Cancer Gene Therapy
https://doi.org/10.1038/s41417-021-00336-2

REVIEW ARTICLE

Delivery technologies to engineer natural killer cells for cancer immunotherapy

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Received: 24 August 2020 / Revised: 9 March 2021 / Accepted: 29 March 2021

Abstract

In recent years, immune cell-based cancer therapeutics have been utilized broadly in the clinic. Through advances in cellular engineering, chimeric antigen receptor (CAR) T-cell therapies have demonstrated substantial success in treating hematological tumors and have become the most prominent cell-based therapy with three commercialized products in the market. However, T-cell-based immunotherapies have certain limitations, including a restriction to autologous cell sources to avoid severe side-effects caused by human leukocyte antigen (HLA) mismatch. This necessity for personalized treatment inevitably results in tremendous manufacturing and time costs, reducing accessibility for many patients. As an alternative strategy, natural killer (NK) cells have emerged as potential candidates for improved cell-based immunotherapies. NK cells are capable of killing cancer cells directly without requiring HLA matching. Furthermore, NK cell-based therapies can use various allogeneic cell sources, allowing for the possibility of "off-the-shelf" immunotherapies with reduced side-effects and shortened manufacturing times. Here we provide an overview of the use of NK cells in cancer immunotherapy, their current status in clinical trials, as well as the design and implementation of delivery technologies—including viral, non-viral, and nanoparticle-based approaches—for engineering NK cell-based immunotherapies.

Introduction

Cell-based immunotherapies have advanced rapidly in the clinic in recent years, manifesting optimistic outcomes for cancer patients [1–4]. At present, clinical studies mainly focus on the use of T cells for treating several B-cell malignancies [5]. In these studies, T cells are engineered to express the chimeric antigen receptor (CAR), a synthetic receptor that endows them with targeting specificity to tumor-associated antigens (TAAs) [6]. CAR T-cells targeting the CD19 antigen on B cells have shown striking therapeutic results in patients with acute lymphoblastic leukemia (ALL) and lymphoma [7]. To date, three CAR T-cell-based immunotherapies have been successfully translated from the laboratory to the marketplace [8–10].

However, T-cell immunotherapy still presents several clinical challenges. The primary safety concerns associated with T-cell therapies include neurotoxicity, cytokine release syndrome (CRS), and graft-versus-host disease (GvHD) [11–13]. T-cell sources are restricted to autologous cells, to prevent donor and host human leukocyte antigen (HLA) mismatch, which may trigger GvHD [5]. Such restriction results in cumbersome manufacturing and time costs, and also reduces the accessibility of T-cell therapy for patients who have low lymphocyte counts from their disease or treatment [14]. In addition, T-cell therapies face several challenges in targeting solid tumors, in part due to the highly heterogeneous and immunosuppressive tumor microenvironment (TME) [6]. To enhance immunotherapy in solid tumors, T cells require (i) CARs targeting suitable TAAs in solid tumors, (ii) enhanced infiltration into tumors,

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Published online: 22 April 2021

and (iii) strategies to prevent T-cell exhaustion caused by immunosuppressive proteins in the TME [11].

Natural killer (NK) cells have been studied extensively in recent years for applications in cancer immunotherapy, due to the combination of their innate immune cell functionalities that allow them to efficiently target tumor cells and their ability to be derived from allogeneic sources [15–18]. Numerous preclinical trials have reported NK cell cytotoxicity towards various types of hematological and solid tumors [19–22]. Unlike T cells, the lack of antigen-specific cell surface receptors has enabled NK cells to achieve an enhanced graft-versus-tumor effect and has eliminated the concern of GvHD in T-cell-depleted adoptive cell transfer (ACT) [23, 24]. In addition, CRS and neurotoxicity pose a lesser concern in NK cell therapies than in CAR T-cell therapies, in part due to a different cytokine secretion profile, which contains low interleukin (IL)-1a, IL-1Ra, IL-2, IL-2Ra, IL-6, tumor necrosis factor-α (TNF-α), MCP-1, IL-8, IL-10, and IL-15 levels, which have been strongly associated with CRS and neurotoxicity in CAR T-cell treatments [25]. As NK cells do not require HLA matching, manufacturing of NK cell therapeutics can be expedited through the use of allogenic cells [14]. Moreover, their scalability allows them to be potentially developed into a cell-based "off-the-shelf" product in the future [14]. Furthermore, although CAR T-cell immunotherapies have resulted in cancer relapse due to the loss of CARs, engineered NK cells retain their full array of native activating and inhibitory receptors, which naturally function to target and kill cancer cells, which can potentially avoid cancer relapse caused by the loss of engineered antigen [18].

NK cells are resistant to genetic engineering approaches and undergo a limited number of cell divisions, thus limiting their proliferation and persistence for clinical use [26, 27]. Hence, it is critical to explore a wide variety of NK cell sources and utilize novel engineering tools to maximize the therapeutic potential of NK cell-based therapeutics. Herein, we review the current clinical advancements of engineered NK cells for treating both hematological and solid tumors, alongside a discussion of various sources of NK cells in immunotherapy. Furthermore, we broadly review various viral, non-viral, and nanoparticle-based strategies for engineering NK cells for cancer immunotherapy (Fig. 1).

