Gene-edited and CAR-NK cells: Opportunities and challenges with engineering of NK cells for immunotherapy

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INTRODUCTION

Natural killer (NK) cell activity in cancer, particularly in solid tumors, does not match the cells’ activity in an environment of unencumbered immune activation, largely because of the suppressive nature of the tumor microenvironment (TME). It has been reported that, in a number of cancers, NK cell presence in tumors may positively correlate with improved patient survival.1,2 It is thus beneficial, from a clinical perspective, for NK cells to not only reach the tumor but be able to perform their anti-tumor functions there. As therapeutic agents, NK cells are very attractive because of their ability to operate alloge-

ically in settings of major histocompatibility complex (MHC) ligand-receptor mismatch as well as their inherent safety, motivating the desire to use them in immunotherapy. Unfortunately, NK cells isolated from patient tumors are not like NK cells from peripheral blood of healthy donors, but are characterized instead by altered patterns of receptor expression and aberrant functional responses, and are often immunometabolically reprogrammed toward dysfunc-
tion.3,4 In other words, even NK cells that do make it to the tumor have an impaired ability to mount meaningful anti-tumor responses, along with abnormal receptor expression levels and altered metabolic fitness. NK cell activity in tumors is primarily driven by interaction of germline-encoded receptors with corresponding ligands on cancer cells. NK cells are regulated simultaneously by activating and inhibitory receptors. Signaling of both types of receptors determines whether NK cells perform effector responses against target cells, as is the case in cancer. When activated, NK cells are able to directly kill tumor cells by releasing granymes and perforins to form tiny pores on membranes of tumor cells and then cause cell apoptosis. This process is regarded as selective for the primary reason that tumor cells lack MHC class I, which is a so-called signal for “self” recognition and is expressed on normal cells. The “missing self” hypothesis is a common but not exclusive theory for NK cell activation in tumors. NK cells are able to function through antibody-dependent cell cytotoxility (ADCC), where they are activated in the presence of the Fc fragment of immunoglobulin G (IgG) antibodies bound to target cells through CD16 receptors.5 Although NK cells do not require MHC to kill, they do require cytokines for activation and persistence, which, in addition, help them communicate with other immune cells, like T cells and dendritic cells. Cytokines facilitate NK cell function by enhancing NK cell cytotoxicity (e.g. interferon [IFN]-α and tumor necrosis factor alpha [TNF-α]) and maintaining persistence (e.g. interleukin-2 [IL-2], IL-15, and IL-18), and can work in combination (e.g. IL-21 and IL-15).

Although exogenous manipulation of NK cell activity with cytokines is an accepted requirement for their ability to not only activate their cytotoxic machinery but also enable them to persist in vivo, it is the potential of genetic engineering to rewire NK cells’ effector responses that has offered the most promise. Not unlike their T cell counterparts, NK cells have been engineered to express chimeric antigen receptors (CARs) or
subjected to CRISPR-Cas9 to eliminate key genes of functional suppression, sometimes even in combination. Combined with antibody therapies or bi- or trispecific functional engagers, these genetic approaches are powerful in eliciting NK cell activity in environments where it would otherwise be suppressed. Engineered NK cell therapy is now a clinical reality, with trials for hematological malignancies and solid tumors underway across the world.

Unfortunately, reality has not been as fast to catch up to technological advances. Engineered NK cell therapy has so far mostly shown results in hematological tumors, with a phase I/II trial of CD19-CAR-NK cells in acute myeloid leukemia showing durable responses in 8 of 11 patients and NK cell persistence for up to 1 year, driven by endogenous expression of IL-15. For many other indications, particularly solid tumors, engineered NK cell therapy has been a challenge. Some of these challenges are intrinsic to the condition treated, largely linked to the broad immunosuppression in the TME, but some are specific to NK cells. For instance, NK cells are difficult to engineer, require cytokines for activation, and persist for a short time. Strategies to address these challenges exist, and include various cytokine stimulation programs, new ways to engineer NK cells, and new NK cell sources, all of which are active areas of investigation with exciting recent advances.

Despite the known challenges, engineered NK cell therapy remains a vastly promising approach to not only enhance NK cell activity in tumors but tackle severe suppressive mechanisms in cancer to achieve sustained and durable responses. In this review, we discuss recent developments in NK cell engineering with specific examples, and present the current state of the art of the field preclinically as well as clinically.

Sourcing NK cells

**Peripheral and cord blood-derived NK cells**

Peripheral blood has so far been the most common source of NK cells for adoptive therapy. This has partly been driven by the need for autologous therapies, which matching between the same donor-patient allows. In peripheral blood, NK cells constitute about 5%–10% of lymphocytes and have typically been distinguished as two functionally distinct subsets based on expression of the surface marker CD56: CD56dim and CD56bright NK cells. The major circulating subset in peripheral blood, representing about 90% of all NK cells, constitutes CD56dim cells, which are considered the more highly cytotoxic subset, and are characterized as expressing CD16, killer immunoglobulin-like receptors (KIRs) and possessing a higher capacity for degranulation. CD56bright NK cells, on the other hand, are considered abundant cytokine producers with a lower cytolytic capacity. Apart from these, CD56neg cells, which are found in low numbers in healthy individuals but more abundant in those with chronic and acute viral infections, lack CD56 but express NKP46. These cells have severely impaired cytolytic ability and antibody-dependent cellular cytotoxicity.

In contrast to peripheral blood, which relies on the availability of blood donors, cord blood has emerged as an alternative, more donor-independent source of functionally mature NK cells. Because cord blood is often banked and is thus easier to source, it has been thought of as an “off-the-shelf” source of allogeneic NK cells. Umbilical cord blood NK cells are typically younger and less mature than their peripheral blood counterparts and express a higher proportion of the homing chemokine receptor CXCR3. Cord blood NK cells have been phenotyped as expressing less CD16, KIRs, DNAM-1, NKG2C, IL-2R, and granzyme B and upregulating NKG2A, which is thought to contribute to their lower cytotoxicity compared with peripheral blood NK cells. Cord blood NK cells are a source of CAR-NK cells, and clinical trials of CD19-expressing cord blood-derived CAR-NK cells have shown good safety profiles when infused into patients.

