MSc Drug Innovation
Writing Assignment

Beyond CAR-T: Advancements in CAR-NK and CAR-NKT Therapies for Cancer Treatment

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January 2025

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Declaration of AI Assistance in the Writing Assignment

In this research project, I utilized GenAI, particularly ChatGPT 4.0, to support the structuring, refinement, and grammatical accuracy of the text. I carefully reviewed all AI-assisted outputs to ensure that the content aligns with high standards of academic integrity and precision. While GenAI significantly enhanced my efficiency, I maintained a critical approach, meticulously verifying all information to uphold the originality and rigor of the work.

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January 30, 2025
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1. *Plain Language Summary*

Cancer is a complex disease where cells grow uncontrollably. The human immune system can recognize and fight cancer cells, but tumors often find ways to evade this defense. One approach to cancer treatment is immunotherapy, which boosts the ability of the immune system to attack cancer cells. One of the most promising immunotherapies uses modified immune cells called CAR-T cells. These cells are engineered to recognize and attack cancer cells more effectively. However, CAR-T therapies can cause side effects, such as inflammation and difficulty targeting solid tumors.

Recently, scientists have developed a new approach using another type of immune cell, called CAR-NK cells, which show promise in treating cancer, especially in solid tumors. CAR-NK cells have some advantages over CAR-T cells, including fewer side effects like inflammation and the ability to target a broader range of tumors. However, there are still challenges in making these therapies more effective, such as improving how long the CAR-NK cells last in the body and overcoming the ability of tumors to hide from the immune system.

A new development, CAR-NKT cells, combines the best features of both CAR-T and CAR-NK cells. These cells are designed to target cancer more efficiently, even in hard-to-treat tumors, and they may have fewer side effects than CAR-T cells. Early research into CAR-NKT cells looks promising, but further studies are needed to confirm their effectiveness and safety.

In conclusion, while CAR-NK and CAR-NKT cells offer exciting new possibilities for cancer treatment, more research is needed to overcome current challenges. Advances in technology and a deeper understanding of how these immune cells work will help make these therapies safer and more effective for patients with cancer.

2. Abstract

Chimeric Antigen Receptor (CAR) technologies have transformed cancer immunotherapy, with CAR-T cell therapies demonstrating remarkable success in hematological malignancies. However, their clinical application is hindered by severe toxicities, high manufacturing costs, antigen heterogeneity, and limited efficacy in solid tumors due to poor infiltration and the immunosuppressive tumor microenvironment (TME). CAR-NK therapies have emerged as a promising alternative, offering an innate immune-based approach with lower toxicity, broader tumor recognition, and the potential for scalable "off-the-shelf" products. Despite these advantages, CAR-NK cells face challenges such as limited persistence, susceptibility to TME suppression, and lower expansion capabilities. Engineering strategies, including IL-15 expression, chemokine receptor upregulation, and CAR-independent mechanisms, are being explored to enhance their therapeutic potential in solid tumors. Additionally, CAR-NKT cells provide a hybrid approach, combining T and NK cell features to target both protein and glycolipid antigens while exhibiting improved infiltration and TME modulation. Early clinical trials suggest that CAR-NK and CAR-NKT therapies may address key limitations of CAR-T therapies, particularly in solid tumors, but further research is required to optimize their persistence, tumor specificity, and clinical scalability. This review explores the mechanistic differences between CAR-T and CAR-NK therapies, their respective strengths and limitations, and the potential of CAR-NKT cells as an emerging strategy to expand the reach of immunotherapy.

Keywords: CAR-T cells, CAR-NK cells, CAR-NKT cells, immunotherapy, tumor microenvironment, hematological malignancies, solid tumors, adoptive cell therapy.

3. Introduction

Cancer is a complex disease characterized by uncontrolled cell growth, genetic instability, and the accumulation of mutations (1). These mutations can lead to the production of tumor-specific antigens, which are recognized as foreign by the immune system (2). Despite the immune system's role in identifying and eliminating malignant cells, tumors often evade immune surveillance, posing a significant challenge to their complete eradication (2).

Cancer cells employ diverse evasion strategies, including downregulating antigen presentation, recruiting immunosuppressive cells including regulatory T cells (Tregs), and exploiting immune checkpoints to inhibit T-cell activity (2,3). Such mechanisms create a tumor microenvironment (TME), which is a complex network of immune cells, fibroblasts, vasculature, and extracellular matrix that suppresses immune responses and, consequently, promotes tumor growth and metastasis (4). This dual role of the immune system, involving both tumor suppression and promotion, underscores its importance in cancer biology (1).

The immune system comprises two interconnected arms: innate and adaptive immune systems. The innate immune system, consisting of natural killer (NK) cells, dendritic cells (DCs), macrophages, and neutrophils, provides the first line of defense through rapid nonspecific responses (1,5). In contrast, the adaptive immune system, mediated by T and B lymphocytes, offers a targeted and long-lasting response. T cells, central to adaptive immunity, recognize tumor antigens presented by antigen-presenting cells (APCs) and mediate cytotoxic effects. B cells contribute through antibody production and antigen presentation. Together, these systems form a complex network that protects against cancer progression (1). Understanding the immune system and its interactions with cancer cells has paved the way for the development of immunotherapy, a revolutionary approach to cancer treatment (1).

Immunotherapy aims to enhance the immune system capacity to recognize and eliminate cancer cells (3). This field encompasses several strategies, each targeting specific aspects of the immune response. Among these, adoptive cell transfer (ACT) has gained considerable attention, particularly through the development of Chimeric Antigen Receptor (CAR) T-cell therapies. CAR-

T cells are engineered from patient-derived T cells to express synthetic receptors that specifically bind to antigens on tumor cells, enabling precise and potent tumor cell killing (1,2,6). Immune checkpoint inhibitors (ICIs), block negative regulatory pathways, such as PD-1/PD-L1 and CTLA-4, which are often exploited by cancer cells to evade immune detection and destruction. By inhibiting these pathways, ICIs restore T-cell activity, enabling the immune system to effectively target and eliminate tumors (1,6). Cancer vaccines stimulate immune responses by presenting tumor antigens, while cytokine therapies boost immune signaling (1,6). Emerging approaches like oncolytic virus therapies use modified viruses to enhance local and systemic antitumor immunity (1,6).

While immunotherapy has achieved remarkable success, particularly in hematologic cancers, challenges remain. These include limited efficacy in solid tumors, variability in patient responses, and potential side effects such as cytokine release syndrome (CRS) (2,7). Furthermore, the TME often fosters immune evasion through mechanisms such as checkpoint pathway upregulation, immunosuppressive cytokines, and the recruitment of regulatory immune cells (1,7). Although CAR-T therapy has revolutionized the treatment of certain malignancies, limitations, such as manufacturing complexity, toxicity, and difficulty in targeting solid tumors, underscore the need for continued innovation in cell-based immunotherapy (1,2,6).

Recent efforts have focused on harnessing the innate immune system, with therapies targeting NK cells showing promise as complementary or standalone treatments. These efforts also include exploring alternative CAR-based modalities, particularly CAR-NK cells, to potentially circumvent some of the challenges observed with CAR-T cells (8–10). By expanding the repertoire of immunotherapeutic strategies, researchers aim to overcome the limitations of existing treatments and extend the benefits of immune-based therapies to a broader range of patients (8–10).

This review explores the role of CAR therapies in cancer immunotherapy, emphasizing both CAR-T and emerging CAR-NK cell approaches. It highlights the limitations of current CAR-T therapies and examines the potential advantages of CAR-NK cells. The review also discusses the significance of NK cells as a therapeutic modality and the key challenges faced by this emerging

therapy. Finally, it evaluates the future prospects of NKT cell-based immunotherapies and the progress toward achieving an effective therapeutic strategy.

4. Mechanistic Insights into CAR-T and CAR-NK Cells

CAR-based therapies have revolutionized cancer treatment, particularly in hematological malignancies (11). CARs are engineered fusion proteins that direct immune cells to recognize and attack tumor cells (8). While CAR-T cells are derived from autologous T lymphocytes, CAR-NK cells often originate from allogeneic NK cells. Despite structural similarities, these therapies differ in biological mechanisms and therapeutic potential due to their distinct immune cell origins and CAR designs (8,11,12).

