Review

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Review

Recent Progress in NKG2D CAR-NK Cells for Cancer Immunotherapy

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Abstract

Adoptive cellular therapy has emerged as one of the most pivotal modalities in cancer immunotherapy. Although chimeric antigen receptor (CAR)-T cell therapy has achieved remarkable clinical success in hematological malignancies, its broad application is hampered by severe immune-related toxicities, autologous manufacturing limitations, and suboptimal efficacy against most solid tumors, highlighting an urgent unmet clinical need for safer and more versatile cellular therapeutics. As a promising alternative, CAR-NK cell therapy exhibits inherent advantages in safety profile, off-the-shelf accessibility, and multimodal antitumor mechanisms. NKG2D, a pivotal activating receptor broadly expressed on NK cells, specifically recognizes stress-inducible ligands such as MICA/B and ULBP family molecules. These ligands are abundantly overexpressed in various hematological and solid malignancies yet rarely detected in normal tissues, thereby rendering NKG2D an ideal candidate for universal targeted immunotherapy. In this review, we systematically examine the structural design and iterative optimization of NKG2D CAR constructs and elucidate the inherent advantages of CAR-NK over conventional CAR-T cells. We also comprehensively review recent research advances in NKG2D CAR-NK across multiple malignancies, and critically analyze three pivotal translational bottlenecks, including tumor antigen escape, the immunosuppressive tumor microenvironment, and inadequate in vivo persistence. Moreover, we highlight current genetic engineering and combinatorial strategies to overcome these limitations, and outline future research directions focusing on the development of universal off-the-shelf products, multifunctional cell engineering, and rational combination regimens. By integrating mechanistic advances, preclinical findings and early clinical evidence, this review provides a systematic theoretical basis and translational guidance for the structural optimization, clinical translation, and widespread clinical adoption of NKG2D CAR-NK therapy, laying a solid foundation for its future development as a standardized universal anticancer cellular therapeutic.

Keywords: NKG2D; CAR-NK; NK cell therapy; cancers; immunotherapy

1. Introduction

Adoptive cellular therapy has catalyzed transformative advances in modern oncotherapy. As a prominent subset, CAR-NK cell therapy integrates the intrinsic broad-spectrum antitumor activity of natural killer (NK) cells with the precise targeting capability of CAR engineering technology, positioning itself as a focal point of research for next-generation immunotherapy. While several reviews have addressed the broader landscape of CAR-NK cell therapy, few have provided a systematic and up-to-date overview specifically focusing on NKG2D-based CAR-NK cells, with specific emphasis on integrating the latest clinical data and engineering advances [1–3]. NKG2D serves as a core activating receptor on NK cells, capable of specifically recognizing stress-related ligands upregulated on transformed and malignant cells [4,5]. Herein, we systematically elucidate the biological characteristics, structural engineering design, clinical applications, existing challenges, and future research directions of NKG2D CAR-NK cell therapy, aiming to provide a comprehensive

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reference framework for the rational development and clinical translation of this promising therapeutic modality.

2. Biological Basis and Engineering Design of NKG2D CAR-NK Cells

2.1. Characteristics of NKG2D and Its Ligands

NKG2D (Natural Killer Group 2 Member D, also designated CD314) is encoded by the *KLRK1* gene, which maps to human chromosome 12 and mouse chromosome 4. It is a member of the C-type lectin-like receptor superfamily and is classified as a highly conserved type II single-pass transmembrane glycoprotein [1–3]. As a key activating receptor expressed on NK cells and $CD8^+$ T cells, NKG2D forms stable homodimers via interchain disulfide bonds. Notably, its intracellular domain lacks independent signaling motifs. In humans, NKG2D assembles with four DAP10 adaptor proteins to form a functional hexameric NKG2D-DAP10 signaling complex for downstream signal transduction, whereas murine NKG2D can interact with either DAP10 or DAP12 to initiate signaling cascades [2,4]. The NKG2D hexameric complex binds to the α1 and α2 extracellular domains of NKG2D ligands (NKG2DLs) on target cells, thereby triggering downstream signaling that promotes effector cell survival and proliferation, as well as the secretion of perforin and granzymes to mediate tumor cell lysis **[3]**. Functionally, NKG2D acts as the master activation switch of NK cells. Signals transduced by the NKG2D-NKG2DL axis can override inhibitory signals from immune checkpoint receptors, enabling direct NK cell activation and target cell killing independent of antigen presentation and co-stimulation. In humans, cytokines including IL-2, IL-7, IL-12, and IL-15 can upregulate NKG2D expression, while TGF-β and IFN-β1 exert suppressive effects on its expression [2,4].

