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Review

Natural killer cell-based Immunotherapy for Solid tumors: A Comprehensive Review

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Abstract

Natural Killer Celle (NK) are innate immune cells with potent cytotoxic activity against tumor cells, making them attractive candidates for cancer immunotherapy. While NK cell-based therapies have shown promise in hematologic malignancies, their efficacy against solid tumors remains challenging due to the immunosuppressive tumor microenvironment (TME) and limited NK cell persistence. This review discusses recent advances in NK cell-based immunotherapies, including adoptive NK cell transfer, NK cell engagers, and genetic modifications to enhance their anti-tumor activity. We also explore the barriers to effective NK cell therapies in solid tumors and potential strategies to overcome these limitations.

1. INTRODUCTION

Solid tumors present significant hurdles for immunotherapy due to their ability to create an immunosuppressive environment and evade immune responses. Unlike hematologic malignancies, which are more accessible to immune cells, solid tumors establish complex barriers that inhibit immune infiltration and function (1). The innate immune system's vital components, NK cells, have shown strong anti-tumor properties. For cancer immunotherapy, they are appealing candidates because of their capacity to eradicate cancerous cells without the need for prior antigen sensitization (2). However, the success of Natural killer therapies based on cell in solid tumors remains limited due to several critical challenges.

The tumor microenvironment (TME) is one of the primary obstacles it a highly dynamic and immunosuppressive milieu that promotes tumor survival and resistance to immune attack (3). Solid tumors are surrounded by dense extracellular matrix (ECM) components, such as fibronectin & Collagen, creating physical barriers restricting NK cell infiltration. Additionally, the rapid proliferation of tumor cells leads to oxygen deprivation, resulting in a hypoxic environment that suppresses NK cell cytotoxicity. Hypoxia inducible factor 1 alpha (HIF-1α) down regulates NK cell-activating receptors, reducing their capacity to identify and eradicate tumor cells (4). Furthermore, the TME is rich in immunosuppressive cytokines, including transforming growth factor beta (TGF-β), interleukin 10 (IL10), and prostaglandin E2 (PGE2), all of them are impaired by NK cell activation, proliferation, and cytotoxicity (5).

Another challenge is Natural killer cells' inefficient homing and persistence in solid tumors. Unlike T cells, which rely on antigen-specific receptors to target tumor cells, NK cells depend on a balance of activating and inhibitory signals (6). However, tumors often evade from NK cell recognition by upregulating immune checkpoint molecules such as programmed death-ligand 1 (PD-L1) and human leukocyte

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antigen (HLA)-E, which engage NK cell inhibitory receptors and reduce their cytotoxic potential (7). NK cells exhibit relatively short-lived persistence in vivo, limiting their sustained antitumor effects. Once inside the tumor site, NK cells often undergo exhaustion due to chronic exposure to inhibitory signals, further reducing their effectiveness (8).

Several strategies are being explored to improve Natural Killer cell-based immunotherapies for solid tumors. Genetic Engineering approaches, such as Chimeric antigen receptors (CARs) NK cells, enhance specificity and persistence by introducing tumor-targeting receptors (9). Additionally, cytokine preconditioning using Interleukin-15 (IL-15) or Interleukin-21 (IL-21) has been shown to enhance NK cell survival and function. Overcoming physical barriers can be achieved through strategies that degrade the ECM, as an example using heparinase or matrix metalloproteinases (MMPs) to enhance NK cell infiltration (10-11). Combining NK cell therapies with Immune checkpoint inhibitors, such as anti-PD-L1 or anti-TGF-β antibodies, can help restore NK cell activity within the TME (12). Furthermore, improving NK cell homing through chemokine receptor modifications, such as CXCR3 and CCR5 expression, may enhance their migration to tumor sites (13).

Despite these challenges, Natural Killer cell-based immunotherapy is evolving rapidly, with on-going clinical trials investigating novel strategies to improve the effectiveness of NK cells in solid tumors. Addressing the barriers TME poses, improving NK cell persistence, and optimising combination therapies will be crucial for successfully applying NK cell-based immunotherapies in solid malignancies.

2. NATURAL KILLER CELL SUBSETS, DIFFERENTIATION, AND FUNCTIONAL PLASTICITY

Natural killer cells are key immune cells that are innate and belong to the group 1 innate lymphoid cells (ILCs), lacking antigen specific receptors. They are primarily recognised for their potent cytotoxic abilities and the production of hefty amount of interferon gamma (IFN-γ), with their development relying on the transcription factor (t bet), though not exclusively. Human NK cells originate from CD34+ hematopoietic progenitors in the bone marrow, progressing through a series of differentiation stages. These progenitors first give rise to common lymphoid progenitors, which differentiate into pre-NK and other innate lymphoid precursors (14). The Natural Killer precursors further develop into functional NK cells, expressing interleukin-15 receptor (IL-15R) and the hallmark surface marker CD56 (15). Based on CD56 & CD16 expression, Natural Killer cells are divided into two significant subpopulations: CD56brightCD16−/low and CD56dimCD16+ NK cells (16) The CD56dimCD16+ subset represents 90– 95% of circulating NK cells and exhibits potent cytotoxicity, primarily mediating direct tumor cell killing. In contrast, the CD56 bright CD16−/low subset, which accounts for 5–10% of NK cells, is less cytotoxic but plays a significant immunoregulatory role by secreting cytokines like IFN-γ, TNF-α, and GM-CSF, contributing to adaptive immune responses (17). While traditionally thought to be distinct lineages, the CD56brightCD16−/low NK cells are also considered precursors to CD56dimCD16+ NK cells, transitioning into a fully mature cytotoxic phenotype under stimulation with IL-2 or IL-15 (18). Instead of homing molecules controlling the thymus and their dispersion throughout the body, NK cells mature in bone marrow and secondary lymphoid tissues, in contrast to T cells.CD56bright NK cells, expressing CCR7 and L-selectin, preferentially migrate to secondary lymphoid organs, while CD56dim NK cells, expressing CX3CR1 and CXCR1, localise to peripheral tissues (19).

