HSCs) within the bone marrow, NK cells are not entirely mature upon departure from the bone marrow. They undergo further maturation influenced by specific signals and the microenvironment where they reside.^{6,7}

Peripheral blood NK cells can be categorized into two primary subpopulations: CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺. The former represents a mere 5 – 10% of the NK cells circulating in peripheral blood and is thought to be at an early stage in NK cell maturation. In contrast, the vast majority of NK cells are CD56^{dim}CD16⁺, which is indicative of a more mature state. Interestingly, the CD56^{bright}CD16⁻ cells, despite their lower abundance, exhibit limited capability for antibody-dependent cell-mediated cytotoxicity (ADCC), a key mechanism through which NK cells mediate their immune response.^{8,9} The cytotoxic effect of NK cells is not restricted by the major histocompatibility complex (MHC),¹⁰ making them promising candidates for allogeneic transplantation with favorable clinical prospects. CD16 (Fcγ receptor III), expressed on the surface of NK cells, facilitates ADCC, synergizing with adaptive immunity^{11,12} and enhancing the efficacy of therapeutic antibody-mediated targeted therapy.¹³ During the late stages of NK cell development, NK cells establish immune tolerance with self-MHC-I molecules¹⁴ through a process known as licensing.¹⁵ The activating and inhibitory receptors, presented on the surface of NK cells are in a delicate state of “dynamic equilibrium”.^{16,17} During the malignant transformation of normal cells, activating receptor ligands are often upregulated, whereas inhibitory receptor ligands are frequently downregulated, rendering these cells more susceptible to NK cell-mediated targeting.¹⁸ NK cells have shown promising clinical prospects in the treatment of hematological cancer.¹⁹⁻²² The graft-versus-leukemia (GVL) effect, primarily mediated by NK cells, has garnered increasing clinical attention and is being applied in the context of human leukocyte antigen (HLA) haplotype-mismatched HSC transplantation.²³ However, the efficacy of NK cell-mediated cytotoxicity against solid tumors is not as ideal as expected.^{24,25}

The microenvironment of solid tumors is characterized by low nutrient content, high acidity,²⁶ and low oxygen,²⁷ which negatively affect the function and activation of NK cells and other immune cells, resulting in poor cytotoxicity

of NK cells. The growth of abnormal blood vessels in solid tumors creates hypoxic areas that can cause degranulation of NK cells and mitochondrial damage, leading to NK cell exhaustion.²⁸ Other tumor-associated cells, such as M2 macrophages or tumor-associated macrophages (TAMs), also weaken NK cytotoxicity.^{29,30} Immunosuppressive cytokines in the solid tumor microenvironment, such as transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10), can reduce the surface expression of activating receptors, including NKp30, NKG2D, and CD16, on NK cells (31 – 33), decrease the levels of transcription factors such as T-bet (34), and potentially reprogram NK cells into ILC1 cells, which possess a weaker anti-tumor capacity.³¹

For clinical therapies, NK cells are primarily obtained from peripheral blood,³² cord blood,³³ or established NK cell lines.³⁴ Although *ex vivo*-expanded or stimulated NK cells have shown promising safety profiles in clinical applications through autologous or allogeneic transplantation, the full extent of their therapeutic efficacy awaits further definitive validation. Previous research on NK cells has unveiled numerous genes that exert positive or negative influences on NK cell maturation, activation, and their capacity to combat tumors. Certain genes positively influence NK cells by fostering their maturation and boosting their cytotoxic abilities,³⁵ whereas others act as negative regulators by impeding NK cell development³⁶ or dampening their activation.³⁷ These insights significantly enrich our comprehension of NK cell biology and suggest innovative avenues for their therapeutic deployment. Targeting these regulatory genes presents an exciting opportunity to devise novel treatments. Through precise gene editing or molecular modulation, it is possible to improve the anti-tumor potency of NK cells or augment their efficacy within specific immunological niches, enhancing the effectiveness of NK cell-based immunotherapies. Strategies that activate or upregulate genes that positively regulate NK cells could enhance their functional activity and prolong their longevity. On the other hand, the inhibition or deletion of genes that negatively regulate NK cells may alleviate constraints on their functionality, thus bolstering their anti-tumor performance. Due to significant challenges in gene transfection and limited expansion lifespan, both *in vitro* and *in vivo*, NK cells encounter constraints that complicate their genetic engineering and clinical utilization.³⁸ In comparison, T cells, especially chimeric antigen receptor (CAR)-engineered T (CAR-T) cells, demonstrate superior viral transfection rates, enable substantial *ex vivo* expansion, and have the capability to establish enduring memory T cells within the host.³⁹ These attributes enable CAR-T cells to persistently identify and eradicate tumor cells that present specific antigens. On

the other hand, genetically engineered NK cells encounter difficulties in achieving adequate cell quantities with the desired genetic payload, maintaining effectiveness *in vivo*, and overcoming the immunosuppressive forces of the tumor microenvironment. These challenges significantly limit the utilization of NK cells in the arena of cancer immunotherapy.

Induced pluripotent stem cells (iPSCs), a type of pluripotent stem cells derived from terminally differentiated cells,⁴⁰ can be efficiently induced to differentiate into NK cells through specific combinations of cytokines.⁴¹ iPSC-derived NK cells have demonstrated promising anti-tumor activity in pre-clinical and clinical experiments, making them a highly potential therapeutic strategy for NK cell-based cancer treatment.⁴² Compared to peripheral blood NK cells, iPSCs are more flexible to gene overexpression, gene silencing, and gene editing. Coupled with the inherent ability of iPSCs for limitless proliferation, this technology supports the feasibility of selecting and purifying genetically modified cells. These advancements not only establish a foundation for precise genetic manipulation of NK cells but also open avenues for further NK cell research and their application in clinical settings. This review explores iPSC-based approaches, particularly genetic modifications, to enhance NK cell-based strategies for combating solid tumors. It covers the biological characteristics of tissue-resident NK cells, challenges related to the resistance of various solid tumors to NK cell-mediated cytotoxicity, and clinical efforts aimed at improving NK cell-mediated killing of solid tumors.

