

Reprogramming natural killer cells for cancer therapy

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The last decade has seen rapid development in the field of cellular immunotherapy, particularly in regard to chimeric antigen receptor (CAR)-modified T cells. However, challenges, such as severe treatment-related toxicities and inconsistent quality of autologous products, have hindered the broader use of CAR-T cell therapy, highlighting the need to explore alternative immune cells for cancer targeting. In this regard, natural killer (NK) cells have been extensively studied in cellular immunotherapy and were found to exert cytotoxic effects without being restricted by human leukocyte antigen and have a lower risk of causing graft-versus-host disease; making them favorable for the development of readily available "off-the-shelf" products. Clinical trials utilizing unedited NK cells or reprogrammed NK cells have shown early signs of their effectiveness against tumors. However, limitations, including limited *in vivo* persistence and expansion potential, remained. To enhance the antitumor function of NK cells, advanced gene-editing technologies and combination approaches have been explored. In this review, we summarize current clinical trials of antitumor NK cell therapy, provide an overview of innovative strategies for reprogramming NK cells, which include improvements in persistence, cytotoxicity, trafficking and the ability to counteract the immunosuppressive tumor micro-environment, and also discuss some potential combination therapies.

INTRODUCTION

Cellular therapy has emerged as a rapidly advancing field in precision cancer treatment.¹ To date, the US Food and Drug Administration (FDA) has approved six chimeric antigen receptor (CAR)-T cell therapies for treating relapsed or refractory (r/r) non-Hodgkin's lymphoma, B-cell acute lymphoblastic leukemia (ALL), and multiple myeloma (MM).^{2–6} However, the production of autologous CAR-T cells has proven to be time consuming and costly. Furthermore, the high incidence of adverse effects, including potentially fatal complications such as irreversible cytokine-release syndrome (CRS), immune effector cell-associated neurotoxicity, and off-target cross-reactions, poses a significant risk to vulnerable patients.⁷ Therefore, there is a pressing clinical need for alternative strategies to generate antitumor immune cells.⁸

In recent years, natural killer (NK) cells have gained recognition as a promising therapeutic option due to their unique biological properties, which can potentially overcome many limitations associated with autologous cellular therapies^{9–11} (Table 1). NK cells can lyse tumor cells in an HLA-unrestricted manner, often without inducing severe graft-versus-host disease (GvHD),¹⁴ which makes them attractive candidates for "off-the-shelf" products with broad clinical application potential. Additionally, activated NK cells tend to produce lower levels of IL-6 compared with CAR-T cells, reducing the likelihood of severe CRS.^{12,15} Accordingly, clinical trials of NK cell therapies have demonstrated a lower incidence of treatment-related toxicities.^{16,17} Furthermore, the presence of various activation receptors on NK cells enables them to exert cytotoxicity against a wide range of cancers, thereby diminishing the risk of antigen-negative relapse.¹⁸ These advantages have made NK cells becoming prominent in the field of cellular immunotherapy.

Mature NK cells serve a crucial role in the innate immune system by actively monitoring and eliminating abnormal cells without the need for prior exposure. This effector function is intricately regulated through a set of receptors. NK cells also possess potent activating receptors that enable them to rapidly eliminate infected, transformed, or stressed cells, while inhibitory receptors such as KIR and NKG2A protect healthy autologous cells¹⁹ (Figure 1A). This mechanism is based on three primary recognition patterns.⁹ First, the "missing self" hypothesis suggests that NK cells remain inactive in environments lacking major histocompatibility complex (MHC)-I molecules, but when cells are

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Table 1. CAR-T vs. CAR-NK cells

	CAR-NK	CAR-T
Persistency	Limited persistence <i>in vivo</i> for 2–3 weeks; CAR-NK cells derived from CB could expand and persist for one year <i>in vivo</i> . ¹²	Most CAR-T cells have robust expansion and cytokine production; CAR-T cells can be detected years post-infusion in some patients. ¹³
Safety	Low incidence of toxicities	Common adverse effects including CRS, ICANS
Universality	No TCR to drive GvHD; Prime candidates for the “off-the-shelf” products.	Universal CAR-T cells require the knockdown or knockout of the T cell receptor, or it may lead to GvHD.
Efficacy	NK-cell innate function expands the target coverage against solid and liquid tumors; Current clinical studies present limited efficacy <i>in vivo</i> owing to limited persistence.	High efficacy in B cell hematological cancers; Limited efficacy in solid and heterogeneous cancer, like AML.

ICANS, immune effector cell associated neurotoxicity syndrome.

downregulated or lack MHC-I in the presence of other cells presenting MHC-I molecules, NK cells recognize and eliminate them by releasing granzyme and cytotoxic perforin (Figure 1B). Second, according to the “inducing self” hypothesis, activating receptors such as NKG2D identify stress ligands induced by infected or transformed cells, initiating an immune response that leads to their lysis while overriding inhibitory signals²⁰ (Figure 1C). NK cells also play a significant role in antibody-dependent cell-mediated cytotoxicity (ADCC).²¹ CD16A, expressed on NK cells and macrophages, facilitates ADCC when it binds to immunoglobulin G antibodies (IgG). Upon detecting mutated cells, the human immune system generates IgG1 antibodies specific to their antigens. Subsequently, CD16 binds to these antibodies, activating NK cells to eliminate target cells coated with antibodies²² (Figure 1D). Importantly, exogenous antibodies can similarly activate NK cell function, offering a flexible therapeutic strategy by altering antibody targets.²³ Furthermore, NK cells produce a range of cytokines and chemokines, including tumor necrosis factor, interferon- γ (IFN- γ) and granulocyte-macrophage colony-stimulating factor, which serve to recruit T and B cells to effectively coordinate secondary adaptive immune responses.

Clinical trials on NK cell therapy have reported satisfactory safety profiles, although only a few have achieved remission rates exceeding 50% (Table 2). Encouragingly, the rapid advancements in bioengineering technology have enabled the development of more effective NK cell-based antitumor therapies, with most research efforts in this field dedicated to enhancing the killing potency and *in vivo* persistence of NK cells. In this review, we provide an overview and analysis of the current landscape of clinical trials involving NK cell-based therapies, and delve into innovative strategies for optimizing the longevity and trafficking of NK cells, such as the overexpression of cytokines and chemokines, as well as potential strategies to overcome the immunosuppressive tumor microenvironment (TME) and enhance cytotoxicity, all aimed at improving treatment efficacy. Additionally, we emphasize the promising potential of combinational NK cell-based therapies, which encompass bite engagers, antibody-drug conjugates (ADCs), small molecule drugs, and oncolytic viruses.

DEVELOPING NK-BASED CELLULAR IMMUNOTHERAPY

Extensive research has been conducted on the adoptive transfer of NK cells for cellular immunotherapy, revealing significant therapeutic potential. Both autologous and allogeneic NK cells have been explored as

viable sources for NK cell therapy. Ongoing investigations have also recognized the suitability of NK cells derived from cord blood (CB-NK) and induced pluripotent stem cells (iPSCs) as valuable cell resources. Although NK cells possess inherent cytolytic capabilities, the introduction of genetic engineering to produce CAR-bearing NK cells has ushered in a new era of NK-based therapies.⁴⁰

Expanding NK resources

Autologous NK cells have demonstrated limited effectiveness against tumors *in vivo*, partly attributable to the compromised function of NK cells in heavily pretreated patients.⁴¹ As a result, haploidentical NK cells have become the primary source in current clinical applications. Typically, mononuclear cells are obtained from healthy HLA-unrestricted donors through apheresis, which simplifies the isolation of the required NK cells for therapeutic purposes. In human peripheral blood lymphocytes, NK cells constitute only about 10% of the population, with most of them being in a resting state. Consequently, *in vitro* stimulation and expansion, typically involving the use of interleukin-2 (IL-2) at a concentration of 100 IU/mL,⁴² are necessary to generate a sufficient number of therapeutic products.⁴³ However, it's worth noting that NK cells extracted from peripheral blood (PB-NK) exhibit inherent antiviral properties, which, to some extent, limit their susceptibility to genetic manipulation and have thus constrained their broader applications.

The umbilical CB serves as another valuable source of primary NK cells (CB-NK), readily available from blood banks in frozen form when required. Researchers have conducted analyses of CB-NK cell phenotypes and observed a higher percentage of NK cells in CB (approximately 30%) compared with PB. Additionally, a significant portion of these NK cells belongs to the CD56^{bright} subset.⁴⁴ Notably, genes associated with the cell cycle and replication show pronounced enrichment in CB-NK cells,⁴⁵ suggesting a potential for enhanced cytotoxicity and *in vivo* persistence. Furthermore, researchers have utilized umbilical CB to obtain CD34⁺ hematopoietic stem cells. For instance, Harry et al. successfully generated scalable NK cell products from CD34⁺ hematopoietic stem and progenitor cells (HSPCs) in the presence of IL-2 and IL-15, using HLA-matched umbilical CB units as a source,⁴⁶ following which they administered HSPC-NK cell infusions to elderly patients with acute myeloid leukemia (AML) and demonstrated that this “off-the-shelf” product could mature and transiently persist *in vivo*.⁴⁶