CAR Structure and Key Components

CARs consist of four key regions: the extracellular antigen recognition domain, hinge region, transmembrane segment, and intracellular signaling elements, each contributing to their functionality (Figure 1). The extracellular domain, commonly a single-chain variable fragment (scFv) derived from antibodies, provides specificity by targeting antigens expressed on tumor cells (8–10). This domain allows CARs to bypass the major histocompatibility complex (MHC) restriction that typically governs natural T-cell activation. In this process, T cells recognize antigens only when they are presented by patient-specific MHC molecules. By bypassing this restriction, CARs broaden their applicability across diverse patient populations regardless of MHC variability (8,11,12). The hinge region provides flexibility to the CAR, optimizing its spatial positioning for effective antigen binding. The transmembrane segment anchors the CAR to the cell membrane, connecting the extracellular recognition domain with the intracellular signaling components (8,11–13). These structural elements are conserved between CAR-T and CAR-NK cells, but the intracellular signaling domains, which are critical for immune cell activation, exhibit substantial differences (Figure 1).

Intracellular Signaling Domains in CAR-T and CAR-NK Cells

The intracellular domains of CAR constructs are designed to initiate immune cell activation upon antigen engagement. In CAR-T cells, CD3ζ, derived from the T-cell receptor (TCR) complex, is the

primary signaling domain. CD3ζ contains immunoreceptor tyrosine-based activation motifs (ITAMs) that trigger TCR-like signaling cascades upon phosphorylation, driving T-cell activation. To enhance activation, persistence, and proliferation, CAR-T constructs also incorporate co-stimulatory domains, such as CD28 or 4-1BB (CD137) (Figure 1). CD28 facilitates rapid expansion and effector functions by activating the PI3K/AKT pathway, while 4-1BB improves mitochondrial fitness and durability by engaging the NF-κB pathway (8,11–13).

CAR-NK cells, while often incorporating CD3ζ to enhance cytotoxicity, rely on NK-specific signaling pathways for activation. CAR-NK constructs include domains such as DAP10 or DAP12, which are adaptor proteins associated with activating NK receptors like NKG2D or NKp44 (Figure 1). DAP10 recruits PI3K, triggering cytotoxic signaling cascades that lead to NK cell activation and cytotoxic responses, while DAP12 signals through its ITAMs to activate Syk/ZAP-70 kinases, promoting calcium mobilization and cytotoxic granule release (14). Co-stimulatory domains such as 2B4 (CD244), which interact with SLAM-associated protein (SAP), further enhance NK cell proliferation and effector functions. These signaling adaptations reflect the distinct biology of NK cell activation compared to T cells (8,9,15).

Activation Mechanisms of T and NK Cells

The activation requirements of T cells and NK cells differ substantially and shape the design of CAR constructs. T-cell activation relies on a two-signal process: recognition of antigens presented by MHC molecules through the TCR-CD3ζ complex and engagement of co-stimulatory receptors, such as CD28 binding to CD80/86. CAR-T cells bypass MHC restriction by using the scFv domain to directly recognize tumor antigens, with CD3ζ transducing the activation signal without requiring additional receptor interactions (Figure 3A) (8,11–13).

In contrast, NK cell activation depends on the balance of activating and inhibitory signals. Activating receptors, such as NKG2D, detect stress-induced ligands on tumor cells, while inhibitory receptors, such as killer-cell immunoglobulin-like receptors (KIRs), recognize self-MHC molecules to prevent unintended cytotoxicity. Because NK cells do not rely on MHC molecules for activation, they can target tumor cells that evade T-cell responses by downregulating MHC expression. This ability is further exploited in CAR-NK cells through the inclusion of domains such

as DAP10 and DAP12, which mimic the signaling cascades of activating receptors and enable the elimination of tumor cells (Figure 3B) (8,9,15).

Effector Functions of CAR-T and CAR-NK Cells

CAR-T and CAR-NK cells share some effector functions but differ in their underlying mechanisms. CAR-T cells eliminate tumor cells primarily through the perforin-granzyme pathway. Perforin forms pores in the target cell membrane, allowing granzymes to enter and induce apoptosis. Activated CAR-T cells also secrete cytokines such as IFN-γ, TNF-α, and IL-2, which enhance the antitumor immune response but can contribute to CRS, a common side effect of CAR-T therapy characterized by an excessive immune response (16). Additionally, CAR-T cells, as part of the adaptive immune system, can differentiate into memory subsets, enabling long-term persistence and rapid reactivation upon antigen re-encounter (8,11–13). These features contribute to the lasting efficacy of CAR-T therapies.

CAR-NK cells also utilize the perforin-granzyme pathway but possess additional cytotoxic mechanisms, such as antibody-dependent cellular cytotoxicity (ADCC). Through their CD16 (FcγRIII) receptor, NK cells recognize antibody-coated tumor cells, triggering degranulation and cytotoxicity. Moreover, CAR-NK cells produce lower levels of pro-inflammatory cytokines than CAR-T cells, reducing the risk of CRS and immune-related toxicities (8,9,15).

Persistence and Lifespan

The persistence and lifespan of CAR-T and CAR-NK cells represent another key distinction. CAR-T cells benefit from their natural capacity for proliferation and long-term survival, particularly when 4-1BB domains are incorporated into their design. This promotes memory formation and mitochondrial fitness, enabling sustained antitumor activity for months or even years post-infusion (17). In contrast, NK cells have a shorter lifespan and limited *in vivo* expansion. To address this limitation, CAR-NK constructs are often engineered to express IL-15, which supports survival, enhances proliferation, and compensates for the intrinsic limitations of NK cells in persistence (8–10,12,13,15).

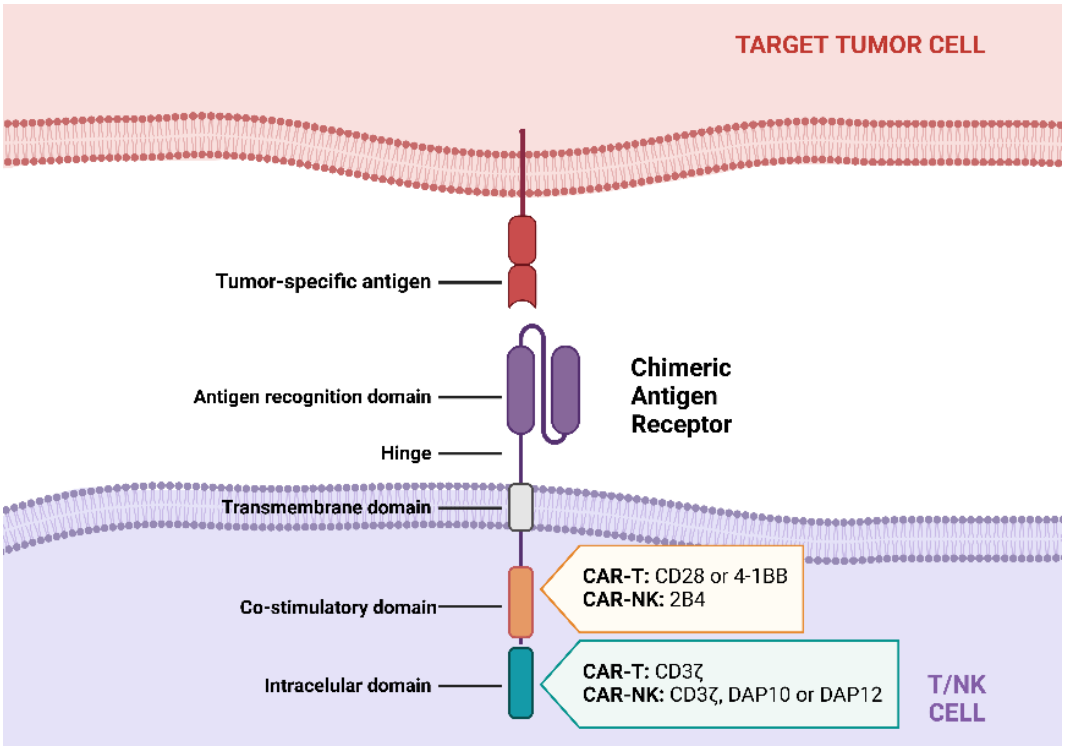

Figure 1. **Structure and Functional Domains of Chimeric Antigen Receptors (CARs) in T and NK Cells.** The CAR structure consists of an antigen recognition domain (scFv) that binds tumor antigens, linked to a hinge and transmembrane region. The intracellular domain includes co-stimulatory signals (e.g., CD28 or 4-1BB in CAR-T cells, and 2B4 in CAR-NK cells) and signaling domains (e.g., CD3ζ in CAR-T cells, and CD3ζ, DAP10, or DAP12 in CAR-NK cells), which activate and amplify the immune cell response (Adapted from June *et al*. 2018 (13)).