2. Mechanisms employed by solid cancer to evade NK cell attack

NK cells and CD8⁺ T cells exhibit parallel cytotoxic capabilities, sharing the ability to eliminate target cells through similar mechanisms, such as employing perforin and granzyme B.⁴³ Despite these shared cytotoxic mechanisms, the activation pathways and tumor cell recognition strategies of NK cells and T cells differ fundamentally, often standing in stark contrast to each other.⁴⁴ This distinct divergence in recognition strategies suggests that T cells and NK cells offer complementary approaches to tumor cell identification. In this context, it is critical to delve into the strategies employed by tumor cells to evade NK cell-mediated destruction. These strategies span a broad spectrum, ranging from evading NK cell detection to manipulating the NK cell activation processes. In addition, the tumor immune microenvironment plays a pivotal role in suppressing NK cells, even after they have recognized tumor cells and are primed to execute their cytotoxic function.⁴⁵ Understanding these evasion and

inhibition mechanisms is vital for the development of targeted therapies that enhance the antitumor efficacy of NK cells. This includes employing genetic modifications or engineering, notably those based on iPSCs, to bolster their tumor-killing capacity.

2.1. Influences on NK cell maturation in peripheral tissues

In human peripheral blood, NK cells can be categorized into two distinct groups based on the intensity of CD56 expression: CD56^{bright} and CD56^{dim}. Cells in the CD56^{dim} subset express higher levels of CD16, a crucial Fc receptor necessary for ADCC mediated by NK cells. CD56^{dim} NK cells are generally considered more mature than their CD56^{bright} counterparts. The population of CD56^{dim}CD16⁺ NK cells, which have cytotoxic function in the peripheral blood, is significantly decreased in hepatocellular carcinoma (HCC) patients. This is accompanied by a reduction in the production of granzyme and interferon (IFN)- γ . Moreover, the number of CD56^{dim}CD16⁺ cells infiltrating liver tumors is much lower than that in non-tumor regions.²⁴ In addition, human NK cells can be further stratified into the four-subset model based on the expression levels of CD11b and CD27.⁴⁶ Non-small cell lung cancer tumors are infiltrated by a large number of CD56⁺CD11b⁻CD27⁻ double negative NK cells. These double-negative NK cells are considered relatively immature, exhibit lower susceptibility to activation, and their presence is positively correlated with tumor malignancy.^{47,48}

2.2. Changes in cytokine levels (including chemokines)

TGF- β stands as a pivotal immunosuppressive factor for NK cells, impeding their capacity to eliminate target cells, release cytokines, and attenuate the expression of activating receptors.^{49,50} On lung cancer cells, TGF- β downregulates the expression of ligands for NK cell activating receptors, such as NKG2DLs, thereby facilitating cancer cells' evasion from NK cell-mediated destruction.^{51,52} Extensive evidence has firmly established that a diverse range of substances contribute to the development, migration, and increased malignancy of lung cancer by modulating the TGF- β signaling pathway.⁵³⁻⁵⁵

IL-10 represents another cytokine that inhibits the functionality of NK cells. Although NK cells and immature dendritic cells (DCs) have the ability to activate each other, it has been observed that NK cells can also kill DCs that express CD40 or are derived from the activation of IL-10.^{56,57} In the lung tumor microenvironment, NK cells not only exhibit reduced cytotoxicity but also play a role in negatively regulating DC maturation, thereby assisting tumors in evading immune surveillance.⁵⁸

A crucial step in the NK cell-mediated eradication of cancer cells is their infiltration into solid tumors. CXCL14, an important chemokine that promotes NK cell migration toward inflammatory or malignant sites,⁵⁹ is significantly underexpressed in head and neck squamous cell carcinoma.⁶⁰ The presence of hypoxia within the tumor microenvironment can induce the expression of podoplanin, which disrupts the interaction between CCL21 and CCR7, ultimately resulting in diminished NK cell migration toward solid tumors and a consequent reduction in NK cell cytotoxicity.⁶¹

2.3. Altered expression levels of activating and inhibitory receptors

NKp30 (CD337) collaborates with NKp46 and plays a pivotal role in the activation of NK cells.⁶² The reduction of NKp30 expression in NK cells from individuals with acute myeloid leukemia (AML) profoundly affects NK cell function.⁶³ This reduction in efficacy extends into the realm of solid tumors, including cervical and breast cancers, where the malignant cells bind to NKp30, inhibiting NK cytotoxicity through the release of galactose lectin (Gal-3).^{64,65} Moreover, colorectal cancer patients exhibit lower levels of NKp30, NKp44, NKp46, and NKG2D in their peripheral blood NK cells compared to healthy individuals.⁶⁶ During malignant transformation, the human BCL2-related protein BAG-6 inhibits NKp30-mediated signal transduction, thereby contributing to immune evasion by tumor cells.⁶⁷

NKG2D, expressed on NK cells, serves as a critical activating receptor.^{68,69} However, both virus infections and tumor cells deploy diverse mechanisms to escape NKG2D-mediated cytotoxicity.^{70,71} In cases of HCC, the expression of NKG2D ligands is diminished through the action of the β -catenin signaling pathway.⁷² The expression of the NK cell activating receptor NKG2D is notably reduced^{73,74} in liver cancer. As a consequence, NK cells are less easily activated by tumor cells, leading to diminished cytotoxic effects and a reduced capacity to release cytokines. Similarly, in non-small cell lung cancer, the enzyme indoleamine 2,3-dioxygenase 1 induces NK cell dysfunction by downregulating NKG2D.⁷⁵ NK cells exposed to activating signals mediated by NKG2D and NKp46 downregulate the expression of activating receptors and upregulate checkpoint molecules. This results in reduced cytokine production and cytotoxicity. Hypoxia also induces diminished expression of ligands that activate NK cell receptors. This includes the downregulation of MICA/B, the ligand for NKG2D, on breast cancer and pancreatic cancer cells. This downregulation enables these cancer cells to evade NK cell-mediated killing.^{76,77}