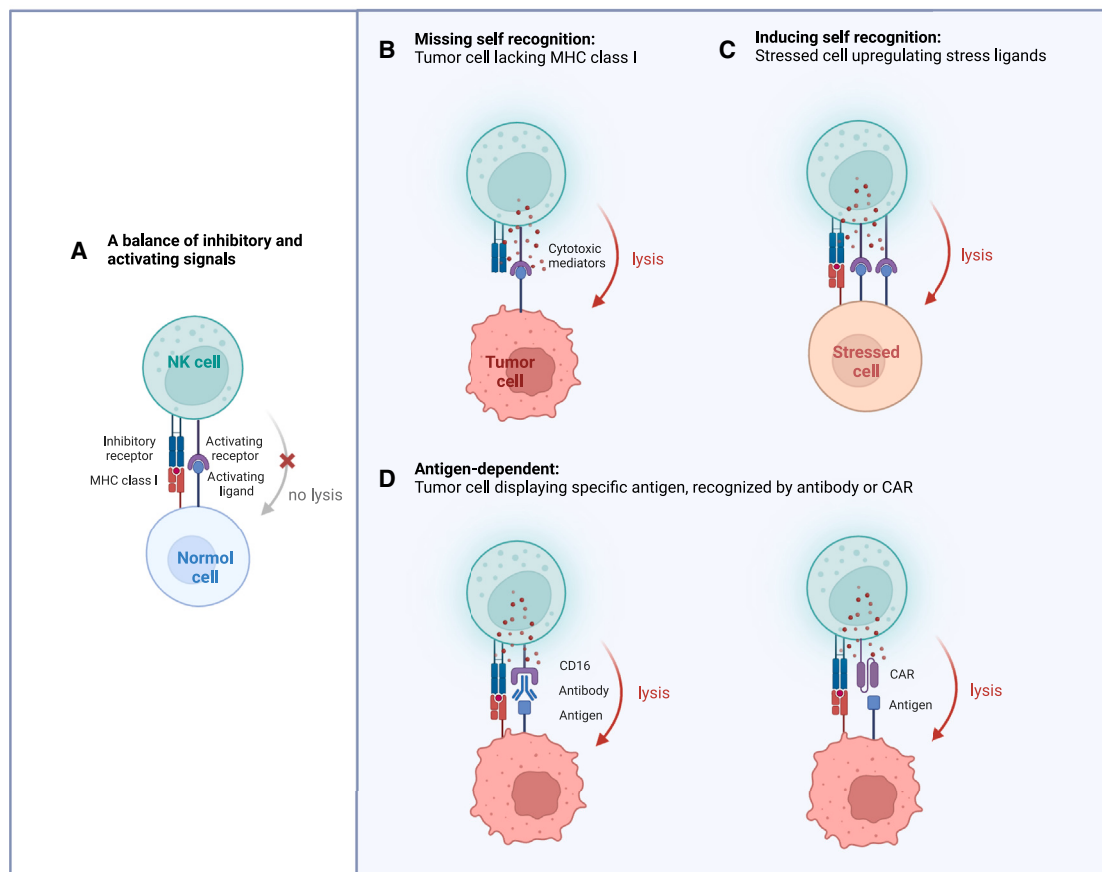


Figure 1. The mechanism of NK-cell recognition and cytotoxicity

NK cells possess a range of activatory and inhibitory receptors that control their recognition and targeted cytotoxicity. (A) Autologous normal cells display ligands for inhibitory receptors on NK cells, preventing NK cell activation. (B) Tumor cells often downregulate or lose MHC-I molecules, resulting in the loss of inhibitory signals and NK cell activation. (C) Stressed cells upregulate stimulatory ligands, tipping the balance toward a predominance of stimulatory signals, leading to "induced lysis." (D) Specific recognition by antibodies or CARs activates NK cells to eliminate targeted cells.

The NK-92 cell line is a well characterized homogeneous NK lymphoma cell line that can be readily expanded using various good manufacturing practice-compliant methods.^{47,48} Following its approval by the FDA for NK-based immunotherapy, NK-92 has been extensively investigated in clinical settings. However, their cancerous origin necessitates irradiation prior to infusion, limiting their *in vivo* persistence to just 48 h, which, in turn, hampers their long-term clinical effectiveness.⁴⁹ Additionally, NK-92 cells exhibit limited ADCC function due to the absence of CD16 expression.⁵⁰ Therefore, an engineering approach involving the expression of exogenous IL-2 and a high-affinity variant of CD16 is crucial to enhance their effector function,⁵¹ which is currently under evaluation in clinical trials targeting various malignancies (NCT03387085, NCT03853317, NCT03387111, and NCT03586869).

iPSCs represent a self-renewing cell source with immense potential for generating unlimited quantities of off-the-shelf immune cells, facilitating on-demand administration. These iPSCs can be repro-

grammed from healthy donor skin and fibroblasts and then differentiated into functional NK cells on a clinical scale.⁵² However, iPSC-derived NK cells display a less mature phenotype compared with PB-derived NK cells and exhibit higher NKG2A expression but lower CD16 and KIR expression.⁵³ One of the notable advantages of iPSCs is their greater amenability to genetic editing compared with primary NK cells, which researchers have exploited by conducting multi-gene engineering on iPSCs before their differentiation into NK cells, resulting in the creation of NK cells with potent cytotoxicity and prolonged persistence. In preclinical studies, iPSC-derived NK cells were shown to effectively target both solid and liquid tumors.^{54,55} The first CAR-NK cell product derived from iPSCs, known as FT596, is currently evaluated in a clinical study (NCT04245722).⁵⁶

Engineering CAR-NK

CARs are synthetic molecules that redirect immune effector cells for precise, enhanced antitumor capabilities. Over the past decade, CAR-NK cell therapy has demonstrated promising antitumor efficacy in preclinical studies.⁵⁷ Importantly, CAR-NK cells can target and

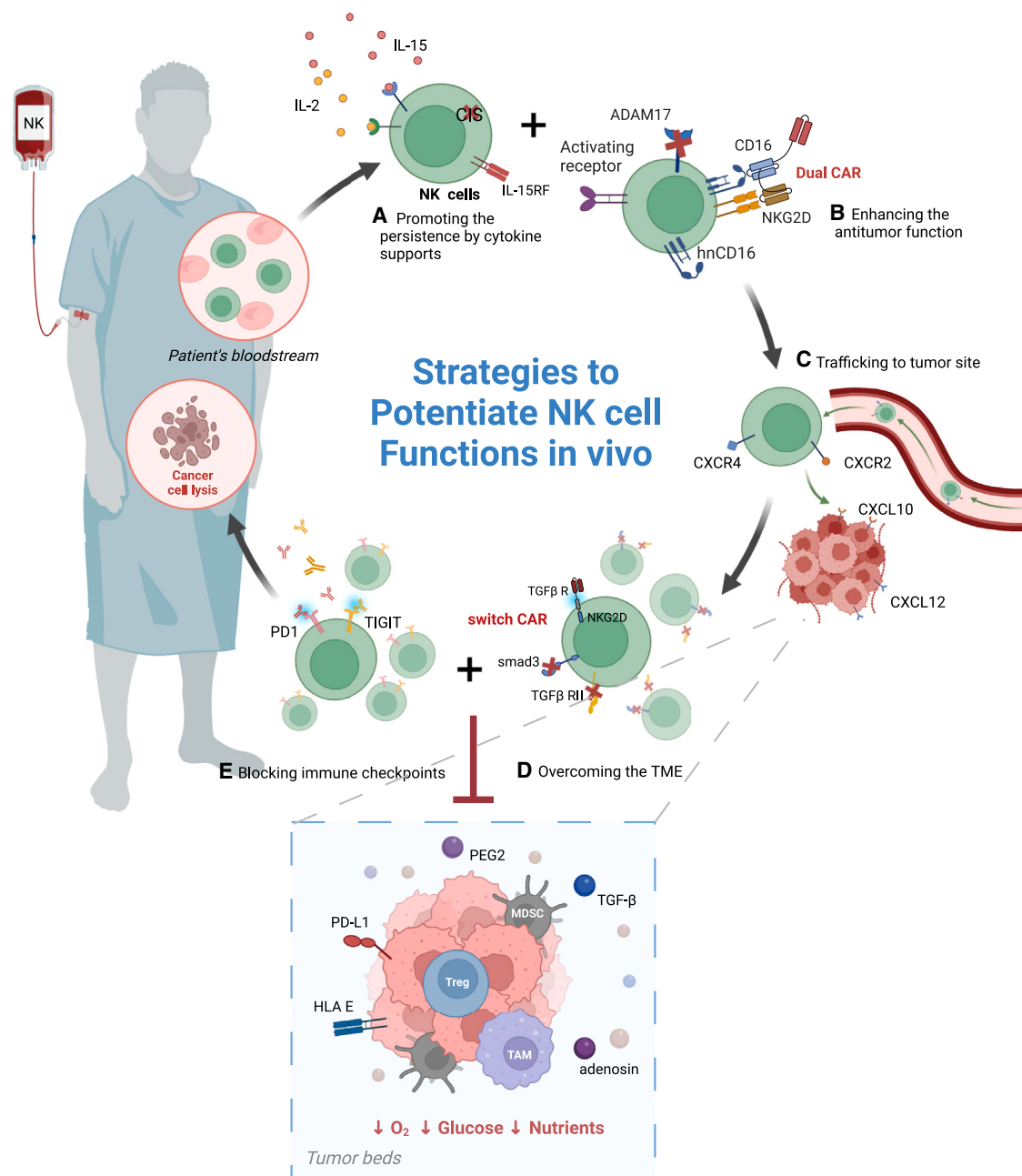


Figure 2. Strategies for enhancing NK cell functions in cancer immunotherapy

Improving NK cell therapy involves two primary aspects: enhancing NK cell antitumor function and overcoming the challenges of the adverse TME. (A) Cytokine support through IL-2 or IL-15 infusions helps maintain NK cell expansion and persistence. Equipping NK cells with a recombinant IL-15 receptor fusion (IL-15RF) for cytokine-autonomous persistence has demonstrated advantages in preclinical studies. Knocking out CISH in human NK cells also enhances their antitumor activity. (B) Armoring NK cells with CAR molecules enhances their cytotoxicity against tumors. Additionally, the use of a high-affinity non-cleavable variant of CD16 (hCD16) or inhibition of the metalloproteinase ADAM17 prevents CD16 shedding, improving ADCC efficacy. (C) Overexpression of chemokine receptors such as CXCR2 and CXCR4 enhances NK cell trafficking and infiltration into tumor sites. Regarding the TME, immunosuppressive factors like TGF- β , prostaglandin E2, and adenosine released by suppressor cells, including TAMs, MDSCs, and Tregs, impair NK cell metabolism and activity. (D) Innovative switch CARs modify the immunosuppressive effect of TGF- β , converting it into activating signals mediated by NKG2D, which can be achieved by combining the extracellular and transmembrane segments of TGF- β RII with the intracellular domains of NKG2D. Engineering NK cells to silence Smad3 and TGF- β RII also improves their cytotoxic function in a TGF- β -rich milieu. (E) Blocking immune checkpoints such as PD1 and TIGIT assists NK cells in killing cancer cells.