In addition to conventional circulating NK cells (cNKs), there is growing recognition of tissue-resident NK cells (trNKs), which differ in phenotype and transcriptional regulation from cNKs. These trNKs are influenced by their microenvironment, displaying tissue-specific characteristics (20). For instance, renal trNKs (CD49a+CD49b−) depend on T-bet, whereas salivary gland trNKs (CD49a+CD49b+) develop independently of T-bet and NFIL3, with TGF-β playing a critical role in their differentiation. These studies imply that NK cells can adapt to distinct tissue contexts, developing unique functional features (21).

Beyond their classical innate immune functions, NK cells also exhibit immunological memory-like properties. Memory-like NK cells (MLNKs) grow via two primary mechanisms (22). The first is about antigen-specific memory NK cells, which, like T and B cells, grow after being stimulated by haptens or viruses. The second pathway generates cytokine-induced memory-like (CIML) NK cells, which arise after exposure to Interleukin-12, Interleukin 15, and Interleukin 18, enabling them to maintain enhanced functional responses for weeks to months (23).

Additional NK cell subsets contribute to immune regulation. HLA-DR+ NK cells exhibit features of both Natural Killer cells and dendritic cells, enabling them to present antigens and activate T cells. Regulatory NK cells (CD27+CD11b+/−) have reduced cytotoxicity but secrete immunosuppressive cytokines such as IL-10 and TGF-β, dampening immune responses (24). NK cells can also act as "helper" cells, aiding in the maturation of type-I polarised

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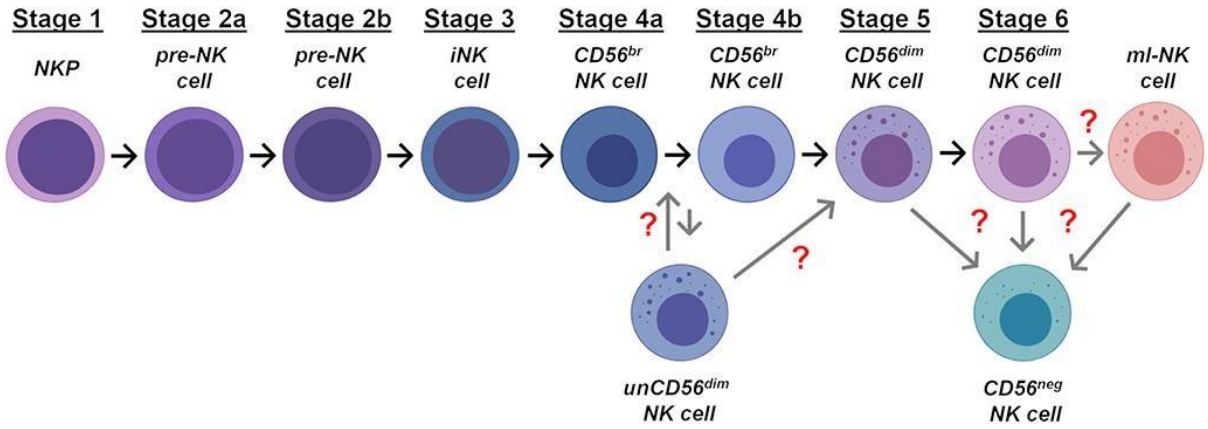

Figure 1. Steps of NK cell ontogenesis. Diagrammatic representation of the many stages of NK cell formation in human bone marrow and secondary lymphoid organs. Where unCD56dim, CD56neg, and ml-NK cells might be found during the NK cell proliferation process is indicated by red question marks and gray arrows (***Courtesy to Shi et al 2024***).

dendritic cells (DCs), particularly under stimulation by IL-18 in combination with type-I interferons or IL-2. Overall, NK cells encompass a diverse range of subtypes, each fulfilling specialised roles in immune defense, immune regulation, and tissue homeostasis (25).

NK cell ontogenesis phases: Grey arrows and red question marks show the potential location of unCD56dim, CD56neg, and ml-NK cells in NK cell development. This diagram illustrates the many stages of NK cell differentiation in human bone marrow and secondary lymphoid organs (26).

3. NATURAL KILLER CELLS AND THEIR ROLE IN CANCER

3.1. Mechanisms of NK Cell-Mediated Tumor Recognition and Cytotoxicity

By destroying tumor cells via a variety of methods, natural killer cells are essential to the immune system's defense against cancer. NK cells are a quick and efficient line of defence against cancers because, in contrast to T cells, they don't require prior sensitisation or antigen presentation (26-27) They can differentiate between healthy and aberrant cells thanks to a complex balance of activating and inhibiting signals, which underpins their cytotoxic action. The primary mechanisms through which NK cells eliminate tumor cells include direct cytotoxicity, antibody-dependent cell-mediated cytotoxicity (ADCC), cytokine secretion, and the granule exocytosis pathway. Among these, direct cytotoxicity is one of the most well-characterized and critical pathways in NK cell-mediated tumor elimination (28).

3.1.1. Direct Cytotoxicity: NK Cell Activation via Stress Ligands

Finding stress-induced ligands that are upregulated on cancer cells is one of the basic ways NK cells identify and eliminate tumor cells. These ligands serve as distress signals, alerting NK cells to cellular abnormalities caused by DNA damage, oncogenic transformation, or viral infections (29). Activating receptors on the surface of NK cells facilitates the identification of these ligands.

NKG2D is one of these receptors that is essential for tumor identification. NKG2D interacts to UL16binding proteins (ULBP1-6) and MHC class I-related chains A and B (MICA/MICB), which are commonly increased on tumor cells in response to cellular stress (30). NKG2D and its ligands combine to trigger a series of intracellular signalling events that activate NK cells and release cytotoxic granules containing granzymes and perforin, which cause the target cell to undergo apoptosis (31). NK cells possess additional activating receptors besides NKG2D, namely DNAM-1 (CD226), which recognizes CD112 (Nectin-2) and CD155 (PVR), which are molecules frequently overexpressed in different kinds of malignancy. The natural cytotoxicity receptors (NCRs)—NKp30, NKp44, and NKp46—also play crucial roles in tumor recognition (32). These receptors interact with ligands such as B7-H6, proliferating cell nuclear antigen (PCNA), and viral hemagglutinins, further enhancing NK cell activation (33).