**Stem cell-derived NK cells**

In recent years, advances have been made in generating functional NK cells from stem cell sources. Differentiation of induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) into NK cells enables routine production of NK cells for use in adoptive transfer therapy under controlled conditions, allowing allogeneic or “off-the-shelf” therapy with an appropriately designed NK cell product because of the ability of these stem cells to grow indefinitely in an undifferentiated state. Such conditions have included use of chemically defined media and lack of feeder cells, enabling generation of NK cells under fully xeno-free conditions.

Because NK cells natively originate from CD34+ hematopoietic stem cells, differentiation protocols have typically included a two-step process to first generate CD34+ hematopoietic progenitors from pluripotent stem cells, followed by a second stage where cytokines are introduced to induce differentiation of these cells into CD45+CD56+ NK cells. Multiple protocols have appeared, optimizing various aspects of this process from differentiation to expansion, including generation of NK cells from erythro-myeloid progenitors through Wnt-dependent and Wnt-independent processes. Stem cell-derived NK cells have been shown to be functionally competent, able to release cytokines such as IFN-γ and degranulate and kill cancer targets in vitro and in vivo, including in combination with anti-PD-1 checkpoint blockade immunotherapy. They have also been genetically engineered to express CARs, introduce a high-affinity, cleavage-resistant CD16 receptor, express an IL-15-IL-15r fusion receptor to improve in vivo NK cell persistence, or knock out the CISH gene and improve the metabolic fitness of NK cells via IL-15. The advantage of using pluripotent stem cells is that they can be engineered with relative ease at the stem cell stage and subsequently clonally selected, if required by the process, and sourced from stem cell banks.

Despite these remarkable advances, generating functional NK cells from pluripotent stem cells is not easy. The requirement for monolayer cultures and generation of embryoid bodies under specialized culture conditions as a first step in the process poses challenges in terms of ease of scale up and manufacturing to create a good manufacturing practice (GMP) product. The heterogeneous maturation and purity of these cells introduces the requirement for clonal selection of target cell subsets.
NK cell lines

NK cell lines have been a common source of NK cells for preclinical evaluation, and some have advanced to the clinic. NK cell lines include NK-92, KHYG-1, NKL, NKG, YT, hNK, NK3.3, and NK-YS. A common feature of these cell lines is their cancer origin, in that they have been established from patients bearing specific malignancies, most notably leukemia or lymphoma. Their utility, however, has found a home in research labs as a model for harder-to-obtain blood-derived NK cells and because of their cell line-like features, such as extensive expansion capabilities and ease of banking.

Although NK cell lines are an attractive source of NK cells for cell therapy, their functional and phenotypic identity needs to be considered when they are used as immune effectors.

Genetic engineering of NK cells

Viral vectors have been thought of as more efficient than non-viral ones when introducing CAR-based genes into immune cells. They have, however, also been marred by potential risks of toxicity and inflammation because of the nature of the vector. Lentiviral and non-lentivirus retroviral vectors have been successful in transducing NK cells, but no universally accepted protocol currently exists. Different labs have found success with different vectors, although some have been more common than others.

Transduction using lentiviral vectors is frequently used to engineer NK cells, typically by producing the vector in the HEK293T cell line. Lentiviruses are a subclass of retroviruses, all of which belong to the Retroviridae family, whose members contain a single strand of RNA that can be reverse-transcribed into DNA and packaged into the cell genome. Lentiviruses are capable of transducing non-dividing cells and can support large gene cargo (up to 10 kb). The lentiviral transduction system is composed of a transfer plasmid, an envelope plasmid, and a packaging plasmid. Multiple generations of lentiviral vector systems have been developed with the aim of improving performance and, most importantly, safety. A retrovirus enters cells through direct fusion. RNA is released while the capsule of the retrovirus is dissolved and then is reverse transcribed into DNA, which is then integrated into the host nucleus by an integrase.

The first example of genetic modification of NK cells with retroviral vectors dates back to 1998, when Nagashima et al. retrovirally delivered an IL-2 gene into two types of NK cells, NK-92 and YT2C2, and 10%–20% of the cells were successfully transduced.

The traditional and most common retroviral packaging system is derived from HIV, with lentiviruses being the most common retroviral subclass to be utilized for transduction of NK cells. The first-generation lentiviral system included all HIV genes (the gag, pol, tat, rev, and env genes and accessory genes). The gag gene encodes for matrix proteins that aid in viral infection. The pol gene encodes for reverse transcription enzymes. Finally, env encodes for proteins that help with viral entry into cells. The second-generation vectors removed the four HIV accessory genes (vif, vpr, vpu, and nef). In the third-generation vectors, gag and pol genes were encoded in different plasmids and tat genes were removed to avoid recombination of the virus. Because of removal of tat, heterologous promoters are used, either viral (like CMV) or cellular (like EF1-α). Recent work aimed at improving the performance of HIV-based lentiviral vectors showed that they can be created using minimal HIV-1 sequences by deleting most of the gag and over 50% of the env regions, ultimately improving the safety of these vectors.

