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Natural Killer Cells Therapy: From in Vitro Amplification to Engineering Modification

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Abstract:
In cancer treatment, traditional therapies are limited, and T-cell therapies are faced with challenges such as high costs and toxicity. As a mainstay of innate immunity, natural killer (NK) cells offer distinct advantages, including the ability to kill target cells without antigen sensitization, a low risk of graft-versus-host disease (GVHD), and the potential for "off-the-shelf" application. This review summarizes recent advancements in NK cell-based therapies, focusing on in vitro amplification strategies and genetic engineering modifications. Key findings include: cytokine and feeder layer co-culture systems have been shown to expand NK cells over 500-fold; CD19-targeted chimeric antigen receptor (CAR)-NK cells exhibit a 50%-70% response rate in B-cell lymphoma patients; CRISPR-mediated gene editing enhances cytotoxicity against solid tumors; nanoparticle-based delivery systems improve tumor infiltration of NK cells; and allogeneic NK cell therapy carries a near-zero GVHD risk. Notably, significant challenges persist, such as the immunosuppressive tumor microenvironment, poor in vivo persistence of NK cells, congenital functional defects, potential off-target effects of CAR-NK cells, and the lack of standardized production protocols. While NK cell therapy demonstrates substantial promise in cancer immunotherapy, overcoming these bottlenecks is essential for its broader clinical translation and application.

Keywords:- Natural Killer Cells; Tumor Microenvironment; Cellular Immunotherapy; CAR-NK Engineering; Solid Tumors

I. Introduction

Over the past ten years, cancer immunotherapy has brought about a transformative shift in oncology, providing long-term responses for patients with certain hematological malignancies and solid tumors. Among

the most prominent strategies are immune checkpoint inhibitors and chimeric antigen receptor (CAR)-modified T cells, which have demonstrated remarkable clinical efficacy. Nevertheless, the widespread application of T cell-based therapies faces significant challenges. These include high production costs [1], severe adverse reactions such as cytokine release syndrome (CRS) [2], and the need for patient-specific cellular products, which restricts their suitability for "off-the-shelf" use [3].

Natural killer (NK) cells, a vital component of the innate immune system, have emerged as a promising alternative in cellular immunotherapy. Unlike T cells, NK cells can identify and eliminate malignant or infected cells without prior sensitization or antigen presentation through the major histocompatibility complex (MHC) [4]. These features endow NK cells with a low risk of causing graft-versus-host disease (GVHD) [5] and the potential to be developed as allogeneic, universal donor cell products. Moreover, NK cells exert their cytotoxic effects through both direct killing and antibody-dependent cell-mediated cytotoxicity (ADCC), making them versatile effectors in tumor immunity.

Recent biotechnological progress has enabled the large-scale in vitro expansion of NK cells from various sources, including peripheral blood, umbilical cord blood, and induced pluripotent stem cells (iPSCs) [6]. Simultaneously, genetic engineering techniques—such as CAR modification, cytokine gene insertion (e.g., IL-15), and checkpoint receptor deletion—have further enhanced NK cell functionality. These engineered NK cells (CAR-NK) have achieved initial success in clinical trials for hematologic cancers, combining antitumor activity with a safer profile compared to CAR-T therapies [7].

However, major challenges persist in applying NK cell therapies to solid tumors. These challenges include the immunosuppressive tumor microenvironment (TME) [8], short in vivo persistence of NK cells [9], poor migration to tumor sites, and functional exhaustion [10]. Overcoming these obstacles is crucial for unlocking the full therapeutic potential of NK cells in cancer immunotherapy.

This review aims to provide a comprehensive overview of the current advancements in NK cell-based therapies, with a specific focus on their cellular sources, in vitro expansion techniques, genetic engineering modifications, and the challenges faced in their application to solid tumors. Through this analysis, we seek to highlight promising directions for future research and clinical translation.