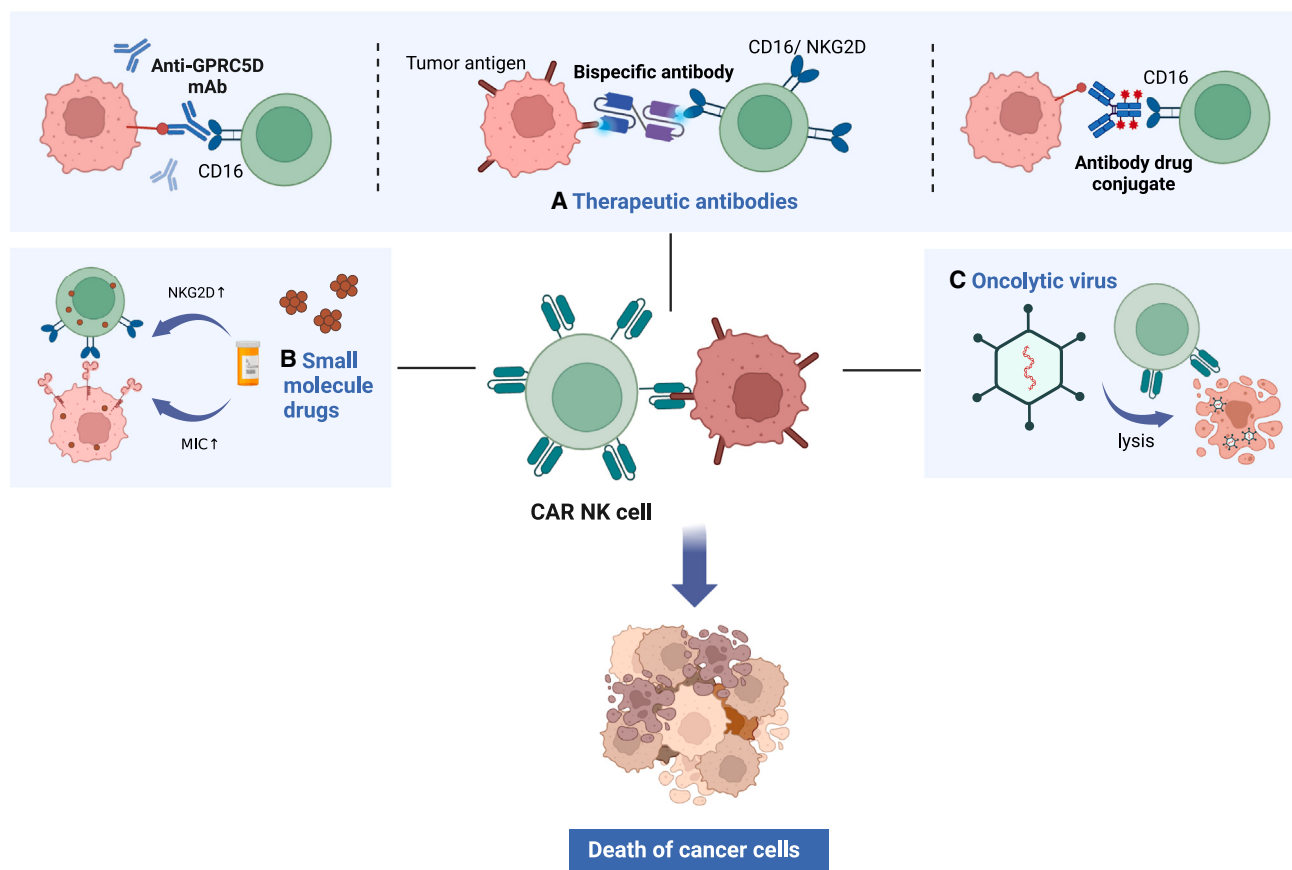


Figure 3. Potential combinational therapies of CAR-NK cells

Therapeutic antibodies, small molecule drugs, and oncolytic viruses offer promising strategies to enhance NK cell-mediated tumor targeting. (A) Recent mAbs targeting GPRC5D have demonstrated significant efficacy against MM cells, independently of BCMA. Bispecific engagers bring together cancer cells and effector cells, redirecting NK cells to target and eliminate tumors. Similarly, ADCs enable precise drug delivery to tumor sites, facilitating NK cell-mediated tumor elimination. (B) Certain specialized agents induce the expression of ligands for NK-cell activating receptors (NKARs) on target tumor cells or increase NKAR levels on NK cells, rendering cancer cells more susceptible to NK-mediated cytotoxicity. (C) oHSVs exert their tumor-killing function through intratumoral replication and induction of host antitumor immunity. Combined administration of CAR-NK cells and oHSV-1 enhances the *in vivo* killing of tumor cells.

eliminate tumor cells through both NKG2D-mediated and CAR-mediated cytotoxicity, rendering them effective against a wide range of tumors, including the ones with substantial heterogeneity such as AML.⁵⁸ In 2017, Liu et al.⁵⁹ introduced genes encoding CAR-CD19, IL-15, and the inducible caspase-9-based suicide gene into CB-NK cells using a retroviral vector. Their research findings demonstrated that these genetically modified cells effectively targeted and eliminated CD19-expressing cell lines and primary leukemia cells in laboratory conditions. Furthermore, in a Raji lymphoma mouse model, they observed a significant extension in survival time.⁵⁹ Moreover, they can generate more than 100 doses of CAR-NK cells from a single CB unit and were investigated in clinical trials in 2020, achieving an impressive 63.6% complete response rate (7/11 patients) and showing no signs of CRS or neurotoxicity.¹²

The design of CARs plays a pivotal role in optimizing effector function. Thus far, most CAR-NK engineering has relied on CARs

initially designed for T cells, which typically include CD3 ζ and co-stimulatory molecules. This design has yielded favorable effector potency in preclinical models.⁶⁰ In addition to these approaches, researchers have developed novel NK-specific CAR structures to enhance the tumor-targeting capabilities of NK cells. For instance, researchers in a previous study designed a CAR that incorporates the transmembrane domain of NKG2D, the DAP10 co-stimulatory domain and the CD3zeta domain to provide potent antigen-specific signaling to NK cells.⁶¹ NK cells that were transduced with the NKG2D-DAP10-CD3zeta CAR using retroviral techniques displayed elevated surface expression of NKG2D and demonstrated enhanced cytotoxic activity against both leukemia and solid tumors in *in vitro* experiments.⁶¹ Another CAR design incorporated the 2B4 co-stimulatory domain to create the NKG2D-2B4-CD3zeta CAR to generate iPSC-derived NK cells, demonstrating their capacity to inhibit tumor growth and extend survival in an ovarian cancer xenograft model.⁶² Furthermore, these CAR-iPSC-NK cells

Table 2. Selected completed NK cell-based clinical trials

Disease	Enrolled patients	Intervention	NK cell source	Response	Long-term efficacy	Toxicity	Reference
Hematological malignancies							
AML	14	NK cell infusion 6 doses	haploidentical	CR 50% ORR 78.6%	OS 8.5months; DFS 4.4months	no CRS, 1 CNS toxicity, 1 GVHD	Silla et al. ²⁴
AML	8	MLNKS +DLI	haploidentical (HCT donor)	CR 50%	NA	no significant toxicity	Bednarski et al. ²⁵
AML/ALL	18	NK cell infusion +IL-2	haploidentical	CR 72%	2-year OS 20%	no CRS/GVHD	Vela et al. ¹⁶
AML/MDS	30	NK cells infusion on Day+13, +20 posttransplant	haploidentical	CR 77%	OS 35%; PFS 33% (30 months)	56% aGVHD; 33% cGVHD	Lee et al. ²⁶
AML/CML/MDS	13	NK cells infusion on day-2, +7, and+28 posttransplant	haploidentical (HSCT donor)	CR 92%	1-year OS 92% 1-year DFS 85%	7 grade 1–2 aGVHD after HSCT	Ciurea et al. ²⁷
Myeloid malignancies	24	NK cells infusion on day-2, +7, and+28 posttransplant	haploidentical (HSCT donor)	NA	2-year OS 70% 2-year DFS 66% relapse rate 4%	10 aGVHD (9 grade 2; 1 grade 3) No cGVHD	Ciurea et al. ²⁸
Plasma cell myeloma	10	NK cell infusion after ASCT	haploidentical	NA	5-year OS 50% 5-year PFS 17%	no significant toxicity	Tschan-Plessl et al. ²⁹
B-NHL	9	NK cell infusion+IL-2 +rituximab	allogenic (3rd-party)	ORR 55.6%	TTP 50days	1 CRS 89% AE (grade<3)	Yoon et al. ³⁰
NHL/CLL	11	CD19 CAR-NK +IL-15	CB	CR 64% ORR 73%	NA	no CRS/GVHD; 10 neutropenia and lymphopenia	Liu et al. ¹²
Solid tumor							
NSCLC (mHsp70+)	6	NK cells infusion + radiochemotherapy	autologous	CR 16.7% PR 16.7%	1-year PFS 67%	no CRS/GVHD 3 SAE	Multhoff et al. ³¹
NSCLC	20	NK cell infusion + sintilimab	autologous	ORR 45%	1-year OS 80% PFS 11.6 months	2 (10%) grade 3 AE (hypertriglyceridemia, neutropenia, and increased creatine kinase)	Jia et al. ³²
Lung/liver/colon cancer	10	NK cells and CTL infusion	autologous	NA	1-year OS 80% 2-year OS 56%	no severe adverse events	Liem et al. ³³
Breast cancer gastric cancer	31	NK-cell infusion +trastuzumab	autologous	PR 54.8%	PFS 3months	no CRS/ICANS no GVHD; 3 SAE	Lee et al. ³⁴
Neuroblastoma	35	NK cells infusion +m3F8 (anti-GD2)	haploidentical	CR 14.2% PR 14.2%	PFS 7.4months OS 30.7months	no GVHD; 32 febrile neutropenia	Modak et al. ³⁵
HCC	11	NK infusion +hepatic arterial infusion chemotherapy	autologous	CR 36.4% ORR 63.6%	PFS 10.3months OS 41.6months	no CRS/ICANS/GVHD; 2 neutropenia; 1thrombocytopenia; 5 anemia; 4 hyperkalemia	Bae et al. ³⁶

