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Historical Evolution and CRISPR-Mediated Gene Editing Applications in NK Cell Therapy

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Abstract: Natural Killer (NK) cell therapy has emerged as a transformative approach in cancer immunotherapy, driven by decades of research since their discovery in the 1970s. NK cells possess a unique ability to target tumor cells through antigen-independent recognition, which has been expanded with the discovery of antigen-specific memory and licensing mechanisms. This review explores the evolution of NK cell biology, highlighting key findings such as the enhanced response of NK cells upon re-exposure to cytomegalovirus (CMV), defying conventional immune paradigms. Recent advancements in gene editing, particularly with CRISPR technology, have led to the development of "armored" NK cells. For example, knock-in strategies have enabled the insertion of chimeric antigen receptors (CAR), enhancing tumor specificity, while knock-out approaches have improved NK cell activation by removing inhibitory molecules like PD-1 and NKG2A. Early clinical trials demonstrate improved tumor-targeting efficacy and reduced resistance. However, challenges remain, including high R&D costs, limited clinical data, and technical uncertainties such as off-target effects. In China, NK cell therapy is in its nascent stages, with increasing interest but limited clinical applications due to regulatory and funding barriers. This review provides a comprehensive analysis of NK cell evolution, CRISPR-based advancements, and future directions for overcoming current limitations and optimizing therapeutic outcomes.

Keywords: Natural killer (NK) cell therapy, cancer immunotherapy, CRISPR gene editing.

1. Introduction

The journey of NK cell therapy began in the 1970s when researchers first identified NK cells as a distinct population of lymphocytes capable of spontaneous cytotoxicity against tumor cells [1]. Over the past five decades, the comprehension of NK cells has significantly evolved, transitioning from their initial identification as innate immune cells to the recognition of their adaptability. Starting from their innate ability for antigen-independent recognition, NK cells were then discovered to possess antigen-specific memory and undergo a licensing process through interactions with self-MHC molecules. This progression underscores their ability to adapt and enhance their functionalities, bolstered by concepts such as licensing and antigen-specific memory-like responses following the initial exposition of the "missing-self" hypothesis. Studies on cytomegalovirus (CMV) infection revealed that NK cells improved their responses with re-exposure, defying conventional wisdom on the immune system [2]. The progression of NK cell biology has significant implications for the development of gene engineering technology in cancer immunotherapy. During clinical trials,

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CRISPR gene editing technology has become a powerful tool to build “armoured” NK cells, particularly through its knock-in and knock-out mechanisms. One promising example is the gene editing of CAR-NK cells under knock-in strategy where chimeric antigen receptors (CAR) are inserted to confer specificity towards particular tumor antigens [3]. The application of CAR-NK therapy has brought new alternatives for cancer treatment, especially in tackling the challenges associated with overcoming drug resistance and tumor heterogeneity. Conversely by knocking out inhibitory checkpoint molecules such as PD-1 and NKG2A, researchers are able to enhance NK cell activation.

This review paper aims to detail the evolution of biological knowledge regarding NK cells and trace their initial characterization to a comprehensive analysis of their expanded functions. Moreover, contemporary technological advancements and potential applications are examined.

2. Historical Evolution of NK Cell Therapy and Clinical Implication

The journey of NK cell discovery began in the early 1970s when researchers identified a unique lymphocyte population capable of non-antigen-specific cytotoxicity. Unlike cytotoxic T cells that recognize antigens via their T-cell receptor (TCR), NK cells detect the lack of Major Histocompatibility Complex (“MHC”) class I molecules, which usually inhibit their activity, and subsequently release cytotoxic agents that target and destroy tumor cells, bypassing the need for antigen recognition. NK cells demonstrated several key advantages over T cells. They are not restricted by human leukocyte antigen (HLA) compatibility, allowing for allogeneic sourcing and making them available as "off-the-shelf" therapeutic options. Furthermore, NK cells exhibit low levels of cytokine secretion, which mitigates the risk of cytokine release syndrome (CRS) and graft-versus-host disease (GvHD), thereby contributing to a more favourable treatment profile. Since the early 1970s, the biological understanding of NK cells has progressed to reveal features of both the innate and adaptive immune systems [4]. Below as the key milestone finding listed out in two stages namely early discoveries (1970s-1980s) and advancements (1990s-2010s).