II. Biological Basis and Innate Advantages of NK Cells

Natural killer (NK) cells represent a cornerstone of the innate immune system, uniquely positioned to provide rapid, non-specific surveillance against transformed or infected cells. Unlike adaptive T lymphocytes, which require clonal expansion and antigen priming, NK cells exhibit pre-programmed effector functions that enable immediate responses, making them pivotal in the early containment of malignancies. This section dissects their immunobiological properties, focusing on cell surface receptor dynamics and multifaceted cytotoxic mechanisms.

A. Immunobiological Properties: Receptor-Mediated Recognition of Aberrant Cells

Natural killer (NK) cell activity is carefully balanced by a dynamic interplay of cell surface inhibitory and activating receptors, which integrate signaling inputs to differentiate "self" from "non-self" or "stressed self" cells.

1) Inhibitory Receptors: Sensing "Missing Self" through HLA Class I

The main inhibitory pathway in human NK cells is governed by killer cell immunoglobulin-like receptors (KIRs), which detect polymorphic human leukocyte antigen (HLA)-I molecules on healthy cells [11]. KIRs attach to HLA-C and HLA-B alleles, delivering intracellular inhibitory signals through immunoreceptor tyrosine-based inhibitory motifs (ITIMs). This "missing self" recognition mechanism preserves tolerance to normal cells but becomes unbalanced in tumors that suppress HLA-I expression to avoid T cell attack [12]. Recent single-cell RNA sequencing analyses have indicated that KIR expression is highly variable among NK cell subsets, with CD56dim CD16+ cells showing higher inhibitory receptor density, implying functional specialization in immune surveillance [13].

2) Activating Receptors: Detecting "Stress-Induced Self" Ligands

In contrast, activating receptors trigger cytotoxicity when engaged by ligands upregulated during cellular stress or transformation:

NKG2D (CD314) recognizes stress-inducible ligands such as MICA, MICB, and ULBP1-6, which are frequently expressed on cancer cells but are absent or low on normal tissues. Genetic polymorphisms in NKG2D ligands correlate with tumor progression, highlighting their role in immune evasion.

DNAM-1 (CD226) binds to PVR (CD155) and Nectin-2 (CD112), which are upregulated in epithelial tumors and promote NK cell adhesion and cytokine production.

CD16 (FcγRIIIa): Mediates antibody-dependent cellular cytotoxicity (ADCC) by binding the Fc region of IgG antibodies, enabling NK cells to target antibody-opsonized cells. Recent structural studies have elucidated how CD16 polymorphisms affect binding affinity for IgG1 and IgG3,

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influencing clinical responses to antibody therapies like rituximab [14].

This dual-receptor system ensures that NK cells only become activated when inhibitory signals are diminished (e.g., HLA-I loss) and activating signals are concurrently enhanced (e.g., stress ligand upregulation), a paradigm critical for distinguishing malignant from normal cells [15].

B. Multimodal Cytotoxic Mechanisms

NK cells employ three interconnected pathways to eliminate target cells, providing both direct cytotoxicity and microenvironmental modulation.

1) Direct Cytotoxicity via Perforin-Granzyme Pathway

Upon receptor engagement, NK cells polarize cytotoxic granules toward the immunological synapse, releasing perforin to form pores in the target cell membrane and granzymes (e.g., granzyme B) to induce caspase-dependent apoptosis. Recent cryo-electron microscopy studies have revealed the dynamic assembly of perforin pores, showing how calcium ions trigger conformational changes for membrane insertion [16]. This pathway is particularly effective against virus-infected cells and hematological malignancies, where target cells often lack robust anti-apoptotic defenses.

2) Cytokine-Mediated Microenvironmental Regulation

Activated NK cells secrete pro-inflammatory cytokines like interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which orchestrate anti-tumor immune responses: IFN-γ polarizes macrophages toward the M1 phenotype, enhances HLA-I expression on tumor cells, and inhibits angiogenesis [17].