(Continued on next page)

Table 2. Continued

Disease	Enrolled patients	Intervention	NK cell source	Response	Long-term efficacy	Toxicity	Reference
Gastric and colorectal cancer	9	NK cells infusion +trastuzumab- or cetuximab-based chemotherapy	autologous	SD 44.4% PD 22.2% DCR 66.7%	NA	no CRS/GVHD	Ishikawa et al. ³⁷
Stage III pancreatic cancer	18	NK cells infusion+ percutaneous irreversible electroporation	autologous	RR 63.2%	OS 13.6 months PFS 9.1 months	no severe adverse events	Lin et al. ³⁸
Stage IV pancreatic cancer	19	NK cells infusion+ percutaneous irreversible electroporation	autologous	DCR 66.7%	OS 10.2 months	no severe adverse events	Lin et al. ³⁸
Recurrent/metastatic nasopharyngeal carcinoma	7	NK cells infusion+ cetuximab	autologous	SD 57.1%	NA	no severe adverse events	Lim et al. ³⁹

Because the number of trials in each category is large, examples of the trials are provided in this review. CML, chronic myeloid leukemia; CTL, cytotoxic T lymphocytes; DCR, disease control rate; DLI, donor lymphocyte infusion; EPN, ependymoma; IV, intravenously; ICANS, immune effector cell-associated neurotoxicity syndrome; mbIL-15, membrane-bound IL-15; MDS, myelodysplastic syndrome; MB, medulloblastoma; NHL, non-Hodgkin lymphoma; ORR, objective response rate; PB-NK, peripheral blood natural killer; PFS, progression-free survival; PR, partial response; SC, subcutaneously; SAE, severe adverse event; TTP, time to progression.

exhibited *in vivo* antitumor efficacy equivalent to that of CAR-T cells but with fewer adverse effects.

To advance "off-the-shelf" allogeneic cell therapy products, iPSCs have been engineered to express additional NK cell activation factors alongside CARs. For example, ongoing clinical trials are evaluating quadruple-engineered NK cells derived from iPSCs. These engineered cells feature a CAR targeting BCMA, a CD38 knockout, a membrane-bound IL-15 receptor, and a high-affinity, uncleavable CD16 signaling complex in patients with r/r MM.⁶³ In the 64th American Society of Hematology Annual Meeting, the interim clinical data have shown the safety and tolerability and initial antitumor activity.⁶⁴ Another approach combines CAR-NK therapy with other antitumor agents. FT536, a CAR-MICA/B NK cell product, has been assessed in preclinical studies for its potential in combination with radiotherapy and antibody treatments as a pan-cancer targeting strategy.⁶⁵ These studies suggest the feasibility of developing "universal" CAR-NK cells that can be produced on a large scale, expanding treatment accessibility to a broader patient population.

LANDSCAPE OF CLINICAL NK CELL-BASED THERAPY

Clinical studies of NK-based cellular therapies were initiated approximately two decades ago. Compared with T cell therapy, NK cell therapy often requires higher doses and multiple infusions^{24,66–69} and is often combined with supportive cytokines to enhance NK cell potency. For instance, the GIMEMA phase 1 trial demonstrated that administering higher NK cell doses led to increased NK cell activity and more significant modulation of the host immune response in patients with Ph+ ALL.¹⁷ Additionally, in some studies, continuous *in vivo* administration of IL-2/IL-15 was employed to sustain NK cell potency following NK cell infusion.^{70–72} Among completed clinical studies involving NK cells, a substantial proportion focused on treating hematological tumors or using NK cell therapy as a consolidation treatment post stem cell transplantation to reduce relapse.²⁸ For solid tumors, NK cells were commonly co-applied with other antitumor agents such as radiochemotherapy,⁷³ locoregional chemotherapy drug infusion³⁶ or monoclonal antibodies (mAbs).^{34,74} While early clinical studies primarily utilized unmodified NK cells, there is a growing number of ongoing trials using CAR-NK cell therapies (Table 3).

Hematological malignancies

NK-based therapies have been rigorously evaluated clinically in hematological cancers, including lymphoma,⁶⁹ ALL,¹⁷ MM,⁷⁵ and myeloid malignancies.⁷⁶ CD19, expressed in the B-cell lineage, has been a primary target for cellular therapies, particularly CAR-T cells.⁷⁷ Additionally, allogeneic CD19 CAR-NK cells, known as NKX019, have demonstrated good initial safety and tolerability in patients with B-cell non-Hodgkin lymphoma, achieving a 70% complete response rate at day 28 following infusion at highest dose levels.⁷⁸ Notably, CD19-targeting CAR-NK cells derived from CB have shown impressive efficacy in patients with B-cell malignancies.¹² Incorporating IL-15 into the CAR design has enabled these CAR-NK cells to expand and maintain *in vivo* for at least 12 months.¹² Clinical trials

targeting CD70 on B cell-originated tumors are currently ongoing (NCT05092451), demonstrating the expanding scope of NK-based therapies as an alternative strategy to treat B cell malignancies.

Notably, the advancement of CAR-NK therapy holds significant importance for patients diagnosed with AML. Patients with AML, especially those experiencing disease after hematopoietic stem cell transplantation (HSCT), usually have limited treatment options other than salvage chemotherapies which offer little potential for a cure. It has been found that AML cells can be recognized by NK cell-activating receptors (NKARs), rendering them susceptible to NK cell-mediated cytotoxicity.⁵⁸ Moreover, due to the aggressive and immunosuppressive nature of AML, CAR-T cell therapy has been suffering from manufacturing failure and poor product quality,^{79,80} making NK cells a feasible alternative approach of AML-targeting cellular therapy. Recent studies of NK cell therapy in AML patients have shown promising results of safety and short-term remission. Compared with conventional consolidation therapy, NK cell products have demonstrated advanced therapeutic efficacy in patients with minimal residual disease (MRD)+ AML after several cycles of intensive chemotherapy, leading to improved overall survival (OS) without adverse events.⁸¹ Particularly, CD33 is expressed on a wide spectrum of AML blasts,⁸² which has been utilized as a CAR-NK cell target. Preliminary data from a trial (NCT05008575) indicate that 60% (6 out of 10) of r/r AML patients achieved MRD-negative complete remission (CR) at the day 28 assessment. Additionally, donor-derived NK cell therapy has shown promise in extending the non-relapse survival of AML patients following haploidentical HSCT.²⁷ In a phase I/II clinical trial, patients who received NK cell infusion after HSCT had a 2-year relapse rate of 4%, compared with a 38% relapse rate in those without NK cell infusion.²⁸ This study also revealed a dose-dependent increase in NK cells in recipient blood, exhibiting a mature, proliferating, and highly cytotoxic phenotype.²⁸ NK cells from different sources, therefore, hold great potential in treating hematological malignancies especially AMLs.

Solid tumors

Unmodified NK cells have been clinically evaluated for treating solid tumors, including non-small cell lung cancer (NSCLC),³¹ brain malignancies,³⁴ gastric or colorectal cancer,³⁷ and pancreatic cancer.³⁸ Confronted with the challenges posed by solid tumors, researchers have explored various combination therapy strategies to advance treatment outcomes. Given their inherent ability to mediate ADCC, NK cells are frequently combined with therapeutic mAbs. In a phase I trial involving patients with HER2-positive malignancies, the combinational treatment of autologous NK cell infusion with trastuzumab was well tolerated, and exhibited initial indications of tumor-killing efficacy.³⁴ Another trial utilized allogeneic NK cells in combination with the programmed cell death (PD)-1-blocking antibody pembrolizumab to treat advanced NSCLC and reported that patients receiving this combination therapy experienced longer median OS (15.5 months) compared with those treated with pembrolizumab alone (13.3 months).⁷⁴ Moreover, patients who received multiple doses of NK cell infusion had even longer OS (18.5 months) than