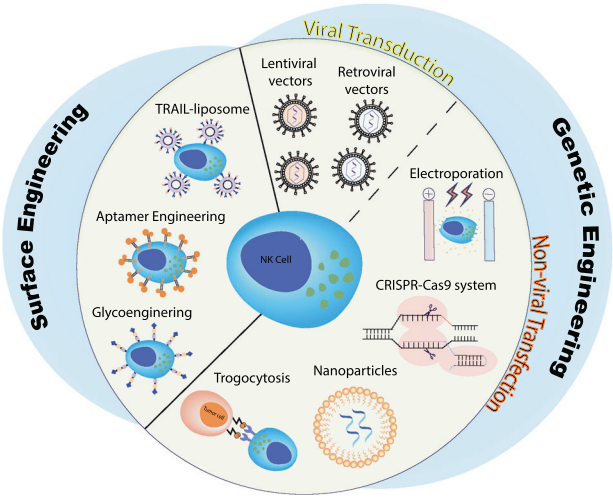

Fig. 1 Delivery technologies for engineering natural killer (NK) cells. Schematic illustrating both genetic and surface engineering methods currently applied to engineer NK cells for cancer immunotherapy. Methods for genetically engineering NK cells can be classified as either viral or non-viral. Viral transduction relies on engineered retroviral vectors or lentiviral vectors to deliver desired genetic constructs into cells. Non-viral transfection technologies include electroporation, CRISPR-Cas9, nanoparticles, and trogocytosis. Surface engineering include TNF-related apoptosis-inducing ligand (TRAIL)-based liposomes, glycoengineering, and aptamer-based engineering of NK cells.

Overview of NK cells

NK cells are part of the innate immune system and are responsible for killing aberrant cells, such as cancer cells and virally infected cells, through a complex interplay of a repertoire of germline-encoded inhibitory and activating receptors [28]. The primary inhibitory receptors include the killer cell immunoglobulin-like receptors (KIRs) and CD94/NKG2A [28]. These inhibitory receptors are involved in NK cell education and maturation into cytotoxic effectors toward malignant cells missing HLA class I, and are involved in preventing NK cells from attacking healthy tissues [29]. Primary activating receptors include the three natural cytotoxicity receptors (NKp44, NKp46, and NKp30) and NKG2D [30]. The corresponding activating ligands of these activating receptors are often upregulated on tumor cells, thus triggering the NK cell antitumor response [28, 31].

To defend the host body, NK cells are highly efficient in immunosurveillance by distinguishing "self" major histocompatibility complex (MHC) class I molecules on target cell surfaces. The vast array of activating and inhibitory receptors on NK cells directly interact with corresponding ligands on tumor cells for spontaneous cell lysis, making NK cells promising candidates for cancer immunotherapy [32]. Malignant cells with MHC class I deficiency (i.e. "missing self") or upregulated with stress ligands are automatically subjected to NK cell-mediated killing [28, 31, 32]. Furthermore, NK cells are highly efficient in tumor killing, because their cytotoxicity is not dependent on pre-sensitization or antigen-specific priming [33]. The cytolytic activity of NK cells is triggered when the net balance of inhibitory and activating receptors is disrupted [34, 35] (Fig. 2).