Inhibitory receptors constitute a pivotal mechanism through which NK cells establish immune tolerance. MHC class I molecules serve as the principal ligands for these receptors. Melanoma cells have evolved various strategies to evade immune system surveillance, with one of the most common being the downregulation of MHC-I expression on their surface, allowing them to evade recognition and elimination by T cells.⁷⁸ However, this downregulation also renders melanoma cells susceptible to recognition by NK cells, as they lack the inhibitory signals typically provided by MHC-I. While classical MHC-I molecules such as HLA-B are down-regulated, other non-classical MHC-I molecules, such as HLA-E or HLA-G, exhibit increased expression in melanoma.^{79,80} HLA-E is a crucial ligand for the CD94/NKG2A inhibitory receptor. Consequently, the increased expression of HLA-E renders the cancer cells less susceptible to NK cell recognition and attack. In contrast to classical MHC-I molecules, non-classical MHC-I molecules usually do not present tumor antigens to T cells. Therefore, increased expression of HLA-E does not make cancer cells more susceptible to T cell-mediated elimination. The concurrent downregulation of classical MHC-I molecules and upregulation of non-classical MHC-I molecules equip melanoma cells with resistance against T cell-mediated elimination and enable them to escape NK cell-mediated killing simultaneously (Figure 1). Melanoma-induced exhaustion of NK cells is another mechanism that impairs their function. This exhaustion is characterized by impaired cytotoxicity, cytokine secretion, and response, decreased expression of activating receptors, and increased expression of inhibitory receptors.⁸¹

2.4. Induction of NK cell exhaustion

In the tumor microenvironment, cancer cells and other surrounding cells can induce a state in NK cells similar to T cell exhaustion. Macrophages and monocytes in HCC tissues express high levels of CD48, which leads to early activation of NK cells through binding to 2B4 and subsequent dysfunction. When cocultured with monocytes, NK cells derived from the peripheral blood of HCC patients show increased expression of Ki67, granzyme B (GzmB), CD69, and TRAIL within a short period; however, these NK cells ultimately undergo substantial apoptosis.⁸²

Killer cell lectin-like receptor G1 (KLRG1) was initially considered one of the markers for the maturation of NK cells (117). Subsequent research, however, revealed that KLRG1 has an inhibitory effect on the activation of NK cells.⁸³ Similar to T cells, NK cells also express certain immune checkpoints. The ligands of KLRG1 primarily include molecules related to cell-cell adhesion, such as cadherin.⁸⁴ The expression of cadherin in tumor cells often