NK cells undergo cytoskeletal reorganization once the activating receptors bind to their respective ligands on tumor cells, creating an immunological synapse between the NK cell and its target (34). The release of cytotoxic granules containing perforin, a pore-forming protein that forms

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channels in the tumor cell membrane to allow granzymes to enter, is facilitated by this synapse (35). Granzymes are serine proteases that cause the target cell to undergo apoptosis, which results in the cell's eventual planned death. One of the most effective ways NK cells eradicate tumors without first sensitizing to antigens is by this direct killing method (36).

3.2. Regulation by Inhibitory Receptors: The Balance Between Killing and Self-Tolerance

While NK cells possess powerful cytotoxic abilities, their activity is tightly regulated by inhibitory receptors to prevent unintended damage to healthy tissues. These inhibitory receptors primarily recognise major histocompatibility complex (MHC) class I molecules, which are ubiquitously expressed on normal cells. The presence of MHC class I molecules on a cell sends an inhibitory signal to NK cells, preventing them from initiating a cytotoxic response. This system ensures that NK cells selectively target abnormal or malignant cells while sparing healthy ones (37). Among the most significant inhibitory receptors are the Killer cell immunoglobulin-like receptors (KIRs), which recognise classical MHC class I molecules such as HLA-A, HLA-B, and HLA-C. When an NK cell engages with a normal, healthy cell expressing MHC class I, the interaction between KIRs and MHC class I molecules suppresses NK cell activation, preventing unnecessary destruction (38). Another key inhibitory receptor is NKG2A, which binds to HLA-E, a non-classical MHC class I molecule. The engagement of NKG2A by HLA-E transmits a strong inhibitory signal, effectively dampening NK cell-mediated cytotoxic (39).

3.3. NK Cell-Mediated Antibody-Dependent Cellular Cytotoxicity (ADCC) Solid Tumor Therapy

Antibody-dependent cellular cytotoxicity (ADCC) is a crucial mechanism through which natural killer (NK) cells eliminate tumor cells, particularly in monoclonal antibody-based cancer therapies. ADCC enhances the ability of NK cells to recognise and destroy solid tumor cells that might otherwise evade direct immune surveillance. This mechanism relies on the interaction between therapeutic antibodies, tumor cell surface antigens, and Fc receptors (FcγRIII, CD16) on NK cells (40).

3.4. Binding of Therapeutic Antibodies to Tumor Cells

Many solid tumors overexpress specific surface antigens, such as HER2 (in breast cancer), EGFR (in colorectal and lung cancer), and PD-L1 (in various cancers). Monoclonal antibodies (mAbs) such as trastuzumab (HER2-targeting) and cetuximab (EGFR-targeting) are designed to bind to these tumor associated antigens (41).

Recognition by NK Cells via CD16 Receptor: Once antibodies coat the tumor cell surface, the Fc region of the bound antibody is recognised by the FcγRIII (CD16) receptor on NK cells. This interaction triggers intracellular signalling cascades, leading to NK cell activation (42).

3.5. NK Cell Activation and Cytotoxic Response

After activation, NK cells release granzymes and perforin, which cause the tumor cells to undergo apoptosis. They also release pro-inflammatory cytokines including TNF-α and IFN-γ, which attract additional immune cells and strengthen antitumor immune responses (43).

3.6. Elimination of Tumor Cells

Through ADCC, NK cells can effectively eliminate antibody-coated tumor cells, providing a powerful immune response against solid tumors. Unlike direct cytotoxicity, which relies on stress-induced ligands, ADCC allows NK cells to target tumors even if they evade other immune recognition pathways (44).

3.7. Cytokine Secretion by NK Cells in Tumor Immunity

In addition to their direct cytotoxicity, natural killer (NK) cells secrete pro-inflammatory cytokines, especially tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), which are essential for anti-tumor immunity. These cytokines contribute to both direct tumor suppression and immune system activation, shaping the tumor microenvironment (TME) to favour immune-mediated tumor rejection (45).

3.7.1. Interferon-Gamma (IFN-γ): This potent cytokine exerts anti-tumor effects by enhancing antigen presentation on tumor cells, making them more recognisable to cytotoxic T lymphocytes (CTLs). IFNγ also upregulates MHC class I expression, facilitating adaptive immune responses and recruiting additional immune cells, such as macrophages and dendritic cells, to the tumor site. Moreover, IFNγ promotes the polarisation of macrophages toward an M1-like phenotype, which supports inflammation and tumor destruction (46).

3.7.2. Tumor necrosis factor alpha (TNF-α): TNF α contributes to direct tumor suppression by inducing apoptosis through death receptor signalling. It also disrupts tumor vasculature,

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limiting blood supply and nutrient availability to malignant cells. Furthermore, TNF-α contributes to maintaining an inflammatory milieu that improves the activation and recruitment of additional immune cells, thereby fortifying the anti-tumor response (47).

By secreting these cytokines, NK cells bridge innate and adaptive immunity, reinforcing immune-mediated tumor elimination. However, tumors often develop immunosuppressive mechanisms to counteract NK cell function, such as secreting TGF-β to inhibit IFN-γ production or recruiting suppressive immune cells like regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) to dampen inflammation (48-49).

3.8. Granule Exocytosis: A Key Mechanism of NK Cell Cytotoxicity

Natural Killer (NK) cells eliminate tumor cells primarily through the granule exocytosis pathway, a highly efficient and tightly regulated cytotoxic mechanism. Within their cytoplasm, NK cells contain specialized secretory granules filled with cytotoxic proteins, including perforin and granzymes (50). Upon recognising a malignant or stressed target cell, NK cells form an immunological synapse, a highly organised interface between the NK cell and its target (51).