Typically, the HIV envelope glycoprotein gains entry by binding to CD4 on target cells. In the absence of CD4, pseudotyping with other envelope glycoproteins has been pursued. One such commonly used envelope is vesicular stomatitis virus G protein (VSV-G), whose capability to bind to the low-density lipoprotein (LDL) receptor allows the virus to transduce a large variety of cells. Optimization of
transduction of NK cells with VSV-G-pseudotyped lentiviral vectors has been attempted by upregulating LDL receptor expression with rosvustatin, yielding a transduction efficiency of over 60% for NK-92 cells.\(^\text{56}\)

In addition to these, alpharetroviral vectors have also been used to engineer NK cells.\(^\text{57}\) With alpharetroviruses, the pro gene is encoded in the gag open reading frame (ORF), and pol is produced via ribosomal frameshifting, and they possess a short leader sequence devoid of splice sites, contributing to their safety profile. Compared with lentiviruses, alpharetroviral vectors have a relatively neutral integration pattern, which makes them less prone to genotoxicity-associated risks.\(^\text{58,59}\) Engineering of CAR-NK cells with alpharetroviral vectors has been successful, with high efficiency of transduction.\(^\text{60}\)

Generally, optimization of viral transduction and development of new vectors has most often relied on use of NK cells lines, which are easier to transduce, source, and expand. Although advantageous in terms of cost and time, primary NK cells present a complex set of challenges not easily recapitated by cell lines. NK-92, YT, NKL, and DERL7 have been widely used in development of engineered NK cell-based products. Early attempts yielded transduction efficiencies of around 15% for NK-92 cells and 30%–40% for YT, NKL, and DERL7 cells.\(^\text{61}\) These have since been improved through use of transduction enhancers such as dextran or by pseudotyping lentiviral vectors with new envelopes. However, transduction efficiencies of primary NK cells can still be as low as less than 20% with lentiviral vectors, and though higher efficiencies have been reported through protocol modification,\(^\text{62}\) there is still a demand for increasing the efficiency or enriching the cells after transduction.\(^\text{63}\)

Although much work has been done with lentiviral vectors, retroviral vectors that are not lentiviruses (e.g., gammaretroviruses) have matured as another approach to deliver genetic cargo to NK cells. Other retroviruses can only infect mitotically active cells, unlike retroviruses. To improve safety by eliminating unwanted gene activation (including of potential oncogenes) upon retroviral integration, generation of self-inactivated (SIN) vectors by deletion of 299 base pairs in the U3 region of the 3' long terminal repeat (LTR) of the DNA has been pursued.\(^\text{51}\) To avoid generation of replication-competent retroviruses (RCRs), trans elements were added and cis elements were reduced in the packaging and envelope constructs.\(^\text{64}\) However, cis-regulatory sequences should be inserted into the backbones to increase transduction efficiency.\(^\text{64}\)

Although NK cells have low transduction efficiency, attempts at increasing the efficiency of transduction are underway. Guven et al.\(^\text{65}\) investigated the potential of multiple rounds of retroviral transduction for enhancing gene expression efficiency on NK cells. Although a first round yielded 27.2% transduction efficiency, a second round of transduction increased the efficiency to 47.1%. Similarly, by performing two rounds of transduction at a different time points (on day 21 of NK cell expansion), they achieved a transduction efficiency of 51.9% (single round) and 75.4% (two rounds).

Despite these advances, there are a number of disadvantages associated with use of viral vectors, many of which have been documented extensively. Most notably, they carry a high risk of insertional gene mutation because of the high titers used and pose potential genotoxicity risks.\(^\text{66}\)

These are in addition to the notorious aversion of NK cells to viral transduction, particularly when using lentiviruses. This has been a major challenge in development of engineered NK cell therapies. Multiple approaches are being investigated to improve the performance of viral gene transfer to NK cells. One involves development of new vectors. To that end, Colamartino et al.\(^\text{67}\) developed baboon envelope-pseudotyped lentiviral vectors, which can bind to the sodium-dependent neutral amino acid transporter (ASCT1) to promote viral entry. Using this envelope, they were able to obtain a transduction efficiency of 38.3% ± 23.8% (mean ± SD) in freshly isolated human NK cells and 58.4% ± 7.8% after sorting and re-expansion, which are considered relatively robust rates of transduction efficiency for human NK cells. Another approach involves use of transduction enhancers such as protamine sulfate, retronectin, BX795 or Polybrene. Nanbakhsh et al.\(^\text{68}\) used dextran as a lentiviral transduction enhancer to successfully enhance the transduction efficiency of human NK cells to 40% after one round or 100% after two rounds. Allan et al.\(^\text{69}\) showed that, in the presence of IL-2 stimulation during transduction, NK cells tend to show improved transduction efficiency with lentiviral vectors. They also co-cultured NK cells with LCL (irradiated lymphoblastoid) feeder cells and IL-2, which reduced the transduction efficiency of cells but achieved higher expansion rates, ultimately leading to a higher total yield of transduced NK cells.

Non-viral engineering of NK cells

The most commonly used non-viral transduction approaches are electroporation, nucleofection, and lipofection.\(^\text{70}\) Electroporation causes pore formation on the recipient cells’ surface, allowing gene entry. The transfection efficiency achieved with electroporation can be higher than lipofection with the right instrument and conditions but depends on the cell type, whereas the resulting cell viability is dependent on cargo, whose size can have an effect on efficiency and, for NK cells, requires the presence of cytokines. For many primary immune cells, such as NK cells, transfection rates are lower because of the loss of viability, which negatively affects the ability of the cell to take up the gene and survive electroporation. Electroporation and its related technique, nucleofection, are suitable for clinical implementation and are generally considered superior to lipofection.

Lipofection, also known as liposome encapsulation, is another fairly established approach for gene transfer. Genes are encapsulated in liposomes, spherical ball-like structures, surrounded by lipid bilayers. The earliest study using lipofection on NK cells involved transfer of a murine IL-2-expressing plasmid into primary NK cells using
1,2-dimyrystoylpropyl-3-dimethyl-hydroxyethyl ammonium bromide/dioleoyl phosphatidylethanol-amine (DMRIE/DOPE). Transfection of IL-2 could help promote the cytotoxicity of engineered NK cells.  