Table 3. Ongoing CAR-NK cell clinical trials

Disease	Country	Agent	NK cell source	NCT number
Hematological malignancies				
B-Cell malignancies	United States	CNTY-101 (CD19 CAR NK)	iPSC	NCT05336409
B-Cell lymphoma/AML/MDS	United States	CD70 CAR NK (IL15-transduced)	CB	NCT05092451
B-Cell malignancies	United States	NKX019(CD19 CAR NK)	allogeneic	NCT05020678
B-Cell NHL	China	CD19 CAR NK	CB	NCT05472558
B-Cell NHL	China	CD19 CAR NK	haploidentical	NCT04887012
B-Cell NHL	China	CD19/CD70 CAR NK	CB	NCT05667155
B-Cell malignancies	China	CD19 CAR NK	allogeneic	NCT05739227
B-Cell malignancies	China	CD19 CAR NK	allogeneic	NCT05645601
B-Cell malignancies	China	CD19 CAR NK	NA	NCT05410041
B-cell Malignancies	China	CD19 CAR NK	CB	NCT04796675
B-Cell malignancies	China	universal CD19 UCAR NK after HSCT	NA	NCT05654038
B-Cell malignancies	China	universal CD19 CAR NK	NA	NCT04796688
B-Cell lymphoma	China	QN-019a (CD19 CAR NK) + rituximab	iPSC	NCT05379647
AML/MDS	United States	NKX101 (NKG2D CAR NK)	allogeneic	NCT04623944
AML	China	CD123 CAR NK	allogeneic	NCT05574608
AML	China	CD33/CLL1 CAR NK	NA	NCT05215015
AML	China	CD33 CAR NK	NA	NCT05008575
AML	China	NKG2D CAR NK	NA	NCT05734898
MM	United States	FT576 (CAR-BCMA + Engineered hnCD16 Fc Receptor + IL15-RF + CD38 Knock-out) +Daratumumab	iPSC	NCT05182073
MM	China	BCMA CAR NK	allogeneic	NCT05652530
MM	China	BCMA CAR NK	NA	NCT05008536
Solid tumor				
Recurrent/metastatic gastric or head and neck cancer	United States	irradiated PD-L1 CAR NK Cells +N-803 + pembrolizumab	NA	NCT04847466
Refractory metastatic colorectal cancer	China	NKG2D CAR NK	NA	NCT05213195
Ovarian cancer	China	NKG2D CAR NK	NA	NCT05776355
Stage IV ovarian cancer, testis cancer, refractory endometrial cancer recurrent	China	CLDN6 CAR NK	autologous	NCT05410717
Extensive stage small cell lung cancer	China	DLL3 CAR NK	NA	NCT05507593
Solid tumors ¹	China	NKG2D CAR NK	NK92	NCT05528341
Solid tumors ²	China	Anti-5T4 CAR NK	NA	NCT05194709

CLDN6, claudin 6; Anti-5T4, oncofetal trophoblast glycoprotein (5T4) conjugated antibody; N-803, IL-15 agonist.

¹There is no specific tumor type in the website of clinicaltrials.gov; one of the inclusion criteria is that histopathology confirmed the diagnosis of malignant tumors (including breast cancer, lung cancer, gastric cancer, ovarian cancer, cervical cancer, renal carcinoma, malignant melanoma, osteosarcoma, and lymphoma).

²There is no specific tumor type in the website of clinicaltrials.gov, and one of the inclusion criteria is patients with advanced malignant solid tumors, histologically or cytologically confirmed.

those who received a single dose (13.5 months),⁷⁴ thereby suggesting that combination therapy with multiple NK cell infusions could improve survival in patients with advanced NSCLC. More recently, CAR-engineered NK cells offer significant advantages for clinical applications against solid tumors. For instance, the NKG2D CAR is commonly used as a target for CAR-NK cell therapy in ovarian cancer (NCT05776355) and refractory metastatic colorectal cancer (NCT05213195). Additionally, a clinical trial involving CAR-NK cells targeting claudin 6 for advanced solid cancers is also ongoing (NCT05410717). More ongoing clinical trials involving CAR-NK therapy against solid tumors are summarized in Table 3.

Toxicity

The adoptive transfer of NK cells has been found to be generally well tolerated and safe in most clinical trials, with minimal toxicity observed. Patients who receive NK cell infusions commonly experience little to no CRS, neurotoxicity, or hemophagocytic syndrome.⁸³ The most common adverse events reported are transient hematological toxicity (including neutropenia, lymphopenia, thrombocytopenia, and anemia) and infections, which are often consistent with the effects of lymphodepletion chemotherapy and the underlying disease. Most of these side effects can be effectively managed with standard supportive treatments, such as blood transfusions and anti-infection therapy. In a clinical trial involving genetic-engineering NK cells, 3 of 11 patients experienced grade 3 adverse events after anti-CD19 CAR-NK infusion (except from hematological events), one hypertension, one electrolyte abnormality and one viral infection, respectively, no grade 4 adverse events.¹² Severe GvHD is also rarely observed following NK cell infusions, largely due to the HLA-unrestricted manner of NK-mediated killing.⁸⁴ Interestingly, NK cells derived from HLA-mismatched donors may exert a more robust graft-versus-tumor effect due to NK-cell alloreactivity.⁸⁵ However, a trial involving the adoptive transfer of donor-derived IL-15/4-1BBL-activated NK cells (aNK-DLI) following HSCT reported a high incidence of acute GvHD, which could be attributed to the enhanced allogeneic T cell responses against the infused NK cells.⁸⁶ Therefore, gaining a better understanding of the interactions between the infused NK cells and the host immune system is crucial for predicting and addressing potential adverse effects.

Lessons from clinical practice

Both unmodified and CAR-engineered NK cell therapies have been extensively evaluated in clinical studies. While some clinical responses have been observed, these studies have revealed that unmodified NK cells exhibit limited antitumor efficacy, primarily due to their relatively short *in vivo* lifespan (typically 2–3 weeks post-transfer),^{68,87,88} which has illustrated the need to further potentiate NK cell function. Genetic engineering approaches, including the engineering of CARs, the incorporation of other NK cell activation factors, and the combination with antibody or chemotherapy, have emerged as promising strategies to enhance the antitumor potency of NK cells (Figure 2). These ongoing clinical trials are expanding the scope of NK cell therapy to a wide range of hematological malignancies and solid tumors.

POTENTIATING NK CELLS FOR CANCER THERAPY

Promoting the persistence by cytokine supports

Cytokines, particularly IL-2 and IL-15, play a crucial role in boosting the proliferation, activation, and longevity of NK cells.⁷⁰ In clinical trials, cytokines have been administered following NK cell infusion to support their function. Moreover, advancements in gene editing technology have enabled the engineering of NK cells with cytokine/cytokine receptor fusion proteins and the capacity for autocrine IL-2/IL-15 production, thereby delivering continuous cytokine signals to NK cells.

IL-2, one of the earliest-defined immune stimulatory cytokines, has been used in combination with NK cells in early clinical trials spanning over 20 years.⁶⁶ However, IL-2R α , the α -chain of the IL-2 receptor, is also expressed on regulatory T cells (Treg cells) and vascular endothelium.⁸⁹ Consequently, high-dose IL-2 administration can activate Treg cells, impairing NK cell antitumor function and may lead to severe complications such as diffuse capillary leak and pulmonary edema.⁹⁰ To address these challenges, researchers have developed an "IL-2 superkine" through a natural conformational switch, increasing its binding affinity for IL-2R β without the functional requirement for IL-2R α (CD25) expression, leading to superior expansion of conventional T cells and fewer Treg cells.⁹¹ Additionally, a fusion protein named OMCP-mutIL-2, comprising a cowpox virus-encoded NKG2D binding protein and a modified version of IL-2, has been designed to selectively activate the IL-2 signal in NKG2D-bearing cells like NK cells while sparing IL-2R α -bearing cells like Tregs.⁹² In preclinical studies, OMCP-mutIL-2 not only demonstrated effective tumor control in murine models of several malignancies, but also exhibited a favorable safety profile, avoiding the adverse effects and vascular complications associated with the original, unmodified IL-2.⁹²

IL-15 is another extensively studied cytokine that shares signaling β - and γ -subunits with the IL-2 receptor.⁹³ In an initial human trial involving recombinant human IL-15 (rhIL-15), the co-administration of rhIL-15 induced robust proliferation of adoptively transferred NK cells in patients with AML.⁹⁴ Besides direct injection of IL-15, there are alternative methods to activate IL-15 signaling in NK cells. In early studies, it was observed that human NK cells transduced with membrane-bound IL-15 exhibited enhanced persistence compared with soluble IL-15.⁹⁵ Other approaches involve using IL-15 agonists to activate endogenous NK cells. For example, ALT-803 (or N-803) is a fusion protein that links a superagonist mutant IL-15 with a dimeric IL-15R α -IgG1-Fc, which activated NK and T cells in syngeneic murine models.⁹⁶ In a multicenter clinical trial of ALT-803, it augmented NK cell activation without increasing Tregs and demonstrated a well tolerated safety profile in patients with hematological malignancies.⁹⁷ Furthermore, scientists have engineered CAR-NK cells to continuously express IL-15 *in vivo* through gene editing strategies. By incorporating IL-15 into the CAR design, the infused CAR-NK cells exhibited heightened antitumor activity and persisted *in vivo* for at least 12 months.¹²

The cytokine-inducible SH2-containing protein (CIS), encoded by the *CISH* gene, belongs to the suppressors of the cytokine signaling protein family and plays a crucial role in negatively regulating IL-15 in NK cells.⁹⁸ Zhu et al. utilized an iPSC-NK platform to eliminate *CISH* in NK cells, resulting in enhanced expansion and cytotoxicity against various tumor cell lines, alongside decreased cytokine production.⁹⁷ Mechanistically, iPSC-NK cells lacking *CISH* (*CISH*^{-/-}) exhibited improved metabolic performance, characterized by increased glycolytic potential and peak mitochondrial respiration, mediated through the mammalian target of rapamycin signaling pathway.⁹⁹ Another study employed CRISPR-Cas9 to delete *CISH* in CB-derived CAR-NK cells engineered to secrete IL-15, leading to superior metabolic fitness and tumor lytic potency.¹⁰⁰ In an assessment using a lymphoma murine model, this combined strategy enhanced NK-cell anti-tumor efficacy without any measurable side effects.¹⁰⁰

Certain cytokine conditions have been identified to induce memory-like properties in NK cells, which could enhance their persistence. In 2012, Fehniger et al. discovered that human NK cells, when preactivated with a short-term combination of cytokines (including IL-12, IL-15, and IL-18), displayed increased IFN- γ production after resting for weeks to months and then being restimulated with these cytokines or K562 leukemia tumor cells.¹⁰¹ These cytokine-preactivated NK cells, referred to as memory-like NK cells (MLNK), were subsequently evaluated in a first-in-human clinical trial, demonstrating efficacy and safety with four CR in five of nine evaluable AML patients.¹⁰² Another clinical trial (NCT03068819) assessed MLNK therapy in patients with post-HSCT relapsed AML, resulting in CR in four out of eight pediatric AML patients, with one patient maintaining a durable remission for 2 years.²⁵ Building on these findings, MLNK engineered to express an anti-CD19 CAR (CAR-ML NK) was shown to effectively control lymphoma burden in xenograft murine models.¹⁰³ Future clinical investigations of CAR-ML NK cells will be important to validate whether their memory-like properties can enhance therapeutic efficacy in patients.

