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Harnessing Natural Killer Cells Engineering for Cancer Immunotherapy

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ABSTRACT

The use of genetically modified immune cells for cancer therapy is one of the most significant achievements in biotechnology in recent years. Although currently approved cell therapies are dominated by CAR-T cells, natural killer (NK) cells, with their unique cancer killing properties and safety of use, set the stage for the development of more convenient allogeneic therapies. Herein, we reviewed the challenges and strategies in harnessing NK cells for cancer immunotherapy. Various sources for NK cell acquisition are discussed, and genetic engineering methods, including retroviral vectors, lentiviral vectors, adeno-associated virus (AAV) vectors, and non-viral transfection, are explored. NK cells have the potential to address limitations associated with CAR-T therapy, and the paper addresses the need for innovative solutions to overcome technical hurdles associated with NK cell immunotherapy.

Introduction

Chimeric antigen receptors (CARs) are modified receptor proteins that specifically recognize target proteins and induce secondary signaling. CARs have mostly been used in T cells to retarget them against tumors, and CAR-T cell therapy has produced remarkably effective and durable clinical responses (June et al., 2018). However, T-cell immunotherapy poses various clinical challenges. Key safety issues linked to T-cell therapies are neurotoxicity, cytokine release syndrome (CRS), and graft-versus-host disease (GvHD) (Wei et al., 2019; Gargett et al., 2014; Graham et al., 2018). Importantly, the current CAR-T therapy is restricted to autologous T cells, which results in a time-consuming manufacturing process and sometimes inaccessibility of T-cell therapy for patients with low lymphocyte counts as result of their disease or treatment (June et al., 2015; Pfefferle et al., 2020).

Natural killer (NK) cells are a critical component of innate immunity that show unprimed cytotoxicity toward tumors and viral infections (Kiessling et al., 1975; Trinchieri et al., 1989). A primary role of NK cells is to eliminate cells exhibiting reduced or lacking expression of major histocompatibility complex class I (MHC-I) molecules. While NK cells possess both activating and inhibitory receptors for MHC molecules, the MHC mismatch between graft and host is typically not enough to contribute to pathology (Gill et al., 2009). NK cells, unlike T cells, have no surface T cell receptors (TCRs), which normally recognize peptide antigens associated with MHC molecules. The insensitivity of NK cells to antigens presented by MHC enables their use in an allogeneic context with minimal risk of graft-versus-host disease (GvHD). This property made NK cells an attractive alternative for generating pre-made therapeutic cell products that could be readily available for immediate clinical use (Han et al., 2015; Li et al., 2017;). Moreover, while CAR-T cell therapy may lead to cancer recurrence attributable to the loss of CAR or down-regulation of CAR antigen expression by tumor cells, engineered NK cells preserve their entire repertoire of activating and inhibitory receptors. These receptors naturally target and eliminate cancer cells, potentially preventing cancer recurrence arising from CAR or CAR antigen loss (Liu et al., 2018; Xu et al., 2020).

NK cells can rapidly kill adjacent cancer cells using multiple strategies. Activated NK cells form a synapse with target cells, releasing lytic granules containing perforin and granzyme, leading to target cell lysis and apoptosis (Orange et al., 2008). NK cell effector functions for therapeutic purposes are further enhanced through antibody-dependent cell-mediated cytotoxicity (ADCC), mediated by CD16a. CD16a on NK cell surfaces binds to the constant region (Fc) of IgG antibodies, resulting in the killing of antibody-coated cancer cells (Chin et al., 2022). Additionally, NK cells rapidly release pro-inflammatory cytokines, chemokines, and growth factors, contributing to early inflammatory responses, including type 1 cytokines, INF-γ, TNF-α, FLT3L, GM-CSF, and others. Therefore, NK cells can influence the activity of other immune cells, including T cells, dendritic cells, and B cells, by secreting cytokines (Vivier et al., 2011).