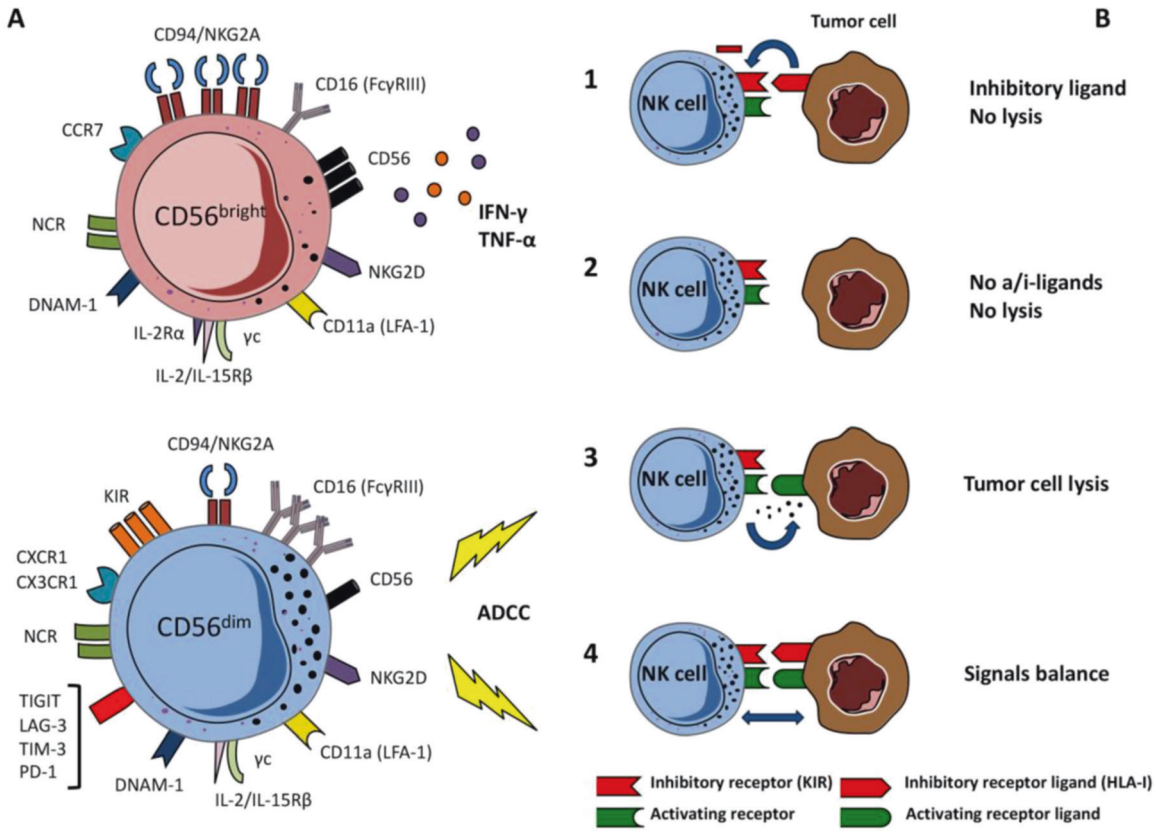

Fig. 2 Natural killer (NK) cell subsets and cytotoxic mechanisms. **A** $CD56^{bright}$ NK cells have high CD56 expression and are capable of producing IFN-γ and TNF-α, and thus are classified as immunomodulatory NK cells; $CD56^{dim}$ NK cells have low CD56 expression. However, they are highly cytotoxic due to the high expression of the CD16 receptor, which allows them to induce antibody-dependent cell-mediated cytotoxicity (ADCC). **B** The cytolytic activity of NK cells is triggered when the net balance of inhibitory and activating receptors is disrupted. (1) Target cells lacking activating receptor ligand but expressing normal levels of HLA-1 blocks NK cell activation. (2) Target cells lacking HLA-1 and activating receptor ligand fail to trigger NK cell cytotoxicity. (3) Target cells lacking HLA-1 and possessing activating ligands trigger NK cell cytotoxicity. (4) Equal amounts of ligand expression on target cells and receptor expression on NK cells leads to a signal balance. Figure adapted from MDPI [30].

In most cases, NK cells lyse cancer cells through the spontaneous release of cytotoxic proteins such as perforin and granzymes [36]. Other tumor-killing mechanisms include interactions between cancer cells and TNF-α, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL) expressed on the surface of NK cells [37–40]. In addition, NK cells are considered the most important cells in inducing antibody-dependent cell-mediated cytotoxicity (ADCC), which provides antitumor cytotoxicity through interaction with monoclonal IgG antibodies. Through this mechanism, CD16a (FcγRIIIa) on NK cells bind the Fc region of IgG, which has bound a tumor-specific antigen. This allows the target cell to be recognized and subsequently lysed by the NK cell [35].

Currently, different allogenic sources of NK cells are utilized in the clinic. The effector functionalities and maturity of NK cells obtained from these various sources are determined by the NK cell subset to which they belong [30]. Highly cytotoxic NK cells are identified as $CD56^{dim}CD16^{+}$, whereas immunomodulatory NK cells are identified as $CD56^{bright}CD16^{-}$[30,41] (Fig. 2). To reinforce NK cell immunosurveillance and effector functionalities, and endow NK cells with target specificity and enhanced in vivo persistence, various engineering methods have been applied to modify NK cells for cancer immunotherapy.

NK cells in the clinic for cancer immunotherapy

Engineered NK cells are at the nascent stage of clinical application compared to engineered T-cell-based therapies. In a recent study examining the current clinical landscape of CAR-based cell therapies, ~96.4% of 520 active trials globally are CAR T-cell-based therapies [42]. A very small fraction of the remaining active trials study CAR-NK cells, as they are still a relatively new field undergoing extensive exploration and experimentation [42]. The demonstrated efficacies and maturation of CAR T-cell therapies have attracted increased attention from academia and industry for

CAR-NK cell research [1, 17, 25, 43, 44]. Currently, NK cells from a variety of sources and engineered with different CAR constructs have advanced into early-stage clinical trials, to test their safety and efficacy towards treating various types of cancer [17, 45].

NK cell line NK-92

Among the many sources for NK cells, the NK-92 cell line is most frequently used, as it is an immortalized cell line that can be readily purchased [46]. The NK-92 cell line, subjected to irradiation before use, has demonstrated safety in various preclinical and clinical trials [47, 48]. Multiple in vivo studies have shown that NK-92 cells are more cytotoxic towards tumor cells than primary NK cells, which can be attributed to the lack of the KIR inhibitory receptor in this cell line [49, 50]. Due to their cytotoxicity and availability, NK-92 cells were the main subjects of early research for NK cell-based therapies, including numerous clinical trials for both hematological and solid tumors. However, NK-92 cells are characterized by a lack of or low CD16 expression, which limits their capability for ADCC-mediated tumor killing. This limitation has resulted in the exploration of other NK cell sources for cell-based immunotherapy. The biotechnology company NantKwest (El Segundo, CA) is developing high-affinity NK-92 cells, which are activated NK-92 cells genetically modified to express CD16 for combination therapy with IgG1 monoclonal antibodies [51]. Clinical trials sponsored by PersonGen BioTherapeutics (Suzhou, China) are currently conducting several CAR-NK-92 cell-based trials targeting receptors CD7, CD19, and CD33 for treating various types of hematological tumors (NCT02742727, NCT02892695, and NCT02944162). The therapeutic potential of CAR-NK-92 cells for treating solid tumors, such as metastatic breast cancer and glioblastoma, has been reported in numerous preclinical trials [15, 52, 53]. Furthermore, several CAR-NK-92 cell treatments for solid tumors that target HER2 and ROBO1 antigen have advanced to clinical trials (NCT03383978 and NCT03940820).