Human NKG2DLs are mainly divided into two subfamilies: MHC class I chain-related proteins A/B (MICA/B) and UL16-binding proteins (ULBP1–6) [4]. All eight NKG2DL subtypes contain conserved extracellular α1 and α2 domains responsible for NKG2D recognition and binding. As the first identified NKG2DL members, MICA/B possess an extracellular structure consisting of α1, α2 and α3 domains. The distal α1/α2 domains mediate NKG2D binding, while the proximal α3 domain contains cleavage sites for matrix metalloproteinases. NKG2DLs are expressed at negligible levels in normal tissues, but are robustly induced under cellular stress conditions such as DNA damage, cellular senescence, and oncogenic transformation [2,5]. Given their broad overexpression in both hematological malignancies and solid tumors, NKG2DLs are considered promising therapeutic targets [6–8]. Clinically, NKG2D CAR-NK treatment rarely induces severe adverse events such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), presenting a favorable safety profile [9–11]. Distinct from personalized CAR-T products that only serve limited patient populations, NKG2D CAR-NK holds great promise for the development of widely accessible off-the-shelf cellular therapeutics [12].

2.2. NKG2D CAR-NK Design

The conventional construction of NKG2D CAR-NK adopts established design frameworks from CAR-T cells, consisting of three core components: an extracellular antigen-recognition domain (NKG2D extracellular region), a transmembrane domain (e.g., the CD8α hinge and transmembrane segment), and an intracellular signaling domain (e.g., CD3ζ) [3]. The extracellular NKG2D domain recognizes tumor surface NKG2DLs and initiates intracellular signaling via the CD3ζ motif [13,14]. Nevertheless, CAR-NK cells equipped with only the CD3ζ domain exhibit limited in vivo persistence and suboptimal antitumor activity, necessitating further structural optimization. Stepwise screening of CAR components revealed that the combination of NKG2D with 2B4 signaling domains elicits stronger NK cell activation than the DNAM-1/2B4 combination. A customized CAR structure integrating DAP10, 2B4 and CD3ζ signaling modules with the CD8α hinge and the CD28 transmembrane regions exerted superior cytotoxicity both in vitro and in vivo, highlighting the necessity of tailored signaling domain assembly for NK cell engineering [15].

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Beyond conventional co-stimulation domain optimization, researchers have incorporated endogenous NK adaptor proteins such as DAP10 and DAP12 into CAR design. The newly constructed NKG2D/DAP10-12 fusion CAR co-expresses NKG2D and DAP10-DAP12 chimeric adaptor, which has achieved robust therapeutic efficacy in multiple tumor xenograft models, enabling tumor regression and durable disease control in NKG2DL-positive malignancies. This design enhances NKG2D-mediated signaling by supplementing exogenous DAP10 and optimizing the membrane localization of signaling complexes [16]. In addition, multiple innovative engineering strategies have been developed to broaden the functional potential of NKG2D CAR-NK, including dual-target design, chemokine receptor co-expression and cytokine arming. For instance, PD-L1/MICA/B dual-target CAR-NK92 cells exert synergistic antitumor effects via OR-logic gating, predominantly triggering GSDME-dependent pyroptosis and effector molecule release [17]. CAR-NK cells co-expressing CXCR1 and NKG2D exhibit enhanced tumor migration and infiltration capacity, while IL-21-armed NKG2D CAR-NK cells improve cell proliferation, cytotoxicity and IFN-γ secretion via the PI3K/AKT pathway with reduced apoptosis and cellular exhaustion [18,19]. Collectively, these iterative design optimizations have significantly improved the antitumor potency of NKG2D CAR-NK cells.

2.3. Advantages of CAR-NK over CAR-T

CAR-NK cell therapy possesses three predominant competitive advantages relative to CAR-T therapy (Table 1). First, CAR-NK exhibits a superior safety profile. CAR-NK cells secrete low levels of pro-inflammatory cytokines and produce negligible amounts of IL-6, the core driver of CRS. Furthermore, NK cells rarely cross the blood-brain barrier, resulting in a low risk of off-target neurological damage and a negligible incidence of fatal CRS or ICANS [20–23]. In contrast, once activated by tumor antigens, CAR-T cells undergo massive clonal expansion and release a cascade of pro-inflammatory cytokines including IFN-γ, TNF-α and GM-CSF, which further activate macrophages and monocytes to secrete abundant IL-6, triggering systemic inflammatory response, vascular injury, tissue edema and multi-organ inflammatory infiltration [24–27]. CAR-T-related neurotoxicity is mainly attributed to peripheral cytokine storm and on-target/off-tumor toxicity against normal neuronal cells expressing targeted antigens [28–31].

Second, CAR-NK presents excellent clinical accessibility and scalable manufacturing potential. Allogeneic NK cell infusion rarely induces graft-versus-host disease (GVHD) or immune rejection [23]. Multiple accessible NK cell sources are available, including peripheral blood, umbilical cord blood, NK-92 cell line and induced pluripotent stem cells (iPSCs), providing abundant seed cell resources. Mature in vitro expansion protocols support industrial large-scale production, and standardized cryopreservation and resuscitation procedures enable long-term storage in liquid nitrogen as ready-to-use off-the-shelf products [32,33]. Due to their logistical stability during transport, CAR-NK cells can be administered immediately after preparation, avoiding disease progression caused by prolonged waiting, which is particularly critical for patients with rapidly progressive advanced tumors [34,35]. In comparison, most clinically approved CAR-T products rely on autologous cell preparation, requiring personalized customization and long production cycles, which limits their application in rapidly progressing malignancies [36].

