Enhancing NK Cells Antitumor Activity by CRISPR-Cas9 Mediated Knockout of Inhibitory Receptors

Minchang Wang *

Aulin College, Northeast Forestry University, Harbin, China

* Corresponding Author Email: minchang1231@outlook.com

Abstract. Cancer is still one of the leading causes of death globally, and current treatment methods such as chemotherapy and CAR-T cell therapy are commonly used but are restricted by systemic toxicity, high costs, and reduced efficacy in many solid tumors. CRISPR-Cas9 technology offers a versatile platform to engineer natural killer cells to overcome these limitations by precise genomic edits that enhance cytotoxicity and enable off the shelf manufacturing. Recent approaches include knockout of inhibitory receptors including NKG2A, TIGIT and TIM 3, high throughput target discovery, and combinatorial strategies such as overexpression of activating receptors or integration of chimeric antigen receptors to broaden antigen specificity. Preclinical studies report improved target cell killing, resistance to tumor microenvironment induced dysfunction, and efficacy in hematologic and solid tumor models, while outstanding challenges remain in delivery efficiency, off target editing, functional persistence and safety. Addressing these challenges will be critical for clinical translation. This review aims to integrate current preclinical and clinical evidence on CRISPR-edited natural killer (NK) cells, assess the efficacy and safety of inhibitory receptor disruption and combined engineering strategies, and outline priorities for translational research to accelerate the process of developing safer and more effective NK cell therapies.

Keywords: NK cells, CRISPR-Cas9, inhibitory receptors, tumor.

1. Introduction

Natural killer cells (NK cells) mainly originate from hematopoietic stem cells in the bone marrow, develop and mature there, and play a significant role in innate immunity. NK cells have natural cytotoxicity and can destroy tumor cells or virus-infected cells [1]. NK cells recognize self and non-self cells by activating surface activating receptors and inhibitory receptors. The main activating receptors include NKG2D, natural cytotoxic receptors (NCRs), CD16, etc. The main inhibitory receptors include CD94/NKG2A, TIGIT, KIRs, etc. Whether NK cells are activated depends on the ratio of signals received by surface-activating receptors and inhibitory receptors. When the activating signal is greater than the inhibitory signal, NK cells are activated, releasing cytotoxic granules and cytokines to attack target cells. Otherwise, the signal is inhibited to maintain stability and avoid accidental damage to normal tissues. Currently, the genes encoding inhibitory receptors on the surface of NK cells can be knocked out by using gene editing technology, thereby removing their inhibitory signals and enhancing their killing ability against tumor cells. Precise double-strand breaks at specific sites determine whether gene editing is successful. The broken ends can be rejoined through non-homologous end joins (NHEJ) and micro-homology-mediated end joins (MMEJ), thereby leading to insertions or deletions (INDELs). At present, in gene editing technology, commonly used nuclease tools include zinc finger nucleases (ZFNs), transcription activator efferent nucleases (TALENs), and regular clustered interspaced short palindromic repeat sequences and associated proteins 9 (CRISPR-Cas9), etc. Among them, CRISPR-Cas9 stands out particularly in terms of scalability, flexibility and ease of operation.

The CRISPR-Cas9 system consists of single-guide RNA (sgRNA) and the DNA endonuclease Cas9. The sgRNA guides Cas9 to a specific DNA sequence, where Cas9 specifically cuts the double-stranded DNA site [2]. Compared with TALENs and ZFNs, designing sgRNA in CRISPR-Cas9 is so convenient that it can target almost all regions of the genome [3]. Recent studies have shown that CRISPR-Cas9 editing knockout of the KLRC1 gene in NK cells and disrupting the inhibitory signal of the NKG2A-HLA-E axis can remarkable enhancement of NK cell cytotoxicity against multiple

myeloma [4]. In addition, when CRISPR-Cas9 was used to knock out TIGIT, NK cells showed increased cytotoxicity and metabolic adaptability, and the cells did not self-destruct [5]. Gene-edited NK cells have demonstrated good efficacy and safety in the treatment of hematological malignancies such as multiple myeloma, lymphoma, and acute myeloid leukemia. However, in solid tumors, their delivery efficiency, off-target effects, and immunosuppressive microenvironment still limit their efficacy, and further optimization is needed. This review aims to systematically summarize strategies for CRISPR-Cas9 gene editing and modification of NK cells, evaluate the enhanced cytotoxicity, safety, and clinical application prospects after inhibitory receptor knockout, and provide theoretical support for the optimization of NK cell immunotherapy.