5. Challenges and Limitations of Current CAR-T Therapies

Currently, six CAR-T therapies have been approved by the FDA and EMA, representing significant progress in the treatment of hematological malignancies (Table 1) (18). These therapies primarily target CD19 and BCMA, antigens which play crucial roles in cancer biology. CD19, a surface protein widely expressed on B cells, serves as an ideal target for treating B-cell malignancies, minimizing off-target effects. Therapies targeting CD19 are approved for diseases such as B-cell Acute Lymphoblastic Leukemia (B-ALL), Diffuse Large B-Cell Lymphoma (DLBCL), and Mantle Cell Lymphoma (MCL) (15). Similarly, BCMA is targeted in therapies designed for relapsed or refractory Multiple Myeloma (RRMM), given its overexpression in malignant plasma cells (18).

Table 1. **FDA and EMA-Approved CAR-T Cell Therapies.** An overview of FDA and EMA-approved CAR-T cell therapies, detailing their names, approval years, molecular targets, and approved indications.

Therapy	Year Approved	Target	Indications
Kymriah® (tisagenlecleucel)	2017	CD19	B-ALL, DLBCL
Yescarta® (axicabtagene ciloleucel)	2017	CD19	DLBCL
Tecartus® (brexucabtagene autoleucel)	2020	CD19	MCL
Breyanzi® (lisocabtagene maraleucel)	2021	CD19	R/R LBCL
Abecma® (idecabtagene vicleucel)	2021	BCMA	RRMM
Carvykti® (ciltacabtagene autoleucel)	2022	BCMA	RRMM

Adverse Effects and Safety Concerns

Despite their transformative impact, CAR-T therapies present significant challenges. One of the most critical is CRS, a potentially life-threatening condition characterized by fever, hypotension, and multi-organ dysfunction. CRS is frequently accompanied by neurotoxicity, which manifests as encephalopathy and seizures, complicating patient management (12,17,19,20).

Another major challenge in T-cell-based therapies, particularly in allogeneic settings, is graft-versus-host disease (GVHD), a severe complication of allogeneic transplants (21). GVHD occurs when donor-derived T cells recognize the recipient's tissues as foreign and mount an immune attack, leading to significant tissue damage, morbidity, and mortality (22,23). In the context of allogeneic CAR-T therapies, donor-derived CAR-T cells may still possess endogenous T-cell receptors (TCRs) capable of recognizing the recipient's MHC molecules as foreign, triggering GVHD (21).

To date, most CAR-T therapies have been developed using autologous T cells, in which the patient's own T cells are genetically modified and reinfused. This approach eliminates the risk of alloreactivity since the cells are genetically identical to the host (21). However, the autologous

approach has significant limitations, including high costs and a time-consuming manufacturing process, which can delay treatment for critically ill patients (21).

To enable allogeneic CAR-T therapies while reducing the risk of GVHD, one of the most widely used strategies is the design of TCR-deficient T cells through genome editing tools such as CRISPR/Cas9 (21). By eliminating endogenous TCR expression, this approach prevents donor-derived CAR-T cells from recognizing host MHC molecules, thereby mitigating the risk of GVHD in allogeneic CAR-T therapies (21).

CAR-T cell therapies have achieved remarkable success in treating hematological malignancies. However, their efficacy in solid tumors has been limited (12,24). This limitation is primarily due to the limited trafficking and infiltration to the TME, T-cell exhaustion, and tumor antigen heterogeneity.

One major challenge is the TME which is highly complex and often inhibits CAR-T cell infiltration and distribution within the tumor mass, thereby reducing their therapeutic effectiveness (12). Furthermore, the immunosuppressive environment withing the TME can induce T-cell exhaustion, characterized by diminished proliferation, cytokine production and cytotoxicity (12).

Another critical factor limiting CAR-T efficacy in solid tumors is antigen heterogeneity. Unlike hematological cancers, which often express a uniform target antigen, solid tumors exhibit significant variability in antigen expression (7). As a result, not all tumor cells express the target antigen uniformly, allowing antigen-negative cells to escape CAR-T cell recognition, leading to incomplete tumor eradication (25).

Several strategies have been explored to enhance CAR-T cell efficacy in solid tumors. Engineering CAR-T cells to express chemokine receptors, such as CXCR2, which interacts with chemokines overexpressed in the TME, can improve migration and infiltration into tumor tissue (12). To counteract the immunosuppressive TME, combining CAR-T therapy with ICIs, such as PD-1 or PD-L1 inhibitors, may enhance CAR-T cell persistence and function (12). Additionally, targeting multiple tumor-associated antigens with dual-signaling or tandem CARs helps overcome antigen

heterogeneity, reducing the likelihood of tumor escape variants and improving therapeutic efficacy (25).

Beyond these barriers, long-term safety concerns remain. A 2023 report by the EMA highlighted risks of secondary T-cell malignancies associated with several CAR-T products, including Abecma®, Breyanzi®, Carvykti®, Kymriah®, Tecartus®, and Yescarta® (26). By April 2024, 38 cases of secondary T-cell malignancies, including T-cell lymphoma and T-cell lymphocytic leukemia, had been reported. These occurred between weeks and years after CAR-T administration, with seven cases revealing the presence of CAR constructs in the malignancy. This finding suggests potential involvement of the therapy in disease development, possibly through mechanisms like insertional mutagenesis. However, the absence of CAR constructs in other cases indicates that additional factors may also contribute. Lifelong monitoring and continued research into the molecular mechanisms underlying these malignancies are crucial to enhancing the safety of CAR-T therapies (26).

High Production Costs and Manufacturing Complexities

Another significant limitation is the high cost of CAR-T therapies, driven by the complexity of their manufacturing process. The individualized production pipeline involves multiple labor-intensive steps, including T-cell isolation, genetic modification, and *ex vivo* expansion, all requiring rigorous quality control. These processes contribute to substantial costs and delays in production, which can be critical for patients with aggressive diseases requiring urgent treatment (18,27).

The manufacturing process begins with the collection of T cells from the patient through leukapheresis, a procedure where peripheral blood is extracted, and white blood cells are isolated. The T cells are then activated *ex vivo* using artificial stimulators, such as anti-CD3/CD28 beads, which mimic APC signals. Once activated, the cells are genetically modified with viral vectors to introduce the CAR gene, enabling tumor antigen recognition. The modified cells are expanded under controlled conditions to achieve sufficient numbers for therapeutic infusion. After passing stringent safety and functional tests, the CAR-T cells are cryopreserved until needed. Before infusion, patients undergo lymphodepleting chemotherapy to enhance CAR-T

engraftment. Once administered, the CAR-T cells target and eliminate tumor cells expressing the designated antigen (Figure 2) (24,28,29).