Third, CAR-NK exhibits multilayered antitumor mechanisms effective within the complex tumor microenvironment. In addition to CAR-mediated targeted killing, CAR-NK retains both the intrinsic broad-spectrum cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) of primary NK cells [37–40]. Native NK cells balance activating and inhibitory signals via surface receptors: activating receptors such as NKG2D, NKp46, NKp44, NKp30 and 2B4 recognize tumor stress ligands, while inhibitory killer immunoglobulin-like receptors (KIR) bind MHC-I molecules on normal cells to avoid autoreactivity [41]. Malignant cells often downregulate MHC-I expression to escape T cell surveillance, which conversely relieves KIR-mediated inhibition and activates NK cell cytotoxicity. In the context of tumor heterogeneity, CAR-NK can eliminate tumor subclones and cancer stem cells via inherent NK recognition pathways [42,43]. Moreover, CAR-NK can directly clear myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment and secrete IFN-γ to recruit and activate dendritic cells (DCs), further initiating endogenous antitumor T cell responses

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[44]. By contrast, CAR-T cells mainly rely on single-antigen targeted killing, which is prone to therapeutic failure and tumor recurrence in heterogeneous solid tumors [45].

Table 1. Advantages of CAR-NK over CAR-T.

Feature	CAR-NK	CAR-T
Safety	Absence of lethal CRS or ICANS; minimal risk of off-target toxicity	High risk of lethal GVHD, CRS and neurotoxicity; severe on-target/off-tumor damage
Accessibility	No apparent risk of GVHD; diverse allogeneic sources; scalable for off-the-shelf manufacturing	Primarily reliant on autologous preparation; complex allogeneic production workflow
Antitumor mechanism	CAR-specific targeting, intrinsic broad-spectrum cytotoxicity, and ADCC	Limited to CAR-mediated cytotoxicity

CRS: Cytokine Release Syndrome; ICANS: Immune effector Cell-Associated Neurotoxicity Syndrome; GVHD: Graft-Versus-Host Disease; CAR: Chimeric Antigen Receptor; ADCC: Antibody-Dependent Cellular Cytotoxicity.

3. Applications of NKG2D CAR-NK in Different Tumors

Advances in NKG2D CAR-NK cell therapy vary considerably based on the type of tumor being targeted. Hematological malignancies represent the most established area of research, with the longest history of clinical evaluation, followed by colorectal cancer. In contrast, studies focused on ovarian cancer, lung cancer, glioma, and pancreatic cancer are still largely limited to preclinical exploration and the clinical recruitment phase, as comprehensive clinical outcomes have not yet been fully reported.

3.1. Hematological Malignancies

Patients with relapsed/refractory (R/R) acute myeloid leukemia (AML) and high-risk myelodysplastic syndrome (MDS) generally face a poor clinical prognosis [46,47]. Conventional CAR-T therapy yields limited efficacy in these populations, largely due to the lack of specific tumor antigens and the suppressive tumor microenvironment [48]. Unmodified NK cells have demonstrated acceptable therapeutic effects and favorable safety profiles without fatal CRS or neurotoxicity in AML treatment [49,50]. To further enhance the antileukemic potency of NK cells, Nkarta lnc. developed NKX101, an allogeneic off-the-shelf NKG2D CAR-NK product. NKX101 is derived from peripheral blood NK cells of healthy donors, engineered to express a CAR comprising the NKG2D extracellular domain, the OX40 co-stimulatory domain, and the CD3ζ signaling domain, alongside membrane-bound IL-15 to sustain in vivo persistence. Preclinical data indicated that NKX101 exerted 4–8-fold stronger antitumor activity than unmodified NK cells in AML xenograft models with prolonged in vivo retention. Optimized cryopreservation protocols enable NKX101 to be manufactured as a standardized product for multicenter clinical trials (NCT04623944) [51].

At the 2023 ASH Annual Meeting, Nkarta lnc. released phase I clinical data of NKX101 for R/R AML treatment. Among six enrolled patients, four achieved complete remission (CR) or complete remission with incomplete hematologic recovery (CRi), including three cases of strict CR. Several patients achieved minimal residual disease (MRD)-negative deep remission after one treatment cycle, and no severe CRS or neurotoxicity was observed during the whole treatment course, confirming the favorable safety and efficacy profile of NKX101 [52]. Mechanistically, NKX101 exerts synergistic antileukemic effects combined with cytarabine (Ara-C) and partially overcomes AML drug resistance. Ara-C pretreatment can significantly upregulate NKG2DL expression on AML cell surfaces, thereby enhancing NKX101-mediated recognition and cytotoxicity by 53%–366%. Multiple

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preclinical models have verified the prominent combined efficacy of NKX101 plus Ara-C [53]. In addition, NKX101 displays broad-spectrum cytotoxicity against both leukemic stem cells and immature blast cells, providing a promising strategy to eradicate MRD and prevent AML relapse [54]. It can also be combined with cetuximab to exert ADCC-mediated killing against EGFR-positive solid tumors [55].