Careful optimization of these techniques has resulted in remarkably high gene transfection efficiency—up to 75% for lipofection and up to 60% for electroporation.

In addition to their use as drug delivery vehicles, nanoparticles have been used for gene transfer as an alternative to non-viral transfection modalities and to enhance the poor efficiency often encountered with other non-viral methods, such as electroporation or lipofection. Nanoparticles are typically engineered to support the stability of the encapsulated genetic cargo and promote entry into the cell. So far, various lipid-based, polymer-based, and inorganic nanoparticles have been developed for gene delivery.  

Iron oxide core magnetic nanoparticles (MNPs) have been shown to be capable of transfecting NK-92 cells, and these MNP-bearing NK cells could be manipulated to efficiently target tumors. A recent modification involved attachment of these nanoparticles to the cell surface to overcome low transfection efficiencies encountered with primary NK cells. Generally, however, nanoparticle toxicity at high doses is still considered a major obstacle in their use as transfection agents.

Our group has described transfection of an NKG2D-CAR into NK-92 cells using a polymer nanoparticle based on the piggyBac transposon system. Such non-virally transfected NK cells showed, in combination with CD73 blockade, enhanced anti-tumor efficacy in vitro and in vivo in a non-small cell lung cancer model. Zheng et al. designed immunomodulating nanoparticles (IMNs), which could accumulate in the target tumor site and enhance absorbance of NK cells by modifying the surface. Their IMNs, in combination with IgG antibody treatment, demonstrated significant inhibition of tumor growth in a 4T1-tumor-bearing breast cancer tumor mouse model. Wu et al. developed Fe3O4 MNP-modified NK cells for treatment of non-small cell lung cancer (NSCLC) and showed that such an approach could facilitate infiltration and recruitment of NK cells to the target site and induce increased apoptosis of A549 cells in vitro and strong inhibition of tumor growth in mice in vivo.

The sleeping beauty transposon system (SBTS) is another transposon-based non-viral gene modification system. It consists of two parts: a DNA transposon for gene expression and a transposase enzyme for inserting the transposon into chromosomes. Compared with the piggyBac transposon system, the SBTS has two major drawbacks: cargo size limitation (up to about 5 kb) and a tendency for lower transposition at higher transposase concentration. The SBTS has been successfully used to generate CD19-CAR-NK cells with high cytotoxicity against acute lymphoblastic leukemia in vitro and in vivo.

Methods and strategies that have been used in developing NK cell therapy using viral and non-viral transduction have also been utilized for different purposes, such as enhancement of NK cell persistence and expansion as well as cytotoxicity (Table 1).

**Gene editing of NK cells via knockout (CRISPR-Cas9) or knockdown (small interfering RNA [siRNA]/short hairpin RNA [shRNA]) approaches**

CRISPR-Cas9 is an established technique for gene editing that works on the principle of formation of double-strand breaks (DSBs), which can cause irreversible genetic modifications. The mechanism behind CRISPR-Cas9 is that, when viral DNA is inserted, it can be incorporated into the host genome between a repeated palindromic sequence to form a spacer. Newly formed sequences then undergo transcription to form CRISPR RNA (crRNA). Subsequently, the Cas9 protein becomes involved. The tracer RNA in the protein contains the sections that pair with the palindromic repeats. In this way, the complex of tracer RNA, crRNA, and Cas9 protein is formed. Each individual complex is cleaved off by a nuclease called RNase III, and it can recognize the crRNA and form DSBs.

Although still under development, the CRISPR-Cas9 system has been utilized to enhance the activity and performance of NK cells for adoptive transfer therapy. These modifications have included engineering NK cells toward more persistence, to modulate their exhaustion/dysfunction, and to enhance their anti-tumor specificity. A number of studies have focused on not only the therapeutic outcomes of such gene edits on NK cells, but on improving the CRISPR-Cas9 platform to more efficiently induce genome edits to NK cells. One such approach involves expanding NK cells to clinical numbers with C9 feeder cells and performing CRISPR-Cas9 genome editing to knock out ADAM17 and PDCD1 via electroporation to up to 90% efficiency using two pulses of 1,850 V and 10-ms pulse width.

The potential of using gene editing to generate safer NK cell therapy products was demonstrated by Hoerster et al., who eliminated expression of the HLA class I molecules by knocking out the B2M gene via CRISPR-Cas9 on NK cells and subsequently co-engineering the NK cells to express a single-chain HLA-E molecule. These cells were not only phenotypically equivalent to native NK cells but also avoided mismatched T cell elimination and NK cell fratricide, thus representing non-HLA-matched primary human NK cells as “off-the-shelf” immune effectors.

CRISPR-Cas9 technology has also been used to knock out expression of the cytokine Inducible SH2 Containing Protein (CISH) gene in human iPSCs by using a pair of guide RNAs (gRNAs) located in direct and complementary strands targeting exon 3 of the CISH gene. These CISH-/- iPSC-derived NK cells demonstrated a more robust metabolic profile via IL-15 activity and efficacy against a model of AML.

Contrary to CRISPR-Cas9 genome editing, gene silencing via siRNA or shRNA is a more commonly established technique to downregulate expression of target genes and has been used to enhance NK cell...
activity in the context of cell therapy. This has been commonly achieved via electroporation or lentiviral transduction. In the context of cancer immunotherapy, siRNA has been shown to be effective in enhancing NK cell anti-tumor function by silencing the inhibitory receptor NKG2A while overcoming NK cell exhaustion. Specifically, Figueiredo et al. used lentiviral vectors bearing shRNA targeting expression of NKG2A on NK cells, which they successfully reduced by 95%. The cytotoxicity assays against K562 and B lymphoblastoid cells showed that NK lysis had been increased by up to 40%.