Once the synapse is established, NK cells undergo polarisation, directing their cytotoxic granules toward the target cell. The degranulation process then releases these granules into the synaptic cleft (52). Perforin, a pore-forming protein, integrates into the tumor cell membrane, creating micropores that disrupt membrane integrity. These pores permit for the entry of granzymes, a family of serine proteases, into the tumor cell's cytoplasm (53). Granzymes, particularly granzyme B, initiate a caspase-dependent apoptosis cascade, leading to DNA fragmentation, mitochondrial damage, and eventual tumor cell death (54). This mechanism enables NK cells to rapidly and efficiently eliminate tumor cells without affecting surrounding healthy tissue. Additionally, granzyme-induced apoptosis prevents inflammation and reduces the risk of tumor cell resistance, making it a critical pathway for innate immune surveillance and cancer immunotherapy.

3.9. NK Cell-Mediated Tumor Killing via Death Receptor Pathway

NK cells express key apoptosis-inducing ligands, such as Fas Ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), as shown in the image below (147). These ligands bind to their respective death receptors Fas (CD95) and TRAIL receptors (DR4/DR5) which are upregulated on tumor cells in response to cellular stress or immune signalling(55) An intracellular cascade is initiated upon ligand- receptor binding, activating caspase-8 and downstream caspase-3, leading to DNA fragmentation, mitochondrial damage, and tumor cell apoptosis(56) This death receptor-mediated killing is particularly effective against tumor cells resistant to perforin/granzyme-mediated apoptosis. The TRAIL pathway is also a favourable target for immunotherapy, as it selectively kills cancer cells while sparing normal tissues (57).

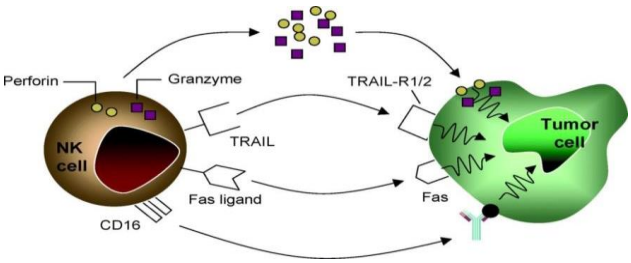

Figure 2. Through the perforin granzyme pathway or death receptor ligands like tumor necrosis, NK cells can also directly cause tumor apoptosis. Their cell surfaces express either Fas ligand or TNF-related apoptosis-inducing ligand (TRAIL), which directly cause tumor death through their corresponding receptors (Courtesy Srivastava S et al 2008).

4. CHALLENGES OF NK CELL THERAPY IN SOLID TUMOR S

Despite the promising potential of natural killer cell therapy in cancer treatment, its effectiveness against solid tumors faces several critical challenges. One of the major hurdles is the tumor microenvironment (TME), which creates an immunosuppressive milieu that limits NK cell infiltration, activation, and persistence (58). NK cell function is actively suppressed by a variety of inhibitory cytokines, including TGF-β, IL-10, and IDO, as well as suppressive immune cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Furthermore, the physical structure of solid tumors, characterised by dense extracellular matrix (ECM) components, prevents efficient NK cell penetration, reducing their ability to reach and eliminate tumor cells effectively (59). Another prominent challenge is the limited persistence and survival of NK cells in vivo. Unlike T cells, NK cells have a shorter lifespan and require continuous cytokines like IL-15 and IL-21 stimulation to maintain their cytotoxic activity (60). However, the absence of these growth factors in the TME often leads to rapid exhaustion and reduced antitumor efficacy (61). Furthermore, tumor cells employ multiple immune evasion mechanisms, including the

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downregulation of stress ligands (such as MICA/MICB) that are recognised by activating NK receptors and the upregulation of inhibitory ligands (such as HLAE and PD-L1) that suppress NK cell activity (62). Some tumors also releases soluble versions of these ligands to interfere with NK cell function at a systemic level.

Another obstacle is the inefficiency of Natural Killer cell homing & infiltration into solid tumors. Unlike hematologic malignancies, where Natural Killer cell have direct access to circulating cancer cells, solid tumors poses a significant barrier to immune cell entry (63). NK cells express limited levels of chemokine receptors required for tumor homing, making it difficult to migrate into the tumor core (64). Engineering NK cells with enhanced CXCR4, CXCR3, or CCR7 expression is an emerging approach to address this limitation, but further optimisation is required for clinical translation (65). Moreover, the challenges in large-scale manufacturing, gene editing, and cryopreservation of NK cells present additional barriers to widespread clinical application (66). Unlike T cell therapies, NK cells are more resistant to viral transduction, making CAR-NK engineering less efficient. Additionally, conventional cryopreservation methods using dimethyl sulfoxide (DMSO) significantly reduce NK cell viability and function after thawing (67). Recent advances in iPSC-derived NK cells and feeder-free expansion methods are being explored to develop standardised, off-the-shelf NK cell products. However, cost and scalability remain significant concerns (68).

4.1. Adoptive NK cell delivery in solid tumor s, both preclinical and clinical

Treating solid tumors remains a significant challenge, as patients often endure multiple treatment modalities, including radiotherapy, chemotherapy, immunotherapy, and even CAR-T therapy. However, recent findings from preclinical studies and clinical trials have demonstrated that adoptive NK cell transfer holds great promise as a prominent therapeutic approach for solid tumors (69).

4.2. Infusion of non-genetically altered NK cells

Numerous solid tumors, such as neuroblastoma and malignancies of the kidneys, ovaries, pancreas, breast, and lungs, have been investigated for treatment using non-genetically modified NK cell infusion (Table 1). Natural killer cell therapy has demonstrated promise in terms of safety and effectiveness, despite the fact that the therapeutic results have differed depending on the type of tumor. The safety profile and clinical efficacy of NK cell-based treatments for solid tumors will be further examined in the sections that follow.

Depending on whether autologous or allogeneic transplantation is utilized, the safety results of NK cell therapy can differ. NK cells made from CD3-depleted peripheral blood mononuclear cells (PBMCs) were reinfused into patients with solid tumors as part of an autologous transplant clinical trial. According to the data, the therapy was well tolerated; no serious side effects or toxicities were noted (70). However, only three out of ten patients exhibited stable disease, and none of them obtained complete remission, indicating that the treatment efficacy was limited (70). Likewise, autologous NK cell therapy did not produce a clinical response but was considered safe for patients with cancer of the digestive system (71). Whether the limited efficiency resulted from the inhibitory interactions between tumor cells and self-derived NK cells (72) or from the temporary activation state of NK cells after ex vivo production [10,70] is still unknown. Notwithstanding these difficulties, autologous NK cell therapy has a favourable safety profile, which implies that combining it with other forms of treatment may improve its anticancer effects (71).