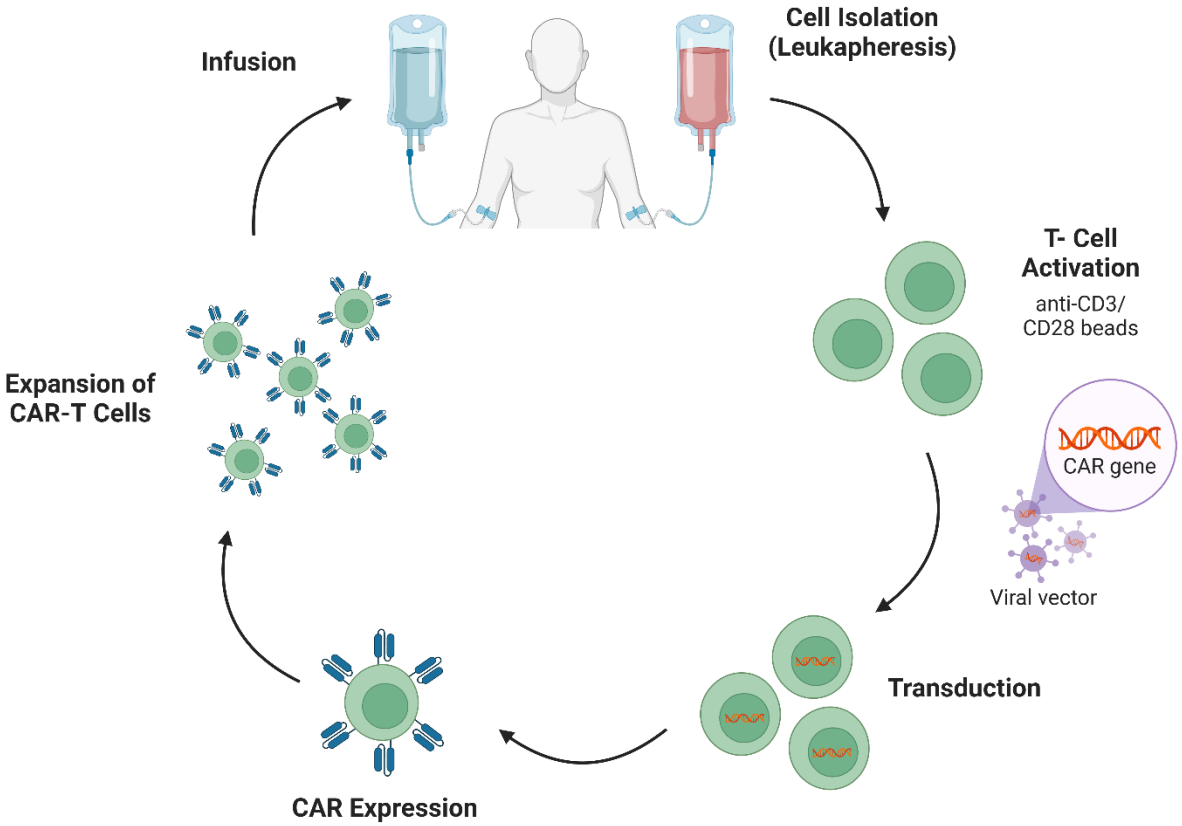

Figure 2. Overview of the CAR-T Cell Autologous Manufacturing Process. This figure illustrates the CAR-T cell production process: T cells are collected from the patient via leukapheresis, activated with anti-CD3/CD28 beads, genetically engineered to express the CAR receptor, expanded to therapeutic levels, and infused back into the patient to target and eliminate tumor cells (Adapted from Faeq *et al*. 2023 (29)).

6. CAR–NK Therapy: A Promising Alternative to CAR–T

The emergence of CAR-NK cells represents a promising alternative to address the limitations of CAR-T therapies (15). NK cells are innate immune cells capable of recognizing and eliminating tumor cells without prior sensitization, playing a critical role in the first line of defense of the immune system (8,30). Representing 5–10% of peripheral blood lymphocytes, NK cells are also present in tissues such as bone marrow, liver, spleen, and lymph nodes (8). Their cytotoxicity is mediated through direct cell killing and cytokine secretion, such as interferon-gamma (IFN-γ),

which amplifies immune responses. NK cells regulate their activity through a balance of activating and inhibitory signals, enabling them to target abnormal cells while maintaining self-tolerance. This "missing-self" mechanism allows NK cells to detect and eliminate cells with downregulated MHC, a strategy often used by tumor cells to evade T-cell responses (8,30). By combining the innate cytotoxicity of NK cells with the tumor-specific targeting capabilities of CAR constructs, CAR-NK cell therapies offer unique advantages over CAR-T cell therapies, particularly in addressing some of their limitations (Table 2) (10).

Reduced Cytotoxic Effects and Toxicity

One of the most notable advantages of CAR-NK cell therapy is the lower incidence of CRS, a severe side effect of CAR-T cell therapy caused by excessive pro-inflammatory cytokine release, particularly IL-1, IL-6, and TNF-α (12). While CAR-T and CAR-NK cells share similar cytotoxic mechanisms, CAR-NK cells exhibit a distinct cytokine profile, characterized by lower IL-6 secretion and increased production of anti-inflammatory cytokines like IL-10, which help mitigate excessive inflammation (8,9,16). Additionally, CAR-NK cells can utilize ADCC, contributing to a more controlled immune response (8,16)

Differences in activation kinetics and proliferation further influence CRS risk. CAR-T cells undergo rapid clonal expansion upon activation, increasing cytokine production and toxicity (12). In contrast, CAR-NK cells exhibit more regulated activation and limited proliferation, reducing the risk of excessive CRS (31). Their shorter lifespan, typically persisting for weeks rather than months, also limits prolonged immune activation and associated toxicities (15).

Allogenic Sources and “Off-the-Shelf” Potential

A significant limitation of CAR-T therapies is their autologous nature, which restricts their application to individual patients. This personalized manufacturing process is time-consuming and costly, presenting challenges for patients with rapidly progressing cancers (12,23,24). The need for autologous CAR-T arises from the risk of GVHD in allogenic settings, as T cells retain their endogenous TCRs, which can recognize host tissues as foreign and initiate immune rejection (21).

In contrast, CAR-NK therapies are often derived from allogeneic sources, such as NK-92 cell line, adult peripheral blood (AB), umbilical cord blood (CB), and induced pluripotent stem cells (iPSCs) (32,33). Unlike T cells, NK cells lack antigen-specific TCRs, preventing GVHD and allowing for "off-the-shelf" CAR-NK therapies that can be readily available for multiple patients (21,32).

Each NK cell source differs in maturation stage, viability, and anti-tumor activity, influencing their clinical potential. NK-92 cells are highly cytotoxic, easy to expand, and express low levels of inhibitory receptors (34). However, since they originate from a non-Hodgkin lymphoma patient, they require irradiation before infusion to prevent tumor engraftment, which negatively affects persistence and proliferation (9,32).

Primary NK cells from AB and CB are more challenging to expand *in vitro* but are effective sources for CAR-NK therapy (34). CB-derived NK cells yield larger numbers but exhibit an immature phenotype with reduced cytotoxic potential, incomplete receptor repertoire, and lower functional activity, requiring *ex vivo* expansion (34). Both AB and CB-derived CAR-NK cells can be expanded using IL-2, IL-12, IL-15, IL-18, IL-21, or APCs, but reaching a therapeutic dose requires several weeks (34,35).

iPSC-derived NK cells offer advantages similar to NK-92 cells, enabling the generation of large numbers of functional NK cells (34). Unlike NK-92 cells, iPSCs do not require irradiation, but they exhibit a less mature phenotype, similar to CB-derived NK cells (34).

The allogenic nature of CAR-NK cells enables the development of "off-the-shelf" products, allowing for the pre-production, expansion, and cryopreservation of CAR-NK cells. This approach reduces production times, logistical complexities, and overall costs, making these therapies more accessible and scalable than CAR-T therapies (32).

Overcoming TME Barriers

Both CAR-T and CAR-NK cells encounter significant barriers in the TME, but CAR-NK cells offer distinct advantages in overcoming immunosuppression and modulating the TME. The TME is rich in immunosuppressive cytokines, such as Transforming Growth Factor-beta (TGF-β), IL-10,

and IL-4, which impair immune cell function. TGF-β, in particular, plays a significant role in suppressing anti-tumor immune responses (36).

CAR-T cells are highly susceptible to TGF-β, which can inhibit their proliferation and effector functions (36). TGF-β achieves this by suppressing TCR signaling and inducing a state of exhaustion in the CAR-T cells (36). This suppression can significantly reduce the efficacy of CAR-T cell therapies (36). Similarly, CAR-NK cells are also affected by TGF-β, which downregulates activating receptors such as NKG2D, leading to reduced cytotoxic activity (37). However, unlike CAR-T cells, CAR-NK cells do not rely on TCR signaling, and studies suggest they exhibit partial resistance to TGF-β–mediated suppression (37). Despite this advantage, TGF-β can still impair CAR-NK function, necessitating strategies to counteract its effects (37). One promising approach involves engineering both CAR-T and CAR-NK cells to express dominant-negative TGF-β receptors, preventing downstream signaling and restoring cytotoxic activity, which has shown therapeutic potential in preclinical models (38).

In addition to immunosuppression, the TME is characterized by antigen heterogeneity, which limits CAR-T cell efficacy due to its dependence on specific antigen recognition for activation (25). Although various strategies have been developed to address this challenge, such as tandem CARs, CAR-NK cells possess an inherent advantage by utilizing both CAR-dependent and CAR-independent mechanisms (8). In addition to targeting tumor cells via engineered receptors, NK cells recognize stressed cells through natural cytotoxicity receptors (NKG2D, NKp30, NKp46) and mediate ADCC via CD16 (8). These innate pathways enable CAR-NK cells to effectively eliminate tumor cells regardless of antigen heterogeneity, enhancing their therapeutic potential in solid tumors (8,39).