NK cells, especially primary NK cells, are difficult to genetically modify, which is a major barrier to efficient deployment of these cells as engineered immunotherapeutic agents. Combining non-viral approaches for gene delivery is also a possibility and has been demonstrated. For instance, Nguyen et al. stabilized Cas9 ribonucleoproteins (RNPs) with polyglutamic acid to transfer into NK cells using electroporation, which enhanced the transfection efficiency in CRISPR-Cas9 genome editing therapy. Therefore, electroporation of polymer-stabilized CRISPR delivery systems might become a promising approach for NK cell immunotherapy.

### CAR-NK versus CAR-T cells

Although many of the CAR architectures originally developed for T cells have been utilized successfully to engineer NK cells, these two immune cell types possess distinct differences that informs their engineering and performance as CAR-based effectors. Most notable among them is the NK cells’ relatively short persistence in vivo compared with T cells. Because they are part of the innate immune system, NK cells’ rapid activation results in modest persistence in the circulation that is typically no longer than about 2–3 weeks, unlike T cells, which last considerably longer. For those reasons, although CAR-T cells have been engineered with suicide switches to mitigate prolonged in vivo activation in adoptive transfer settings, NK cells require cytokines to promote their survival. The rapid activation of NK cells is driven by their ability to respond and degranulate in response to targets via their germline-encoded activating and inhibitory receptors, unlike T cells, which require antigen presentation for activation. CAR-NK cells can thus kill targets via CAR-dependent

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### Table 1. Summary of representative approaches focused on engineering NK cells for improved function

| Purpose                      | Strategy                          | Gene        | Transfer vector | Disease                              | Cell type | Efficiency | References                          |
|------------------------------|-----------------------------------|-------------|----------------|--------------------------------------|-----------|------------|-------------------------------------|
| Persistence/ proliferation   | cytokine stimulation             | IL-2        | retrovirus      | liver metastasis of gastric carcinoma| NK-92, YT | 10%–20%   | Miller et al.                        |
|                              |                                   | IL-15       | lentivirus, electroporation, retrovirus | leukemia, hepatocellular carcinoma, AML | NK-92, NKL, pNK | <4%, up to 71% | Nagashima et al.                      |
|                              | CAR                               | CD19 EGFR   | lentivirus, retrovirus | leukemia, glioblastoma | pNK, pNK, NK92, NKL | 49%, 12.9%, 30%–50%, <30% | Liu et al., Müller et al., Herrera et al., Gang et al., Han et al. |
|                              | CAR                               | CD5         | lentivirus      | B cell malignancies                 | NK-92     | 88.9%     | Xu et al.                           |
|                              | CAR                               | CD20        | lentivirus      | B cell malignancies                 | pNK       | 66.7%     | Chu et al.                          |
|                              | CAR                               | CD123       | RD114 retrovirus | AML                                 | pNK       | Up to 98% | Sinha et al.                        |
|                              | CAR                               | CD138       | lentivirus      | MM                                   | NK-92MI   | >95%     | Jiang et al.                        |
|                              | ADCC suppressive pathway blockade | CD73        | lentivirus      | lung cancer                         | pNK       | 100%     | Chambers et al.                     |
|                              | chemokine receptor engineering   | CD16        | retrovirus      | lymphoma                            | NK-92     | 5%–10%   | Binyamin et al., Zhao et al.         |
|                              | elimination of metabolic suppression | CCR5       | lentivirus      | colon cancer                         | pNK       | 30%–70% | Li et al.                           |
|                              | targeting the TME                 | CXCR1 + NKG2D CAR | mRNA | ovarian cancer | pNK | 95.6% | Ng et al.                           |
|                              | multispecific targeting           | GSTh knockout | CRISPR-Cas9 mRNA | AML                                 | iPSC-NK   | 100%     | Zhu et al.                          |
|                              |                                   | dual CAR + CD73 single-chain variable fragment (scFv) release | lentivirus | glioblastoma | pNK | 38.1% | Wang et al.                          |
and CAR-independent mechanisms. When engineered to express a CAR, NK cells are typically considered harder to transduce or transfect, although difficulties are associated with engineering any primary immune cell. Lentiviral and retroviral vectors have been used to engineer both cell types. To date, all CAR-T cell therapies that have been commercially approved use gammaretroviruses or lentiviruses for engineering T cells. Other distinctions between CAR-NK and CAR-T cells include the ability to manufacture CAR-NK cells allogeneically from cell lines or by sourcing these cells from donors based on unmatched MHC. Related to this is the CAR-NK cells’ higher safety and lower reported risks for toxicity associated with graft versus host disease (GvHD), cytokine release syndrome, or neurotoxicity, which NK cells are thought to not directly induce, unlike T cells.

ENGINEERED NK CELLS FOR HEMATOLOGICAL MALIGNANCIES

CAR-NK cells have been shown pre-clinically and clinically to hold significant promise for treatment of hematological cancers. Clinical data have often substantiated such claims and supported the notion that enhancing NK cell activity can have positive clinical effects. For instance, the presence of NK cells in the bone marrow of patients with acute lymphoblastic leukemia (ALL) has been associated with better responses to treatment.

NK cell CAR targets in hematologic malignancies have included CD19, CD20, CD7, and CD5, among others (Table 1). These have been primarily built with signaling architectures analogous to those used in CAR-T therapies, although NK-specific CARs engaging signaling via CD16 or NKG2D cascades have also emerged. A recent review discusses in more detail the development of the architecture for NK cell engineering.