Given the intrinsic antiviral characteristics of NK cells, viral vector-mediated transfection often results in low CAR engineering efficiency, while large-scale viral production and quality control also bring high costs, limiting the widespread application of viral-based CAR-NK preparation [56–58]. Non-viral transposon systems such as PiggyBac (PB) serve as an effective alternative strategy. PB-engineered NKG2D CAR-NK cells with autonomous IL-15 secretion exhibited potent anti-AML activity in vitro and improved in vivo persistence and survival benefits in xenograft models [59,60].

NKG2D CAR-NK has also been explored for multiple myeloma (MM) treatment. Autologous NK cells isolated from MM patients were transduced with lentiviral vectors carrying the NKG2D extracellular domain, the 4-1BB co-stimulatory domain, and the CD3ζ signaling domain. The engineered CAR-NK cells exerted potent and specific cytotoxicity against multiple MM cell lines and primary patient-derived tumor cells with negligible damage to normal cells. In MM mouse models, approximately one-quarter of tumor-bearing mice achieved complete tumor regression in the absence of overt treatment-related adverse events [61].

3.2. Colorectal Cancer

Xiao et al. constructed NKG2D CAR-NK cells via mRNA electroporation by fusing the NKG2D extracellular domain with the DAP12 intracellular signaling region [14]. Compared with traditional CD3ζ-based CAR-NK cells, this design exhibited higher cytotoxicity against colorectal, ovarian, and pharyngeal cancer cell lines, and significantly prolonged survival in HCT116 colorectal cancer xenograft models. In a pilot clinical trial involving three chemotherapy-refractory metastatic colorectal cancer patients, intraperitoneal injection reduced tumor cell counts in ascites and controlled malignant effusion accumulation, while ultrasound-guided intratumoral injection achieved complete metabolic regression of liver metastases in one patient.

Li et al. treated nine heavily pretreated advanced metastatic colorectal cancer patients with intraperitoneal infusion of mbIL-15-expressing NKG2D CAR-NK cells. The regimen demonstrated a favorable safety profile without CRS or neurotoxicity, with a disease control rate of 33.3% and a median overall survival of 10.8 months. Local CAR-NK infusion not only directly eliminated tumor cells but also remodeled the immune microenvironment and activated endogenous $CD38^{+}HLA\text{-}DR^{+}CD8^{+}$ T cells with enhanced proliferative and antitumor capacity. The high expression of PD-1 on activated endogenous T cells provides a rational basis for combination therapy with a PD-1 antibody [62].

Wang et al. further conducted a phase II exploratory study, enrolling six advanced colorectal cancer patients to receive single-agent CAR-NK or combination therapy with a PD-1 antibody. One patient achieved stable disease, with a median progression-free survival of 28 days and median overall survival of 292.5 days; two patients survived for more than two years. Pharmacokinetic analysis confirmed that PD-1 inhibition significantly elevated peripheral CAR copy number and prolonged CAR-NK in vivo persistence, verifying the clinical potential of this combinatorial strategy [63].

3.3. Ovarian Cancer

Over 80% of ovarian cancer patients are diagnosed at advanced clinical stages. Standard clinical regimens include cytoreductive surgery and platinum-based chemotherapy, whereas PARP inhibitors are routinely used as maintenance therapy for BRCA-mutated or HRD-positive patients [64,65]. Novel therapeutic options are urgently needed for patients with advanced disease with few remaining therapeutic options. Insufficient tumor homing and infiltration remain major bottlenecks restricting CAR-NK efficacy in solid tumors. The ovarian cancer microenvironment is characterized

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by high levels of secreted IL-8, and co-expression of its receptor CXCR1 can markedly enhance the tumor tropism of engineered NK cells [66]. mRNA-electroporated NKG2D CAR-NK cells co-expressing CXCR1 showed enhanced migratory capacity toward IL-8-secreting tumor cells in vitro. In subcutaneous FaDu and intraperitoneal SKOV3 xenograft models, CXCR1-modified CAR-NK cells exhibited nearly 10-fold higher tumor infiltration extent. In the ovarian cancer xenograft model, CXCR1 overexpression extended median survival from 50 to 60 days, demonstrating that optimizing tumor homing can effectively improve the in vivo antitumor efficacy of CAR-NK [18].