Tumor infiltration is another critical barrier for both CAR-T and CAR-NK cells in solid tumors. To improve CAR-T cell migration, various strategies have been explored to engineer these cells to express chemokine receptors that match tumor-secreted chemokines, thereby enhancing their infiltration (12). NK cells naturally express a variety of chemokine receptors, including CXCR3 and CXCR4, which facilitate their migration toward tumor sites (4). Further genetic engineering to overexpress these receptors enhances their ability to follow chemokine gradients and infiltrate

tumors more effectively (4). Additionally, CAR-NK cells can actively recruit other immune cells, including DCs and cytotoxic T cells, by releasing chemokines such as CCL5 and XCL1 (4). This recruitment amplifies the overall immune response against the tumor.

Ongoing Clinical Trials in Hematological and Solid Tumors

CAR-NK therapies are gaining attention as a safer and more versatile alternative to CAR-T therapies. While many targets under investigation for CAR-NK therapies are also explored in CAR-T cell approaches, CAR-NK cells offer distinct advantages. Their lower risk of CRS, neurotoxicity, GVHD, ability to function as off-the-shelf therapies, and natural CAR-independent cytotoxicity make them a promising platform for treating both hematological malignancies and solid tumors (39). These advantages allow CAR-NK cells to target tumors more effectively, even in cases of antigen downregulation or heterogeneity.

Ongoing clinical trials are actively investigating their potential in both hematological malignancies and solid tumors. Preliminary results have demonstrated favorable safety profiles with minimal risks of CRS, neurotoxicity, and GVHD (40). Among the most studied targets, CAR-NK therapies targeting CD19 for B-cell malignancies, BCMA for multiple myeloma (MM), and NKG2D ligands for acute myeloid leukemia (AML) and solid tumors have shown significant potential. Other notable targets include CD123, frequently expressed in AML and blastic plasmacytoid dendritic cell neoplasm (BPDCN), and CD72, an emerging target for T-cell lymphomas and AML.

Solid tumors, a long-standing challenge for cellular therapies due to the immunosuppressive TME, are also being addressed by CAR-NK cells. For instance, CAR-NK therapies targeting Trop2 are currently under investigation for a variety of solid tumors, including non-small cell lung cancer (NSCLC) and pancreatic cancer. Similarly, ROBO1-targeted CAR-NK cells are being studied in pancreatic and other solid tumors, reflecting innovative approaches to address the unique biology of these malignancies. The potential of CAR-NK cells in solid tumors extends beyond target selection. Their ability to infiltrate the TME via chemokine gradients, bypass antigen heterogeneity through CAR-independent mechanisms, and recruit immune cells to amplify the response makes them a promising strategy for solid tumor therapy.

Trials investigating multiple novel antigens, such as Claudin6, GPC3, Mesothelin, and AXL, are underway for ovarian, testicular, and endometrial cancers. These efforts highlight the versatility of CAR-NK therapies in targeting diverse tumor types.

These advancements underscore the versatility and potential of CAR-NK therapies in addressing diverse cancer types and overcoming the limitations of CAR-T therapies. Continued research and further clinical trials will be essential to fully unlocking their therapeutic potential and scalability. For detailed information on clinical trial case numbers, targets, and indications, refer to Table A.1 in the Appendix.

7. *Key Challenges in the Development of CAR-NK Therapies*

CAR-NK therapy holds significant promise in cancer treatment. However, multiple challenges must be addressed to optimize its clinical efficacy and safety. Although early studies demonstrate favorable safety profiles, with reduced risk of CRS, neurotoxicity, and GVHD, the clinical application of CAR-NK therapies remains in its infancy (41).

Limited robust clinical trial data impedes thorough evaluation of adverse effects, refinement of protocols, and the development of strategies to enhance therapeutic efficacy, particularly against solid tumors. For example, while IL-15-engineered CAR-NK cells have shown improved persistence and cytotoxicity, their long-term safety and risks of systemic effects remain underexplored in larger patient cohorts (41). Future research should prioritize well-designed trials to address these challenges, focusing on improving persistence, scalability, and overcoming the immunosuppressive TME.

Challenges in Manufacturing and Genetic Modification

The production of CAR-NK cells involves complex, labor-intensive, and costly processes, including genetic modification and *ex vivo* expansion. One significant challenge is the relatively low transduction efficiency of NK cells compared to T cells, especially when using viral vectors (32). This inefficiency arises from several factors, including the intrinsic antiviral mechanisms of NK cells, their quiescent state, and differences in surface receptor expression that reduce vector uptake (31). NK cells possess antiviral mechanisms that can inhibit viral vector-mediated gene

transfer, making them inherently resistant to common transduction approaches. Furthermore, NK cells are typically in a quiescent state and lack active proliferation, which reduces the effectiveness of viral vectors that rely on cell division for effective gene integration. Additionally, differences in surface receptor expression between NK and T cells further complicate the uptake and incorporation of viral vectors into NK cells (31).

In addition to these genetic modification challenges, NK cells are difficult to culture and expand *in vitro*, leading to variability and inconsistencies in genetic modification outcomes. Viral vector designs often require specific optimization, and culture conditions must be adapted to the unique biological characteristics of NK cells, which adds another layer of complexity (31).

To address these challenges, efforts are underway to optimize viral vector designs, explore non-viral transduction methods such as electroporation, and refine culture conditions to improve NK cell transduction and expansion (31). Standardized, scalable, and cost-effective manufacturing protocols are critical for the widespread clinical application of CAR-NK therapies.

Limited Persistence and Durability

A notable limitation of CAR-NK therapy is the transient *in vivo* persistence of NK cells, which constrains their therapeutic potential. As innate immune effectors, NK cells generally exhibit shorter lifespans than T cells, reducing the durability of therapeutic effects (15). This limitation is particularly problematic in solid tumors, where prolonged immune activity is essential to overcome the immunosuppressive TME and sustain anti-tumor responses (4).

To address this, engineering strategies such as incorporating IL-15 into CAR constructs have shown promise. IL-15 enhances NK cell survival, proliferation, and cytotoxic activity, prolonging their lifespan and improving their therapeutic efficacy. Further advancements in engineering persistence-enhancing strategies will be crucial for expanding the clinical applicability of CAR-NK therapies (8,30).

Challenges and Strategies for Enhancing CAR-NK Cell Therapy in Solid Tumors

Despite the availability of multiple strategies to enhance their efficacy against solid tumors, CAR-NK cells still face significant challenges in achieving optimal targeting and overcoming the

barriers imposed by the TME (39). Key obstacles include hypoxia within the TME, which reduces granzyme activity and downregulates activating receptors, as well as metabolic byproducts that suppress NK cell cytotoxicity, such as TGF-β (37,39). Additionally, poor tumor infiltration remains a major limitation, exacerbated by mismatched chemokine receptor expression and abnormal vasculature, which is disorganized, highly permeable, and restricts blood flow and immune cell trafficking (4,7).

To address these challenges, several approaches have been explored, including the expression of dominant-negative TGF-β receptors to sustain cytotoxic activity, upregulation of chemokine receptors to enhance tumor infiltration, and leveraging CAR-independent mechanisms through CAR design to bypass antigen heterogeneity and target tumor cells more effectively (8,34,38). Furthermore, CAR-NK cells can recruit other immune cells by secreting chemokines, helping to remodel the TME and overcome its immunosuppressive effects (4). Their persistence *in vivo* can also be improved through IL-15 expression, supporting a more durable anti-tumor response in solid malignancies (8,30).

In addition to these intrinsic modifications, combining CAR-NK therapy with immune checkpoint inhibitors and anti-angiogenic drugs holds promise for further mitigating TME-mediated suppression and enhancing therapeutic efficacy (39). Such combinatorial strategies could help unlock the full potential of CAR-NK cells in the treatment of solid tumors.

Unclear NK Cell Classifications Block Full Therapeutic Potential

Not all challenges in the development of CAR-NK therapies are directly linked to the therapeutic approaches themselves, some arise from gaps in our fundamental understanding of NK cell biology. A significant hurdle lies in the lack of a unified NK cell classification system, which complicates our ability to fully exploit their therapeutic potential. For decades, NK cells have been categorized into two primary subsets based on CD56 expression: $CD56^{bright}$ and $CD56^{dim}$ (42,43).