2.1. Early Discoveries (1970s-1980s)

In 1974, Herberman and colleagues demonstrated that peripheral blood lymphocytes from healthy individuals could kill certain tumor cells. This was further substantiated by Kiessling et al. in 1975 [5], who coined the term "natural killing" to describe this phenomenon. They established that NK cells could reject tumors in vivo, leading to the identification of NK1.1 as a prototypic NK cell marker. Then in the early 1980s, the first clinical applications of NK cells began with lymphokine-activated killer (LAK) cells generated ex vivo, developed by Steven Rosenberg. These cells were generated from peripheral blood mononuclear cells (PBMCs) cultured with interleukin-2 (IL-2). This was followed by the “missing-self” hypothesis [6], proposed by Klas Kärre in the seminal letter in Nature in 1986 [7]. Back then, the immunological community was trying to solve the intriguing question on how NK cells selectively target and kill tumor cells without harming healthy cells. As such, the missing-self hypothesis is centred on the concept that NK cells are activated when they detect a lack of “self” molecules on target cells, thereby providing a logical explanation to address this question. Klas and his team described how target cells become vulnerable to NK cell-mediated lysis in absence of MHC class I molecules. Their studies revealed that NK cells are affected by the presence or lack of these molecules on target cells since they could reject tumor cells with lost MHC class I expression. Usually expressing on the surface of all nucleated cells, MHC class I molecules provide peptides produced from intracellular proteins to the immune system. This presentation is crucial for self-recognition and for signalling to the immune system that the cell is healthy and not infected or transformed.

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Two mechanisms were proposed to elucidate the role of MHC class I molecules in influencing the resistance or susceptibility of target cells to lysis by NK cells. The first mechanism, known as “the receptor inhibition recognition model,” suggests that NK cells have inhibitory receptors that identify self-MHC class I molecules. When engaging with MHC class I molecules found on healthy cells, these receptors convey inhibitory signals that function to suppress the activation of NK cells. In contrast, the absence or downregulation of MHC class I, frequently observed in tumor or virally infected cells, results in the loss of these inhibitory signals. This loss permits the activation of NK cells, leading to the initiation of cytotoxic responses. The second mechanism, referred to as “the target interference model,” posits that ligands located on target cells, which play a crucial role in activating NK cell receptors, are obscured by the expression of MHC class I molecules, consequently inhibiting NK cell activation. On comparative basis, the first one has been supported by more extensive laboratory research in the 1990s. Below as the summarized key findings during 1990s-2010s.

2.2. Advancements in NK Cell Therapies (1990s-2010s)

The initial foundational study underpinning the missing-self hypothesis was conducted by Ljunggren and Kärre [8]. They presented compelling in vivo evidence indicating that NK cells are capable of effectively eliminating H-2-deficient target cells due to the lack of MHC class I molecule expression. Subsequently, Karlhofer et al. [9] elucidated the presence of the Ly49 family of receptors on murine NK cells, which exhibit specificity for MHC class I molecules. This study demonstrated a direct correlation between the expression of MHC class I and the tolerance of NK cells, indicating that Ly49+ NK cells experienced inhibition due to interactions with self-MHC class I. This suggests that the presence of MHC class I on target cells is crucial for inhibiting NK cell activation in healthy tissues. In further support of this concept, Colonna and Samaridis [10] identified human Killer Immunoglobulin-like Receptors (KIRs) as essential inhibitory receptors on NK cells that additionally recognize MHC class I molecules. Their research emphasized the significance of KIRs in sustaining tolerance towards self-cells while facilitating NK cell activation against atypical targets that lack MHC class I, thus reinforcing the missing-self hypothesis within the context of human immunology. Furthermore, Lanier elaborated on the missing-self hypothesis by investigating the interactions between inhibitory receptors such as NKG2A and MHC class I in the regulation of NK cell activity [11]. The engagement of these receptors has been shown to be essential for the establishment of self-tolerance in NK cells, thereby reinforcing the notion that the recognition of self-MHC is critical for the prevention of autoimmunity while facilitating effective responses against infected or transformed cells.

Starting from the early 2000s, the concepts of licensing and memory have been raised, further contributing to our understanding of NK cell functionality. Licensing denotes the mechanism by which NK cells attain functional competence via interactions with self-MHC class I molecules throughout their developmental stages. This interaction enhances their ability to respond to activating signals from stressed or abnormal cells. NK cells can identify "missing self" by sensing the lack of MHC class I and can also be positively modulated by interactions with cells that express MHC class I. This positive control serves as a sort of "licensing" that affects host resistance to infections by influencing cytokine production and cytotoxicity [12]. Licensed NK cells are prepared to respond more robustly to future interactions with target cells, including tumor cells that display diminished or absent MHC class I expression, by engaging with the inhibitory receptors, such as Killer Immunoglobulin-like Receptors (KIRs) in humans or Ly49 receptors in mice, alongside MHC class I on dendritic cells (DCs).