3.4. Lung Cancer

Lung cancer ranks first in global morbidity and mortality, with an overall five-year survival rate lower than 25% despite continuous therapeutic advances [67]. NKG2DLs are frequently overexpressed in lung cancer tissues, making NKG2D an ideal targeted therapeutic candidate. IL-21 modulates immune cell proliferation, cytotoxicity and cytokine secretion through JAK/STAT, MAPK and PI3K/AKT signaling pathways [68]. Zhang et al. engineered IL-21-co-expressing NKG2D CAR-NK cells, which displayed superior proliferation, cytotoxicity and IFN-γ secretion, along with reduced apoptosis and cell exhaustion via PI3K/AKT activation [19]. To overcome tumor immune escape, Zhi et al. developed dual-specific CAR-NK92 cells targeting PD-L1 and MICA/B [17]. The dual-target CAR operated via OR-logic gating and exerted synergistic killing effects on H1299 lung cancer cells mainly through GSDME-dependent pyroptosis and effector molecule release. In xenograft models, the dual-specific CAR-NK92 cells significantly inhibited tumor growth and upregulated serum levels of antitumor effector cytokines. [17]. Fukutani et al. engineered functionally enhanced super NK cells (18H5-eNK) from hiPSCs by integrating six genes: CCL19 for DC recruitment, CCR2B for tumor homing, FCGR3A encoding high-affinity CD16 for ADCC, HCST (DAP10), IL15 for persistence, and KLRK1 (NKG2D). 18H5-eNK cells exhibited superior cytotoxicity against a panel of solid tumor cell lines in vitro and demonstrated sustained tumor control in orthotopic lung cancer models without exogenous cytokine support. These cells lack NKG2A expression, conferring resistance to HLA-E-mediated inhibition, and exhibit autocrine IL-15 production to ensure prolonged persistence [69].

3.5. Glioma

Glioblastoma is the most aggressive primary intracranial tumor with limited curative treatment options currently. Myeloid-derived suppressor cells (MDSCs) exert core immunosuppressive functions in the glioma microenvironment by secreting abundant TGF-β, which downregulates DAP10 expression and impairs endogenous NKG2D signaling [70,71]. NKG2D.ζ-engineered NK cells can specifically eliminate NKG2DL-positive MDSCs, maintain functional activity under TGF-β and soluble NKG2DL stimulation, and secrete chemokines such as CCL5 and CCL3 to recruit GD2 CAR-T cells. Sequential infusion of NKG2D.ζ-NK followed by GD2 CAR-T significantly improved antitumor efficacy and prolonged survival in neuroblastoma models [44].

Look et al. systematically compared the therapeutic effects of NKG2D CAR-T, CAR-NK and CAR-macrophages in orthotopic glioma models. CAR-T cells demonstrated optimal tumor homing ability but limited survival benefit as monotherapy. CAR-NK retained potent cytotoxicity under immunosuppressive conditions and induced broad immune activation. CAR-macrophages mainly inhibited tumor growth via non-lytic pathways, albeit with insufficient deep tumor infiltration. Further optimization showed that multifunctional CAR-NK co-expressing IL-12 and IFN-α2 achieved the best therapeutic outcome, curing 4/6 tumor-bearing mice by remodeling immunologically "cold" tumors into immunologically "hot" microenvironments [72].

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3.6. Pancreatic Cancer

Pancreatic cancer is widely known for its extremely poor prognosis, and these cells abundantly overexpress MICA/B, rendering them susceptible to NKG2D CAR-NK targeted therapy [73]. Jin et al. constructed a novel CAR-NK platform expressing two anti-MICB scFv fragments linked by F2A peptides. These engineered NK cells not only target MICB-positive pancreatic cancer cells but also secrete soluble anti-MICB scFv to block ligand shedding. In pancreatic cancer models, this strategy upregulated MICA/B expression on tumor cells and increased intratumoral NK infiltration, thereby enhancing overall antitumor efficacy [74]. Guo et al. identified GPR116 as a key negative regulator of NK cell function. GPR116 knockout inhibited pancreatic tumor growth, increased intratumoral NK infiltration and upregulated granzyme B and IFN-γ expression. Mechanistically, GPR116 downregulation suppresses HIF1α and activates the NF-κB pathway. GPR116-knockdown NKG2D CAR-NK exhibited markedly enhanced anti-pancreatic cancer activity both in vitro and in vivo [75].

Table 2. Clinical Trials of NKG2D CAR-NK Cell Therapy.