Peripheral blood-derived NK cells

Ninety percent of the total population of NK cells derived from peripheral blood are characterized as $CD56^{dim}CD16^{+}$ [54]. These NK cells are highly cytotoxic but proliferate to a lesser extent than cells obtained from cord blood [54]. Furthermore, as NK cells comprise about 10% of all circulating lymphocytes in peripheral blood cells, a major drawback of peripheral blood-derived NK (PB-NK) cells is that they require extensive ex vivo proliferation before achieving a clinically relevant number of cells [45]. Thus, after apheresis, NK cells are subjected to either cytokine stimulation with IL-15 or IL-2, or co-culture with feeder cells for ex vivo expansion [45]. Nevertheless, PB-NK cells have been widely used in clinical settings due to their safety in both autologous and allogeneic uses with cells acquired from either HLA-mismatched or matched donors [26, 55]. Currently registered clinical trials using CAR-PB-NK cells target MUC1, NKG2D, and mesothelin for treating $MUC1^{+}$ solid tumors, $NKG2D^{+}$ solid tumors, and epithelial ovarian cancer, respectively (NCT02839954, NCT03415100, and NCT03692637). The biotechnology company Nkarta Therapeutics (South San Francisco, CA) is currently testing CAR-PB-NK cells targeting NKG2D and CD19 ligands for treating both hematological and solid tumors, and is currently conducting clinical trials. The manufacturing constraints of PB-NK cells, however, have led some researchers and biotechnology companies to utilize cell sources with more relaxed manufacturing requirements.

Cord blood-derived NK cells

Lymphocytes derived from cord blood consist of ~15–30% NK cells. Cord blood-derived NK (CB-NK) cells have limited ADCC function and are functionally less mature when compared to PB-NK cells, somewhat limiting their cytotoxicity and clinical translation [56, 57]. However, CB-NK cells proliferate to a greater extent and are more sensitive to cytokine stimulation [58], thus eliminating the need for feeder cells and improving manufacturing outlook. Furthermore, cord blood contains fewer T and B cells than that in peripheral blood, and thus poses a greatly reduced risk of clinically significant GvHD [43], making this cell source more appealing for clinical use. Recently, Liu et al. [17] reported promising results from a phase I/II clinical trial conducted using CB-NK cells transduced with genes for anti-CD19-CAR, IL-15, and the iCasp9 suicide molecule for treating non-Hodgkin's lymphoma or chronic lymphocytic leukemia. Among the 11 patients treated in this trial, 73% of patients responded and seven patients had complete remission. Moreover, there was no occurrence of any critical side-effects such as GvHD, CRS, and neurotoxicity [17] (NCT03056339). All told, although CB-NK cells are less efficient in tumor killing, they are easier to manufacture and pose a lesser risk of GvHD, making them appealing for both manufacturing and safety considerations.

Induced pluripotent stem cell-derived NK cells

Unlike other primary NK cell sources, induced pluripotent stem cells present an unlimited proliferative potential for induced pluripotent stem cell-derived NK (iNK) cell generation and a greatly improved manufacturing process [59, 60]. These cells also demonstrate enhanced transfection efficiency [61, 62]. However, iNK cells possess limitations,

such as low CD16 receptor surface expression and poor in vivo persistence [21], leading to decreased tumor-killing effectiveness. To mitigate these drawbacks and improve the therapeutic outcomes of iNK cells, several biotechnology companies are engineering iNK cells to enhance their immunosurveillance, cytotoxicity, and persistence. FATE Therapeutics (La Jolla, CA) was recently approved to assess the safety and tolerability of iNK cells in a phase 1 clinical study (NCT03841110), indicating the potential of iNK cells for becoming an "off-the-shelf" therapeutic product. The FT596 program, which developed CD19-CAR-iNK cells with hnCD16 and IL-15 receptor fusion protein, has shown promising data on cell persistence and antitumor cytotoxicity, and is currently under phase I/II investigation for treating advanced B-cell malignancies (NCT04245722). Allife Medical Science and Technology, Co. is currently conducting an early phase I clinical trial, investigating the therapeutic potential of anti-CD19-CAR-iNK cells against refractory B-cell lymphoma (NCT03690310). Another company, Cytovia Therapeutics (Miami, FL), is developing CAR-iNK cells targeting epidermal growth factor receptor (EGFR) [63]. Cytovia is planning to start multiple clinical trials using NK cell-based cancer immunotherapies in 2021/2022, although it is unclear whether these trials will evaluate EGFR-CAR-iNK cells or other NK cell-based therapeutics [64].

Engineering NK cells for cancer immunotherapy

Several limitations currently exist for NK cell-based immunotherapies that need to be addressed to enable broad clinical translation. First, the in vivo persistence of NK cells needs to be enhanced for long-term therapeutic regimes. Second, it is critical to improve both the length of duration and the stability of the engineered antigen receptors for a robust therapeutic response. Third, more effective and safer systems for NK cell engineering need to be developed to replace the broadly utilized viral transduction strategies, to achieve high transduction efficiency and improve NK cell viability after modification. To tackle these limitations, numerous genetic and surface engineering methods for enhancing NK cell immunotherapy have been investigated.

Genetic engineering of NK cells using viral vectors

Techniques for genetically engineering NK cells can be classified as either viral or non-viral. These strategies aim to bestow NK cells with improved in vivo persistence and expansion capabilities, homing and migration to tumor tissues, and tumor-targeting capabilities for adoptive cancer immunotherapy. Viral transduction renders long-term expression of a stable transgene and is currently utilized in clinical settings [65]. However, to transduce a clinically relevant number of NK cells, a large quantity of virus is required, which raises manufacturing complexities and costs [66]. Further, the potential risk of insertional mutagenesis remains a significant concern with viral vectors [67, 68].