On the other hand, allogeneic NK cell therapy, though generally not associated with graft-versus-host disease (GvHD) (72), has been reported to exacerbate T-cell alloreactivity. In one study, 5 out of 9 transplant recipients developed acute GvHD following allogeneic NK cell infusion, with 3 patients experiencing severe Grade 4 GvHD. The incidence of GvHD was exceptionally high in patients who received NK cells from matched unrelated or matched sibling donors with high CD3 chimerism (73). Nevertheless, successful cases have been observed in hematologic malignancies following haploidentical NK cell transplantation, particularly when the donor's HLA ligands were mismatched with the recipient's inhibitory KIRs, leading to enhanced NK cell activity (KIR-HLA receptor-ligand mismatch) (74).

Regarding therapeutic efficacy, the expansion of adoptively transferred NK cells in vivo plays a critical role in attaining tumor remission. This expansion is influenced by the patient's lymphocyte levels and the degree of immunosuppression induced by preconditioning regimens such as cyclophosphamide and fludarabine. In a research on acute myeloid leukaemia (AML), patients who received NK cell infusion after high-dose cyclophosphamide and fludarabine exhibited elevated endogenous IL-15 levels, indicating enhanced NK cell expansion. This led to complete hematologic remission in 5 of 19 individuals with a dismal prognosis. In the same trial, solid tumors did not,

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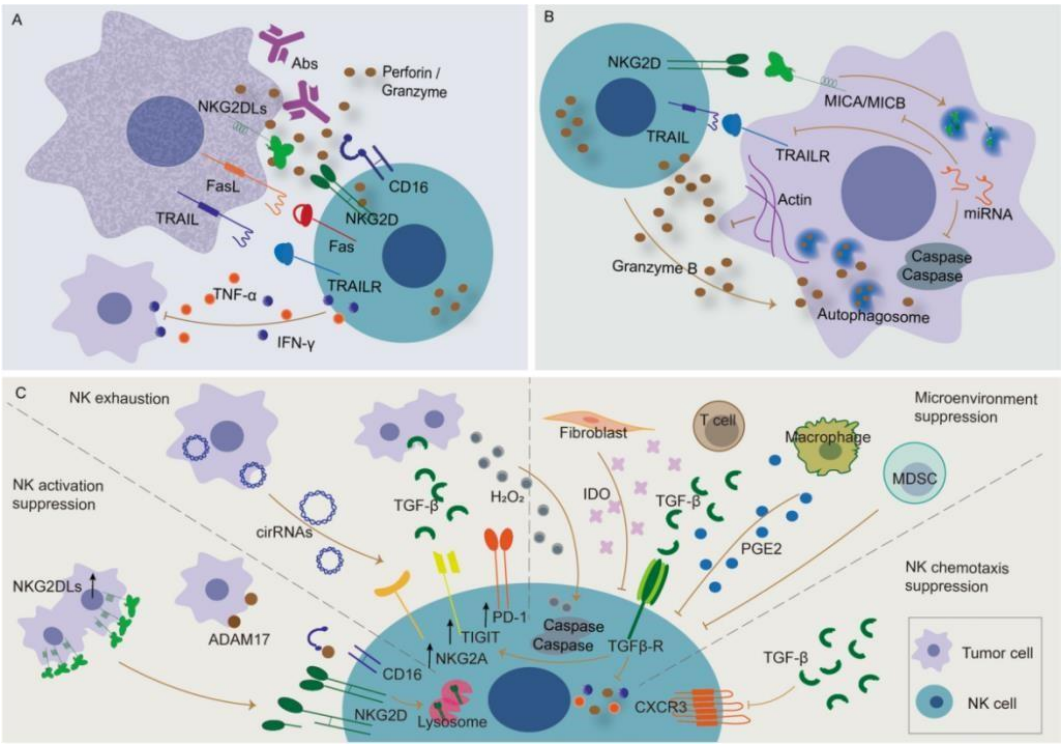

Figure 3. **The relationship between tumor cells and NK cells**. A. NK cells inhibit tumors by four ways. **A.** Through CD16-dependent ADCC, the release of perforin and granzyme, the secretion of TNF-α and IFN-γ, or the induction of apoptosis via Fas/FasL and TRAIL/TRAILR signals, NK cells can promote the cell death of the target tumor. **B.** The resistance of tumor cells against NK cell cytotoxicity. Tumor cells initiate anti-apoptotic pathways, inactivate/eliminate toxicity granules, and shield corresponding activating ligands of NK cells. **C**. Illustration of NK cell immunosuppression in the TME. By producing soluble modulators and creating hypoxic conditions that adversely affect NK cell activation, motility, and effector function, the TME suppresses NK cell activity and encourages NK cell exhaustion and apoptosis. B and C both help tumor cells evade the immune system (***Courtesy Shi Y 2024***).

however, show any appreciable improvement (75). Additionally, host immunological responses had an impact on the durability of infused allogeneic NK cells. Allogeneic NK cells remained viable for up to four days following a single infusion, however their durability following several injections varied from a few hours to three days. Donor NK cell-specific antibodies were found more frequently following multiple injections than following a single injection. Clinical outcomes were better for patients who had multiple high-dose injections than for those who received a single low-dose injection, but the difference was not statistically significant (76). To further enhance NK cell efficacy, non-genetically modified NK cells are combined with monoclonal antibodies (mAbs) in clinical trials targeting solid tumors. One promising approach involves the combination of NK cells with anti-GD2 mAb, which binds to the disialoganglioside GD2 expressed on neuroblastoma cells. In a trial involving 13 patients with recurrent or refractory neuroblastoma, 11 received both NK cells and anti-GD2 mAb, achieving an overall response rate of 61.5%, with 5 patients maintaining stable disease (77). Similarly, NK cells plus the anti-PD-1 drug pembrolizumab increased survival rates in patients with advanced PD-L1+ non-small cell lung cancer (NSCLC). When compared to patients treated with pembrolizumab alone, patients who got the combo therapy had a longer overall survival. Interestingly, patients who had several sessions of NK cell injection lived longer overall—18.5 months—than those who only received one treatment (13.5 months) (78). Furthermore, patients on combination therapy showed decreased PD-1 expression, indicating enhanced NK cell activity and activation (78). These results demonstrate how adoptive NK cell treatment and monoclonal antibodies can work together to increase antitumor responses and promote patient outcomes.