Despite their unique potential for cancer treatment, NK cells pose special problems for therapeutic development. In order to yield a proper effector-to-target ratio, large numbers of NK cells, ranging from $5x10^6$ to $1x10^8$ cells per kilogram body weight, are required to achieve a considerable reduction of tumor burden (Wu et al., 2020). Unlike T cells, NK cells are not usually clonally expanded. Kinetic analysis shows that the turnover of human NK cells in blood is about 2 weeks (Zhang et al., 2007). The limited proliferative potential makes the maintenance and expansion of engineered NK cells challenging. Except for that, a key step in the generation of CAR-NK cells is to introduce the modified genetic element into the NK cells. As the first line of defense against viral infections, NK-cells are usually resistant to transduction and hold robust foreign DNA- and RNA-sensing mechanisms that limit the efficiency of traditional gene-delivery methods (Shaffer et al., 2016; Bachanova et al., 2010). The challenge of delivering genetic modifiers is a major technical hurdle for developing NK-cell immunotherapy.

NK Cells from Different Sources and Their Properties in Genetic Engineering

NK cells for therapy can be acquired from various sources such as peripheral blood (PB) (Miller et al., 2005; Romee et al., 2016), umbilical cord blood (UCB) (Verneris et al., 2009), human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) (Huang et al., 2019) as well as cells lines such as NK-92 (Klingemann et al., 2016). Compared properties of NK cells from different sources are listed in table 1.

Table 1. Therapeutic properties of NK cells from different sources. High/low characterization is relative and based on comparisons within NK cell types. *NK cell line has to be irradiated before infusion into the patient to avoid tumorigenicity.

	Cytotoxicity	ADCC activity	In vitro expansion	Transfectability	In vivo persistence
PB-NK	high	high	low	low	low
UCB-NK	low	low	high	high	high
iPSC-NK	high	low	high	high	low
NK cell line*	high	low	high	high	low *

Peripheral Blood NK Cells

In peripheral blood, ~10% of mononuclear cells are NK cells; thus they are readily isolated from density gradient preparations of peripheral blood mononuclear cells (PBMC). Allogeneic NK cells from healthy adults can

be isolated from PBMCs by either CD3/CD19 depletion (Williams et al., 2018) or CD3 depletion and subsequent CD56 enrichment (Fujisaki et al., 2009). PB-NK cells are highly cytotoxic with potent ADCC activity, but proliferate to a lesser extent than cells obtained from other resources, such as umbilical cord blood (Pomeroy et al., 2019). Another drawback for PB-NK cells is that they are usually difficult to engineer. The factors causing this are unclear but include low transduction efficiency with poor expansion. Except for that, cryopreservation of PB NK cells lowers the cytotoxic ability (Shankar et al., 2020). Despite the challenges, PB-NK cells have found extensive use in clinical settings due to their safety profile in both allogeneic and autologous applications, sourced from either HLA-mismatched or matched donors (Shaffer et al., 2016; Yang et al., 2016.).

Umbilical Cord Blood NK Cells

Lymphocytes taken from umbilical cord blood (UCB) are composed of ~15–30% NK cells. UCB-NK cells can be obtained from cord blood post-birth through umbilical cord venipuncture and subsequent purification using density gradient centrifugation (Shah et al., 2013). Alternatively, CD34 hematopoietic stem cells can be isolated from UCB and then differentiated into NK cells (Araki et al., 2007). Compared to PB-NK cells, UCB-NK cells are limited in ADCC function and are functionally less mature in terms of cytotoxicity. On the flip side, UCB NK cells have higher proliferation potential than their PB counterparts and large quantities can be more easily manufactured for clinical use (Herrera et al., 2019; Kundu et al., 2021). One study indicated that over 100 doses of NK cells could be produced from a single UCB unit for clinical use, suggesting that UCB-derived NK cells are highly capable of proliferating in vitro (Liu et al. 2018). Moreover, because of the proliferation potency of UCB-NK cells, their transfection efficiency is usually higher than that of primary NK cells. Meanwhile, a study showed that extended cryopreservation did not impact the expansion capability and activity of UCB-derived NK cells, which is another significant advantage (Nham et al., 2018). For clinical applications, the existence of UCB libraries ensures that donors can be selected according to specific HLA types and specific NK receptor profiles. A recent review by Zhao X and colleagues summarized clinical trials being performed to evaluate the safety, feasibility, and efficacy of UCB-derived NK cells in the treatment of malignant tumors (Zhao et al., 2020).