Retroviral vectors

Retroviral vectors were one of the first vectors to be used for NK cell engineering, where NK cells were transduced with IL-2 cDNA for enhancing cell persistence in vivo [69]. However, retroviral vectors exhibit low transduction efficiency in NK cells, which could be partially attributed to the inherent defense mechanism of NK cells toward viral vectors [70]. Retroviral transduction thus requires actively and rapidly dividing cells for maximum efficiency [71]. Other means of improving retroviral transduction efficiency often include pre-activation of NK cells using IL-2 and K562 cells, and multiple rounds of transduction [71, 72]. With these enhancements, retroviral transduction efficiency for NK-92 cells can range from 60% to 90% [73], whereas the transfection efficiency for primary NK cells is lower, at approximately 50% [71].

Lentiviral vectors

Lentiviruses, unlike retroviruses, do not require actively dividing cells, thus presenting some potential advantages over retroviral transduction by being able to transduce more types of NK cells [62]. Lentiviral transduction efficiency of primary NK cells commonly relies on chemical reagents, such as cationic polybrene and protamine sulfate, which promote viral entry [74, 75]. However, these reagents have shown toxicity towards NK-92 cells and thus alternative reagents, such as DEAE-dextran and poly-L-lysine, have also been used [69]. Moreover, nontoxic cationic peptides, such as Retronectin, have been used to transfect hematopoietic stem cell-derived NK cells and have demonstrated increased transfection efficacy compared to the chemical reagents [76, 77].

Genetic engineering of NK cells using non-viral vectors

To mitigate the concerns of viral transduction, novel non-viral delivery methods have been explored and developed for NK cell engineering. Currently known non-viral transfection strategies used for NK cell genetic engineering include electroporation, trogocytosis-mediated methods, and several nanoparticle-based delivery systems, such as charge-altering releasable transporters (CARTs) and lipid nanoparticles (LNPs).

Electroporation

Electroporation is one of the earliest and most extensively used non-viral transfection strategies. A short electrical pulse is applied to cells, to create temporary permeability and allow for the infusion of DNA or RNA encoding the gene of interest [78]. Studies have shown that electroporation of NK cells with mRNA over plasmid DNA dramatically increased transfection efficiency, reaching 80–90% transfection in both resting human primary NK cells and ex vivo expanded NK cells [79]. Furthermore, Boissel et al. [80] observed that electroporation results in higher NK-92 cell transfection over lentiviral transduction. CD19- and CD20-CAR-NK-92 cells generated by electroporation-mediated mRNA delivery possess significantly higher cytotoxicity against malignant lymphoid cell lines than that of lentiviral vector-transduced NK-92 cells [80]. Shimasaki et al. [65] reported promising results of electroporated human NK cells in combating B-cell leukemia in a pre-clinical study. They also observed that the median cell viability was maintained at around 90% after electroporation, and that NK cells expressing anti-CD19-CAR secreted interferon-γ upon interacting with $CD19^+$ target cells exhibited enhanced cytotoxicity [65].

In addition to hematological cancers, electroporated NK cells were shown to be effective in treating solid tumors [52, 81, 82]. Liu et al. [52] genetically modified NK-92 cells with plasmid DNA encoding HER2-CAR via electroporation to treat breast cancer cells. Results showed that the HER2-CAR-NK-92 cells retained 60–90% cell viability after electroporation and exhibited significant antigen-specific tumor tissue infiltration and tumor growth inhibition in a mouse model of breast cancer [52]. This study highlights the potential clinical translatability of electroporated NK-92 cells for treating solid tumors [52]. However, although electroporation is safer than viral transduction, there is potential for cell death and irreversible damage to the cell membrane, thus limiting its clinical potential [83]. Therefore, less toxic strategies are being widely explored.

Trogocytosis-mediated methods

Trogocytosis is a phenomenon that occurs when lymphocytes, such as B, T, and NK cells, interact with antigen-presenting cells and inherit surface molecules from these cells through immunological synapses, which they can express on their own surface [84]. Studies showed that trogocytosis also occurs between lymphocytes and target cells, such as cancer cells [85]. Somanchi et al. [86] demonstrated the potential of trogocytosis in engineering human NK cells for enhanced homing to the lymph nodes, by co-culturing NK cells with a K562 "donor" cell line expressing chemokine receptor CCR7. Results showed that 80% of NK cells co-cultured with K562 cells expressed CCR7 after 1 h, and enhanced NK cell migration to the lymph nodes was observed in mice [86].

The potential use of trogocytosis as a non-viral delivery method for transferring the CAR construct to NK cells has also been explored [66, 86, 87]. Cho et al. [66] co-cultured K562 "donor" cells expressing high levels of anti-CD19 CARs with PB-NK cells and observed that 18.6% of NK cells expressed anti-CD19-CAR after 1 h of co-culture, and these NK cells demonstrated enhanced cytotoxicity towards B-ALL cells. However, trogocytosis-mediated receptor transfer was easily lost; specifically, CCR7 and CD19 expression ceased after 72 h and 2 h, respectively [66, 86, 87]. This rapid loss of acquired protein has significantly limited the use of trogocytosis-engineered NK cells in clinical settings. Thus, further studies are essential for improving the persistence and stability of trogocytosis-based modification of NK cells.

CRISPR-Cas9 system

The clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated protein 9 (Cas9) system has been broadly utilized as a cost-effective and highly efficient tool for targeted gene editing [44, 88, 89]. The CRISPR-Cas9 system achieves customizable specificity through induction of double-strand breaks by the Cas9 nuclease under the direction of a guide RNA. The system then inserts the genetic sequence of interest with nonhomologous end-joining or homology-directed repair pathways [88, 89]. A recent study by Pomeroy et al. [54] reported promising proof-of-concept results of CRISPR-Cas9-mediated knockout of the key inhibitory signaling molecules, ADAM17 and PDCD1, for improving PB-NK cell functionalities. The method efficiently modified 90% of the PB-NK cells, with increased cytokine production and tumor cytotoxicity in the immunosuppressive TME [54]. Furthermore, the group also expanded the edited PB-NK cells to clinically relevant cell numbers without loss of cell activity, demonstrating the clinical translatability of CRISPR-Cas9-engineered NK cells [54].