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Table 1. Clinical studies using NK cell-based therapies in solid tumors.

Condition	Source	Ex-vivo Preparation	Combination therapy	# of patient	Outcome	Phase	Identifiers
Advanced Solid tumors	Allogeneic/PB	Autologous feeder cells triggered by irradiation	Chemotherapy	17	SD in 7 patients PD in 8 patients PFS in patients with SD was 4 months	I	NCT01212341 (80)
Advanced Solid tumors	NK92 cell	IL-2	Chemotherapy (1 with HSCT)	15	SD in 1 patient MR in 2 patients PD in 12 patients 2 patients died after HSCT	I	(81)
Digestive cancer	Autologous/PB	OK432, IL2/FNCH296 induced T cells	–	14	NR in all patients	I	[71) us NK cell
Neuroblastoma	Haploidentical	IL-2 preactivated ed	anti-GD2 mAb/ cyclophosphamide	35	CR in 5 patients;	I	NCT00877110 [82)
Solid tumors	Allogenic/PB	K562mb1541BBL activated	HSCT	9	CR in 2 patients; 4 patients remain alive	I	NCT01287104 [83)
Solid tumors	Autologous/PB	IL-2 preactivated ed	Chemotherapy	10	SD in 3 patients; PR in 1 patient; PD in 6 patients	I/IIa	(84)

CR: completed remission; NR: no response; MR: mixed response; mAb: monoclonal antibody; OS: overall survival; PFS: progress free survival; PD: progressive disease; PR: partial response; PBSC: peripheral blood stem cell; SD: stable disease

4.3. NK cells derived extracellular vesicles as an alternative to cell therapy

Although NK cell infusion has demonstrated initial success in treating solid tumors, challenges such as limited infiltration into the tumor core and tumor microenvironment (TME) continue to hinder its clinical application (85). As a cell-free therapeutic option, extracellular vehicles (EVs) derived from NK cells offer a potential alternative or complementary approach, aiming to overcome these obstacles, particularly in solid tumor treatment (86). Extracellular vesicles are membrane-bound structures that carry various bioactive molecules. They function as intercellular messengers, released by donor cells and received by recipient cells (87). NK cell-derived EVs have been successfully isolated from the culture supernatant of NK-92 cells, PBMCs, and primary NK cells (148). Human NK cells are capable of releasing EVs under both stimulated and unstimulated conditions. These EVs express key NK biomarkers, including CD56, the NK-activating receptor NKG2D, and natural cytotoxicity receptors NKp30, NKp44, and NKp46, as well as cytotoxic proteins such as FasL and perforin (88). Studies have shown that receptor-ligand interactions, such as Fas/FasL binding, can induce apoptosis in tumor cells through a caspase-dependent mechanism (89). Additionally, perforin and granzyme delivered by NK cell-derived EVs trigger apoptosis in target cells through both caspase-dependent and caspase-independent pathways. Notably, NK cell-derived EVs selectively target activated immune cells, suggesting that they possess both antitumor and immune-regulatory properties (90). Furthermore, NK EVs have been explored as nanoscale carriers for chemotherapy drugs and therapeutic nucleic acids, enhancing their anticancer efficacy in both hematologic malignancies and solid tumors (91 -92). The functional properties of NK cell-derived EVs may vary depending on the stimuli used during their generation, providing a means to regulate and optimize their therapeutic potential (93-94).

4.4. Comparison of NK and T cell based-cancer therapies

Currently, adoptive cellular immunotherapies primarily encompass CAR-T cells, tumor -infiltrating lymphocytes, T-cell receptor-engineered T cells, and genetically modified NK or dendritic cells (95). With the extensive research focus on T-cell therapies, particularly CAR-T cell therapy, we will compare the procedural aspects of T and NK cell-based cancer treatments, highlighting their respective benefits and drawbacks, as well as the challenges they face and potential strategies for improvement.

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4.5. Clinical protocols for cancer treatments based on adoptive T and NK cells

At present, clinical trials for adoptive cell therapies are only approved for patients who have not responded to existing treatments or have no other therapeutic options available (96). Before undergoing clinical trials, patients scheduled to receive autologous immune cell transplantation must undergo a risk evaluation. Challenges may arise in obtaining cells of adequate quality or quantity, and some patients may struggle to tolerate the required lymphodepletion prior to cell reinfusion. In the case of allogeneic immune cell transplantation, both the donor and recipient must undergo human histocompatibility antigen matching assessments (97). For NK cell transfer, an additional evaluation of KIR and HLA-I antigen mismatching is required, as these genes play a crucial role in determining the efficacy of allogeneic NK cell therapy. Mismatches in these genes can directly impact clinical outcomes (98). For instance, Patients with missing KIR-HLA pairings had a better progression-free survival (PFS) rate after receiving hematopoietic stem cell transplantation (HSCT) from unrelated donors for hematological malignancies (99).

Following these evaluations, apheresis products are collected and purified, after which CAR genes are introduced via retroviral or lentiviral transduction. The resulting CAR-expressing products are then sorted, activated, and expanded in vitro (100). Typically, the production of CAR products takes approximately 2–4 weeks (101).

Before receiving CAR products, patients are hospitalized at least a week in advance for lymphodepletion. Chemotherapy agents such as fludarabine phosphate and cyclophosphamide, along with total body irradiation, help suppress the patient's immune system, reducing the likelihood of donor cell rejection (102). While immune resistance is not an issue with autologous cells, lymphodepletion enhances the persistence of re-infused cells and diminishes immunosuppressive myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), creating a more favourable environment for adoptive cell therapy (103). At this stage, patients must undergo a second evaluation to determine their continued eligibility, as some may no longer qualify by the time of administration. For example, disease progression during the cell preparation phase could render a patient too weak to tolerate the procedure, or their survival expectancy may fall below the study duration (104).