As per Goldszmid’s research published in 2007, TAP-1 plays a role in inducing IFN-γ-producing NK cells, highlighting the impact of NK cell licensing on host defense mechanisms [13]. Furthermore, MHC class I alleles is demonstrated to affect the licensing of Ly49A+ NK cells, suggesting that

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licensing establishes a safety margin against NK cell autoreactivity [14]. Around the same time, a pathway was identified by which licensed human NK cells aid dendritic cell maturation, emphasizing the role of inhibitory receptors specific for self-MHC class I molecules in licensing. Human diseases depend on the interaction between HLA and KIR receptors since HLA alleles define variations in human NK cell responsiveness and potency [15]. Carlomagno showed in 2017 that KIR3DS1 controls positive signals upon recognition of HLA-B*51 surface molecules, therefore influencing NK cell licensing in those with particular KIR/HLA combination settings [16]. Recent findings have further strengthened the view that licensed NK cells were shown to produce higher levels of IFN-γ and exhibit enhanced cytotoxicity against infected targets compared to unlicensed NK cells.

Moreover, the results obtained during the same time period indicate that NK cells may demonstrate memory-like antigen-specific characteristics following activation with cytokines including IL-12, IL-15, and IL-18 34. These memory-like NK cells demonstrate enhanced functionality upon re-exposure to tumor targets, indicating a more sophisticated role for NK cells in immune responses than previously understood. Von Adrian and colleagues' innovative work in 2006 questioned the missing-self hypothesis. The study demonstrated that mouse NK cells display a form of memory response upon re-exposure to the identical antigen, which can initiate hapten-specific contact hypersensitivity reactions. The notion of antigen-specific memory within NK cells challenges the traditional view that NK cells operate solely as innate lymphocytes.

In the 2010s, research on Memory Responses Against CMV infection—a common viral infection capable of producing NK cell memory—solidified this idea even more. First of all, CMV infection causes a clear increase of NK cell counts [17]. This development causes NK cells to differentiate into memory phenotypes, which can last long after the first CMV infection has cleared. These memory NK cells have increased cytotoxic activity, which helps them to identify and destroy CMV-infected cells upon re-exposure, therefore offering a quick and strong immune reaction. Fascinatingly, memory NK cells particular for CMV can show a unique repertoire of activating and inhibitory receptors. They often upregulate receptors like NKG2C, for example, which lets them identify ligands brought on by stress on CMV-infected cells. Their increased sensitivity and potency in immunological responses depend on this special receptor expression [2]. Moreover, the memory NK cells produced during CMV infection might be long-term persistent; some can stay in the body for several years. Maintaining constant protection against re-infection depends on this lifetime, which also enables the immune system to keep strong and responsive to CMV and associated infections [18,19]. Apart from their particular purposes against CMV, these memory NK cells can show cross-reactivity. This indicates that they can identify and react to other related viruses, therefore improving general immune monitoring and extending the protection against a range of infections. This amazing adaptability emphasizes the important part memory NK cells perform in the specific and general immune reactions of the organism.

Historically, it was believed that antigen-specific responses to pathogens were solely the domain of T cells that possess the ability to perform somatic rearrangement of their antigen receptor gene loci. However, the findings from lab research indicate that antigen specificity has been established for the germline-encoded activating receptor Ly49H, which is present on a subset of NK cells in mice, and specifically identifies the mouse cytomegalovirus (MCMV) -encoded glycoprotein m157 [20,21]. The interaction between the receptor and ligand leads to the activation and proliferation of Ly49H+ NK cells. Subsequent to the adoptive transfer of Ly49H+ NK cells into receptor-deficient mice, the virus-specific NK cell population exhibited an expansion of up to 1000-fold within a week post-MCMV infection, followed by a contraction phase that resulted in the formation of a memory cell pool. This observation indicates that NK cells undergo differentiation through distinct expansion and contraction phases, ultimately leading to the establishment of long-lived memory cells in response to viral infections.