Charge-altering releasable transporters

Recently, McKinlay et al. [90, 91] reported a new delivery system for the delivery of mRNA to immune cells known as charge-altering releasable transporters (CARTs). Specifically, CARTs initially serve as cations, noncovalently complexing, protecting, and delivering mRNA to immune cells. Upon entering the cell membrane, CARTs undergo biological degradation and break apart into small, nontoxic, neutral molecules, releasing functional mRNA to induce protein expression [91] (Fig. 3).

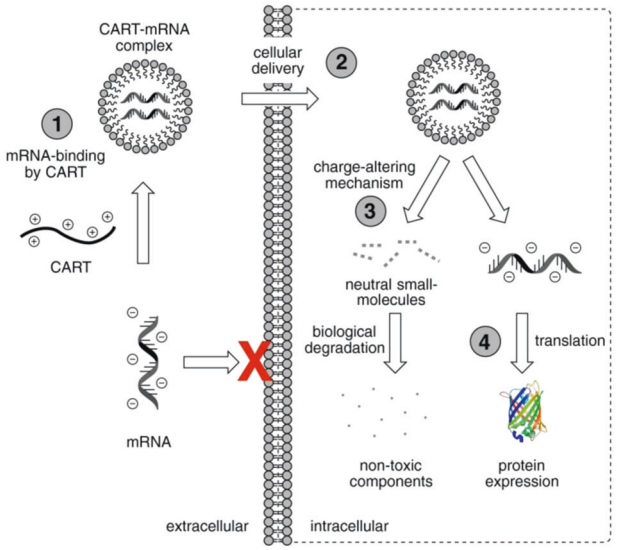

Fig. 3 Functional delivery of mRNA by charge-altering releasable transports (CARTs). (1) CARTs initially serve as cations to bind with mRNA, forming CART–mRNA complex. (2) Intracellular delivery of CART–mRNA complex. (3) CARTs undergo biological degradation, releasing functional mRNA. (4) Protein expression is induced. Figure adapted with permission from ref. [91], PNAS.

Using CARTs, Wilk et al. [92] delivered mRNA encoding anti-CD19-CAR to primary human NK cells. Wilk et al. [92] showed that CART-mediated mRNA transfection was as efficient as a high dose of electroporation-mediated transfection, but with higher overall cell viability. In addition, the resulting transfected primary NK cells had superior cytotoxicity towards leukemia cell lines [92]. Moreover, CART-mediated transfection minimally altered NK cell phenotype and proteomic expression compared to electroporation [90, 92]. Thus, this CART-mediated transfection strategy is promising in terms of both their application in clinical settings for cancer immunotherapy and for furthering the understanding of NK cell biology [92].

Lipid nanoparticles

To mediate the cytosolic delivery of nucleic acids for modulating gene expression, lipid nanoparticles (LNPs) have been developed as a non-viral delivery system that is able to protect nucleic acid cargo from degradation by nucleases and mediate endosomal escape [93, 94]. LNPs are comprised of an ionizable lipid component that is neutrally charged at physiological pH, which becomes positively charged in the acidic endosomal compartment to allow for release of cargo into the cytosol [95, 96]. In addition to the ionizable lipid, LNPs are commonly formulated with a combination of three additional excipients: a cholesterol component to improve stability and enhance membrane fusion, a helper phospholipid component to aid in endosomal escape and encapsulation of cargo, and a lipid-anchored polyethylene-glycol conjugate to minimize LNP aggregation [97, 98] (Fig. 4). Several research studies have examined the use of LNPs in transfecting a variety of immune cells [99–101]. In the context of CAR-based immunotherapy, Billingsley et al. [101] reported the use of ionizable LNPs to generate human CAR T-cells using mRNA. The approach delivers CAR mRNA to human T cells via LNPs, to induce transient CAR expression [101] (Fig. 4). This approach demonstrated a transfection efficiency comparable to traditional electroporation technology, but with substantially higher cell viability and improved retention of antitumor cell cytotoxicity in vitro [101]. In light of LNP-mediated CAR T-cell generation, similar LNP systems can be designed to engineer CAR-NK cells for cancer immunotherapy.

Surface engineering of NK cells for cancer immunotherapy

Recognizing the need to address the clinical obstacles faced by genetic engineering, there are several studies devoted to establishing an effective and less toxic engineering method as an alternative to the genetic engineering of NK cells. Currently explored surface engineering strategies include TRAIL-based liposomes, glycoengineering, and aptamer engineering of NK cells [37, 38, 41, 102–107]. These methods eliminate the mutagenic risks associated with genetic alteration and also minimize cellular damage.

Liposomes

Cytokine-mediated cytotoxicity is one of the major tumor-killing mechanisms of NK cells [102]. The release of cytokines and chemokines is triggered when tumor cells activate NK cell immune effector functions, by interacting with key effector molecules such as TRAIL expressed on the surface of NK cells [38, 41, 102, 108, 109]. Chandrasekaran et al. [110] reported the development of "super" NK cells, which were functionalized with TRAIL and anti-NK1.1 protein-coated liposomes that significantly enhanced the therapeutic potential of NK cells within tumor-draining lymph nodes in animal models, by directly presenting TRAIL to induce cancer cell apoptosis and prevent tumor metastasis (Fig. 5). Moreover, Siegler et al. [103] reported a CAR-NK cell functionalized with cross-linked multilamellar liposomal vesicles, which encapsulated the conventional small-molecule chemotherapeutic paclitaxel (PTX). The physical conjugation of PTX to CAR-NK cells showed significantly increased cytotoxicity in HER2- and CD19-expressing tumors in vivo compared to that of CAR-NK cells or PTX alone [103].