Following cell infusion, the treatment's objective response and disease progression are assessed using standardized guidelines such as the Response Evaluation Criteria in Solid Tumors (RECIST) (116). Since CAR-based therapies function as "living drugs," long-term monitoring is necessary to evaluate the survival and persistence of immune cells in vivo (105). Additionally, technologies that allow physicians to regulate infused cells in case of adverse events are essential. One such strategy involves engineering CAR constructs to express suicide genes like inducible caspase 9 (iCasp9), enabling selective depletion of administered cells in response to toxicity (106).

Significant differences exist between the clinical procedures of CAR-T and CAR-NK cell therapies. In autologous CAR-T therapy, T-cell collection may be insufficient due to prior chemotherapy. While allogeneic CAR-T cells could address this limitation and reduce production delays for patients with rapidly progressing diseases, they are associated with severe side effects, including cytokine release syndrome (CRS), graft-versus-host disease (GvHD), and immune effector cell-associated neurotoxicity syndrome (ICANS) (107). These complications necessitate intensive hospitalization, significantly increasing treatment costs and hindering the widespread clinical application of CAR-T therapy (108).

In contrast, CAR-NK therapy mitigates several of these challenges. Allogeneic CAR-NK cells can recognize tumor s through MHC-independent mechanisms with a lower incidence of CRS (109). Moreover, allogeneic NK cells expressing KIRs that do not recognize HLA-I molecules on patient cells can prevent GvHD (109). The availability of multiple allogeneic NK cell sources—including the NK-92 cell line, umbilical cord blood, human embryonic stem cells, and induced pluripotent stem cells (iPSCs)—expands the NK cell supply, allowing for rapid production of CAR-NK products without reliance on a single donor source (110-111). Additionally, unlike CAR-T cells, NK cells can be administered in multiple doses as "off-the-shelf" products, as they do not require MHC-dependent antigen presentation. This enables their use in multiple patients without the need for HLA matching (112). However, CAR-NK therapy faces technical challenges related to in vitro cell expansion, gene engineering, and cryopreservation (113). Two primary methods for expanding NK cells for clinical use have been developed. One approach involves co-culturing NK cells with the leukaemia feeder cell line K562, which expresses membrane-bound IL-21 and 4-1BBL, resulting in a 47,567-fold expansion in vitro (114). Alternatively, cytokine-induced memory-like (CIML) NK cells, which do not require feeder cells, serve as a reliable source for NK cell therapy. In a phase I trial, CIML NK cells accounted for more than 90% of blood NK cells at day 7, with a mean expansion of (419 ± 166)-fold compared to day 1 (115).

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Gene delivery technologies, including murine retroviral and HIV-derived lentiviral vectors, have advanced CAR product research (116). These techniques have been successfully applied to generate high-efficiency, stable CAR-T cells, some of which are undergoing clinical trials (117). However, NK cells exhibit natural resistance to viral infection, resulting in relatively low transduction efficiency (118). To obtain sufficient CAR-NK cells, multiple rounds of transduction and the use of adjuvants are necessary (116, 125). Chemical reagents such as TBK1/IKKε inhibitors (e.g., BX-795, MRT67303, and amlexanox) have improved lentiviral transduction efficiency in primary NK cells from 4.49% to 34.22% (119). Additionally, mRNA electroporation has been explored as a gene delivery method. For example, electroporation of anti-CD19-BB-z mRNA into NK cells achieved an efficiency of ~52%, though gene expression remained transient (120).

Advancements in immune cell-delivery nano-systems, such as cell surface engineering, nano-bioengagers, and nanocarriers, have yielded promising preclinical results (121-122). A significant example is a non-viral lipid nanoparticle technology designed to deliver small interfering RNAs (siRNAs) targeting intrinsic inhibitory NK cell molecules. These nanoparticles successfully inhibited checkpoint signalling molecules in NK cells, boosting their tumor-killing capacity in vivo (122). More research is required to confirm their effectiveness in clinical settings.

iPSC technology offers an alternative way to produce CAR-NK cells from stem cells, eliminating the need for direct NK cell manipulation (123). This method enhances the efficiency of CAR engineering while improving NK cell expansion and persistence (124). iPSCs can be genetically modified to express CARs and differentiate into a uniform population of CAR-NK cells. As a result, iPSC-derived CAR-NK cells could serve as standardized "off-the-shelf" immunotherapy products, though further technical refinements are required to reduce costs (125-126). An essential step in preserving the availability of CAR-NK cells for commercial application is cryopreservation. NK cells, on the other hand, are especially vulnerable to cryopreservation (127). Cell viability in traditional cryopreservation conditions, which contain 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO), is only about 50% after a month and just 15% after five years (117,128–129). IF-M, a newly created DMSO-free cryopreservation medium, uses glycerol and human serum albumin to enhance NK cell viability and recovery throughout the short and long term (129). Short-term cryopreservation (90 days) showed an 18.85% higher viability and 23.16% higher recovery rate in IF-M compared to DMSO. Long-term (oneyear) cryopreservation preserved NK cell function, with significantly greater CD107a expression in IFM-stored cells than those stored in DMSO (127). Other promising strategies, such as cryopreserving NK cells pre-complexed with innate cell engagers (130) and optimizing cryopreservation conditions (e.g., cooling rates and cryoprotectant formulations) (131) warrant further investigation to enhance the clinical viability of off-the-shelf CAR-NK therapies.

5. METHODS FOR IMPROVING NK CELL THERAPY FOR SOLID TUMORS

To improve the efficacy of natural killer (NK) cell therapy against solid tumors, multiple strategies have been developed to overcome the immunosuppressive tumor microenvironment (TME), enhance NK cell persistence, improve tumor infiltration, and boost cytotoxic activity (132). These strategies involve genetic engineering, cytokine support, combination therapies, and novel delivery methods to optimize NK cell function and longevity in solid tumor settings.