2. Interactions Between NK Cells and Tumors

The NK cells are categorized as innate lymphoid cells and play a vital role in autoimmunity, possessing powerful cytolytic activity and the ability to secrete a broad spectrum of cytokines and chemokines [6]. Unlike adaptive immunity, NK cells do not rely on classic antigen presentation for recognition and killing, thus demonstrating unique therapeutic potential and rapid effects in anti-tumor immunity. NK cells represent two major classes of molecules on their surface: activating and inhibitory receptors. Their cellular activity is regulated by the balance of signals from these two types of receptors, enabling them to distinguish between healthy cells and transformed or stressed cells [7]. Activating receptors can activate NK cells and promote their cytolytic and proinflammatory activity, mainly including NCRs (NKp30, NKp46, and NKp44), NKG2D, DNM-1, CD16, etc. NKG2D is the core activating receptor of NK cells, and this receptor can recognize a variety of different NKG2D ligands (NKG2DL) [8], such as major histocompatibility complex (MHC) Class I chain-associated protein A (MICA), MHC Class I chain-associated protein B (MICB), UL16-binding protein (ulbp). Their expression can initiate NK cell activation and promote cytotoxicity [9]. MICA and MICB are less well expressed on normal cells, but are upregulated on the surface of damaged, transformed or infected cells due to post-transcriptional or post-translation mechanisms and intracellular pathways. However, tumors can achieve tumor immune escape by reducing the expression of NKG2DL.

Inhibitory receptors are a type of transmembrane protein expressed on the surface of NK cells. The main function is to identify markers composed mainly of MHC-I molecules and transmit inhibitory signals to NK cells, thus preventing their killing of normal healthy cells and maintaining immune resistance. The KIRs, NKG2A, T cell immunoglobulin and mucin domain-3 (TIM-3), T cell immunoglobulin and ITIM domain (TIGIT), T cell activation inhibitory factor (VISTA), programmed death-1 (PD-1), cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and lymphocyte activation gene 3 (LAG-3) are all common inhibitory receptors [10]. According to the missing self theory, sufficient MHC-I and inhibitory receptors on the NK cell surface are expressed in normal cells, delivering inhibitory signals to prevent autoimmune attacks [11]. When these receptors bind to ligands, their intracellular immune receptor tyrosine inhibitory motif (ITIM) undergoes phosphorylation, which then recruits SHP-1/SHP-2 phosphatases. Through dephosphorylation, these phosphatases block downstream signaling molecules, thereby stopping NK cells from producing cytotoxicity. Under normal circumstances, tumor or virus-infected cells downregulate MHC-I expression. Thus, the lack of inhibitory signals results in NK cell activation and clearance of these abnormal cells. However, some tumors successfully evade recognition and elimination by NK cells through upregulation of non-classical MHC-I molecules, such as HLA-E and HLA-G, or immune checkpoint ligands, such as PD-L1, which continuously activate inhibitory receptor signaling pathways. Therefore, targeting inhibitory receptors has become one of the core strategies for restoring or amplifying the anti-tumor activity of NK cells.

3. Application of CRISPR-Cas9 Editing in NK Cells Modification

The CRISPR-Cas9 system is composed of sgRNA and Cas9 DNA endonuclease. Among them, sgRNA pairs with the target DNA through a sequence of approximately 20 nucleotides at its 5' end to precisely identify specific targeted sites in the genome. This process requires Cas9 to pair with the adjacent motif of the original interval sequence downstream of the target sequence (PAM). The identification is usually 5'-NGG-3' to ensure the specificity of the cutting site. Afterwards, the HNH and RuvC nuclease domains of Cas9 cleaved the target DNA's complementary strand and non-complementary strand, respectively, introducing precise double-strand breaks (DSBs) approximately 3 nucleotides above the PAM sequence. This break triggers the cell's own repair mechanism, where efficient non-homologous end junctions (NHEJ) directly connect to the broken ends. Due to the susceptibility to random insertions or deletions (indels), it eventually leads to gene knockout. Homologous directed repair (HDR) that relies on exogenous DNA templates achieves precise gene insertion or replacement through donor templates, but its efficiency is significantly lower than that of NHEJ [2, 12].