Enhancing ADCC function

ADCC plays a pivotal role in the clinical application of therapeutic antibodies as a fundamental antitumor mechanism. CD16 directs NK cells to produce cytokines for target eradication upon recognition of antibody-coated cells. However, it is not consistently expressed on NK cells, and its downregulation can limit ADCC. A reduction in the surface density of CD16 on NK cells has been observed in cancer patients,¹⁰⁴ and research has shown that the metalloproteinase ADAM17 can shed CD16 from the surface of NK cells, reducing CD16 surface expression after activation.¹⁰⁵ This finding is supported by case reports, such as that of a patient with rare ADAM17 deficiency who did not show downregulated CD16A expression on the surface of NK cells during ADCC.¹⁰⁶ Consistent with these observations, ADAM17 inhibitors have demonstrated the ability to enhance NK cell proliferation and improve ADCC function against breast cancer cells *in vitro*.¹⁰⁷ Similarly, another study indicated that MEDI3622, an anti-ADAM17 mAb, could prevent CD16 shedding and enhance IFN γ production in the presence of antibody-bound tumor cells.¹⁰⁸

These findings highlight a promising approach to prevent CD16 shedding by targeting ADAM17, thereby enhancing the function of therapeutic antibodies, which could potentially be extended to NK cell engineering.

The inhibition of CD16 shedding on NK cells can also be achieved by modifying its cleavage domain⁵¹. Kaufman et al. developed a high-affinity uncleavable variant of CD16 (hnCD16) through a point mutation and introduced it into NK92 and iPSC-derived primary NK cells¹⁰⁹ and demonstrated iPSC-derived NK cells with hnCD16 (hnCD16-iNK) were highly resistant to CD16 cleavage, enabling them to exhibit potent ADCC function against multiple tumors.⁵⁵ Furthermore, when compared with unmodified NK cells, combining hnCD16-iNK cells with mAbs resulted in prolonged survival in ovarian cancer and lymphoma xenograft models.⁵⁵

Trafficking to tumor beds

The ability of NK cells to infiltrate tumors is an important requirement for their antitumor functions, particularly for solid tumors, and the deficiency in immune cell ability for such infiltrating has been associated with metastatic melanoma.¹¹⁰ It is well established that chemokine receptors are expressed on various immune cells, enabling their migration and adhesion in response to specific chemotactic cytokines.¹¹¹ Previous studies have shown that CAR T cells equipped with modified chemokine receptors have enhanced trafficking capabilities and improved efficacy against solid tumors, including hepatocellular carcinoma (HCC),¹¹² melanoma,¹¹³ and NSCLC.¹¹⁴ Over the past decade, researchers have developed various engineering techniques to enhance NK cell trafficking for improved antitumor responses.

Current strategies aimed at enhancing NK cell infiltration are primarily focused on two key aspects: engineering NK cells to overexpress chemokine receptors or elevating intratumoral chemokine levels. Related research has demonstrated that robust expression of chemokine receptors on NK cells can increase the migration of these cells to tumor sites, thereby enhancing the clinical efficacy in cancer patients.¹¹⁵ For instance, the CXCL12/CXCR4 axis has been demonstrated to play a crucial role in immune cell homing and tumor-related metastasis.¹¹⁶ Other studies have shown that engineering CAR NK cells to overexpress CXCR4 improves tumor regression compared with regular CAR-NK cells as it enhances NK cell trafficking to glioblastoma tumor sites in murine models.¹¹⁷ Furthermore, CXCR4 gene overexpression enhances NK cell homing to the bone marrow, and by introducing the human CXCR4 gene into huCAR19 NK cells, researchers have been able to create transgenically enhanced CAR NK cells, known as TRACKs.¹¹⁸ When interacting with bone marrow stromal cells, TRACKs exhibit superior migration capacity compared with conventional CAR NK cells while maintaining their cytolytic activity against target tumors.¹¹⁸ Additionally, human NK cells transduced to overexpress CXCR2 have demonstrated enhanced migration ability in renal cell carcinoma, particularly along chemokine gradients produced by tumors, enabling effective tumor eradication.¹¹⁹

Another strategy to enhance NK cell trafficking involves increasing intratumoral chemokine levels. Analysis of data from The Cancer Genome Atlas databases revealed that melanoma patients with high CCL5 expression levels had significantly improved prognoses.¹²⁰ Mgrditchian et al.¹²⁰ demonstrated that targeting autophagy genes such as BECN1 and ATG5, or pharmacologically inhibiting autophagy with chloroquine, increased the expression of CCL5 in melanoma cells, making the tumor cells more susceptible to NK cell infiltration. Additionally, CXCL9 and CXCL10 are ligands for CXCR3 on activated NK cells, and their migration activity is associated with the expression of these ligands.¹²¹ Early studies reported that NK cells migrated more toward melanoma tumors transfected with CXCL10 than CXCL10-negative tumors in xenograft models.¹²² Therefore, increasing the expression of CXCL9 and CXCL10 within tumor sites could be a strategy to enhance chemokine-dependent recruitment of NK cells. Interestingly, researchers found that regional delivery of IFN- γ stimulated the production of CXCL10 by tumor cells, resulting in increased NK cell infiltration.¹²³ These studies collectively suggest that engineering NK cells to overexpress chemokine receptors or boosting intratumoral chemokine levels are both viable approaches to improve therapeutic efficacy by enhancing NK cell trafficking, especially against solid tumors.

Overcoming the immunosuppressive TME

Dysfunction of NK cells in the TME represents a significant barrier to their effective antitumor immunity.¹²⁴ The TME contains soluble immunosuppressants such as transforming growth factor (TGF)- β , adenosine, prostaglandin E2, and indoleamine 2,3-dioxygenase, which can impair the cytotoxicity of NK cells and are primarily secreted by myeloid-derived suppressor cells (MDSCs), Treg, tumor-associated macrophages, as well as tumor cells.²² Additionally, the metabolic reprogramming of tumors can increase the levels of immunosuppressive metabolites, including lactic acid and reactive oxygen species, which in turn impair the normal metabolism and activity of NK cells.¹²⁵ Thus, overcoming these immunosuppressive effects is therefore essential to maintain NK cell function *in vivo*.

Treg cells have been shown to impede the protective immunosurveillance of neoplasms and suppress effective antitumor immunity, including the activity of effector cells like NK cells.¹²⁶ An early clinical trial demonstrated the benefits of host Treg cell depletion before haploidentical NK cell transfer.¹²⁷ In patients who received the IL-2-diphtheria fusion protein along with host Treg cell depletion, the donor NK cell expansion rate was higher compared with the control group (27% vs. 10%), which led to improvements in CR rates at day 28 (53% vs. 21%) and disease-free survival (DFS) at 6 months (33% vs. 5%).¹²⁷ Furthermore, Treg cells can be recruited by other immunosuppressive cells. In this regard, Pan et al.¹²⁸ discovered that the expression of the immune stimulatory receptor CD40 on MDSCs could induce and recruit Treg cells via CD40-CD40L interaction. Therefore, blocking the CD40-CD40L interaction between Treg cells and MDSCs may offer a novel approach to enhancing responses to immunotherapy. Additionally, MDSCs represent a heterogeneous group of immature myeloid cells that suppress antitumor immune re-

sponses.¹²⁹ Several studies have shown that targeting MDSCs can improve the antitumor effects of other immunotherapies, which can be achieved by blocking their recruitment to the TME, inducing their differentiation or inhibiting their immunosuppressive functions.¹³⁰

The TME also reprograms the metabolic profile of infiltrating NK cells to compromise their effector functions.¹³¹ For example, the expression of glutamine-dependent cMyc plays a crucial role in NK cell metabolism and function. Loftus et al.¹³² discovered that the absence of glutamine resulted in the loss of cMyc protein, leading to impaired NK cell growth and effector function, and also revealed that cMyc protein levels are regulated through glycogen synthase kinase 3 (GSK3)-mediated degradation. Interestingly, other researchers observed increased GSK3 expression in NK cells from AML patients and that inhibiting GSK3 genetically or pharmacologically promoted NK cell maturation and enhanced their antitumor activity.^{133,134} These findings suggest that stabilizing cMyc expression in NK cells can improve antitumor responses. Furthermore, glucose metabolism plays a critical role. Ashkar et al.¹³⁵ compared the function of PB NK cells with those isolated from the ascites fluid of ovarian cancer patients and discovered that oxidative stress associated with lipid peroxidation suppresses glucose metabolism, leading to the dysfunction of human NK cells in the TME.¹³⁵ Moreover, they demonstrated that Nrf2 activation could restore NK cell metabolism and antitumor activity through the antioxidant pathway, presenting an attractive approach to bolster the resilience of NK cells in challenging environments.¹³⁵ Similarly, in the hypoxic microenvironment created by solid tumors, immunosuppressive adenosine, produced by CD73 from precursor ATP, can inhibit the antitumor function of NK cells, and blocking CD73 effectively reprogrammed NK cells by alleviating adenosine immunometabolism suppression.¹³⁶