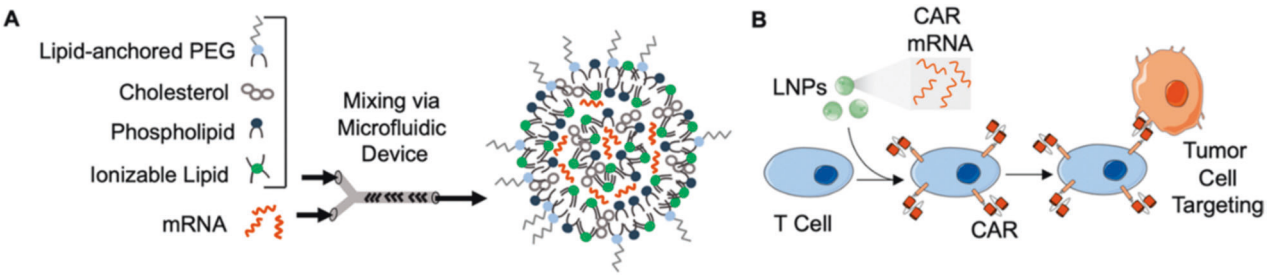

Fig. 4 Ionizable lipid nanoparticle (LNP)-mediated delivery of CAR mRNA for CAR T-cell engineering. A Schematic of the four excipients and the final structure of CAR mRNA-loaded LNP. **B** LNPs releasing CAR mRNA in T cells to engineer CAR T-cells for tumor cell targeting and killing. LNP-mediated genetic engineering of T-cells may be similarly applicable to NK cells. Figure adapted with permission from ref. [101]. Copyright 2020, American Chemical Society.

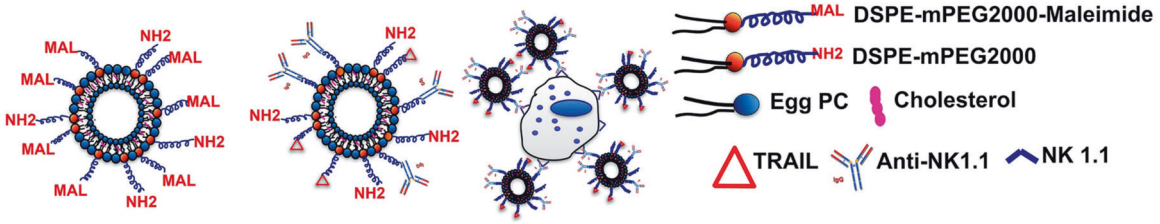

Fig. 5 "Super" natural killer (NK) cell formation. Liposomes decorated with TRAIL and Anti-NK1.1 protein were formed via maleimide-thiol chemistry. NK1.1-expressing NK cells conjugate with anti-NK1.1 on liposomes to form "super" NK cells. Figure adapted with permission from ref. [110], Elsevier.

Glycoengineered NK cells

Recently, several research labs have reported the therapeutic potential of glycoengineered NK cells [104, 105]. Glycoengineering aims to synthesize glycoprotein antibodies by conjugating specific sugars to the cell surface. Wang et al. [104] established a glycoengineering strategy that modified the NK cell surface with high-affinity CD22 ligands, a B-cell restricted antigen, for improved targeting towards B-cell lymphomas isolated from patients (Fig. 6). This study addressed the translational potential of glycoengineered NK cells, showing glycoengineering to be a potent alternative or complementary method to gene editing for the modification of NK cells. Similarly, Hong et al. [105] reported that by using chemoenzymatic glycan editing, they were able to conjugate high-affinity CD22 ligands onto the surface of NK-92MI and cytokine-inducing killer cells. Enhanced targeting specificity towards CD22 overexpressing B-lymphoma cells was shown in in vitro experiments and significant inhibition of B-lymphoma proliferation was achieved after further modifying the NK cell surface with E-selectin [105].

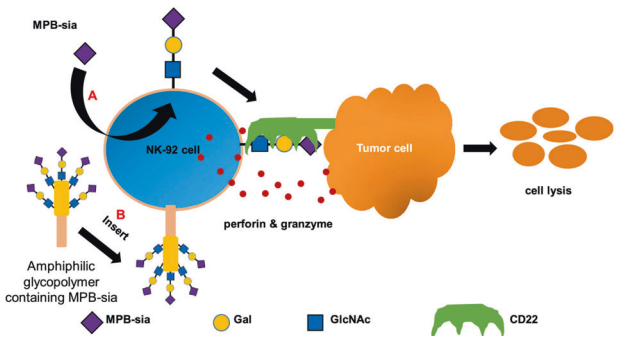

Fig. 6 Glycoengineering NK-92 cells with CD22 ligands for effective targeting and lysis of CD22-positive cancer cells. Method A metabolically engineered MPB-sia, a sialic acid derivative, onto NK-92 cell surface through the sialic acid biosynthetic pathway. Method B utilizes the amphiphilicity of cell membrane, inserts glycol-polymer containing MPB-sia into the NK-92 cell membrane. Figure adapted with permission from ref. [104]. Copyright 2020, American Chemical Society.