Trial number	Study Start	Indication	Title	Status	Sponsor
NCT04623944	2020	R/R AML or high-risk MDS	NKX101, Intravenous Allogeneic CAR NK Cells, in Adults With AML or MDS	Active, not recruiting	Nkarta, Inc.
NCT05734898	2023	R/R AML	NKG2D CAR-NK & r/rAML	Unknown status	Zhejiang University
NCT05247957	2021	R/R AML	NKG2D CAR-NK Cell Therapy in Patients With Relapsed or Refractory Acute Myeloid Leukemia	Terminated	Hangzhou Cheetah Cell Therapeutics Co., Ltd
NCT06379451	2024	R/R Multiple Myeloma	An Clinical Study of NKG2D-CAR-NK Cells for the Treatment of Refractory Recurrent Multiple Myeloma	Not yet recruiting	Changzhou No.2 People's Hospital
NCT03415100	2018	Metastatic Solid Tumors	Pilot Study of NKG2D-Ligand Targeted CAR-NK Cells in Patients With Metastatic Solid Tumours	Unknown status	The Third Affiliated Hospital of Guangzhou Medical University
NCT05213195	2021	Refractory Metastatic Colorectal Cancer	NKG2D CAR-NK Cell Therapy in Patients With Refractory Metastatic Colorectal Cancer	Recruiting	Zhejiang University
NCT06503497	2024	Pancreatic Cancer	A Trail of Second-line Chemotherapy Sequential NKG2D CAR-NK Cell Therapy for Pancreatic Cancer	Recruiting	Zhejiang University
NCT06478459	2024	Advanced Pancreatic Cancer	Endoscopic Ultrasound (EUS) Intratumoral Injection of CAR-NK Cells in the Treatment of Advanced Pancreatic Cancer	Recruiting	Zhejiang University

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NCT07021534	2025	Advanced Solid Tumors with Liver Metastases	Hepatic Artery Transfusion of NKG2D CAR-NK Cells Followed by Intravenous Infusion of NKG2D CAR-T Cells to Treat Patients With Advanced Solid Tumors With Liver Metastases Who Have Failed Standard Treatments: a Phase I Exploratory Clinical Trial	Recruiting	Zhejiang University
NCT05776355	2023	Ovarian Cancer	NKG2D CAR-NK & Ovarian Cancer	Unknown status	Hangzhou Cheetah Cell Therapeutics Co., Ltd
NCT05528341	2023	R/R solid tumor	NKG2D-CAR-NK92 Cells Immunotherapy for Solid Tumors	Recruiting	Xinxiang medical university
NCT06856278	2025	Anaplastic Thyroid Cancer	Clinical Study of NKG2D CAR-NK Combined with PD-1 Monoclonal Antibody in the Treatment of ATC	Not yet recruiting	Zhejiang Provincial People's Hospital

4. Challenges and Solutions for NKG2D CAR-NK Cell Therapy

Despite significant progress, the clinical translation of NKG2D CAR-NK therapy remains hindered by three interconnected critical barriers: tumor antigen escape, the immunosuppressive tumor microenvironment, and inadequate in vivo persistence. These challenges create a self-reinforcing cycle that collectively constrains the therapeutic efficacy of NKG2D CAR-NK cells, especially within the context of solid tumors.

4.1. Tumor Antigen Escape

Tumor cells evade NKG2D-based immune surveillance primarily through two interconnected mechanisms: the downregulation or proteolytic shedding of NKG2D ligands (NKG2DLs) from their cell surface, and inherent tumor heterogeneity. Soluble MICA/B generated by shedding not only fails to trigger NK cell activation but also competitively binds to NKG2D receptors on NK cells, inducing their functional exhaustion. Compounding this challenge, both inter-tumor heterogeneity (inter-patient variations) and intra-tumor heterogeneity (genetic and phenotypic diversity within a single tumor) allow resistant subclones to survive and expand following single-target therapy, ultimately leading to treatment failure and disease recurrence [76,77]. To address these challenges, researchers have developed multiple complementary strategies. First, drug-mediated ligand upregulation provides a strategy to reverse NKG2DL downregulation; specifically, epigenetic regulators and conventional chemotherapeutic agents have been shown to induce re-expression of NKG2DLs on tumor cells, restoring tumor cell susceptibility to NKG2D-mediated recognition and killing [78–80]. Second, CARs targeting the highly conserved α3 domain of MICA/B overcome two major limitations of traditional NKG2D-targeted therapies: allele polymorphism and inhibition by soluble ligands [81,82]. For example, 3MICA/B CAR iNK cells achieved durable tumor control across multiple hematological and solid tumor models and exhibited synergistic efficacy when combined with therapeutic monoclonal antibodies [83]. Similarly, 1D5-CAR specifically binds to the α3 domain without interfering with endogenous NKG2D signaling [84]. Third, shedding inhibition strategies directly block ligand cleavage: MICB-targeted CARs engineered to secrete anti-MICB single-chain variable fragments (scFv) retain MICB on the tumor cell surface, preventing its release and enhancing persistent immune recognition [74]. Finally, bispecific antibodies such as NKAB-ErbB2 and MS-Ig act

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as molecular bridges to redirect endogenous NKG2D-positive lymphocytes to tumor cells, broadening the therapeutic scope and overcoming antigen escape [84,85].