TGF- β is a primary immunosuppressive cytokine in the TME.¹³⁷ Studies have demonstrated that TGF- β could reduce the expression of activation receptors on NK cells and decrease their cytotoxicity against glioblastoma cells by inhibiting perforin secretion.¹³⁸ Targeting the TGF- β pathway can be achieved by engineered novel receptors as well as modification of downstream signal. Yvon et al.¹³⁸ introduced a dominant-negative TGF- β receptor II (DNRII) into CB-derived NK cells using retrovirus transduction, resulting in the sustained expression of NKG2D/DNMA1 and improved killing activity against glioblastoma cells in the presence of TGF- β . Another group of researchers utilized electroporation to modify NK-92 cells with the same DNRII, and their preclinical study demonstrated that the introduction of TGF- β -insensitive NK-92 cells through adoptive transfer into mouse models with lung cancer suppressed tumor proliferation and lung metastasis led to increased survival rates.¹³⁹ One major downstream target of TGF- β is Smad3, which negatively influences the differentiation and tumor-suppressive activities of NK cells through the Smad3-E4BP4 axis.¹⁴⁰ The development of a Smad3-silenced NK-92 cell line has been shown to enhance cytokine production capacity and cytotoxicity, even in a TGF β 1-rich context.¹⁴¹ Consequently, the NK-92-S3KD cell line effectively inhibited cancer

progression in both human hepatoma and melanoma xenograft murine models.¹⁴¹ These results highlight the potential of engineering NK cells to block the TGF- β signaling pathway as an effective therapeutic strategy.

Switch receptors have proven to be powerful tools for converting suppressive signals into stimulatory ones, thereby enhancing effector functions in the immunosuppressive TME and overcoming tumor immune evasion.¹⁴² For instance, pancreatic tumors produce inhibitory cytokines like IL-4 to facilitate immune evasion. In response, researchers developed an inverted cytokine receptor by fusing the IL-4R exodomain with the IL-7R endodomain (4/7 ICR).¹⁴² When transgenically expressed in CAR-T cells, this 4/7 ICR molecule protected immune cells from the immunosuppressive effects of IL-4, promoting T cell proliferation within the pancreatic cancer TME.¹⁴² In NK engineering, switch receptors usually contain extracellular domains of immunosuppressive receptors such as TGF- β and the intracellular domains of NKARs like NKG2D. Wang et al.¹⁴³ genetically modified NK-92 cells with a TGF- β RII-NKG2D switch receptor, demonstrating its ability to transform the immunosuppressive signaling of TGF- β into activating signaling via NKG2D, thereby enhancing NK cell IFN- γ production and cytotoxicity. In an HCC tumor model, the transfer of these modified NK-92 cells significantly inhibited tumor growth and prolonged survival.¹⁴³ Similarly, switch receptors have been employed to counteract the negative signal of PD-1. Lu et al.¹⁴⁴ designed a chimeric PD-1-NKG2D-41BB receptor capable of converting the suppressive PD-1 signal into an activating one. Moreover, when NK92 cells were transduced with this novel CAR, they exhibited significantly enhanced antitumor activity against human lung cancer in both *in vitro* and *in vivo* settings. Collectively, these findings indicate the promising immunotherapeutic potential of switch receptors, although further validation through clinical studies is necessary.

Blocking immune checkpoints

The activation and dysfunction of NK cells are stringently regulated by a network of activatory and inhibitory receptors.¹⁴⁵ Under normal physiological conditions, NK cells express MHC-I-specific inhibitors such as KIRs and NKG2A to prevent the killing of healthy autologous cells. However, some tumor cells may upregulate the ligands for these inhibitory receptors as a strategy to evade recognition by NK cells. Additionally, PD-1, CTLA-4, TIGIT, and LAG3 are prominent inhibitory receptors that suppress the function of both T and NK cells.¹⁴⁶ Numerous strategies have been developed and tested in clinical trials to target these immune checkpoint molecules with the goal of enhancing the effectiveness of NK cell-based therapy. A gene profiling analysis encompassing approximately 10,000 tumor samples revealed that the most common mechanism by which tumors develop resistance to NK cell immune surveillance is the upregulation of HLA-E.¹⁴⁷ HLA-E can suppress NK cell function by binding with CD94/NKG2A.¹⁴⁸ Kamiya et al.¹⁴⁷ generated NKG2A^{null} NK by retroviral transduction of NKG2A protein expression blockers into human PB-NK cells and demonstrated

that these NKG2A^{null} NK were significantly more effective against tumors expressing HLA-E. Targeting this immune checkpoint in cancer, a humanized NKG2A-blocking mAb (monalizumab) has shown efficacy by enhancing NK cell and CD8⁺ T cell tumor immunity in both mice and humans.¹⁴⁹ Similarly, anti-KIR antibodies have been developed to disrupt tolerogenic interactions and have been shown to augment the spontaneous cytotoxicity of NK cells. In a KIR2DL3 transgenic mouse model, KIR-targeting antibodies enhanced the anti-lymphoma function of NK cells when used as monotherapy or in combination with anti-CD20 antibodies.¹⁵⁰ Lirilumab is an example of a mAb targeting KIR. In a phase II study, dual immune checkpoint inhibition involving anti-PD-1 (nivolumab) and anti-KIR (lirilumab) was well tolerated and achieved a 43% response rate among 28 patients with squamous cell carcinoma of the head and neck.¹⁵¹ Furthermore, this study observed favorable DFS and excellent 2-year OS.

Immune checkpoint inhibitors (ICIs) targeting the PD-1/PD ligand 1 (PD-L1) axis have become a hallmark in cancer treatment.¹⁵² Previous studies showed that, in addition to T cells, NK cells also express PD-1, and its expression is further elevated in tumor-infiltrating NK cells, leading to a functionally exhausted state characterized by reduced cytokine production, diminished proliferative responses, and impaired cytotoxicity.^{153,154} Hsu et al. reported that PD-1 serves as a critical checkpoint molecule that inhibits NK cell activation, and blocking PD-1 can induce an antitumor response mediated by NK cells.¹⁵⁵ Furthermore, these immune checkpoint molecules may mediate novel functions in NK cells. Dong et al.¹⁵⁶ introduced a novel perspective, demonstrating that anti-PD-L1 mAbs elicit antitumor mechanisms by activating PD-L1-positive NK cells through a p38 pathway, which operates independent of tumor PD-L1 expression, explaining why PD-L1-negative tumor cells can also exhibit unexpected clinical responses to anti-PD-L1 mAbs. The study revealed that increased PD-L1 expression on NK cells enhances their effector function and prevents cell exhaustion through AKT signaling.¹⁵⁶ Additionally, Zhang et al.¹⁵⁷ identified TIGIT as a checkpoint receptor on NK cells, and blocking it can prevent NK cell exhaustion in several tumor-bearing mouse models. In the same study, TIGIT blockade demonstrated enhanced therapeutic efficacy when combined with anti-PD-L1 mAbs in tumor rechallenge models, highlighting the potential of simultaneously targeting multiple immune checkpoints to potentiate NK cell function.

Significant success has been achieved using ICIs in the context of T cells, particularly in tumors with high immunogenicity.¹⁵⁸ While T cells have traditionally been viewed as the primary responders to these treatments, recent attention has turned to NK cells and how their function can be enhanced through ICI. The blockade of immune checkpoints specific to NK cells or shared between NK and T cells has been investigated in combination with NK cell therapy, and determining the optimal combination of ICI agents capable of synergistically activating NK cells could improve the future development of these strategies.

PROSPECTIVE COMBINATORIAL STRATEGIES

Therapeutic antibodies

Given the important role of NK cells in ADCC, the combination of adoptive NK cell transfer and antibody-mediated therapies represents a potent approach against various cancers, particularly those with consistent antigen expression. In addition to the ICIs discussed earlier, therapeutic mAbs that directly target tumor-associated antigens, as well as ADCs, have also demonstrated promising antitumor efficacy when used in conjunction with NK cell therapy (Figure 3A).¹⁵⁹

The synergistic cytotoxicity of NK cells, when combined with mAbs, has been demonstrated against lymphoma cell lines. Deng et al. showed that *ex vivo* expanded NK cells produced increased levels of IFN- γ and INF- α in the presence of rituximab, resulting in enhanced cytotoxicity against CD20⁺ B lymphoma.¹⁶⁰ In addition to that, anti-BCMA CAR-T¹⁶¹ and daratumumab¹⁶² (anti-CD38) have shown excellent remission rates in MM patients, although relapse may still occur. To address antigen escape and relapse, ongoing research efforts by both academic and biopharmaceutical institutions are focused on identifying new targets and improving the pharmacological properties of mAbs. G-protein-coupled receptor, class C, group 5, member D (GPC5D) has emerged as an alternative immunotherapeutic target antigen highly expressed on myeloma cells independently of BCMA.¹⁶³ In the 2022 American Society of Hematology Meeting, Fate Therapeutics Inc. reported that FT555, a CAR-iNK cell designed to target GPRC5D, demonstrated robust and sustained antigen-mediated tumor cytotoxicity *in vivo* when combined with daratumumab.¹⁶⁴ Therefore, combining NK cell therapy with these novel mAbs can enhance ADCC function, reduce the risk of tumor antigen escape, and mitigate relapse by targeting multiple antigens simultaneously.