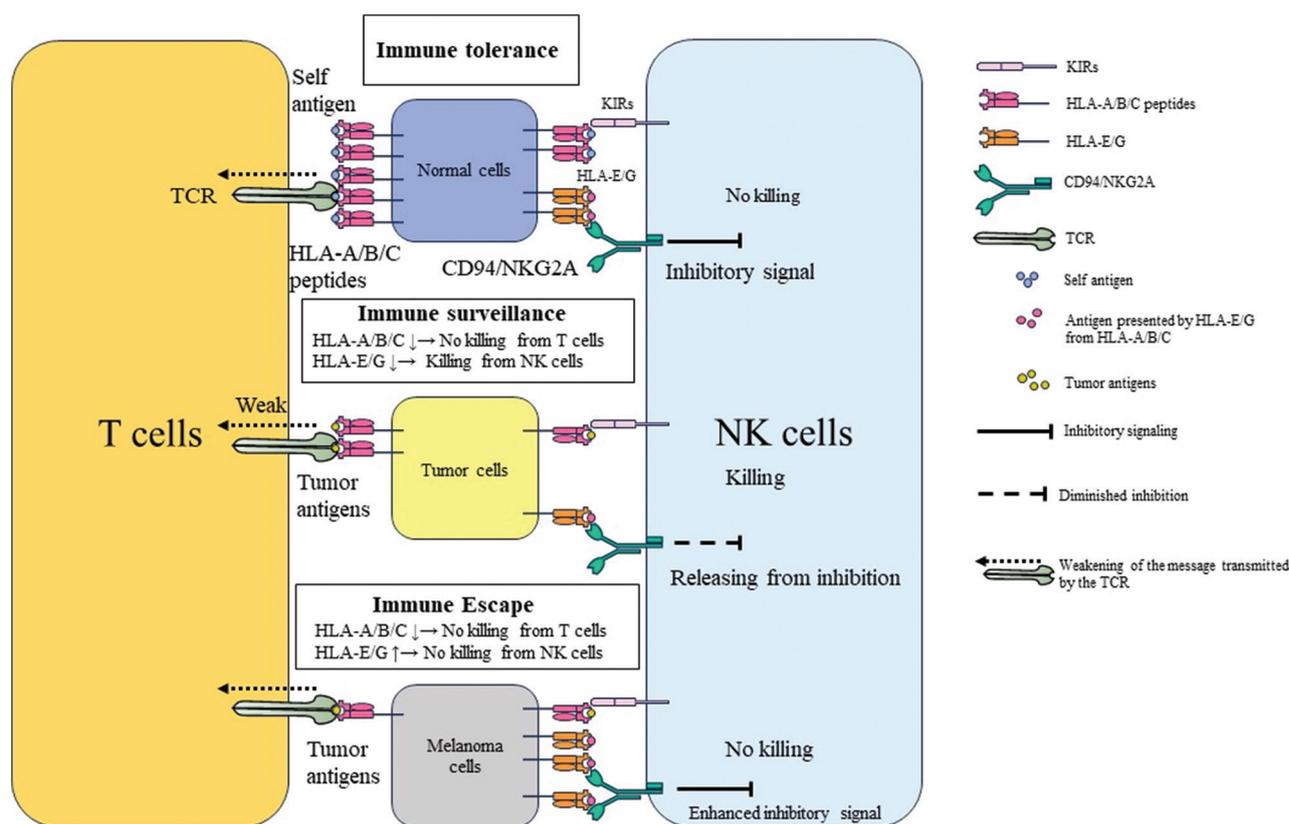


Figure 1. The role of MHC in T cells and NK cell-mediated immune surveillance and escape
 Abbreviations: HLA: Human leukocyte antigen; NK: Natural killer; MHC: major histocompatibility complex; KIR: killer-cell immunoglobulin-like receptor; TCR: T cell receptor. Figure created by the authors.

affects the function of NK cells. Elevated expression of neural cadherin (N-cadherin) in oral cancer cells is also believed to induce NK cell exhaustion through KLRG1.⁸⁵ In some oncolytic virus studies targeting solid tumors, ectopic expression of epithelial cadherin (E-cadherin) has been employed to help evade NK cell cytotoxicity, leading to prolonged tumor-killing effects.⁸⁶

T cell immunoglobulin (Ig) and immunoreceptor tyrosine-based inhibition motifs (ITIM) domain (TIGIT) is an important immune checkpoint in NK cells and plays an inhibitory role in NK cell function. By binding to CD155 expressed on tumor cells, TIGIT transmits signals of exhaustion into lymphocytes.⁸⁷ In patients with multiple myeloma, the levels of CD155 expressed by bone marrow mesenchymal stromal cells are negatively correlated with the levels of effector molecules such as IFN- γ and perforin in NK cells. This exhaustion of NK cells is caused by their interaction with TIGIT.⁸⁸ In adaptive NK cells, reducing the expression of TIGIT can confer resistance to immune suppression originating from myeloid-derived suppressor cells (MDSC).⁸⁹ Some immune therapies targeting TIGIT have also entered clinical trials for cancer treatment.⁹⁰

Interestingly, when inhibitory signals coexist with activating signals, they help mitigate NK cell exhaustion.⁹¹

3. iPSCs-derived NK cells for cancer therapy

3.1. Overcoming genetic engineering challenges in NK cells with iPSCs

Genetic manipulation of NK cells is challenging. Many gene-editing techniques that are highly efficient in other cell types are difficult to implement in NK cells. Moreover, NK cells have limited capacity for *ex vivo* expansion and, unlike T cells, cannot form long-lasting memory cells *in vivo*, presenting a significant barrier to their clinical application. Compared to NK cells, iPSCs are relatively easier to genetically manipulate and have the capability for extensive *in vitro* expansion. Therefore, iPSC-derived NK cells, especially those genetically modified at the iPSC stage, have a very promising clinical application in cancer therapy.

Differentiation of NK cells from iPSCs typically follows a two-step process. First, iPSCs are differentiated into HSCs. Various methodologies have been established for

differentiating pluripotent stem cells, including embryonic stem cells and iPSCs, into HSCs. One such method involves culturing iPSCs on the top of feeder cells, which provides a sufficient microenvironment for the differentiation into HSCs without the need for additional cytokines. For instance, OP9 cells can directly facilitate the differentiation of iPSCs into HSCs. In this process, iPSCs are digested into small aggregates, and the cell suspension containing these iPSC aggregates is then seeded onto OP cells in alpha minimum essential medium supplemented with fetal bovine serum. Within this culture setup, the optimal timeframe for HSC collection is typically between 7 and 8 days.⁹² For iPSC-derived NK cells intended for clinical applications, it is preferable to establish a differentiation protocol that operates without the requirement for feeder cells. This need led to the development of the spin embryoid body (EB) method. In this approach, iPSCs are first separated into single cells and seeded at a density of approximately 4,000 cells per well into a low-attachment 96-well plate. The plate is then centrifuged to promote the formation of a cell aggregate at the bottom of each well. The culture medium is based on APEL medium, supplemented with cytokines such as stem cell factor (SCF), bone morphogenetic protein-4, and vascular endothelial growth factor, and includes the addition of a Rho-associated, coiled-coil containing protein kinase inhibitor during the first 2 – 3 days of EB formation. By days 11 – 13 of hematopoietic induction, HSCs can be harvested for analysis or further differentiation.⁹³ Before differentiating from HSCs into NK cells, the purity of the HSCs is usually assessed. If the percentage of CD43⁺CD34⁺ (or CD45⁺CD34⁺) cells is around 30%, the process can proceed to NK cell differentiation. It is generally believed that the presence of some non-hematopoietic stromal cells will not impede the differentiation of HSCs into NK cells. If the HSC ratio is too low, magnetic bead purification can be employed to enrich the HSC population before proceeding to NK cell differentiation.⁹⁴ The second step initiates the differentiation of HSCs into NK cells. The cytokines necessary for the maturation and differentiation of NK cells have been extensively identified through prior basic research. Therefore, these cytokines are often employed in the process of differentiating HSCs into NK cells. While OP9 cells expressing delta-like 1 (DL1) are utilized in studies of HSC differentiation into NK cells, methods that do not involve feeder cells may hold greater potential for clinical applications. In the feeder-free differentiation system, the EBs formed from HSC differentiation steps are transferred to other cell culture devices, such as 6-well plates, and the medium is replaced with one that facilitates NK cell production, containing IL-3, SCF, IL-7, and IL-15. These cytokines can induce the

differentiation of HSCs into NK cells. After approximately 4 weeks of induced differentiation toward NK cells, the majority of the culture system will become CD45⁺CD56⁺ NK cells. The differentiated NK cells can be expanded several hundred-fold with the support of IL-2 and artificial antigen-presenting cells, achieving increased purity during the expansion process.⁴¹ In recent reports, 3D culture systems have been introduced in the preparation of NK cells derived from iPSCs, which will further enhance the scalability of NK cell production and improve the efficiency of clinical applications.⁹⁵