$CD56^{bright}$ NK cells, which represent less than 15% of NK cells in peripheral blood, are characterized by high CD56 and low or absent CD16 expression (42,43). These cells are primarily

immunomodulatory, producing cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) in response to interleukins IL-2, IL-15, and IL-12 (44). Their low cytotoxic potential, marked by limited perforin and granzyme expression, aligns with their roles in secondary lymphoid tissues (SLTs) where they interact with dendritic cells and T cells to modulate adaptive immunity (43,44). In contrast, $CD56^{dim}$ NK cells, the predominant subset in peripheral blood, excel in direct cytotoxicity, facilitated by their high perforin and granzyme levels and robust antibody-dependent cellular cytotoxicity (ADCC) activity mediated by CD16 (42–44). These cells dominate tissues such as the bone marrow, lungs, and adipose tissue (42,43).

Recent advancements in single-cell technologies challenge the classical CD56-based dichotomy (45). Emerging data suggests a more refined classification, identifying subsets such as NK1, NK2, and NK3 (45). These subsets display distinct molecular profiles, with NK1 linked to cytotoxic functions, NK2 associated with protein synthesis and proliferation, and NK3 resembling adaptive NK cells, particularly in response to human cytomegalovirus (HCMV) (45). These findings highlight the limitations of current frameworks in capturing NK cell diversity and their dynamic roles across different tissues and tumor microenvironments.

Tissue-specific NK cell populations further complicate this landscape (43). Tissue-resident NK cells in organs such as the liver, lungs, and uterus adapt to local microenvironments, acquiring unique receptor profiles distinct from circulating NK cells (43). For example, liver-resident NK cells express receptors like NKG2A, crucial for maintaining immune tolerance (46). This underscores the need for incorporating tissue-specific markers into NK cell classification systems to better reflect their functional specialization.

The lack of a unified NK cell classification system limits the advancement of NK cell-based therapies by complicating cross-study comparisons, reducing reproducibility, and obstructing the translation of preclinical insights into standardized clinical protocols. Emerging subsets, such as adaptive and tissue-resident NK cells, highlight significant gaps in understanding their roles in tumor immunotherapy and how microenvironmental influences shape their functions. Developing a standardized classification framework through single-cell technologies and tissue-

specific profiling is essential to address these gaps, refine therapeutic designs, and fully harness the potential of NK cells in cancer immunotherapy and beyond.

8. Exploring NK–T Cells as a Therapeutic Strategy in Cancer

Natural Killer T (NKT) cells are a unique subset of T cells that combine biological characteristics of both T cells and NK cells, placing them at the intersection of innate and adaptive immunity (47,48). NKT cells originate in the thymus, where they follow a distinct developmental pathway. This process involves the selection of double-positive thymocytes (CD4+CD8+) that interact with CD1d molecules presenting self-glycolipid antigens, a mechanism critical for shaping their distinct semi-invariant T cell receptor (TCR) repertoire (47,49).

Unique Characteristics of NKT Cells

Unlike conventional T cells, which recognize peptide antigens presented by classical MHC molecules, NKT cells recognize glycolipid antigens presented by the non-classical MHC-like molecule CD1d, enabling rapid and diverse immune responses (47,48). The TCRs of NKT cells are uniquely structured, with an invariant alpha chain paired with a limited selection of beta chains (47,48). This semi-invariant nature of their TCRs distinguishes them from the highly diverse TCRs of conventional T cells, contributing to their rapid activation and cytokine production (Figure 3) (47,50).

NKT cells are further characterized by the expression of markers shared with both T cells and NK cells, which reflects their hybrid nature (48,49). Their ability to produce a broad array of cytokines, including IFN-γ, IL-4, IL-17, and TNF-α, enables NKT cells to modulate diverse immune responses, making them key regulators of immune homeostasis (47,50).

The functional diversity of NKT cells is underscored by their classification into two primary subsets: Type I invariant NKT (iNKT) cells and Type II variant NKT (vNKT) cells. iNKT cells are defined by their invariant TCRs and their strong response to glycolipid antigens (47,48). They are potent producers of cytokines and play a critical role in activating dendritic cells, NK cells, and CD8+ T cells, thereby amplifying anti-tumor immune activity (47,50). In contrast, vNKT cells, which exhibit a more diverse TCR repertoire, are primarily involved in immunoregulatory functions

(49,51). While their role in tumor immunity is less defined, vNKT cells are thought to counterbalance the pro-inflammatory activity of iNKT cells in certain contexts (48,50).

NKT Cells in Tumor Immunity

Functionally, NKT cells bridge innate and adaptive immunity through their rapid activation and ability to orchestrate downstream immune responses (48,51). Unlike conventional T cells, which often require days to mount an immune response, NKT cells respond within hours, producing cytokines that influence both innate and adaptive immune cells (47,48). Additionally, NKT cells demonstrate cytotoxic capabilities similar to NK cells, utilizing perforin and granzyme pathways to directly kill tumor cells (50,52). However, their cytotoxicity is tightly regulated by their TCR interactions with CD1d, distinguishing their activity from the MHC-independent targeting mechanisms of NK cells (50,52).

NKT cells also play a significant role in shaping the TME. By producing IFN-γ, they enhance the recruitment and activation of cytotoxic lymphocytes, including NK cells and CD8+ T cells, creating an immune-permissive environment (47,49). Furthermore, NKT cells suppress the activity of Tregs and myeloid-derived suppressor cells (MDSCs), both of which contribute to immunosuppression within the TME (51). This dual role of direct tumor cytotoxicity and immune modulation makes NKT cells particularly effective in anti-tumor immunity (47,49).

Advancing Cancer Immunotherapy with CAR-NKT Cells

Given their unique properties, NKT cells are attractive candidates for cancer immunotherapy, particularly in the form of CAR-engineered NKT cells. By leveraging the distinct biological characteristics of NKT cells, CAR-NKT therapy integrates the benefits of CAR-T and CAR-NK therapies while addressing their key limitations (Table 2) (47).

CAR-T and CAR-NK therapies have individually demonstrated substantial promise in treating cancer. CAR-T therapy, the first to reach clinical success, has shown remarkable efficacy in hematological malignancies but faces significant limitations in solid tumors, including poor tumor infiltration, severe CRS, and immune evasion by tumors (12,24). CAR-NK therapy addresses some of these issues, offering improved safety and tumor tropism due to the natural properties of NK

cells (33,39). However, CAR-NK therapies face challenges related to persistence, cytotoxicity, and scalability (15). CAR-NKT therapy has the potential to address most of the critical limitations of CAR-T and CAR-NK therapies.

CAR-NKT cells exhibit a dual mechanism of action that distinguishes them from CAR-T and CAR-NK cells (Figure 3). On the one hand, the engineered CAR enables precise targeting of tumor-specific antigens, much like CAR-T and CAR-NK cells (47,48). However, NKT cells bring an additional layer of specificity through their invariant TCR, which recognizes glycolipid antigens presented by CD1d molecules (48,50). This dual targeting capability reduces the risk of tumor immune escape, a major limitation of CAR-T and CAR-NK therapies that depend solely on CAR-mediated recognition of protein antigens (47,48). Consequently, CAR-NKT cells achieve broader anti-tumor activity, particularly in heterogeneous tumor environments (47–49).

Beyond their unique targeting mechanisms, CAR-NKT cells naturally express chemokine receptors, including CXCR3 and CCR5, that facilitate effective migration to tumor sites, similarly to CAR-NK cells (47,52). This tumor tropism allows CAR-NKT cells to infiltrate solid tumors more effectively than CAR-T cells, which often struggle in this regard (48,50). Furthermore, within the TME, CAR-NKT cells produce cytokines such as IFN-γ, which not only enhance their own cytotoxicity but also reprogram the immune environment by suppressing regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (50,52). This TME-modulating capability is less pronounced in CAR-NK cells, which lack the same cytokine-driven remodeling capacity (48,50).