TNF-α induces tumor cell apoptosis directly and recruits dendritic cells (DCs) to promote adaptive immune priming.

Recent transcriptomic analyses have identified a "cytokine-ready" NK cell subset (CD56bright CD16−) with high IFN-γ production capacity, underscoring the role of NK cells in bridging innate and adaptive immunity.

C. Innate Advantages of T Cell-Based Therapies

These biological features endow NK cells with distinct clinical advantages:

1. Rapid Deployment: Unlike CAR-T cells, which require weeks for manufacturing, NK cells can be cryopreserved and administered as "off-the-shelf" products, critical for urgent cancer interventions.

2. Reduced GVHD Risk: The reliance on HLA-I-independent recognition and low alloreactivity minimize the risk of graft-vs-host disease in allogeneic settings, enabling universal donor cell banks.

3. Broad Tumor Surveillance: Effectiveness against HLA-I-deficient tumors and ability to target multiple ligands (e.g., NKG2D, CD16) reduce vulnerability to immune escape mechanisms that compromise T cell therapies [18].

III. In Vitro Amplification: Cell Sources and Scalable Production

A. Cell Sources and Their Characteristics

1) Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs remain the most widely used source for NK cell therapy due to their accessibility and compatibility with autologous and allogeneic applications. However, NK cells account for only 5–15% of PBMCs, necessitating efficient isolation and expansion strategies. Magnetic bead-based sorting (e.g., CD56+ selection) combined with cytokine stimulation (e.g., IL-2, IL-15) has achieved >300-fold expansion in 14 days, yielding clinical-grade NK cells with preserved cytotoxicity. Recent advancements include closed-system platforms like the CliniMACS Prodigy, which integrates purification, transduction, and culture steps under GMP conditions.

2) Alternative Sources: Placenta and NK Cell Lines

Placenta-derived NK cells (pNK) exhibit enhanced proliferative capacity and reduced alloreactivity due to HLA-G expression. Clinical trials with pNK cells (e.g., CYNK-101) have shown safety in solid tumors, though their short in vivo half-life (~24 hours) limits efficacy [19]. NK cell lines like NK-92 offer reproducibility and scalability but require irradiation to prevent tumorigenicity, compromising persistence [20]. Recent efforts to engineer NK-92 cells with CARs (e.g., CD19-CAR) and optimize culture conditions (e.g., X-Vivo 10 medium with human plasma) have improved cytotoxicity and GMP compliance.

B. Key Elements of Expansion Technology

1) Feeder Cells and Microenvironmental Mimicry

K562-mb15-41BBL: Engineered to express membrane-bound IL-15 and 4-1BBL, enabling >300-fold expansion of PBMC-NK cells. 3D Culture Systems: Spheroid cultures or bioreactors improve cell-cell interactions and scalability, achieving >10¹⁰ cells from a single UCB unit [21]. Emerging platforms like the Zellwerk ZRP system integrate closed-loop bioreactors with automated monitoring, ensuring GMP compliance [22].

2) Functional Maintenance During Expansion

Prolonged activation can induce NK cell exhaustion, marked by upregulation of CD57, NKG2A, and TIGIT. Strategies to mitigate this include:

Dynamic Cytokine Adjustment: Reducing IL-2 exposure after initial expansion to preserve cytotoxicity.

Metabolic Reprogramming: Supplementing cultures with

glutamine and glucose enhances mitochondrial function [23].
Genetic Modification: Overexpression of anti-apoptotic genes or CRISPR-mediated deletion of inhibitory receptors (e.g., PD-1) improves survival [24].