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3. Application of Gene Editing Technologies in NK Cell Therapy

The evolution of NK cell therapy has significant implications for the development of gene editing and modification technologies in cancer immunotherapy. Since its discovery in 2012, CRISPR-Cas9 technology has revolutionized molecular biology by offering a precise tool for DNA modification. In cancer immunotherapy, CRISPR has been increasingly used to enhance NK cell function and address challenges like tumor evasion mechanisms and short in vivo lifespan. CRISPR-Cas9, with its simplicity and high precision, has enabled the researchers to perform both knock-out and knock-in strategies to overcome these challenges. Knock-out approaches target genes that inhibit NK cell activity or render them susceptibility to tumor evasion, such as SHP1 or MHC class I molecules. By disabling these genes, NK cells become more potent and less susceptible to immune suppression in the tumor microenvironment. Conversely, knock-in strategies involve introducing new genetic elements into NK cells to endow them with enhanced capabilities. One promising example is the engineering of CAR-NK cells, where chimeric antigen receptors are inserted to confer specificity towards particular tumor antigens. This approach redirects NK cells to target cancer cells more effectively, similar to CAR-T cells but with potentially fewer side effects. Moreover, gene editing allows for the introduction of genes that improve NK cell survival, proliferation, and homing to tumors. For instance, inserting genes for cytokines like IL-15 can enhance NK cell persistence and in vivo life span. Additionally, modifying NK cells to express specific chemokine receptors can augment their trafficking to tumor sites, further enhancing therapeutic efficacy.

Several applications of NK cell therapy are analyzed below, with their working mechanisms detailed. Moving forward, innovations like CAR-NK technology, targeting of checkpoint molecules, and strategies to overcome the immunosuppressive tumor microenvironment (TME) are establishing a strong foundation for more effective treatments. These technological advancements represent a dynamic and evolving process, which may be further optimized based on emerging insights from clinical applications. Continued research holds the promise of significantly enhancing patient outcomes across various malignancies.

3.1. Chimeric Antigen Receptor-NK Cell Therapy (CAR-NK Cell Therapy)

As alluded above, CAR-NK cell therapy represents an innovative strategy that employs genetically modified NK cells to specifically target and eradicate tumor cells. This therapeutic approach integrates the inherent cytotoxic properties of natural killer (NK) cells with the targeted specificity provided by chimeric antigen receptor (CAR) technology, thereby facilitating a more efficient and safer treatment alternative for a range of malignancies. The notion of CAR-NK cells emerged in the early 2010s, shaped by the successes of CAR-T cells in addressing hematological malignancies. During the mid-2010s, preliminary clinical trials began to evaluate the effectiveness of CAR-NK cells in addressing acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL). The trials exhibited encouraging outcomes with reduced occurrences of severe adverse effects such as CRS and neurotoxicity in comparison to CAR-T cells [22,23]. A notable advantage of CAR-NK cells over CAR-T cells is their derivation from allogeneic sources, such as umbilical cord blood or induced pluripotent stem cells, which eliminates the need for patient-specific manufacturing processes. To summarize, CAR-NK cell therapy presents a promising avenue for a more straightforward and cost-effective “off-the-shelf” treatment, potentially associated with reduced toxicities. Several critical areas of focus are essential for the advancement of CAR-NK cell therapy.

Optimizing CAR Constructs: Up to date, a significant portion of the research aimed at optimizing CAR constructs for NK cells has concentrated on enhancing cytotoxicity rather than assessing persistence. The initial iteration of CARs incorporated a single-chain variable fragment (scFv) derived from an antibody targeting a tumor antigen, which was connected to a CD3ζ signaling domain

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to facilitate NK cell activation. Nonetheless, concerns regarding efficacy and durability emerged, as CAR-NK cells exhibited signs of exhaustion or anergy following multiple activations. With these constraints, later versions of CARs have integrated improved design elements that encompass supplementary co-stimulatory domains, including CD28, 4-1BB, or OX40a. These modifications significantly improve the survival, proliferation, and functionalities of CAR-NK cells [24].

Identification of Innovative Therapeutic Targets: The effectiveness and safety of CAR-NK cell therapy are significantly influenced by the choice of tumor antigens. The optimal targets are those that exhibit high expression levels on tumor cells, thereby reducing the likelihood of adverse effects on normal tissues. Nevertheless, this presents a considerable challenge, given that numerous tumor antigens are also found on healthy cells, albeit in reduced quantities. In response to this challenge, scholars have concentrated their efforts on pinpointing more tumor-specific targets. Certain targets for the genetic engineering of NK cells exhibit unique expression patterns specifically within tumor-associated conditions, such as by Hypoxia-inducible factor 1 (HIF-1) in hypoxic tumor microenvironments.