Nowadays, the KLRC1 gene in the NKG2A inhibitory receptor can be knocked out through CRISPR-Cas9 technology. By designing specific Sgrnas to target the KLRC1 locus, Cas9 nuclease is guided to induce DSB at the target site, thereby triggering the NHEJ repair mechanism and leading to insertion or deletion mutations. Genotype testing revealed that the destruction frequency of the KLRC1 gene exceeded 90%, but the inhibition of surface receptor expression only decreased by approximately 50%, indicating that some frameshift variations in the indel mutation did not completely eliminate the expression of the NKG2A protein. In the acute myeloid leukemia (AML) model, the combination of KLRC1 knockout and CD33-CAR engineering (CAR33-KLRC1ko-NK) significantly enhanced NK cytotoxicity. By inhibiting the axis of NKG2A-HLA-E immune checkpoint, the initiating cell load of leukemia can be reduced, survival can be prolonged, and the exhaustion induced by the AML microenvironment can be avoided [4]. In addition, frameshift mutations were successfully introduced at exon 2 of the KLRC1 gene which exists in human pluripotent stem cells (hPSCs) containing H9 ESCs and iPSCs. Flow cytometry verification of differentiated NK cells showed that the expression of NKG2A protein was completely eliminated, that is, the knockout efficiency reached 100%. Moreover, the expression of its heterodimer companion CD94 also decreased significantly, while the other activating or inhibitory receptors (such as NKG2D, NKp46, and TRAIL) are not affected. *In vitro* glioblastoma models (GBM), B-cell leukemia models, and mice carrying GBM transplanted tumors, the deletion of NKG2A can block HLA-E-mediated inhibitory signals, relieve NK cell functional inhibition, and enhance cytotoxicity [13].

In addition to NKG2A, inhibitory receptors such as PD-1, TIGIT, and TIM-3 are also important targets. For instance, knocking out the PD-1 target through CRISPR-Cas9 technology can not only relieve the inhibition of immune checkpoints, significantly increase the secretion of effector cytokines such as IFN-γ and TNF-α, and enhance cytotoxicity, but also reduce the proportion of regulatory T cells. Moreover, directly knocking out PD-L1 in tumor cells can synergistically enhance antigen presentation. This technology has been widely applied to various types of tumors, including hematological malignancies (such as leukemia, multiple myeloma) and solid tumors (such as liver cancer, triple-negative breast cancer, cervical cancer, colorectal cancer, lung cancer, etc.) [14]. In addition, knocking out the TIGIT target using CRISPR-Cas9 technology can enhance the direct killing ability by NK cells against tumor cells by releasing TIGIT's inhibition of activating receptors (such as DNAM-1), and can also avoid NK cell killing of itself caused by Fc-mediated anti-TIGIT antibodies. It is now being used to combat lung cancer, neuroblastoma, osteosarcoma, SJBM2, etc [5]. In addition, knocking out TIM3 using CRISPR-Cas9 technology can reverse the functional impairment in the tumor microenvironment of NK cells, block the interaction with TIM3 and its ligands, and significantly increase the cytotoxic effect of NK cells on tumor cells. This technology has been applied to the treatment of glioblastoma [15].

4. Conclusion

Targeted knockout of NK cell inhibitory receptors mediated by CRISPR-Cas9 (such as NKG2A, TIGIT, TIM-3) can significantly break through the inhibition of immune cells by the tumor microenvironment. By removing the so-called immune brake, these interventions can enhance the cytotoxicity of NK cells against solid tumors (such as glioblastoma) and hematological malignancies (AML, lymphoma) with high expression of PD-L1. Secondly, the combination of CAR-NK can construct multiple targets for synergistic killing of tumor cells, and can also avoid the individualized preparation bottleneck of CAR-T therapy, significantly reducing the treatment cost and time. Although gene-edited NK cells have demonstrated considerable efficacy and preliminary safety in hematological tumors such as multiple myeloma, lymphoma and acute myeloid leukemia, the clinical transformation of solid tumors still faces challenges such as delivery efficiency, off-target risk, tumor immunosuppressive microenvironment and long-term functional maintenance. Future research needs to make progress in optimizing sgRNA and Cas9 tools, improving delivery platforms, enhancing the assessment of off-target and immune-related adverse events, and verifying functional persistence in more relevant in vivo models, in order to promote the robust clinical translation of this strategy.

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