iPSC Derived NK Cells

Induced pluripotent stem cell-derived NK cells (iPSC-NK) offer an inexhaustible source for human NK cell therapy. Derived from reprogrammed somatic cells like fibroblasts and blood cells, iPSCs are readily accessible, and their pluripotent nature provides substantial expansion and differentiation capacity for NK cells. In addition to individual iPSC production, master cell banks of iPSCs ensure a consistent supply of genetically identical donor material, allowing for product standardization and process robustness (Karagiannis et al., 2021). Reports indicate that iPSC-NK cells produce inflammatory cytokines and exhibit potent cytotoxicity against various hematologic and solid tumors (Cichocki et al., 2020). In terms of genetic engineering, iPSC-NK cells also exhibit improved transfection efficiency (Kaufman et al., 2018). Nonetheless, they have limitations, including low CD16 receptor surface expression and suboptimal in vivo persistence (Li et al., 2017), resulting in reduced effectiveness in killing tumors.

NK Cancer Cell Lines

In response to the challenges associated with primary allogeneic NK cells, researchers are investigating the use of stable NK cell lines. Among these, the NK-92 cell line has demonstrated antitumor activity across various tumors in pre-clinical studies (Klingemann et al., 2016; Chen et al., 2017). In multiple in vivo studies, NK-92 cells exhibited higher cytotoxicity against tumor cells compared to primary NK cells, attributed to the absence of the KIR inhibitory receptor and elevated levels of granzyme B and perforin (Yan et al., 1998; Klingemann et al., 1996; Gong et al., 1994). However, NK-92 cells are characterized by a lack of or low CD16 expression, limiting their ability for ADCC-mediated tumor killing. While NK-92 cells can be genetically engineered with variable efficiency (4%-95% depending on the transfection method) and expanded in substantial numbers (Matosevic et al., 2018), concerns about their tumorigenicity exist. Consequently, they undergo irradiation before infusion into patients to prevent undesired clonal expansion. However, irradiation treatment may impact the in vivo persistence and efficacy of engineered NK-92 cells (Tam et al., 2003).

Methods of Genetic Engineering of NK Cells for Immunotherapy

NK cell activation is regulated by a combination of activating, co-stimulatory, and inhibitory receptors, with the recognition of target cells achieved through a balance between these signals (Morvan et al., 2015; Chiossone et al., 2018). Genetic modification of therapeutic NK cells can enhance their function in the tumor microenvironment, improve trafficking to tumors, or increase persistence after adoptive transfer (Childs et al., 2015). Additionally, NK cells can be redirected to recognize different tumor antigens through the expression of CARs (Imai et al. 2005). However, modifying human NK cells using viral or non-viral vectors has been challenging due to robust foreign DNA- and RNA-sensing mechanisms, limiting the efficiency of gene-delivery methods (Liu et al., 2020). Systematic optimization is often required to achieve a good manufacturing practice (GMP)-compliant solution.

Retroviral Vector

Retroviruses are enveloped RNA viruses that package two copies of the same capped and polyadenylated positive-sense RNA molecule, ranging in size from 8,000 to 11,000 nucleotides. Accordingly, engineered retroviral vectors allow inserts of up to 7–8 kb long as genes of interest for transduction. This capacity is enough to cover the length of most CAR constructs. During the life cycle of retroviruses, viral RNA is reverse transcribed into double-stranded cDNA which is then semi-randomly integrated into the genome of the infected cell (Cepko et al., 2001). Retroviral vectors integrate into the host genome during transduction, allowing the modified cells to be sustained in the host over extended periods.

Gamma-retroviral vectors have been extensively utilized in the generation of CAR-T cells for both preclinical and clinical applications (Watanabe et al., 2022). However, the challenges in viral modification of NK cells, compared to T cells, have hindered CAR-NK research. Significant efforts have been devoted to enhancing the modification of NK cells using gamma retroviral vectors (Xu et al., 2020; Imai et al., 2005; Guven et al., 2005; Kremer et al., 2017; Lapteva et al., 2016; Streltsova et al., 2017). The use of gamma retroviral vectors is constrained by their reliance on cell proliferation, as they can only integrate into the cell's genome when the nuclear membrane is dissolved (Micucci et al., 2006). To address this limitation, many protocols include conditions to induce NK cell proliferation, such as co-culture with activated T cells (Kremer et al., 2017) or irradiated K562 feeder cells modified with cell surface-expressed cytokines and the 4-1BBL costimulatory molecule (Streltsova et al., 2017; Yoon et al., 2015).