Aptamer-engineered NK cells

Another NK cell surface engineering method currently under investigation is the creation of aptamer-engineered NK (ApEn-NK) cells. Aptamers are short, single-stranded oligonucleotides that are anchored to the cell surface for high-affinity targeting specificity [37, 106, 107]. Yang et al. [37], using aptamer technology, generated $CD30^{-}$-specific ApEn-NK cells to target lymphoma cells (Fig. 7). In vitro results showed significant enhancement of NK cell-targeting specificity and killing towards $CD30^{+}$ T-cell lymphoma, and similar promising results were seen with aptamer-modified primary NK cells [37]. Compared to antibodies, aptamers demonstrated higher tissue permeability with significantly

lower molecular weights (8–25 kDa) and are also easier to modify at a low cost due to their simple structure, which can be easily modified through chemical processes [37].

Perspective on the future of engineered NK cells for cancer immunotherapy

Genetic and surface engineering strategies for redirecting or boosting NK cell cytotoxicity for cancer immunotherapy are rapidly expanding and have attracted increased attention in recent years. Currently, CAR-NK cells represent the majority of the investigated and developed NK cell-based approaches that have reached clinical trials (Table 1).

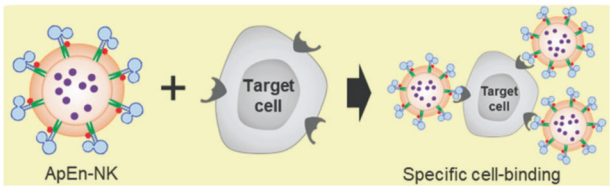

Fig. 7 Schematic of target-specific aptamers anchored on the surface of NK cells forming aptamer-engineered NK (ApEn-NK) cells. The resulting ApEn-NK cells demonstrated specific cell-binding and higher cytotoxicity towards lymphoma cells compared to normal NK cells. Figure adapted with permission from ref. [37]. Copyright 2020, John Wiley and Sons.

Accumulating evidence demonstrates the safety and efficacy of engineering NK cells for treating various types of cancers. With the rapid advancements in drug delivery and gene-editing systems in recent years, both the CRISPR-Cas9 technology and nanoparticle delivery systems serve as highly promising fields that could potentially rapidly advance NK cell-based cancer immunotherapies in the near future.

Further research efforts are essential for NK cell-based cancer immunotherapy to achieve widespread translation from bench to bedside. Currently, several clinical trials have shown the poor proliferative potential and persistence of NK cells in vivo, typically lasting only 1–2 weeks post ACT [26, 27]. Moreover, the therapeutic potential of NK cells towards solid tumors are still under-investigated due to the complexity of the TME. Exploring more optimal therapeutic targets with broad tumor-targeting coverage may efficiently increase clinical effectiveness for tackling solid tumors that manifest complex TME and may also potentially avoid toxic off-target effects. We anticipate that advances in non-viral engineering technologies, in combination with a deeper understanding of the fundamentals of cancer and NK cell biology will allow these cells to be modified effectively and serve as promising candidates for cancer immunotherapy.

Table 1 Current clinical trials of CAR-NK cells in hematological and solid tumors .

Target	Indication	Phase	Reference	Cell source
CD7	Lymphoma, leukemia	Phase I/II	NCT02742727	NK-92
CD19	Lymphoma, leukemia	Phase I/II	NCT02892695	NK-92
CD33	Adult acute myeloid leukemia	Phase I/II	NCT02944162	NK-92
HER2	$HER2^+$ glioblastoma	Phase I	NCT03383978	NK-92
ROBO1	$ROBO1^+$ solid tumor	Phase I/II	NCT03940820	NK-92
BCMA	Multiple myeloma	Phase I/II	NCT03940833	NK-92
MUC1	$MUC1^+$ solid tumors	Phase I/II	NCT02839954	PB-NK cells
NKG2D	$NKG2D^+$ solid tumors	Phase I	NCT03415100	PB-NK cells
Mesothelin	Epithelial ovarian cancer	Early Phase I	NCT03692637	PB-NK cells
CD19	B-lymphoid malignancies; acute lymphocytic leukemia; chronic lymphocytic leukemia; non-Hodgkin lymphoma	Phase I/II	NCT03056339	CB-NK cells
CD19	Lymphoma; B-cell chronic lymphocytic leukemia	Phase I/II	NCT04245722	iNK
CD19	Refractory B-cell lymphoma	Early phase I	NCT03690310	iNK
Unknown	Non-small cell lung cancer	Phase I	NCT03656705	NK-92
CD22	Refractory B-cell lymphoma	Early Phase I	NCT03692767	Unknown
PSMA	Castration-resistant prostate cancer	Early Phase I	NCT03692663	Unknown
CD19/CD22	Refractory B-cell lymphoma	Early Phase I	NCT03824964	Unknown

Acknowledgements M.J.M. acknowledges support from a U.S. National Institutes of Health (NIH) Director's New Innovator Award (DP2 TR002776), a Burroughs Wellcome Fund Career Award at the Scientific Interface (CASI), the National Institutes of Health (NCI R01 CA241661, NCI R37 CA244911, and NIDDK R01 DK123049), an Abramson Cancer Center (ACC)-School of Engineering and Applied Sciences (SEAS) Discovery Grant (P30 CA016520), and a 2018 AACR-Bayer Innovation and Discovery Grant, Grant Number 18-80-44-MITC (to M.J.M.). A.G.H. is supported by a National Science Foundation (NSF) Graduate Research Fellowship (DGE 1845298).

Author contributions R.E., Z.Z., A.G.H., and M.J.M. conceived the ideas, researched the data for the manuscript, discussed the manuscript content, and wrote the manuscript. Z.Z. designed the display items. All authors reviewed and edited the article before submission.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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