5.1. Enhancing NK Cell Persistence and Expansion

One major limitation of NK cell therapy is their short lifespan and reduced survival in vivo. Strategies to prolong NK cell persistence include cytokine pre activation with interleukin 15, interleukin -21, and interleukin 2, which can boost their expansion and survival. Additionally, genetic modifications to introduce cytokine receptors, such as IL-15 receptors or IL-12/IL-18 autocrine loops, can enable NK cells to sustain their function without external cytokine supplementation (133). Another promising approach is the use of memory-like NK cells (CIML NKs), which exhibit enhanced proliferation, persistence, and recall responses after activation.

5.2. Improving NK Cell Tumor Infiltration

Because chemokine receptor expression is required for tumor migration, NK cells have a difficult time homing to solid tumors. NK cell engineering to express tumor-homing chemokine receptors, such as CXCR4 (for CXCL12-expressing tumors), CCR7, and CX3CR1, can improve their ability to infiltrate the tumor core. Additionally, combination therapies with extracellular matrix-degrading enzymes, such as hyaluronidase or heparanase, can break down dense stromal barriers and facilitate NK cell penetration into solid tumors (134).

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5.3. Overcoming Tumor -Induced Immunosuppression

The TME contains various immunosuppressive molecules, including TGF-β, IL-10, and IDO, which inhibit NK cell function. One approach to counteract this is engineering NK cells to be resistant to TGFβ by knocking out TGF-β receptors or introducing dominant-negative TGF-β receptors that block its signalling. Another strategy involves combining NK cell therapy with immune checkpoint inhibitors (ICIs), such as anti-PD-1, anti-TIGIT, or anti-NKG2A antibodies, to restore NK cell activity. Additionally, targeting MDSCs and regulatory T cells (Tregs) through small-molecule inhibitors or depletion strategies can help create a more favourable immune environment for NK cells (135).

5.4. Enhancing Tumor Recognition and Cytotoxicity

Tumor cells often evade NK cell killing by downregulating stress ligands or upregulating inhibitory ligands. One strategy to enhance tumor recognition is chimeric antigen receptor (CAR)-engineered NK cells (CAR-NK cells), which are designed to specifically target tumor antigens like HER2, EGFR, GD2, and MUC1. CAR-NK cells not only provide antigen-specific cytotoxicity but also secrete cytokines and maintain a lower risk of cytokine release syndrome (CRS) compared to CAR-T cells. Furthermore, NK cell engagers (NKCEs) and bispecific T-cell engagers (BiTEs) are examples of bispecific or Tri specific engagers that can aid in bridging NK cells to tumor cells, hence improving their activation and killing capacity (136).

5.5. Developing Novel Delivery Approaches

Cell surface engineering and nanotechnology are being investigated to increase the accuracy and effectiveness of NK cell treatment. Targeted distribution to NK cells without systemic toxicity is possible with nanoparticle-based delivery methods that incorporate cytokines, immune checkpoint inhibitors, or gene-editing tools. Cell surface alterations can also improve NK cells' targeting and retention in the TME, such as binding them to antibodies coated with nanoparticles or immune-stimulatory ligands. A standardized and scalable NK cell source for therapeutic applications is being offered by the development of iPSC-derived NK cells as an off-the-shelf solution (137).

5.6. Combination Therapies for Synergistic Effects

•Given the complexity of solid tumor s, combination therapies integrating NK cell therapy with other treatments can improve efficacy. These include:

•Checkpoint Inhibitors (e.g., anti-PD-1, anti-CTLA-4) to restore NK cell activity

•Chemotherapy or Radiotherapy to create an immunogenic tumor environment

•Oncolytic Viruses that enhance NK cell recruitment and activation

•Targeted Small Molecule Inhibitors (e.g., IDO inhibitors, TGF-β blockers) to reduce immune suppression (138).

Conclusion

Natural killer (NK) cells, which are MHC-independent cytotoxic lymphocytes, are vital elements of the innate immune system. NK cells are rapidly activated and react by releasing cytotoxic granules to kill tumor cells when they come into contact with them. NK cell-based cancer therapy has become a viable approach in both experimental and clinical medicine because to its many benefits, including its wide availability and low incidence of adverse effects (139). While preclinical animal studies have showed promising outcomes in the treatment of solid tumors, NK cell-directed immunotherapy has shown safe and effective in treating people with advanced hematologic malignancies (140).

A number of issues need to be resolved in order to optimize the therapeutic potential of NK cells. First, it is crucial to have a better knowledge of the immunosuppressive tumor microenvironment (TME), specifically how suppressive cells (mostly regulatory T cells and myeloid-derived suppressor cells) and inhibitory chemicals (such TGF-β and IDO) impede the antitumor activity of NK cells (141). Overcoming these barriers remains a key challenge for the successful implementation of NK cell-based therapies.

Second, another factor limiting NK cells' antitumor potency is the scarcity of growth factors that promote their survival and multiplication in the TME. Growth factors like IL-15 should be incorporated into future treatments to improve NK cell persistence in vivo (132). Third, it is essential to enhance NK cell penetration into tumors. In order to maximize the effectiveness of tumor-targeting, future clinical trials should carefully assess genetic alterations, such as the introduction of chemokine receptors or chimeric antigen receptors (CARs), as well as combination therapy involving antibodies, engagers, and proteins (142). Finally, NK cell therapies have advanced significantly in large-scale production; numerous research have used memory-like NK (MLNK) cells and NK cells produced from induced pluripotent stem cells (iPSCs) or hematopoietic progenitor cells (HPCs) for clinical purposes (143). The reduction of genetic alterations and enhanced specificity and homogeneity for customized treatments are further advantages of employing iPSC-derived NK cells. These

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developments might get over present restrictions and offer a standardized, commercially accessible NK cell therapy for the treatment of cancer (144). To improve NK cell activation and proliferation, ex vivo manipulation, in vivo persistence and selectivity, and effective solid tumor targeting, more work is urgently needed despite the enormous achievements (145). In order to fully realize the therapeutic promise of NK cell-based cancer treatments, these issues must be resolved.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding this case report.

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