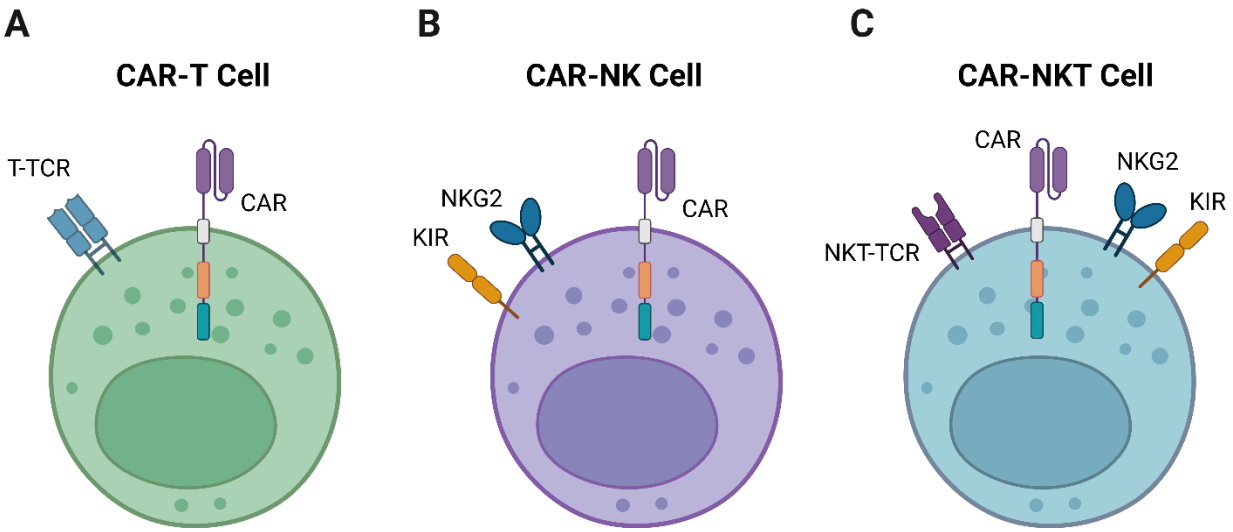

Figure 3. Distinct Features of CAR-T, CAR-NK, and CAR-NKT Cells. **A:** CAR-T Cells feature a CAR for tumor targeting and an endogenous TCR, which does not contribute to tumor killing and limits allogenic use due to risk of GVHD. **B:** CAR-NK Cells possess a CAR alongside activating (e.g., NKG2) and inhibitory (e.g., KIR) receptors, both of which contribute to their tumor-killing activity. **C:** CAR-NKT Cells combine a CAR, an invariant TCR that recognizes glycolipids presented by CD1d (bypassing MHC), and activating/inhibitory receptors, enabling enhanced tumor targeting and elimination (Adapted from Wolf *et al*. 2018 (53)).

Safety and Persistance of CAR-NKT Cells

CAR-NKT therapy offers a safer alternative to CAR-T therapy, which is often associated with severe CRS and neurotoxicity (47,48). CAR-NKT cells maintain a balanced cytokine profile, minimizing excessive inflammatory responses and making them suitable for a broader range of patients (48,50). Additionally, their reduced dependence on MHC presentation lowers the likelihood of immune evasion by tumors (47,49).

CAR-NKT therapy builds on the strengths of CAR-NK therapy while addressing its limitations. While CAR-NK cells have a favorable safety profile and can be derived from allogeneic sources, they face limitations in persistence and durability, often requiring repeated dosing. In contrast, CAR-NKT cells, with their T cell lineage, naturally exhibit greater persistence in vivo (47,49,51). Genetic engineering strategies, such as the incorporation of IL-15 into CAR constructs, further enhance CAR-NKT cell survival and proliferation, making them more effective for long-term cancer control (47,49,51).

Preclinical and Clinical Progress

Preclinical studies have demonstrated the superior performance of CAR-NKT cells in solid tumor models such as neuroblastoma and lymphoma (47,49). These studies highlight their enhanced tumor infiltration, greater resistance to TME suppression, and sustained anti-tumor activity compared to CAR-T and CAR-NK cells (47,48). Early-phase clinical trials are currently evaluating the safety and efficacy of CAR-NKT therapy in human patients, with initial results demonstrating promising anti-tumor responses and minimal toxicity (49,50). These findings reinforce the potential of CAR-NKT therapy as a next-generation treatment for both hematological and solid malignancies (47,48,51).

Table 2. Comparison Between the Features of CAR-T, CAR-NK, and CAR-NKT Cell Therapies.

	CAR-T Cells	CAR-NK Cells	CAR-NKT Cells
Cell Source	Autologous (patient-derived T cells)	Allogenic (NK cells from donors, UCB, PB, NK-92 cell lines)	Allogenic (UCB, PB)
Mechanism of Action	Direct cytotoxicity through CAR-mediated antigen recognition and cytokine production	Direct cytotoxicity via CAR and natural NK mechanisms (e.g., ADCC)	Dual action: CAR-mediated targeting and invariant TCR recognizing CD1d-presented glycolipids
CAR Structure	Includes CD3ζ, CD28, or 4-1BB signaling domains	Includes CD3ζ, DAP10, or DAP12, with optional 2B4 signaling domains	CAR plus invariant TCR
MHC Restriction	Bypasses MHC requirement using scFv for antigen recognition	Independent of MHC: targets stress-induced ligands or antibody-coated cells	Dual specificity: CAR bypasses MHC; invariant TCR recognizes CD1d-presented glycolipids
Manufacturing	Complex and individualized; requires T cell isolation, activation, genetic modification, and expansion	Simplified and scalable using allogeneic sources; allows for off-the-shelf production	Hybrid approach; autologous or allogeneic manufacturing

Scalability	Limited due to patient-specific production	High scalability; potential off-the-shelf CAR-cell products	High scalability; potential off-the-shelf CAR-cell products
Toxicity	High risk of CRS, neurotoxicity, and GVHD	Lower risk of CRS and GVHD; more controlled cytokine profile	Minimal CRS and neurotoxicity; balanced cytokine profile
Persistence	Long-lived; memory T cell formation enables extended responses	Short-lived; requires IL-15 engineering for extended survival	Long-lived; T cell lineage supports persistence; IL-15 engineering enhances durability
Efficacy	High in hematological malignancies; limited in solid tumors due to poor infiltration	Effective against hematological malignancies; moderate efficacy in solid tumors	High efficacy in both solid and hematological malignancies; strong resistance to TME suppression
Therapeutic Window	Narrow; toxicity limits use in some patients	Broad; lower toxicity expands patient eligibility	Broad; reduced toxicity and enhanced safety profile
Application in Solid Tumors	Limited by poor tumor infiltration and antigen heterogeneity	Enhanced by innate tropism and ability to target MHC-deficient cells	Effective; superior infiltration due to chemokine receptors (e.g., CXCR3, CCR5) and TME modulation
TME Modulation	Minimal; limited reprogramming capacity	Moderate; some chemokine and cytokine release	Strong; suppresses Tregs and MDSCs, reprograms immune responses

9. Conclusion

The landscape of cancer immunotherapy has undergone significant transformation with the advent of CAR technologies. CAR-T therapies, the first to achieve clinical success, have demonstrated remarkable efficacy in hematological malignancies, particularly in targeting antigens like CD19 and BCMA (18). However, these therapies face limitations including severe toxicity, manufacturing complexity, and limited success in solid tumors (12,25). These challenges necessitate continuous innovation in CAR-based therapies to extend their benefits to a broader patient population and address unmet clinical needs.

CAR-NK therapies have emerged as a promising alternative, leveraging the innate cytotoxicity, MHC-independent tumor recognition, and broader safety profile of NK cells (22). Compared to CAR-T therapies, CAR-NK cells exhibit a lower incidence of CRS, neurotoxicity, and GVHD (22). Additionally, the ability to derive CAR-NK cells from allogeneic sources enables the development of "off-the-shelf" therapies, improving scalability and cost-effectiveness (32). Despite these advantages, CAR-NK therapies still face challenges, including limited *in vivo* persistence, suboptimal tumor infiltration, and susceptibility to the immunosuppressive effects of the TME (15,33). Advances such as the expression of IL-15 to enhance survival, upregulation of chemokine receptors to improve tumor infiltration, and the harnessing of CAR-independent mechanisms to bypass antigen heterogeneity have shown promise in overcoming these barriers (8,34,38). However, further refinement in these engineering strategies and optimization of combination therapies remain critical to fully realize their therapeutic potential.