4.2. Immunosuppressive Tumor Microenvironment

The immunosuppressive tumor microenvironment (TME) of solid tumors constitutes the most formidable barrier to effective NKG2D CAR-NK therapy. This hostile microenvironment comprises immunosuppressive cells (including tumor-associated macrophages, regulatory T cells, and myeloid-derived suppressor cells), inhibitory cytokines (such as TGF-β and IL-10), and metabolites (such as lactic acid and adenosine). Collectively, these factors severely impair CAR-NK cell infiltration, survival, and cytotoxic function [86–89]. Furthermore, the upregulation of inhibitory receptors, such as TIGIT, on CAR-NK cells further diminishes their anti-tumor efficacy within the TME [90].

Several innovative approaches have been developed to overcome TME-mediated immunosuppression. Cytokine engineering endows CAR-NK cells with intrinsic resistance to TME suppression. Specifically, CAR-NK cells expressing Neo-2/15, a novel IL-2Rβγ agonist, exhibit enhanced mitochondrial adaptability and resistance to exhaustion via activation of the c-Myc/NRF1 signaling pathway [91,92], while IL-15 superagonists engineered to be specifically released upon tumor recognition provide localized support for CAR-NK function without systemic toxicity [93]. A particularly elegant strategy involves converting inhibitory signals into activating signals: the chimeric cytokine receptor TRII/21R, constructed by fusing the extracellular domain of TGF-β receptor II with the transmembrane and intracellular domains of the IL-21 receptor, redirects TGF-β signaling to activate the IL-21R-STAT3 pathway, significantly enhancing CAR-NK anti-tumor activity in gastric cancer models [94]. Furthermore, targeted inhibition of key immunosuppressive pathways in the TME has demonstrated potential: the use of combination therapy with anti-CD73 antibodies blocks adenosine-mediated immunosuppression, substantially improving the efficacy of NKG2D CAR-NK cells against CD73-positive solid tumors [95].

4.3. Inadequate In Vivo Persistence

The relatively short lifespan and limited intrinsic expansion capacity of NK cells in vivo in the absence of exogenous cytokine support pose another major obstacle to the sustained clinical efficacy of NKG2D CAR-NK therapy. Unlike T cells, unmodified NK cells survive for only a few days in vivo, thereby restricting the capacity of a single infusion to sustain an effective effector cell population long enough to eradicate all tumor cells and prevent recurrence [96]. To enhance the in vivo persistence, strategies have centered on optimizing cytokine support and introducing function-enhancing genetic modifications. Membrane-bound IL-15 (mbIL-15) has emerged as the gold standard for improving CAR-NK survival: co-expression of mbIL-15 significantly enhances both the in vitro expansion and the in vivo persistence of CAR-NK cells, while mitigating the systemic toxicities associated with secreted IL-15 [97–99]. Other cytokine engineering approaches, including expression of IL-27 and tethered IL-2, have also demonstrated efficacy in prolonging CAR-NK survival and function [100,101]. Most recently, overexpression of the OR7A10 G protein-coupled receptor has been identified as a transformative strategy to comprehensively enhance CAR-NK function. These OR7A10-engineered CAR-NK cells exhibit markedly improved cytotoxicity, proliferation, metabolic adaptability, and resistance to TME immunosuppression, achieving complete tumor clearance and long-term survival in multiple solid tumor models in the absence of increased genomic instability or risk of cytokine release syndrome [102].

5. Future Directions

5.1. Universal Off-the-Shelf Products

The development of low-cost, ready-to-use off-the-shelf CAR-NK products represents a pivotal strategy for the clinical translation of NKG2D CAR-NK therapy, owing to their potential to significantly reduce treatment costs, shorten patient waiting times, and promote widespread clinical

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application. Currently, four main sources of NK cells are being explored for off-the-shelf manufacturing, each with distinct advantages and limitations. Peripheral blood (PB) is the most accessible and cost-effective source of NK cells for clinical development. However, PB-derived CAR-NK cells are associated with notable limitations. First, the anti-tumor efficacy of autologous PB-NK cells is generally inferior to that of allogeneic NK cells in clinical settings. This limitation is attributed to KIR-HLA interactions between NK cells and tumor cells, as well as the immunosuppressive nature of the autologous microenvironment, both of which collectively impair NK cell activation and effector function. Second, while allogeneic PB-NK cells circumvent this inhibitory signaling, their clinical application requires rigorous depletion of contaminating T cells prior to infusion to prevent the development of graft-versus-host disease (GVHD) [103,104]. Umbilical cord blood (CB) offers several compelling advantages: it poses a low risk of graft-versus-host disease (GVHD), facilitates easy collection and cryopreservation, and yields highly pure NK cells (97.1%) with an average 192-fold expansion after 2 weeks of culture. Notably, cryopreserved CB-NK cells retain more than 97% cytotoxicity against K562 leukemia cells even after 1 year of storage. Furthermore, CD34+ hematopoietic stem and progenitor cells (HSPCs) from a single CB unit can generate up to 1.4×10^7 induced NK (iNK) cells, enabling large-scale production of clinical-grade cell products [105–108]. The NK-92 cell line is another promising platform, notable for its ease of large-scale expansion and genetic modification; however, it requires irradiation before infusion to minimize tumorigenic risk and has limited in vivo persistence [109–111]. Induced pluripotent stem cell (iPSC)-derived NK cells represent the most transformative platform for universal off-the-shelf therapy, with unlimited self-renewal capacity and the potential for highly standardized production. These cells can be genetically edited to reduce immunogenicity via B2M or HLA gene knockout; additionally, the single-factor reprogramming of peripheral blood mononuclear cells (PBMCs) into NK cells through BCL11B knockdown offers a complementary strategy for scalable manufacturing [112–115].