Targeting tumor-specific antigens while simultaneously initiating NK cell activity with bispecific engagers is a promising approach. Previous research efforts have led to the development of bispecific antibodies, such as those targeting CD16 and the cancer marker epithelial cell adhesion molecule,¹⁶⁵ and a trispecific engager with a modified IL-15 crosslinker.¹⁶⁶ These innovations not only improve NK cell proliferation, activation, and survival, but also enhance ADCC against various human carcinoma cell lines, including those from the breast, colon, neck, and prostate, even at low effector-to-target ratios *in vitro*. AFM13 is a bispecific antibody designed to target both CD30 and CD16A, effectively engaging NK cells through CD16A as immune effector cells.¹⁶⁷ In a phase I study of AFM13 in patients with r/r Hodgkin lymphoma, 13 of 26 patients achieved stable disease, resulting in an overall disease control rate of 61.5%.¹⁶⁸ Another clinical trial evaluated the efficacy of AFM13 in combination with pembrolizumab, showing an impressive 83% overall response rate (ORR).¹⁶⁹ Furthermore, CB-derived NK cells complexed with AFM13 induced complete responses in CD30⁺ r/r lymphomas, with very good treatment tolerability, as indicated by preliminary results from a phase I/II trial.¹⁷⁰ Similarly, the bispecific antibody NKAB-HER2 was designed to redirect NKG2D-expressing effector cells to HER2-positive

tumor cells.¹⁷¹ It has also been demonstrated that NKAB-HER2 synergized with NKG2D-CAR NK cells *in vivo*, effectively suppressing the growth of HER2⁺ tumors, even in the absence of NKG2D ligand expression.

ADCs represent a unique class of therapeutic biological products that combine cytotoxic agents with antibody fragments. The FDA has approved a series of ADCs for the treatment of various solid and hematological malignancies.¹⁷² Due to their targeted delivery of chemotherapy within tumor tissues, scientists have made significant efforts to develop rational combinations involving ADCs.¹⁷³ For example, the crystallizable fragment (Fc) region of ADCs can interact with NK cells and mediate the effector function of ADCC,¹⁷⁴ and different subclasses of human IgG could determine different immune function of ADCs.¹⁷⁵ By using IgG1 as the predominant antibody backbone in ADCs, which has the highest Fc γ R-binding affinity, T-DM1¹⁷⁶ and T-DXd¹⁷⁷ have demonstrated to retain the ADCC function of unconjugated trastuzumab in preclinical models. In this regard, engineering the Fc region for improving binding affinity to effector cells might be a possible method to enhance the combined effect of ADCs and NK therapy. While there is limited literature on the use of ADCs in combination with NK cell therapy, researchers have explored the role of ADCs in combination with CAR-T therapy or as a preliminary treatment before CAR-T infusion.¹⁷⁸ Tomas et al.¹⁷⁹ reported that polatumumab vedotin (pola) may be an effective salvage treatment after CAR T cell therapy. In their study involving patients who received pola in their first therapy following CD19-CAR-T treatment, they observed promising ORR (48%) in 26 patients when pola was combined with bendamustine. Another study reported similar results, showing that a pola-containing regimen was successful in 7 of 12 patients who had previously experienced treatment failures with CAR-T therapy.¹⁷⁸ These findings suggest that pola could be a valuable salvage or bridging agent when combined with CAR-NK cells for patients with large B-cell lymphoma. Monotherapies have shown clinical benefits, while the development of ADC-based combinations faces challenges regarding pharmacological interactions and overlapping toxicities.¹⁷³

Small molecule drugs

Some specific agents can induce the expression of NKARs on targeted tumor cells or increase NKAR levels on NK cells, making cancer cells more susceptible to NK-mediated cytotoxicity (Figure 3B). NKG2D, an important activating receptor on NK cell surfaces, plays a crucial role in tumor immunosurveillance. Preclinical studies have shown that tumor cells can regulate the expression of NKG2D ligands (NKG2DLs) as they grow to evade NK cell recognition,¹⁸⁰ leading to the exploration of the pharmacological modulation of NKG2DL expression.¹⁸¹ Diermayr et al.¹⁸² demonstrated that pharmacologically upregulating NKG2DL expression on AML cells using valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, resulted in enhanced NK-mediated lysis of tumor cells. Another study reported similar effects with the narrow-spectrum HDAC inhibitor entinostat,¹⁸³ revealing a time-dependent increase in MIC expression in colon carcinoma or sarcoma cells after entinostat pretreatment.

Furthermore, entinostat can increase NKG2D expression in NK cells to improve cancer cell recognition. Mechanistically, both of these effects were associated with increased acetylated histone 3 binding to related promoters.¹⁸³ However, VPA can suppress the expression of NKG2D in NK cells. Ni et al. reported that STAT3 phosphorylation regulated NKG2D transcription and that VPA selectively inhibited the phosphorylation of STAT3 at tyrosine 705, whereas entinostat did not,¹⁸⁴ suggesting that STAT3 phosphorylation and histone acetylation can cooperatively regulate NKG2D expression in NK cells.¹⁸⁴ In contrast, small molecules such as galunisertib can restore NKAR expression in immunosuppressive TMEs. Galunisertib is an inhibitor of TGF- β R1, and a related preclinical study demonstrated that galunisertib suppressed SMAD activation in a human neuroblastoma NSG mouse model, which protected NK cells from the inhibitory effects of TGF- β .¹⁸⁵ In the same study, the researchers demonstrated that galunisertib increased NK cytotoxicity by enhancing the expression of NKG2D, Nkp30 and DNAM-1 receptors, as well as the release of perforin and granzyme A.¹⁸⁵

Oncolytic virus

The oncolytic herpes simplex virus (oHSV) has emerged as a novel therapeutic agent that has shown survival improvements compared with standard care for the treatment of solid tumors.¹⁸⁶ Recently, Japan approved G47 Δ , a third-generation oHSV type 1 that has been triple-mutated, as the first oHSV product. This approval was based on the remarkable efficacy observed in a phase 2 trial where intratumoral G47 Δ was administered to 19 adult patients with residual or recurrent glioblastoma.¹⁸⁷ The trial demonstrated a survival benefit and a favorable safety profile for oHSV in the treatment of solid cancers, highlighting the potential for combination therapy with immunotherapy (Figure 3C).

Preclinical investigations revealed that oHSVs exert their tumor-killing function through intratumoral replication and by inducing host antitumor immunity,¹⁸⁸ and it was demonstrated that EGFR-CAR-NK-92 cells, in combination with oHSV-1, exhibited superior cytotoxicity and IFN- γ secretion compared with either therapy alone *in vitro*.¹⁸⁹ Furthermore, in a murine model of breast cancer intracranial metastasis, the intratumoral administration of EGFR-CAR-NK-92 cells and oHSV-1 enhanced tumor cell killing and prolonged the survival of tumor-bearing mice.¹⁸⁹ To investigate the mechanism underlying the synergistic effects of oncolytic viruses and CAR-NK cells, Ma et al.¹⁹⁰ treated an immunocompetent model of glioblastoma using a combinational treatment and observed increased intracranial infiltration of NK and CD8⁺ T cells, as well as enhanced persistence of CAR-NK cells *in vivo* after treatment with OV-IL15C (an oncolytic virus expressing human IL15/IL15R α) in combination with EGFR-CAR NK cells, which significantly reduced tumor growth and improved survival. In another study, Ding et al. generated an NK cell-mediated oncolytic adenoviruses (Ad) delivery system (Ad@NK) by combining immunotherapy and virotherapy of cancer.¹⁹¹ In their system, NK cells play the role as bioreactors and shelters for Ads, and Ads upregulate type I IFN signaling to augment NK-cell antitumor immunity. Furthermore, they performed a series

of experiments and showed that Ad@NK treatment induced a higher anticancer activity than treatment with NK cells or Ads alone in *in vitro* and *in vivo* settings. This study showed the complementary interplay between NK cells and Ads in cancer therapy, which provides a clinical basis for promising applications in the future. Although clinical data supporting this combination strategy is limited, the association of CAR-engineered effector cells and oncolytic viruses holds promise and may provide new opportunities for heavily pretreated patients.

CONCLUSIONS

In clinical trials, the limitations of NK cell therapy have become apparent, and synthetic biology is now being used to enhance the potency of NK cells, going beyond the optimization of clinical regimens. Several targeted strategies have been developed and validated to improve the antitumor activity of NK cells, taking into account factors such as proliferation, persistence, trafficking, cytotoxicity and adverse TME. Besides the intrinsic ability of NK cells to target tumor cells without the constraints of MHC recognition, the typical modification involves the use of CARs to increase cytotoxicity and expand the range of cancer types that can be targeted. Cytokine-based interventions extend the longevity of NK cells in the body, while chemokines facilitate their migration to tumor sites, optimizing their localization and efficacy. To counteract the inhibitory effects of the adverse TME, immune checkpoint and inhibitory receptors on NK cells are either deleted or strategically modified to enhance their antitumor function. In addition, novel mAbs, such as ADCs and immune ICIs, as well as bispecific engagers and oncolytic viruses, hold promise as complementary tools to enhance NK cell-mediated tumor eradication.

Moreover, the limited treatment window for advanced cancers has driven the transformation of cellular therapy into an accessible "off-the-shelf" drug option for patients. Among the most promising sources, human stem cell-derived NK cells stand out due to their compatibility with multiplex gene engineering, the ability to generate homogeneous clones, and facilitate large-scale production.

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AUTHOR CONTRIBUTIONS

K.W. wrote the original manuscript and generated the tables and figures. L.W., Y.W., and J.W. conducted the literature review and wrote the original outline. K.W. and L.X. performed literature review and writing for manuscript revision. Y.H., D.W., and H.H. provided conceptual guides to the structure and content of the manuscript. K.W. and L.W. contributed equally to this work. All authors approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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