3.2. iPSC-derived vs. primary NK cells

The killer-cell immunoglobulin-like receptor (KIR) is not only pivotal for NK cells in establishing immune tolerance but also plays a critical role in providing licensing signals that are essential for NK cell development and maturation.⁹⁶ In the clinical context of leukemia treatment through HSC suppression, mismatches in KIR and KIR ligands between donors and recipients can enhance GVL effects and reduce relapse rates, particularly in patients with AML.²³ Although NK cell licensing is modulated by signals from KIR ligands *in vivo*, the expression and functionality of KIR on NK cells following *ex vivo* expansion remain subjects of debate. Some studies have observed that *ex vivo* expansion does not alter the expression state of KIRs on NK cells, thereby preserving the inhibitory impact mediated by their ligands.⁹⁷ Conversely, other studies have highlighted that *ex vivo* cytokine stimulation can license previously unlicensed NK cell subsets, which lacked high reactivity *in vivo*, enabling them to become licensed.⁹⁸ Once these *ex vivo* activated NK cells are reintroduced into the patients, they might become activated due to KIR and KIR ligand mismatches, thus activating previously tolerant NK cells to target tumor cells more effectively. During the *ex vivo* culturing and expansion process of NK cells, whether derived from iPSCs or peripheral blood, a high cytokine concentration is essential. As a result, the cytotoxic capacity of *ex vivo* expanded NK cells might transcend the limitations imposed by KIRs or KIR ligands, effectively bypassing the KIR-mediated inhibition through KIR ligands. Notably, research indicates that NK cells from different iPSC lines show varied KIR expression patterns. Among iPSC-derived NK cells, there is no significant difference in the levels of activating receptor expression between KIR⁺ and KIR⁻ cells, except for CD16, which is expressed at higher levels in KIR⁺ cells. Importantly, iPSC-derived KIR⁺ and KIR⁻ cells do not exhibit significant differences in tumor cytotoxicity, underscoring the nuanced role of KIR in modulating NK cell function.⁹⁹ Therefore, maximizing the anti-tumor capability mediated by KIR in iPSC-derived NK cells likely requires further research and more elegant designs.

One notable advantage of NK cells derived from iPSCs is the feasibility of applying genetic modifications. This advantage, combined with the iPSCs' capability for unlimited proliferation, facilitates the screening of positive clones, leading to the production of genetically modified NK cells of exceptional homogeneity. Various nucleic acid delivery methods and gene-editing technologies have shown significant effectiveness in iPSCs. For example, through transcription activator-like effector nucleases (TALEN) gene-editing technology, the introduction of IL-15 and the concurrent deletion of TGF β 2 not only amplify the NK cells' anti-tumor potency but also equip them to counteract the immunosuppressive impact of TGF- β within the tumor microenvironment.¹⁰⁰ Moreover, the potent clustered regularly interspaced palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system has been employed in iPSC-derived NK cells to remarkable effect. Beyond developing CAR-engineered NK (CAR-NK) cells using this technique, it enables the simultaneous editing of multiple genes in NK cells.¹⁰¹ It is important to note that editing multiple genes in one cell does not significantly affect the genomic stability of NK cells, highlighting the technique's potential to significantly advance cancer immunotherapy.

3.3. Clinical trials of iPSCs-derived NK cells

NK cells derived from iPSCs have progressively entered clinical trials as their safety and efficacy in treating various diseases, including malignant tumors, are being assessed. It is interesting to note that a wide array of NK cells is currently undergoing clinical trials including both genetically modified and unmodified NK cells, those sourced from peripheral blood and derived from iPSCs, as well as autologous and allogeneic NK cells. However, clinical trials involving iPSC-derived NK cells predominantly utilize genetically modified versions of these cells. In a case report, a 76 years old patient with diffuse large B-cell lymphoma, who had previously undergone eight different treatments including *ex vivo* expanded autologous NK cells, autologous stem cell transplant, and engineered autologous T cells, was treated with iPSC-derived NK cells.¹⁰² These cells were engineered to express a CAR targeting CD19, a non-cleavable CD16, and enhanced cytokine autocrine signaling mediated by IL-15 and its receptor. The treatment not only proved safe but also showed a partial response, with a 50% reduction in tumor size. Another case report involved iPSC-derived CAR-NK cells that also targeted CD19 and augmented the IL-15 signaling pathway.¹⁰³ This study additionally engineered the cells to knock out class I MHC molecules and enhance HLA-E expression, aiming to minimize host rejection. The intervention led to an enhanced immune

response within the tumor microenvironment and a reduction in tumor size.

In a trial involving 15 patients with advanced solid tumors and lymphomas, including non-small cell lung cancer and classical Hodgkin lymphoma, the therapeutic efficacy of iPSC-derived NK cells was evaluated. During preparation, NK cells were amplified from hematopoietic progenitor stages by over one million-fold.¹⁰⁴ Although the trial is ongoing, initial results indicate a response to treatment in some patients, and the iPSC-derived NK cells were deemed safe at a dose of 3×10^8 cells.¹⁰⁵ In another clinical trial involving 13 subjects with B-cell lymphoma, iPSC-derived NK cells were engineered to express a high-affinity, non-cleavable CD16 Fc receptor, enhancing their capacity for NK cell-mediated ADCC. The trial administered doses of up to 300 million cells, which were well-tolerated without any dose-limiting toxicities. Remarkably, seven of the patients achieved a complete response.¹⁰⁶ Whether *ex vivo* expanded from peripheral blood or derived from iPSCs, and regardless of genetic modification, current clinical trial outcomes primarily provide safety data. The effectiveness of these NK cells in treating tumors still requires further clinical evidence to be substantiated.