CAR-NK cells in leukemia

Treatments for various types of leukemias using CAR-NK cell therapy are underway pre-clinically and clinically. In a University of Texas MD Anderson Cancer Center phase I/IIa clinical trial of patients with CD19-positive cancer (non-Hodgkin’s lymphoma or chronic lymphocytic leukemia [CLL]), CD19-CAR NK cell therapy using IL-15-expressing cord blood-derived NK cells resulted in a response in 73% (8 of 11) of treated patients, with no evidence of disease found over 13.8 months of follow-up. Of the 8 patients, 4 had CLL, and 3 of them achieved complete remission. Sinha et al. developed a CD123-based CAR construct containing 41BB and CD3 signaling domains to generate CD123-CAR-NK-92 cells. Using in vitro and in vivo AML models, they managed to achieve a significant reduction in tumor burden in mice. In a similar way, Oelsner et al. engineered NK-92 cells expressing an FLT-3 CAR construct and used them to treat a B cell ALL mouse tumor model, ultimately achieving inhibition of disease progression.

When developing CARs for AML, CAR structure has been shown to affect performance. By engineering and comparing a panel of CAR signaling domain architectures for a CD123-targeting CAR, Christo-
ENGINEERED NK CELLS FOR SOLID TUMORS

Extensive research efforts have been aimed at developing therapies for solid tumor using NK cells (Table 2). CARs could selectively target surface antigens associated with such tumors, and activating these targets could facilitate cell lysis by degranulation, aided by expression of activating receptors on NK cells, including activating KIRs, NKG2D, and DNAM-1. In principle, this theory has been proven successful in various in vitro or in vivo scenarios and particularly in the context of blood cancers. Solid tumors, however, are a particularly challenging environment, requiring additional strategies of NK cell activation besides mere CAR-based antigen recognition.

Heterogeneity originating from cellular diversity in cancer is another major concern with the treatment of solid tumors using CARs. For example, cancer stem-like cells (CSCs), which contribute to treatment resistance by promoting tumor initiation, do not typically express the same specific antigens as mature cancer cells, so they are often not be recognized by typical CARs and ultimately contribute to immune evasion. In glioblastoma, for instance, resistance to immune recognition has been attributed to the interaction between glioblastoma (GBM) stem cells (GSCs) and NK cells via $\alpha v$ integrin-mediated transforming growth factor $\beta$ (TGF-$\beta$) activation. In turn, inhibiting such interaction via adoptive transfer of allogeneic TGFBR2 gene-edited allogeneic NK cells in combination with inhibitors of integrin or TGF-$\beta$ signaling prevent GSC-induced NK cell dysfunction and contributed to effective tumor growth.3

Cellular heterogeneity extends beyond tumor cells and encompasses other immune cells, such as immunosuppressive myeloid cells. To effectively reduce their numbers and target PD-L1 immunosuppressive activity in the CAR-NK cell therapy setting, Lee et al. developed PDL-1 CAR-NK cells that could target T cell-resistant tumor cells in head and neck squamous cell carcinoma (HNSCC) cells in vitro. In addition to effective tumor control that was PD-L1 and CD8$^+$ cell dependent, this approach resulted in significantly reduced numbers of peripheral and tumor-infiltrating macrophages and neutrophilic and monocytic myeloid cells expressing PD-L1.

However, it has been known that NK cells have limited therapeutic effects against solid tumors because of the many immunosuppressive effects exerted on them by the solid TME. These effects are caused by a variety of factors. One such mechanism of immunosuppression is up-regulation of the checkpoint molecules PD-1 and PD-L1. In addition, tumor cells secrete immunosuppressive factors such as adenosine, TGF-$\beta$, and indoleamine 2,3-dioxygenase (IDO). These factors further downregulate activating NK cell receptors and reduce their killing ability. To address PD-1 suppression in the TME, Lu et al. developed a CAR-NK cell therapy using a co-stimulatory converting receptor (CCCR) containing the extracellular domain of PD-1, the transmembrane and cytoplasmic domains of PD-L1, and the cytoplasmic domain of 41BB. CAR-NK cells engineered with this construct could inhibit tumor growth in a lung cancer mouse model.

In preclinical and clinical studies, combination therapy with CAR-NK cells and tumor targeting using small molecules has shown to be able to increase and enhance the cytotoxicity and infiltration of NK cells into tumors. Zhang et al. developed a combination therapy using regorafenib, an FDA-approved multi-receptor tyrosine kinase inhibitor, and epithelial cell adhesion molecule (EpCAM)-CAR-NK-92 cells, which showed improved anti-tumor responses compared to monotherapy in colorectal cancer xenograft mouse models. Chen et al. developed EGFR-CAR-NK-92 cells in combination with oncolytic herpes simplex virus 1 (oHSV-1) and showed a higher cytolytic effect in breast cancer brain metastasis (BCBM) mouse models.

Chemokines also play essential roles in NK cell therapy because they are closely related to facilitating NK cell trafficking toward the target site. Chemokine receptors that are commonly expressed on NK cells include CXCR3, CXCR4, CXC2, and CXCR1, which are mainly expressed on CD56$^{bright}$, and CCR7 and CCR2, which are primarily expressed on CD56$^{dim}$ NK cells. The expression of chemokine receptors largely

**Table 2. Clinically and preclinically evaluated CAR-NK cell therapies in solid tumors**

| CAR target | Transfer vector | Disease | Clinical trial | References |
|------------|-----------------|---------|----------------|------------|
| EGFRyIII   | lentivirus       | glioblastoma | phase II        | Muralakami et al. |
| EGFR       | lentivirus       | triple-negative breast cancer | phase I | Liu et al. |
| EpCAM      | lentivirus       | breast cancer | phase I | 100 |
| TF         | lentivirus       | triple-negative breast cancer | phase I | Hu |
| Robe1      | N/A             | pancreatic cancer | phase II | Xia et al. |
| MSLN       | N/A             | pancreatic cancer | phase I | Cao et al. |
| MSLN CLDN6 | lentivirus       | ovarian cancer | early phase I | Second Affiliated Hospital of Guangzhou Medical University |
| GD2/ NKG2D | lentivirus       | glioblastoma | phase I | Wang et al. |
| PSA/PSMA   | lentivirus       | prostate cancer | phase I | Töpfer et al. |
| HER2       | lentivirus       | breast cancer/ renal cell carcinoma | phase I | Schönfeld et al. |
| GPA7       | electroporation | melanoma | phase I | Zhang et al. |
| HER2       | lentivirus       | gastric cancer | phase I | Wu et al. |
| ErbB2      | lentivirus       | glioblastoma | phase I | Zhang et al. |
| GPC3 NKG2D | electroporation | colorectal cancer | phase I | Yu et al. |
| DLL3       | lentivirus       | small cell lung cancer | phase I | Xiao et al. |
| PD-L1      | N/A             | metastatic colorectal cancer | phase II | Liu et al. |