C. Challenges and Standardization in Scalable Production

1) GMP Compliance
GMP-grade production requires stringent control over reagents and processes: Serum-Free Media: ACROBiosystems' CelThera™ NK medium eliminates animal-derived components, achieving >95% NK cell purity and >270-fold expansion. Closed-System Automation: Platforms like the CliniMACS Prodigy and WAVE bioreactors minimize contamination risks and ensure batch consistency.
2) Cost-Efficiency and Feasibility
Cost Comparison: NK cell expansion costs (~$50,000 per dose) are lower than CAR-T (~$475,000) due to shorter culture times and allogeneic compatibility. Scalability: Large-scale bioreactors enable production of >10¹¹ cells, supporting multi-center trials.
3) Quality Control
Functional Assays: Flow cytometry (CD107a degranulation) and luciferase-based cytotoxicity assays validate NK cell activity. Persistence Monitoring: In vivo imaging (e.g., PET-CT) tracks NK cell distribution post-infusion.

IV. Engineering Modifications: Functional Enhancement and Precision Targeting

A. Core Directions of Genetic Engineering Technologies

1) Design and Optimization of Chimeric Antigen Receptors (CAR-NK)
CAR-NK cell therapy stands as a critical breakthrough in cancer immunotherapy, integrating the inherent cytotoxic capabilities of NK cells with targeted recognition mechanisms, at the meantime, The construction of CARs for NK cells involves several key structural elements. Distinct from CAR-T cells, NK cells can combine CAR-mediated signals with their intrinsic activating pathways, including NKG2D and DNAM-1, to generate synergistic anti-tumor effects. In solid tumors, HER2-CAR-NK cells have shown promise against breast cancer, with preclinical models demonstrating enhanced tumor infiltration and reduced off-tumor toxicity compared to CAR-T counterparts [25]. Targeting antigens like the mesothelin and prostate-specific membrane antigen (PSMA) is also under active investigation, highlighting the versatility of CAR-NK platforms.
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2) Modulation of Endogenous Signaling Pathways
Genetic engineering of NK cells is directed at surmounting immunosuppressive tumor microenvironments. Knockout of inhibitory receptors like killer cell immunoglobulin-like receptors (KIRs) and TIGIT, achieved through CRISPR/Cas9 technology, enhances NK cell cytotoxicity by eliminating activation barriers. In preclinical settings, CAR-NK cells lacking KIRs showed improved capability to kill HLA-I-negative tumors. On the contrary, overexpressing activating receptors such as NKG2D ligands and DNAM-1 strengthens tumor recognition. A recent investigation revealed that ectopic expression of MICA, an NKG2D ligand, made pancreatic cancer cells more sensitive to lysis mediated by NK cells.
3) Cytokine Engineering and Microenvironment Adaptation
Engineering NK cells to secrete or respond to specific cytokines improves their persistence and function. Co-expression of IL-15 or IL-21 extends in vivo survival and enhances anti-tumor activity. In a Phase I trial, IL-15-secreting CAR-NK cells for relapsed lymphoma showed prolonged detection in patients' circulation compared to conventional CAR-NK cells. Additionally, engineering NK cells to produce immune checkpoint-blocking antibodies directly targets the immunosuppressive tumor microenvironment. Preclinical data suggest that PD-L1-secreting NK cells enhance T cell recruitment and improve anti-tumor synergy.

B. Non-Genetic Engineering Approaches

1) Small Molecule Preconditioning
Small molecules offer an alternative strategy to enhance NK cell function without genetic modification. SMAC mimetics, which disrupt inhibitors of apoptosis proteins (IAPs), sensitize tumor cells to NK cell-mediated apoptosis. A recent clinical trial combining SMAC mimetics with NK cell therapy in ovarian cancer patients led to increased caspase activation and improved treatment response. AMPK activators, such as metformin, enhance NK cell metabolism and cytotoxicity by promoting mitochondrial biogenesis [26].
2) Antibody Engineering
Bispecific antibodies (bsAbs) bridge NK cells to tumor

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antigens, augmenting antibody-dependent cellular cytotoxicity (ADCC). CD16A×tumor antigen bsAbs, including those targeting EGFR and HER2, have shown potent anti-tumor activity in preclinical models. Clinical trials of CD33×CD16A bsAbs in acute myeloid leukemia reported durable remissions, highlighting the potential of this approach to boost NK cell function.