Currently, retroviral vectors have been used in several clinical trials for UCB-NK cell engineering with acceptable efficiency. For example, MD Anderson Cancer Center reported retrovirus transduction efficiencies between 22%-66% for their NK cells engineering in study NCT03056339 (Liu et al., 2020).

Lentiviral Vector

Lentivirus is a subtype of retroviruses. Similar to retrovirus, Lentiviral vectors with insert fragments of approximately 8.0 kb in size can be integrated into the genome of host cells to produce long-term effects. Compared to retrovirus, lentiviral vectors are more versatile and allow transduction of primary and non-activated NK cells. Even though, single lentiviral transduction usually results in lower transduction efficiencies (Micucci et al., 2006). Recent studies showed that that the efficacy of lentiviral vectors in producing CAR-NK cells is influenced by the envelope protein they express. For example, the VSV-G lentivirus, traditionally employed to generate chimeric antigen receptor (CAR)-T cells (Levine et al., 2006), does not efficiently transduce NK cells. This is attributed to VSV-G's binding to the low-density lipid receptor (LDL-R), which is poorly expressed on activated NK cells (Bari et al., 2019). RD114-pseudotype lentiviral vectors present an attractive alternative since their entry receptor ASCT2 is widely expressed in the hematopoietic lineage (Sandrin et al., 2002). Additionally, a Baboon envelope-pseudotyped lentiviral vector, BaEV-LV, binding to both ASCT1 and ASCT2 for viral entry, demonstrates significantly improved NK cell transduction compared to both RD-114-TR and VSV-G pseudotyped lentiviral vectors (Colamartino et al., 2019). Reports suggest that lentiviral transduction can be further optimized using spinfection, a method involving centrifugation at a low RPM (Boissel et al., 2012). An alternative strategy is to enhance the colocalization of cells and viruses using crosslinking agents such as Retronectin or Vectofusin-1 (Suerth et al., 2016). Multiple manufacturers have used lentiviral vectors in CAR-NK production with high transfection efficiency. For example, preclinical data from Fate Therapeutics report over 99% of CD16-positive clonal for iPSC-NKs upon lentiviral transduction (Zhu et al., 2020). PersonGen BioTherapeutics showed over 90% CD33 expression for their CAR-engineered NK-92 cells with lentiviral transduction (Tang et al., 2018).

Adeno Associated Virus Vectors

Adeno associated virus (AAV) is a non-enveloped, single-stranded DNA virus. It can effectively infect humans and other vertebrates without causing any disease. Safety and efficiency make AAV an ideal vehicle for in vivo gene therapy (Burdett et al., 2023).

There are a variety of AAV serotypes available with tropism for different tissues, which greatly expand the landscape for their optimal use for therapeutic purposes (Mingozzi et al., 2011). AAV serotype 6 (AAV6) is identified as an effective serotype for transduction ex vivo in human hematopoietic stem and progenitor cells (HSPCs) (Song et al., 2013), T cells (Wang et al., 2015), and B cells (Hung et al., 2018). It has been used safely as delivery vectors in preclinical or clinical trials for engineering T cells and B cells (Eyquem et al., 2017; Liu et al., 2019; Rogers et al., 2021). However, AAV transduction of NK cells has suffered low transduction rates, high cell death, and loss of transgene expression after expansion. Another concern with AAV vector is its compact packaging capacity, which limits the size of transferred genes within 4.7 kb.

Currently, there is no report of using AAV vector in CAR-NK production for clinical testing. A major concern is that AAV-transmitted genes persist as episome outside of chromatin, which limits the maintenance of the transgene in proliferating cells. For this reason, an established method for using AAV in NK cell engineering is gene editing. This method involves the transient delivery of a targeted nuclease, such as CRISPR (Cas9/RNP), along with the transduction of a homology donor DNA template packaged in an AAV vector. The targeted nuclease is typically designed for transient expression, usually achieved through mRNA electroporation. After introducing a site-specific break in the targeted chromosomal site, the cellular homology-directed repair (HDR) pathway utilizes the supplied AAV genome to permanently incorporate modified DNA at that site (Pomeroy et al., 2019). Although still in the early stages, these methods demonstrate an efficient strategy for gene editing and delivery using AAV vectors for NK cell therapies (Naeimi et al., 2022).