5.2. Combination Therapy

Single-agent NKG2D CAR-NK therapy is constrained by several inherent limitations in the treatment of solid tumors, including tumor microenvironment (TME) immunosuppression, target antigen heterogeneity, and ligand shedding. Therefore, the development of multi-dimensional combination strategies has emerged as a pivotal approach to improve therapeutic efficacy and overcome drug resistance. Immune checkpoint blockade represents the most widely explored combination strategy. For instance, in metastatic colorectal cancer, PD-1 inhibition has been shown to significantly enhance the in vivo persistence of NKG2D CAR-NK cells, thereby improving clinical outcomes [63]. Small molecule drugs represent an alternative complementary strategy by sensitizing tumor cells to NK cell-mediated killing. Specifically, whole-genome CRISPR/Cas9 screening has revealed that inhibiting PKMYT1 upregulates the expression of NKG2D ligands (MICA/B) and the chemokine CX3CL1 on the surface of pancreatic cancer cells, resulting in synergistic anti-tumor effects when the PKMYT1 inhibitor (RP6306) is combined with NKG2D CAR-NK therapy [79,115]. Cytokine-based combination strategies further optimize CAR-NK function: co-expression of IL-21 in NKG2D CAR-NK cells enhances their proliferation, cytotoxicity, and in vivo persistence in lung cancer models via activation of the PI3K/AKT signaling pathway [19,69]. Together, these combination approaches constitute a multi-faceted synergistic framework that addresses the multiple barriers to effective solid tumor immunotherapy.

5.3. Multifunctional Engineering

To overcome tumor antigen heterogeneity, antigen escape, and TME immunosuppression, the design of NKG2D CAR-NK cell therapy is evolving rapidly toward multi-targeting and intelligent architectures, leveraging strategies derived from modifications developed for other CAR targets and CAR-T cells. Traditional single-target CAR-NK cells have limited efficacy against tumor cells with antigen downregulation or loss, prompting the development of dual- and multi-target CAR structures. For example, dual CAR NK-92 cells simultaneously targeting CD19 and BCMA exhibit

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superior cytotoxicity against malignant B-cell lines and primary tumor cells compared to single-target CAR-NK cells [116,117]. More advanced logic-gated CAR designs, incorporating OR gates for multi-antigen recognition and NOT gates to mitigate off-target toxicity toward healthy cells, enable precise tumor targeting while protecting normal tissues [118,119]. Beyond simple multi-target co-expression, multifunctional CAR designs integrate multiple synergistic functional modules into a single cell product. A paradigmatic example is the 18H5-eNK cell, generated by integrating six functional genes (CCL19, CCR2B, FCGR3A, HCST, IL15, KLRK1) into human induced pluripotent stem cells; this strategy confers comprehensive anti-tumor capabilities upon the resulting NK cells, including enhanced tumor homing, ADCC, in vivo persistence, and TME remodeling [69]. These innovative engineering strategies are transforming NKG2D CAR-NK cells from simple "directed killing" tools into sophisticated "living drugs" that can sense their environment, perform complex logical operations, and dynamically modulate their activity in response to changing tumor conditions.

Conclusion

NKG2D CAR-NK cell therapy represents a promising emerging frontier in tumor immunotherapy, combining the inherent rapid and broad-spectrum cytotoxic capacity of NK cells with the precise targeting of CAR technology. Incorporating NKG2D, which serves as a master switch for NK cell activation, further enhances NK cell functionality. Coupled with the off-the-shelf nature of NK cells, NKG2D CAR-NK products have the potential to become universal cellular therapies for cancer applications.

To realize this potential, future efforts should focus on: (1) conducting well-designed, staged clinical trials to generate high-level clinical evidence across different tumor types and stages; (2) developing large-scale, GMP-compliant cell production processes featuring enhanced standardization and automation to ensure product homogeneity, stability, and safety. Through rigorous clinical validation and mature process development, NKG2D CAR-NK cell therapy will become a standard, universal, off-the-shelf mainstay of tumor immunotherapy, bringing new hope to patients worldwide.

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