4. Boosting anti-tumor efficacy of iPSCs-derived NK cells through genetic engineering

4.1. CAR enhances the targeting of NK cells

Thanks to the success of CAR-T cells in treating hematological disorders and their potential, along with challenges, in solid tumors, CAR-NK cells have emerged as a pivotal focus in the field of genetically modified NK cells. In addition to traditional CAR-T constructs, efforts have been made to invent CAR constructs that are better suited for NK cells. CAR-NK cells based on 2B4,¹⁰⁷ DAP-12,¹⁰⁸ and NKG2D¹⁰⁹ structures have demonstrated superior efficacy, particularly in terms of proliferation, cytokine secretion, and cytotoxicity. These NK cell-specific activation motifs offer a promising avenue for the development of CAR-NK cell therapies and have the potential to significantly enhance the clinical effectiveness of iPSC-derived NK cells, especially in the context of solid tumors.

NK cells sourced from various tissues, including peripheral blood,¹¹⁰ cord blood,¹¹¹ NK-92 cell line,¹¹² and iPSC-derived NK cells,¹¹⁰ have all been utilized in the preparation of CAR-NK cells, yielding promising results in pre-clinical studies. Notably, several CAR-NK cell products have progressed to clinical trials.³³ Current CAR-NK cell research predominantly targets CD19, commonly

expressed in B-cell leukemia and lymphoma (37), B-cell maturation antigen, found in multiple myeloma (117), and HER2, associated with lung cancer.¹¹³ Importantly, these targets for CAR-NK cells align with the same spectrum of antigens typically targeted by CAR-T cell therapies. However, it is also important to recognize the differences between CAR-NK and CAR-T therapies. Given the distinct activation mechanisms of NK and T cells, the effects of CAR in enhancing T cell-mediated tumor cell killing cannot be expected to elevate NK cell performance to the same extent. In fact, some studies have found that in certain contexts, CAR-NK cells do not demonstrate a significantly stronger ability to kill tumor cells compared to unmodified NK cells.

4.2. Transcription factors are good candidates for optimizing the anti-tumor ability of NK cell

Elevating the expression of key transcription factors is a common strategy to enhance the efficiency of generating immune cells from iPSCs. For example, overexpression of SPI1 and CEBPA enhances the differentiation efficiency of microglia, one of the important immune cells in the neural system.¹¹⁴ Precise control of the expression of Runx1 and Hoxa9 during T-cell development can significantly improve the efficiency of generating T cells from iPSCs.¹¹⁵ Numerous transcription factors demonstrate promising potential as targets for optimizing NK cell-based cancer immunotherapies, yet only a select few have been proven in pre-clinical studies to enhance the tumoricidal capabilities of NK cells.

Aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that is widely expressed in immune and non-immune cells. After binding to exogenous or endogenous ligands, AhR translocates to the nucleus and regulates the expression of target genes.¹¹⁶ Many metabolic products in the tumor microenvironment can serve as ligands for AhR.^{117,118} Although resting NK cells do not express AhR at a considerable level, its expression level is greatly increased in response to cytokine stimulation.¹¹⁹ It is worth noting that co-culturing with IL-21-expressing K562 cells, a common expansion process for iPSC-derived NK cells, significantly increases the expression level of AhR.¹²⁰ In human peripheral blood NK cells, CD56^{bright} cells possess higher cytokine secretion capacity, lower cytotoxicity, and higher expression of AhR, compared to CD56^{dim} NK cells. Activation of the AhR signaling pathway increases the cytokine-secreting capacity of CD56^{bright} NK cells.¹²¹ Animal experiments have confirmed that AhR is not only a cell-intrinsic requirement for maintaining the population of liver resident NK cells (possibly ILC1 based on the markers used in flow cytometry assay) but is also necessary for the memory function of liver resident NK

cells.¹²² Experimental melanoma metastasis also shows that AhR is indispensable for NK cell-mediated cancer surveillance.¹¹⁹ However, some studies have found different roles of AhR. Activating AhR in NK cells promotes the secretion of IL-10, a suppressive factor for NK cells,¹²³ which acts on IL-10 receptor-positive NK cells. This mechanism maintains the homeostasis of NK cells in an autocrine and negative feedback manner.¹²⁴ Activation of AhR reduces the cytotoxicity of NK cells by affecting metabolism-related signaling pathways.¹²⁰ AML can evade NK cell-mediated killing by secreting AhR agonists, which inhibit the development and function of NK cells.^{124,125} In some solid tumors, the activation of AhR is believed to inhibit the anti-tumor activity of T cells, DCs, and TAMs.¹²⁶ Therefore, although AhR is an easily activatable or inhibitable transcription factor, only a few NK cell-based immunotherapeutic strategies specifically target it. More investigation is needed to realize the therapeutic potential of AhR in cancer immunology.

Eomesodermin (EOMES) and T-box expressed in T cells (T-BET) are two important transcription factors that promote the development, function, and cancer-immune surveillance of NK cells, especially for liver-resident NK cells.^{127,128} A recent study showed that remarkable improvements can be achieved in the anti-tumor activity of NK cells by enhancing the expression of T-BET or EOMES through genetic modifications. Notably, overexpression of EOMES has also been found to enhance the ADCC function of NK cells.¹²⁹ However, the NK cells in this study are derived from HSCs, and the genetic modifications are also achieved in CD34⁺ HSCs. If comparable results can be replicated in NK cells derived from iPSCs, it would undoubtedly enhance the potential clinical application of tumor immunotherapy based on NK cells.