Non-Viral DNA Transfection

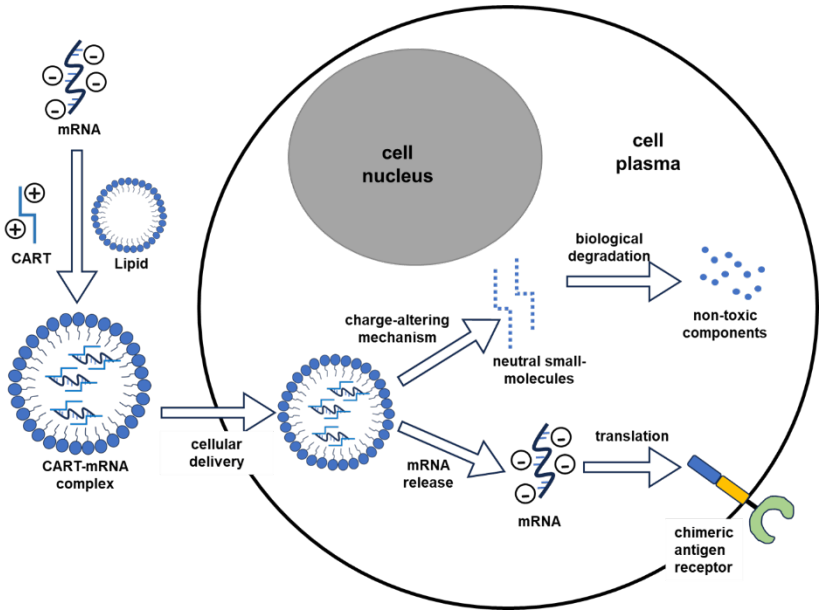
NK cells are highly resistant to transfection by conventional electroporation or lipofection protocols. The transfected DNA in the cytosol must enter the nucleus for transcription, thus it usually has no effect on the transfection of non-proliferating cells (Haraguchi et al., 2022). Trompeter et al introduced nucleofection as an applicable non-viral strategy for NK cell transfection. Nucleofection is an electroporation-based method that uses specialized solutions and specific electric parameters to achieve direct delivery of plasmid DNA into the cell nucleus, leading to enhanced gene expression in NK cells (Trompeter et al., 2003). It was noticed that proliferating NK cells give better results in DNA transfection than resting NK cells. Accordingly, activating NK cells prior to electroporation makes them more amenable to nucleic acid delivery. An improved method using NK cells expanded with IL-2 was reported, which can increase the efficiency of DNA electroporation from 10% to 50% (Ingegnere et al., 2019). Compared with the long-term effect of viral transduction, DNA transfection is a transient expression with a limited durable time of about 15 days. Another drawback of the method, the harsh electroporation condition usually reduces the viability of NK cells. Currently, most DNA gene delivery methods are only used for mechanistic studies, and there is no report about using DNA transfection for NK cell clinical trials.

Non-Viral RNA Transfection

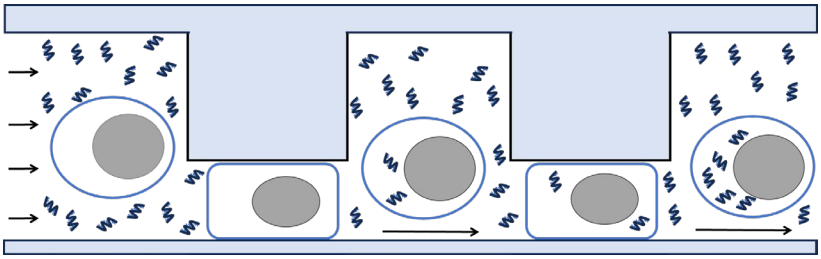
Theoretically, mRNA can be transfected more efficiently than DNA, because it does not need to enter the nucleus for transcription. mRNA electroporation provides an efficient alternative for NK cell engineering. Under optimized conditions including NK cell activation, proper mRNA dosing, and adjusted electroporation parameters, people have achieved > 90% transfection efficiency for cytokine stimulated primary NK cells (Pomeroy et al., 2019). Furthermore, the availability of large-scale cGMP-compliant electroporation systems, such as MaxCyte or CliniMacs, makes it possible to generate large amount of mRNA-engineered NK cells for therapeutic purpose (Shimasaki et al., 2020).