**References**

1. Heterogeneity originating from cellular diversity in cancer is another major concern with the treatment of solid tumors using CARs. For example, cancer stem-like cells (CSCs), which contribute to treatment resistance by promoting tumor initiation, do not typically express the same specific antigens as mature cancer cells, so they are often not be recognized by typical CARs and ultimately contribute to immune evasion. In glioblastoma, for instance, resistance to immune recognition has been attributed to the interaction between glioblastoma (GBM) stem cells (GSCs) and NK cells via $\alpha v$ integrin-mediated transforming growth factor $\beta$ (TGF-$\beta$) activation. In turn, inhibiting such interaction via adoptive transfer of allogeneic TGFBR2 gene-edited allogeneic NK cells in combination with inhibitors of integrin or TGF-$\beta$ signaling prevent GSC-induced NK cell dysfunction and contributed to effective tumor growth.

2. Cellular heterogeneity extends beyond tumor cells and encompasses other immune cells, such as immunosuppressive myeloid cells. To effectively reduce their numbers and target PD-L1 immunosuppressive activity in the CAR-NK cell therapy setting, Lee et al. developed PDL-1 CAR-NK cells that could target T cell-resistant tumor cells in head and neck squamous cell carcinoma (HNSCC) cells in vitro. In addition to effective tumor control that was PD-L1 and CD8$^+$ cell dependent, this approach resulted in significantly reduced numbers of peripheral and tumor-infiltrating macrophages and neutrophilic and monocytic myeloid cells expressing PD-L1.

3. However, it has been known that NK cells have limited therapeutic effects against solid tumors because of the many immunosuppressive effects exerted on them by the solid TME. These effects are caused by a variety of factors. One such mechanism of immunosuppression is up-regulation of the checkpoint molecules PD-1 and PD-L1. In addition, tumor cells secrete immunosuppressive factors such as adenosine, TGF-$\beta$, and indoleamine 2,3-dioxygenase (IDO). These factors further downregulate activating NK cell receptors and reduce their killing ability. To address PD-1 suppression in the TME, Lu et al. developed a CAR-NK cell therapy using a co-stimulatory converting receptor (CCCR) containing the extracellular domain of PD-1, the transmembrane and cytoplasmic domains of PD-L1, and the cytoplasmic domain of 41BB. CAR-NK cells engineered with this construct could inhibit tumor growth in a lung cancer mouse model.

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depends on activation of NK cells; NK cells treated with IL-2 showed a significant decrease in CXCR3 expression and a simultaneous decrease in CXCL10.\(^{153}\) Engineered CAR-NK cells targeting chemokine receptors could show enhanced NK cell activity by promoting their migration toward tumors.\(^{154}\)

**ENGINEERED NK CELLS IN THE CLINIC**

It is clear by now that CAR expression on NK cells gives them strong selectivity toward cancer cells expressing the antigens specific to the CAR and can allow these cells to mediate strong anti-tumor responses when primed appropriately.

Compared with CAR-T cells, CAR-NK cells have a relatively smaller risk of inducing GvHD, a frequent side effect in allogenic transplant settings, and one that has been observed often with CAR-T cell therapy.\(^{155}\) CAR-NK cells also carry a lower risk of inducing neurotoxicity, unlike CAR-T cells, which generate high levels of inflammatory cytokines, such as IL-1α, IL-1Ra, IL-2, IL-2Ra, IL-6, TNF-α, MCP-1, IL-8, IL-10, and IL-15, and risk causing severe toxicity as well as cytokine release syndrome (CRS).\(^{155}\) Unlike CAR-T cells, CAR-NK cells can be administered without the requirement for full HLA matching. Clinical targets for CAR-NK cells have included CD7 (ClinicalTrials.gov: NCT02742272), MUC1 (ClinicalTrials.gov: NCT02839954), HER2 (ClinicalTrials.gov: NCT03383978), ligands to NKG2D (ClinicalTrials.gov: NCT03415100), CCCR (ClinicalTrials.gov: NCT03656705), BCMA (ClinicalTrials.gov: NCT03940833), ROBO1 (ClinicalTrials.gov: NCT03941457 and NCT03940820), CD22 (ClinicalTrials.gov: NCT03692767), mesothelin (ClinicalTrials.gov: NCT03692637), PCMA (ClinicalTrials.gov: NCT03692663), and CD19/CD22 (ClinicalTrials.gov: NCT03824964).

For these reasons, it is not surprising that some CAR-NK cell therapies have progressed to the clinic. Most of these therapeutic platforms have so far been primarily designed to test safety and efficacy against hematological tumors. They have also mostly focused on treating relapsed/refractory patients. Various studies have demonstrated the safety and efficacy of adoptive transfer of CAR-NK cells into human recipients. A durable 73% response rate with no incidence of CRS has so far been observed in a phase I/II trial of cord blood-derived CD19-CAR-NK cells expressing IL-15 in AML.