4.3. Enhanced chemokine response guides NK cells into solid tumors

As mentioned earlier, one of the factors contributing to the limited efficacy of NK cell-mediated killing in solid tumors is reduced migration. Lymphocyte migration and tissue residency are regulated by the interaction between chemokines and their receptors. Chemokine receptor C-X-C motif Chemokine Receptor 2 (CXCR2) is expressed on many leukocytes but is often lost during the *in vitro* culture of NK cells. NK cells overexpressing CXCR2 are similar to control cells in terms of cytotoxicity and IFN- γ secretion. Furthermore, this overexpression enhances adhesion properties, calcium mobilization specifically in response to CXCR2 ligands, and the ability to migrate down the CXCR2 ligand gradient.¹³⁰ CXCR4 is also a vital chemokine receptor expressed in both immune cells and non-hematopoietic cells.¹³¹ The ligand of CXCR4, CXCL12,

is highly expressed in multiple types of tumors and plays a stimulatory role in the proliferation of cancer cells.¹³² Through the overexpression of the CXCR4 receptor, it is possible to significantly facilitate CAR-NK cells targeting EGFRvIII, enabling them to migrate to CXCL12-secreting glioblastomas. The effectiveness of this approach has been validated through *in vivo* xenograft experiments with solid tumors. Intravenously administration of CXCR4 expressing CAR-modified NK cells further improves survival of tumor-bearing animals compared to NK cells only expressing EGFRvIII-specific CAR.¹³³ To strengthen the argument for the enhanced anti-tumor effects achieved through ectopic expression of chemokine receptors, acquiring more *in vivo* data and reinforcing the existing preclinical evidence is essential.

4.4. Other methods utilizing iPSCs to enhance anti-tumor functions of NK cell

CD16 (FcγRIIIA) is uniformly expressed in peripheral CD56^{dim} NK cells and plays a crucial role in ADCC.¹³⁴ When cells are labeled with antibodies, NK cell cytotoxicity is triggered through the Fc segment of the antibody, representing a notable example of adaptive immunity augmenting innate immunity. In pre-clinical *in vivo* experiments, the combination of anti-GD2 antibodies with NK cells has shown promising therapeutic efficacy.¹³⁵ In a clinical trial targeting neuroblastoma, the use of anti-GD2 monoclonal antibodies in conjunction with haploidentical NK cells exhibited anti-cancer activity, particularly at higher doses.¹³⁶ Given the unique properties of CD16, it has risen as a key target for genetic engineering in the iPSC-derived NK cell development.^{137,138} Although CD16 can mediate ADCC, it is not a high-affinity Fc receptor. CD64 (FcγRI), on the other hand, has a higher affinity for IgG, but it is not expressed on NK cells. Studies have combined CD64 and CD16 to create a new recombinant receptor. This receptor consists of a high-affinity antibody binding to the extracellular region of human CD64, whereas the transmembrane and intracellular regions of human CD16A mediate NK cell signaling.¹³⁹ After being equipped with CD64/16A, NK cells demonstrated significantly enhanced abilities in mediating ADCC, increased production of IFN-γ, and improved killing of target cells.¹⁴⁰ CD16, however, is vulnerable to cleavage by metalloproteinases. This cleavage results in a somewhat reduced but still retained sensitivity of NK cells to other activating receptors. For instance, metalloproteinase ADAM17, also known as TNF-α converting enzyme (131), can cause the loss of CD16 when overexpressed on NK cells (132). To mitigate the issue of CD16 cleavage, a non-cleavable version of human CD16 (hnCD16) was engineered.¹⁴¹ However, it is important to note that CD16, by mediating the break after ADCC,

actually helps NK cells to detach from the dying target cells and prepare them to kill the next target cells.¹⁴² Whether this non-cleavable CD16 will have a better tumor-killing effect in the real world compared to the cleavable CD16 remains to be investigated further.

IL-15 is an important cytokine for maintaining NK cell homeostasis and function, increasing the number of CD56^{bright} NK cells and enhancing the cytotoxicity of CD56^{dim} NK cells.^{143,144} It is also an important target of NK cell-centered cancer immunotherapy. However, the IL-15 is a short-lived, low-activity cytokine under normal physiological conditions.¹⁴⁵ Previous studies have shown that the IL-15/IL-15R complex can release the function of NK cells from TGF-β inhibition.¹⁴⁶ Activation of the IL-15 signaling pathway in iPSC-derived NK cells can be achieved through the fusion expression of IL-15Rα with IL-15, mimicking transpresentation.¹⁴⁷ Although NK cells overexpressing IL-15 are freed from autocrine cytokine dependence, their true tumor-killing ability to resist immune suppression in solid tumors remains to be further investigated. The combination of IL-15, IL-18, and IL-12 has been used to stimulate NK cells, enhance NK cell proliferation and activation, and produce IFN-γ, as well as to enhance the anti-tumor properties of NK cells.^{143,148} CIS protein (cytokine-inducible SH2-containing protein), encoded by the CISH gene, can negatively regulate IL-15 signaling in NK cells, and its deletion can improve the metabolic activity of iPSC-derived NK cells.¹⁴⁹ However, recent studies have revealed that IL-15 may also contribute to the downregulation of certain chemokines, thereby influencing NK cell migration and function. CX3CR1 is present in nearly all CD16⁺CD56^{bright} NK cells and is crucial for NK cell adhesion, chemotaxis, degranulation, and tumor cell killing. Both the surface expression and mRNA level of CX3CR1 are reduced in the presence of IL-15.^{150,151} In proliferating NK cells activated by IL-15, CX3CR1 expression is very low and may not function properly as a chemokine receptor. The reason for this is not clear but it could be to protect normal endothelial cells from NK cell attack.¹⁵² Moreover, the enhancement of NK cell migration and cytotoxic functions through short-term hypoxia in conjunction with IL-15 suggests a potential synergy that could be leveraged to augment anti-tumor responses.¹⁵³ This insight underscores the necessity for a more advanced approach to utilizing IL-15 within iPSC-derived NK cell therapies. Such strategies may need to carefully consider the intricate dynamics between IL-15, NK cell biology, and the tumor microenvironment to optimize the therapeutic potential of NK cells against cancer. Methods for augmenting the anti-tumor capabilities of NK cells through genetic modification are summarized in [Figure 2](#).