3.2. Targeting Checkpoint Molecules

CRISPR technology has been utilized to knockout inhibitory receptors, such as CD38 and NKG2A, on NK cells, which are acknowledged for their function in suppressing cytotoxic activities. Clinical studies demonstrate that eliminating CD38 in NK cells markedly enhances their persistence and cytotoxic abilities when subjected to daratumumab (DARA), a monoclonal antibody employed in the treatment of multiple myeloma (MM). In the experiment conducted, the CRISPR/Cas9 system was utilized to facilitate the deletion of CD38 (CD38KO) in ex vivo expanded peripheral blood NK cells. The CD38KO NK cells exhibited total resistance to DARA-induced fratricide, showed improved persistence in immune-deficient mice previously treated with DARA, and demonstrated increased antibody-dependent cellular cytotoxicity against CD38-expressing multiple myeloma cell lines as well as primary multiple myeloma cells. This suggests that the removal of CD38 not only safeguards NK cells from fratricide but also enhances their anti-tumor effectiveness when used in conjunction with daratumumab treatment [25,26]. Similarly, the removal of NKG2A has demonstrated an enhancement in NK cell-mediated cytotoxicity towards leukemia cells that express HLA-E, suggesting that CRISPR-mediated editing can significantly bolster the efficacy of NK cells in the targeting of malignancies [27]. Clinical evidence indicates that the NKG2A-HLA-E axis may significantly influence CAR-NK cell exhaustion. Consequently, the CRISPR/Cas9-mediated disruption of the NKG2A-coding gene could enhance their efficacy in targeting acute myeloid leukemia (AML).

3.3. Modulating Metabolic Pathways

Gene editing can also target metabolic pathways that contribute to NK cell exhaustion within the TME. By knocking out genes associated with metabolic suppression, researchers aim to reinvigorate NK cell function and persistence. One of the key metabolic pathways involved in NK cell exhaustion is purinergic signaling, particularly through the adenosine A2A receptor (A2AR). Studies show A2AR-deficient, terminally mature NK cells maintained their proliferation during the reconstitution and within tumor macro environment. Utilizing CRISPR/Cas9 technology, researchers have successfully knocked out A2AR in NK cells, resulting in enhanced effector functions and improved anti-tumor responses. This modification allows NK cells to maintain their activity even in the presence of high levels of adenosine, a metabolite that typically suppresses immune responses within the TME [28].

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Furthermore, CRISPR has the capability to target additional metabolic regulators, including Casitas B-lineage lymphoma pro-oncogene-b (CBLB), which functions as an intracellular negative regulator of NK cell signalling. Recent research indicates that the downregulation of CBLB in primary human NK cells leads to an increase in granzyme B and perforin expression, as well as enhanced production of interferon gamma and cytotoxicity towards leukemia cell lines. The knockdown of CBLB in the human NK cell line enhances cytotoxicity towards Jurkat cells and increases in vivo anticancer efficacy, consequently diminishing lung metastasis by mitigating the effects of negative receptors. As such, CBLB deletion emerges as a promising strategy to lower activation thresholds and amplify NK cell antitumor efficacy [29]. Reflected by clinical research findings, 94%efficacy of CBLB knockout was attained through the application of CRISPR/Cas9 gene editing technology.

4. Future Perspectives of NK Cell Therapy

NK cell therapy presents significant potential in cancer immunotherapy. Looking ahead, a personalized therapeutic approach will be crucial in enhancing the effectiveness of these treatments. Such personalized therapies are designed to cater to individual patients by considering their unique tumor characteristics, genetic profiles, and immune responses. By harnessing advancements in genetic engineering, particularly CRISPR technology, researchers can modify NK cells to enhance their targeting capabilities and overall anti-tumor efficacy. Additionally ongoing clinical trials are exploring various combination strategies aimed at improving treatment efficacy by leveraging the strengths of multiple therapeutic agents tailored to a patient's specific cancer type and genetic makeup. For example, combining CAR-NK cell therapy with checkpoint inhibitors may help counteract TME-induced immunosuppression while maximizing anti-tumor responses.

5. Conclusion

Despite all of the recent technological advancement, several challenges and uncertainties must be addressed to realize the full potential of NK cell therapy. One primary concern is the limited clinical data available for new technologies, including CRISPR-engineered NK cells. Although preliminary results from a few clinical trials have shown promise, larger-scale studies are essential to validate their safety and efficacy. Another significant hurdle is the substantial funding required for research and development (R&D) expenditures necessary to develop and scale up NK cell therapies. The complex processes involved in gene editing, cell expansion, and manufacturing bottlenecks demand considerable financial resources, potentially limiting accessibility for a broader patient population. Moreover, there are inherent technical uncertainties associated with the precise manipulation of NK cells, such as potential off-target effects and the stability of engineered cells in vivo.

In China, NK cell therapy is still in its early stages. While there is governmental support and increasing interest from both academia and industry, the implementation of NK cell therapies in clinical practice remains very limited. There is a need for further development of regulatory frameworks to ensure the safe and efficient transition of research findings into clinical applications. Additionally, more funding is required to support such initiatives within the Chinese healthcare system.

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