Traditional methods for mRNA electroporation into NK cell requires cell activation, either by engineered feeder cell lines or interleukin 2 (IL-2) pretreatment, because NK cell activation seems to be required for post electroporation viability (Shimasaki et al., 2012; Nguyen et al., 2020). Potentially, the cell activation may alter NK cell biology. With this concern, people have established new strategies to deliver mRNA into NK cells without relying on NK cell activation. A non-viral chemical method called charge-altering releasable transporters (CARTs) has been reported to deliver CAR mRNA into non-dividing NK cells successfully (McKinlay et al., 2018; Wilk et al., 2020). CARTs are initially cationic to noncovalently complex with mRNA and deliver mRNA to NK cells. After entering the cell plasma, the CARTs undergo biodegradation and facilitate the release of the mRNA for protein expression (Figure 1A). CART-mediated mRNA transfection is as efficient as electroporation, but minimally altered NK cell phenotypes compared to electroporation (McKinlay et al., 2018; Wilk et al., 2020).

Recently, Miguel Calero-Garcia and colleagues introduced a novel microfluidic device designed for delivering mRNA cargo into NK cells through a process known as Volume Exchange for Convective Transfection (VECT). In this device, cells pass through a ridged channel that enforces a series of ultra-fast and high-intensity deformations, transiently opening pores and inducing convective transport of mRNA into the cell (Figure 1B) (Loo et al., 2021). The authors demonstrated the device's versatility, showing its operation with different types of human primary immune cells, including NK cells, for the advancement of ex vivo mRNA-based gene therapies.



1A



1B

Figure 1. New strategies for transporting mRNA into NK cells independent of cell activation. (A) Charge-Altering Releasable Transporters (CARTs) (82). (B) Volume Exchange for Convective Transfection (VECT) (84). Figures are adjusted from original articles with permission.

The major challenge for mRNA transfection is the very short period of expression. The durable time of in vitro transcribed mRNA is about 72 hours after transfection; thus, it only allows for transient expression of the transgenes (Ng et al., 2019; Naeimi et al., 2020). To overcome this drawback, mRNA-based transient gene delivery has been used in CRISPR-mediated gene editing for human primary NK cells. Except for that the gene knockout can be easily accomplished in NK cells using electroporation of mRNA-encoded Cas9/RNP (Pomeroy et al., 2019), site-specific gene insertion in NK cells was recently achieved using mRNA-encoded Cas9/RNP in combination with AAVs encoding a gene of interest for insertion. By this strategy, site-directed gene insertion of CD33-specific CARs was achieved in primary human NK cells, which showed improved efficacy against AML targets (Naeimi et al., 2022).

Perspective

Current attempts in NK cell therapies have been focused on cell proliferation and genetic modification in vitro. With the practices of optimized clinical designs and sophisticated quality controls, immune therapies using genetically modified NK cells are expected to be on the horizon. A more optimistic expectation is the progress of in vivo CAR gene therapy. Nawaz and colleagues have reported generation of CAR-T cells within the mouse

model upon injecting an AAV vector carrying the CAR gene (Nawaz et al., 2021). Although it's still in the conceptual stage, a similar possibility exists with in vivo CAR-NK therapy. The success of engineering NK cells or T cells in vivo relies on the availability of genetic vehicles that can efficiently transduce these cells with high specificity. In the last decade, lots of efforts have been made to re-engineer AAV capsid for new tropism (Büning et al., 2018). The successful development of immune cell type-specific AAV capsids will make in vivo cell therapy an option.

Conclusion

NK cells present a hopeful alternative for cancer immunotherapy, surpassing some limitations of CAR-T cell therapies. Many strategies have been developed to utilize NK cells from various sources, including peripheral blood, umbilical cord blood, iPSCs cells, and NK cell lines. The exploration of genetic engineering methods, such as retroviral and lentiviral vectors, AAV vectors, and non-viral transfections, highlight ongoing efforts to enhance therapeutic applications. Despite challenges of limited proliferation and precise targeting, innovative solutions, including gene editing and non-viral transection, offer potential advancements. Ongoing clinical trials assessing NK cell therapies, coupled with exploration of in vivo CAR-NK approaches and improvements in genetic delivery, signify a dynamic landscape for NK cell immunotherapy, advancing towards safer and more effective cancer treatments.

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