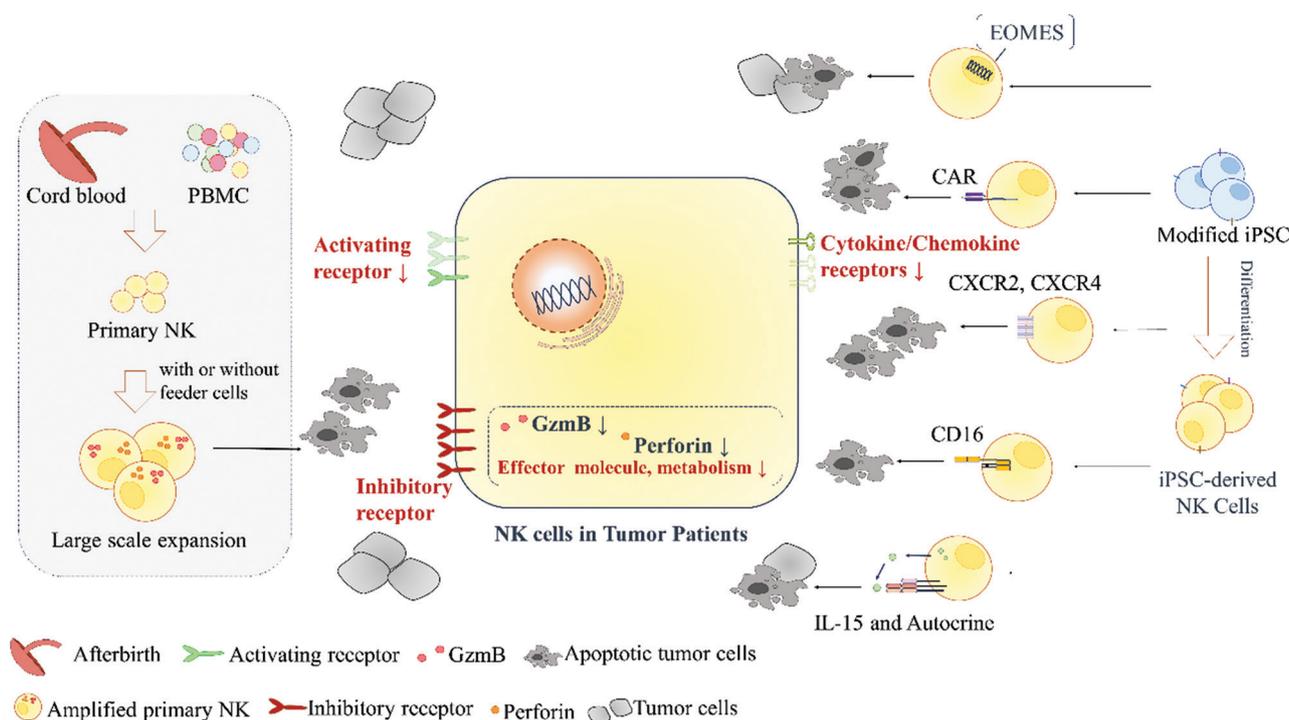


Figure 2. Overcoming microenvironmental barriers and enhancing NK cell antitumor activity through gene editing
 Abbreviations: CAR: Chimeric antigen receptor; CXCR: C-X-C motif chemokine receptor; GzmB: granzyme B; IL: Interleukin; iPSC: Induced pluripotent stem cells; NK: Natural killer; KIR: killer-cell immunoglobulin-like receptor; PBMC: peripheral blood mononuclear cells. Figure created by the authors.

5. Concluding remarks

NK cells possess the remarkable ability to target and eliminate both tumor cells and virus-infected cells. Adoptive transplantation of NK cells exhibits exceptional efficacy in combatting hematologic tumors. However, their application in the treatment of solid tumors is limited. Recent years have witnessed the development of numerous strategies aimed at bolstering and adapting NK cells to surmount this limitation. Even though CAR-T cell therapy has yielded promising clinical results, CAR-NK cells have also gained prominence within the realm of cancer immunotherapy. Nonetheless, it is becoming increasingly apparent that CAR-NK cells may not represent the ultimate solution for adoptive NK cell therapy. The imperative lies in tailoring NK cell therapies to fully exploit their distinctive attributes.

NK cells derived from iPSCs have pioneered a new frontier in NK cell-based tumor immunotherapy strategies. These cells have been genetically engineered to incorporate CARs, enhance their ADCC, and enable autocrine secretion of cytokine. However, these cells also face numerous challenges in clinical applications, including issues with allogeneic rejection. While autologous iPSCs could be employed, the reprogramming, selection, establishment,

quality control, and differentiation processes are complex and time-consuming, making personalized production prohibitively expensive. Consequently, allogeneic iPSCs-derived NK cells are primarily used, but they may cause potential damage to the recipient's normal cells and face rejection by the recipient. The extended differentiation time of iPSC-derived NK cells leads to prolonged preparation cycles, presenting challenges in maintaining process stability and batch consistency. In addition, variability among iPSCs from different sources affects the comparability of data across various laboratories. Addressing these challenges is crucial, as it would greatly enhance the potential of NK cells in cancer treatment by improving their safety and efficacy, thus offering more effective therapeutic options for patients.

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Conflict of interest

The authors declare no conflicts of interest.

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