A phase I trial (ClinicalTrials.gov: NCT04245722) evaluated the safety and efficacy of iPSC-derived, off-the-shelf CD19 CAR-NK cells engineered with a high-affinity 158V, non-cleavable CD16 (hnCD16) Fc receptor. Efficacy was tested in 20 patients with relapsed/refractory B cell lymphoma or CLL with the iPSC-CAR-NK cell product alone or in combination with rituximab. Of the 20 patients, no GvHD or incidences of immune effector cell-associated neurotoxicity syndrome (ICAN) were reported. 2 cases of CRS were reported, and responses were observed in 8 of 11 efficacy-evaluable patients.\(^{156}\)

Despite positive results reported in patients treated with CAR-NK cells derived from cord blood or iPSCs, most of the CAR-NK trials to date have utilized NK-92 cells. Currently registered clinical trials utilizing peripheral blood-derived NK cells target just two antigens: CD19 (ClinicalTrials.gov: NCT01974479) and ligands to NKG2D (ClinicalTrials.gov: NCT03415100). In the latter case, NK cells are engineered by mRNA electroporation.

**CHALLENGES WITH NK CELL THERAPY AND STRATEGIES TO ENHANCE NK CELL ACTIVITY IN TUMORS**

As discussed, engineered NK cell therapy has a series of advantages. NK cell engineering can successfully avoid GvHD and other side effects, such as CRS, encountered with CAR-T therapy.\(^{157}\) Because NK cells have a comparably shorter lifespan than other immune cells, the possibility of long-term cell deficiency caused by memory cell responses such as B cells is reduced. NK cells engineered to express CARs can retain the ability to activate in the presence of target cells based on their intrinsic activating and inhibitory receptors, whereas CAR-T cells can only recognize receptors linked to their scFv sequences.\(^{141}\) Although CAR-NK cells have been used in clinical trials, there are still obstacles and challenges to overcome. First, there are very few techniques to expand NK cells to sufficient numbers for cell transfer.\(^{157}\) NK cells are often expanded under stimulation of feeder cells, which are typically cancer cells, so there is the potential risk that the NK cell product could be transferred along with some feeder cells. Second, the transfection and transduction efficiencies of NK cells are much lower compared with those of other cells; thus, typical engineering techniques have struggled to achieve consistently high levels of genetically-modified NK cells. Third, NK cells have been reported to lose some of their cytotoxic functions after freeze-thaw cycles, limiting the yield of active cells after cryopreservation. More generally, identification of potential targets has been a particularly challenging aspect of CAR-NK cell therapy.\(^{158}\)

Beyond manufacturability challenges, CAR-NK cells face significant obstacles in targeting of many cancers, particularly solid tumors. It has been reported that many solid malignancies have very few infiltrative NK cells. Although engineering NK cells to express CARs is able to enhance their specificity, trafficking and infiltration of CAR-NK cells into solid tumors remain difficult. Multiple immunosuppressive factors contribute to the poor trafficking and activity of NK cells in the TME: tumor heterogeneity, NK cell exhaustion upon TME interaction,\(^{159}\) soluble factors (e.g., TGF-β), metabolic inhibition\(^{160}\) of NK cell responses because of high concentrations of immunosuppressive metabolites (e.g., adenosine, lactic acid)\(^{161-163}\) and nutrient scarcity,\(^{164}\) and upregulation of inhibitory receptors that impair NK cell function (such as NKG2A and PD-1).\(^{165,166}\) As a consequence, NK cell lose their ability to degranulate and secrete cytokines, resulting in overall lower cytolytic capacity.

Although it remains unclear whether exhaustion, anergy, and senescence\(^{167}\) are separate or related mechanisms that contribute to NK cell dysfunction in tumors, it is clear that the TME of many tumors
presents NK cells with significant challenges. Although CAR engineering has been able to enhance the specificity of NK cells and result in improved treatment responses, durable anti-tumor function will likely come from combining multiple approaches targeting TME immunosuppression. Enhancing NK cell activity in tumors has also turned to use of antibodies to boost NK cell activity via ADCC, expansion of NK cells with small molecules such as nicotinamide to enhance their persistence, use of bispecific or trispecific NK cell engagers (BiKEs or TriKEs, respectively), and combining NK cells with immune checkpoint blockade. Targeting chemokine receptor-ligand axes has been employed as a means to enhance intratumoral infiltration of NK cells.

As discussed, NK cells have been engineered to express CARs to primarily enhance their specificity and to target antigens that are over-expressed in cancer. Although this has resulted in remarkable clinical responses in hematological malignancies, many cancers, particularly solid tumors, present a more sophisticated set of challenges that mere CAR-based targeting is unable to overcome.

Strategies to mitigate NK cell dysfunction have included enhancing the persistence of CAR-NK cells through cytokine-induced memory-like phenotypes, or engineering NK cells to express IL-15 to support CAR-NK cell durability, engineering chemokine receptors to improve the trafficking of CAR-NK cells to tumors, engineering dual and multifunctional CAR-NK cells to mitigate antigen escape and tumor immunosuppression, and combining CAR-NK cells with other immunotherapies, such as immune checkpoint blockade.

Although it is not yet clear which of these approaches will result in the optimal response, it is likely that contributions from multiple targeting mechanisms will be necessary to achieve sustained anti-tumor responses, particularly in solid tumors.

Concluding remarks
CAR-NK cell therapy has been demonstrated to hold remarkable promise for treatment of many cancers for which use of wild-type NK cells is inadequate. Simple and more complex engineering architectures are emerging that have been shown to have a unique ability to stimulate NK cell activation, killing, and persistence. As our understanding of the complexities of cancer evolves, so does incorporation of concepts and therapeutic targets in areas as broad as metabolism, senescence, and basic cancer biology with CAR-NK-based therapy. Such combination and multi-targeting approaches hold perhaps the most promise for tackling tumors for which conventional therapies have failed and continue to fail. As a therapeutic modality, there is much to be excited about regarding the potential of NK cells and much to still learn about them.

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X.W. and S.M. wrote and edited the manuscript.

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The authors declare no competing interests.

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