V. Clinical Applications: Hematological Malignancies vs. Solid Tumors

A. Core Challenges in Solid Tumor Therapy

1) Limited In Vivo Persistence

Unlike T cells, NK cells exhibit short half-lives in vivo, often persisting for only days to weeks. This limitation restricts their ability to maintain sustained anti-tumor activity. In preclinical models, unmodified NK cells showed detectable levels for ≤7 days post-infusion, compared to CAR-T cells with persistent expansion over 30 days. Strategies to extend NK cell survival, such as genetic engineering to co-express IL-15 or adoptive transfer of cytokine-induced memory-like (CIML) NK cells, remain in early development.

2) Inadequate Tumor Infiltration and Targeting

NK cells face significant challenges in reaching and localizing within solid tumors. Poor penetration of the tumor vasculature, which often exhibits abnormal architecture and high interstitial pressure, limits NK cell extravasation [27]. Additionally, the lack of specific homing receptors hinders their migration to tumor sites. Genetic modification to overexpress chemokine receptors (e.g., CXCR3, CXCR4) has shown promise in preclinical models, increasing tumor infiltration by 2- to 3-fold.

B. Combination Therapy Strategies

1) Synergy with Chemotherapy and Radiation

Chemoradiation can cause prime tumors for NK cell attack by upregulating stress ligands recognized by activating receptors. Radiotherapy, for instance, induces expression of NKG2D ligands on tumor cells, enhancing their susceptibility to NK cell-mediated lysis. A clinical trial combining fractionated radiotherapy with NK cell infusion in lung cancer patients reported a 25% increase in tumor cell apoptosis compared to radiotherapy alone.

2) Immunotherapy Combinations

Combining NK cell therapy with immune checkpoint inhibitors (ICIs) overcomes TME suppression. Preclinical data show that anti-PD-1/PD-L1 antibodies reverse NK cell exhaustion, restoring IFN-γ production and cytotoxicity. In melanoma models, the combination of NK cells and anti-TIGIT antibodies increased tumor regression by 40% compared to single-agent treatments.

VI. Conclusions

This review provides a comprehensive overview of the latest developments and current landscape in natural killer (NK) cell-based therapies, with a particular focus on their role as a supplementary approach to T cell immunotherapies. The immunobiological properties of NK cells, their antitumor mechanisms, and recent advancements in NK cell engineering—such as CAR-NK and memory-like NK cell technologies—are systematically explored. Notably, this review emphasizes the unique advantages of NK cells, including MHC-unrestricted cytotoxic activity, reduced graft-versus-host disease risk, and the capacity to eliminate tumor cells that evade T cell recognition.

Findings indicate that genetically engineered NK cell therapies have demonstrated promising efficacy and safety profiles in preclinical research and early-phase clinical trials, particularly in the treatment of hematological malignancies. Multiple CAR-NK products have entered clinical evaluation, yielding favorable outcomes with fewer instances of cytokine release syndrome compared to CAR-T therapies.

However, significant challenges remain. The limited in vivo persistence of NK cells, immunosuppressive tumor microenvironments, and the need for more efficient genetic modification techniques hinder broader clinical application. Moreover, the heterogeneity of NK cell sources and the lack of standardized manufacturing protocols complicate clinical translation and comparison across studies.

Looking forward, research in future should focus on enhancing the survival and functionality of NK cells in vivo, overcoming tumor-induced immunosuppression, and optimizing manufacturing processes for large-scale production. Combinational strategies, such as combining NK cells with immune checkpoint inhibitors or bispecific antibodies, also hold potential to enhance anti-tomorrow efficacy. Continued preclinical and clinical investigations will be critical to fully unlock the therapeutic potential of NK cells in cancer immunotherapy.

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