One of the major obstacles for CAR-NK therapy in solid tumors is the complex TME, which is characterized by hypoxia, metabolic suppression, and an abnormal vasculature that restricts immune cell trafficking (7). Hypoxia reduces granzyme activity and downregulates activating receptors, while metabolic byproducts further suppress NK cell cytotoxicity (39). The abnormal tumor vasculature, often disorganized and highly permeable, limits effective immune infiltration (4,7). Strategies such as engineering CAR-NK cells to express dominant-negative TGF-β receptors, combining therapy with ICIs and anti-angiogenic drugs, and exploiting the innate chemokine secretion of CAR-NK cells to recruit additional immune cells have demonstrated potential in mitigating these suppressive effects (37,38).

A major challenge in optimizing CAR-NK therapies is the lack of a unified classification system for NK cell subsets. The traditional $CD56^{bright}$ and $CD56^{dim}$ classifications fail to capture the full spectrum of NK cell diversity, including tissue-resident and adaptive NK cells, which may exhibit distinct anti-tumor properties (42,43). Advancements in single-cell technologies have revealed more refined classifications, underscoring the need for standardized frameworks to improve reproducibility and therapeutic optimization (45). Future research should focus on

characterizing these subsets in both physiological and pathological conditions to refine CAR-NK therapy design and improve clinical outcomes.

CAR-NKT therapies represent a hybrid approach that combines the advantages of both CAR-T and CAR-NK cells. By leveraging the invariant TCR, CAR-NKT cells can target glycolipid antigens presented by CD1d, in addition to protein antigens recognized by the engineered CAR (47,48). This dual targeting mechanism enhances tumor recognition and reduces the risk of immune escape. Furthermore, CAR-NKT cells display superior tumor infiltration due to their natural expression of chemokine receptors and possess a more balanced cytokine profile, lowering the risk of severe inflammatory toxicities (47,49). Preclinical and early-phase clinical trials have demonstrated promising efficacy in both hematological and solid tumors, positioning CAR-NKT cells as a next-generation immunotherapeutic approach (49,50). Further research should explore how CAR-NKT cells could be integrated into current treatment paradigms and whether their potential synergy with existing immunotherapies can be maximized.

Beyond scientific and clinical implications, CAR-based immunotherapies hold profound societal significance. The development of more effective, safer, and accessible CAR therapies could reduce the impact of cancer-related morbidity and mortality. The scalability of CAR-NK therapies, particularly in their allogeneic "off-the-shelf" form, has the potential to democratize access to advanced cancer treatments, reducing geographic and economic disparities in healthcare. Furthermore, advancements in CAR-based strategies could pave the way for novel therapeutic applications beyond oncology, including autoimmune diseases and infectious diseases

In conclusion, CAR-NK and CAR-NKT therapies offer promising solutions to the limitations of CAR-T therapies, particularly in terms of safety, scalability, and applicability in solid tumors. While significant challenges remain, the continued refinement of these technologies, supported by robust clinical research and advances in genetic engineering, holds the potential to revolutionize cancer treatment and provide more effective and accessible immunotherapeutic options for patients worldwide.

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11. Appendix

Table A.1. Overview of CAR-NK Cell Clinical Trials Listed on ClinicalTrials.gov.

NCT Number	Study status	Phases	Target	Indication
NCT05247957	Complete		NKG2D	r/r AML
NCT06696846	Active	I	CD72	r/r TCL, r/r AML
NCT06045091	Active	I	BCMA	MM, PCL
NCT05574608	Active	I	CD123	r/r AML
NCT06454890	Active	I/II	Trop2	NSCLC
NCT05776355	Active		NKG2D	Ovarian Cancer
NCT06478459	Active	I	NKG2D	NRPC
NCT05645601	Active	I	CD13	r/r B-cell HM
NCT05734898	Active		NKG2D	AML
NCT05213195	Active	I	NKG2D	r/m CRC
NCT06690827	Active	I	CD123	AML, BPDCN
NCT05673447	Active	I	CD19	DLBCL
NCT06707259	Active	I	CD19	B-NHL
NCT06307054	Active	I	CLL1	r/r AML
NCT05507593	Unknown	I	DLL3	SCLC
NCT05410041	Unknown	I	CD19	ALL, CLL, NHL
NCT03692767	Unknown	I	CD22	r/BCL
NCT03690310	Unknown	I	CD19	r/BCL
NCT05987696	Active	I	CD33/CLL1	AML
NCT04887012	Unknown	I	CD19	B-NHL
NCT05739227	Active	I	CD19	ALL, BCL, CLL
NCT05652530	Unknown	I	BCMA	MM
NCT05410717	Active	I	Claudin6, GPC3, Me sothelin, or AXL	Ovarian Cancer, Testis Cancer, Endometrial Cancer
NCT05472558	Active	I	CD19	B-NHL
NCT05563545	Complete	I	CD19	ALL
NCT03415100	Unknown	I	NKG2D	Solid Tumors
NCT06594211	Active		BCMA	MM
NCT05248048	Unknown	I	NKG2D	r/m CRC
NCT03941457	Unknown	I/II	ROBO1	Pancreatic Cancer
NCT03940820	Unknown	I/II	ROBO1	Solid Tumors
NCT06027853	Active	I	CLL1	AML
NCT06006403	Active	I/II	CD123	AML, BPDCN, r/r Leukemia
NCT05570188	Unknown	I/II	CD19	BCL, B-CL
NCT02944162	Unknown	I/II	CD33/CLL1	AML
NCT06503497	Active	I	NKG2D	Pancreatic Cancer
NCT06379451	Active	I	NKG2D	MM

NCT03931720	Unknown	I/II	ROBO1	Solid Tumors
NCT03940833	Unknown	I/II	BCMA	MM
NCT05845502	Active		GPC3	HCC
NCT05194709	Unknown	I	5T4	Solid Tumors
NCT03824964	Unknown	I	CD29/CD22	r/BCL
NCT02892695	Unknown	I/II	CD19	ALL, CLL, MCL, DLBCL
NCT05215015	Unknown	I	CD33/CLL1	AML
NCT06242249	Active	I/II	BCMA	MM
NCT04796675	Unknown	I	CD19	ALL, CLL, NHL
NCT05008575	Unknown	I	CD33	AML
NCT06631040	Active	I/II	CD19	ALL
NCT05008536	Unknown	I	BCMA	MM
NCT05922930	Active	I/II	TROP2	Pancreatic Cancer, Ovarian Cancer, Adenocarcinoma
NCT06066424	Active	I	TROP2	Solid Tumors
NCT06464965	Active	I	CLDN18.2	Gastric Cancer, Pancreas Adenocarcinoma
NCT02742727	Unknown	I/II	CD7	AML
NCT06464861	Active	I	CD19	MCL, DLBCL
NCT06201247	Active	I	CD123	r/r AML
NCT06652243	Active	I	GPC3	HCC
NCT04623944	Active	I	NKG2D	AML, MDS
NCT05092451	Active	I/II	CD70	BCL, MDS, AML
NCT03056339	Complete	I/II	CD19	B-LM, CLL, NHL
NCT05110742	Active	I/II	CD5	Hematological Malignancy
NCT05667155	Active	I	CD19/CD70	B-NHL
NCT05941156	Active	II	CD56	NKCL
NCT04847466	Active	II	PD-L1	GEJ Cancer, HNSCC
NCT05020015	Active	II	CD19	r/r B-NHL
NCT05020678	Active	I	CD19	ALL, NHL, LBCL, MCL, CLL
NCT05654038	Active	I/II	CD19	BCL, B-CL
NCT06358430	Active	I	TROP2	Colorectal Cancer
NCT06367673	Active	I	CD33/CLL1	AML
NCT04747093	Unknown	I/II	CD19	BCL, B-CL, NK/TCL, B-ALL
NCT04004637	Unknown	I	CD7	T-LBL, NK/TCL, ALL
NCT04639739	Unknown	I	CD19	NHL
NCT05182073	Active	I	BCMA	MM
NCT06325748	Active	I	CD33	AML
NCT05703854	Active	I/II	CD70	Renal Cell Carcinoma,

				Mesothelioma, Osteosarcoma
NCT03692637	Unknown	I	MESO	Epithelial Ovarian Cancer
NCT04796688	Unknown	I	CD19	ALL, CLL, BCL
NCT02839954	Unknown	I/II	MUC1	Solid Tumors
NCT05856643	Active	I	MESO	Ovarian Epithelial Carcinoma
NCT05842707	Active	I/II	CD19/CD70	r/r B-NHL
NCT05686720	Active	I	MESO	Breast cancer
NCT05487651	Active	I	CD19	NHL, BCL, B-CL, DLBCL, ALL