From Traditional CRISPR-Cas9 to Advanced Precision: Harnessing Base Editing and Prime Editing Technologies in the Precision Genetic Engineering of Natural Killer (NK) Cells

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Abstract

The field of genetic engineering for immune cells represents a promising frontier in treating cancer and autoimmune diseases. While CRISPR-Cas9 has revolutionized genome editing by enabling high-efficiency modifications, its reliance on double-strand breaks (DSBs) raises concerns regarding off-target effects and random indel formations. This review evaluates the transformative potential of next-generation tools, specifically Base Editing and Prime Editing, which overcome these limitations by enabling precise modifications requiring only single-strand nicks. Focusing on Natural Killer (NK) cells, ideal candidates due to their dual role in tumor surveillance and immune regulation, we assess the applications of these advanced technologies. Recent literature indicates that Prime Editing offers a unique balance of flexibility and precision, allowing for targeted insertions, deletions, and substitutions to correct point mutations and enhance NK cell survival and function. However, significant challenges persist, notably the large molecular size of the editing complex limiting delivery efficiency and the need to mitigate immunogenicity against bacterial-derived editing proteins. We analyze the mechanical foundations, clinical applications in oncology and autoimmunity, and critical delivery challenges associated with these platforms, providing a comprehensive overview of the transition from gene disruption to precise correction in NK cell engineering.

Keywords: Gene Editing; CRISPR-Cas Systems; Prime Editing; Base Editing; Killer Cells, Natural; Immunotherapy; Autoimmunity.

Introduction

Autoimmune diseases pose a major clinical challenge, as they lead the immune system to attack healthy body tissues, requiring innovative therapeutic strategies that go beyond traditional reliance on immunosuppressive drugs. Conversely, malignant tumors represent a counter-model of immune dysfunction, where immune cells fail to recognize or eliminate cancer cells due to Immune Evasion mechanisms. In this context, the field of Genetic Engineering has emerged as a promising prospect for reprogramming immune cells in a precise and controllable way.

The CRISPR-Cas9 system is considered a revolutionary tool that ushered in a new era in genome editing, as it provides an effective mechanism for creating a Double-Strand Break (DSB) with the aim of editing or disabling genes (Gene Knockout).[1-3] However, the relatively random nature of the repair following the break, along with the risks of Off-Target Effects, has imposed substantial limitations on expanding the clinical use of this platform, which requires a high degree of precision and safety.[3] To address these challenges, improved generations of genome editing systems have emerged. Base Editing technology allows for the correction of Point Mutations by changing a single nitrogenous base without creating double-strand breaks in the DNA, which increases the level of safety and reduces reliance on random repair mechanisms.[4,5] As for Prime Editing, it represents a qualitative leap in flexibility and precision; it allows for the insertion, deletion, or replacement of short segments of DNA with high accuracy, while sufficing with a Single Strand Nick.[6] This development opens the door to precise and controlled gene editing strategies to treat a wide range of complex genetic disorders.

Natural Killer (NK) cells come at the forefront of immune cells nominated for genetic engineering due to their pivotal role in monitoring the immune system and their innate ability to kill diseased or transformed cells, including tumor cells that escape the adaptive immune response.[7,8] This literature review focuses on evaluating the transformative potential of Base Editing and Prime Editing as tools for the precision engineering of NK cells to enhance their function or reprogram their genetic pathways in the context of treating autoimmune diseases and potentially tumors as well. It also addresses an analysis of the trade-offs between these two techniques, assessing their efficiency and safety, and exploring the key challenges facing the transition from proof-of-concept in the lab to clinical applications.

Body of the Review:

1. Mechanical Foundations of Gene Editing Technologies

The fundamental difference between successive generations of genome editing tools lies in how they target DNA and the degree of control over subsequent cellular repair mechanisms. The basic mechanisms for CRISPR-Cas9, Base Editing, and Prime Editing can be summarized as follows, starting with the traditional CRISPR-Cas9 system:

1.1 Traditional CRISPR-Cas9 System

Basic Components: The Cas9 protein, which is a Nuclease enzyme that acts as "molecular scissors" capable of cleaving DNA at a specific site.

Guide RNA (sgRNA): A hybrid molecule that guides the Cas9 protein to the target genetic sequence with high precision through Base Pairing with the target sequence.

Mechanism of Action: The sgRNA guides the Cas9-sgRNA complex to the specified site in the genome, provided that a neighboring activator sequence known as the Protospacer Adjacent Motif (PAM) is present. Upon successful binding to the target, the Cas9 protein creates a Double Strand Break (DSB) at the targeted site.[1,2]

Editing Outcome: The fate of this double-strand break depends on two main cellular repair pathways:

Non-Homologous End Joining (NHEJ): The most common pathway, which often leads to random insertions or deletions (Indels), resulting in the disruption of the gene's reading frame and a functional Gene Knockout .

Homology Directed Repair (HDR): A less efficient pathway that requires a donor DNA template carrying the desired sequence for insertion; it is used to integrate new sequences or make specific modifications at the target site.[3]

Main Limitation: The fundamental constraint of this system lies in its reliance on creating double-strand breaks in the DNA, which may cause undesirable Chromosomal Rearrangements and increases the probability of Off-Target Effects, raising safety concerns in sensitive clinical applications.[3,9]

1.2 Base Editing

Basic Components: A Cas protein of the Cas Nickase type (often a partially disabled Cas9); it performs a cut in only one strand of the DNA (Single Strand Nick). Deaminase enzyme: The enzyme responsible for the chemical modification of the target nitrogenous base within a specific range of the sequence.[4,5]

Mechanism of Action: The base editing complex (Cas Nickase Deaminase) is guided to the genetic site to be modified by the Guide RNA (sgRNA) . The deaminase enzyme performs a specific chemical modification on a nitrogenous base; for example, Cytosine (C) can be converted to Uracil (U), which is later read as a Thymine (T) base after cycles of replication and transcription, without the need to cut both strands of the DNA. Subsequently, the Cas protein creates a Single Strand Nick in the non-modified strand to stimulate cellular repair mechanisms that use the modified strand as a template, leading to the fixation of the required base change in the

genome. Despite the relatively high precision of this technique in targeting a single base, an important challenge arises known as Bystander Editing, which occurs when more than one editable base exists within the limited Activity Window of the deaminase enzyme. In this case, more than one base may be modified simultaneously and undesirably, which imposes strict constraints on the design of the sgRNA and must be taken into account to avoid introducing unintended additional mutations at the same target genetic site.[5]

Main Advantage: Base editing provides an effective and high-precision means to correct common point mutations, such as converting C to T or A to G, while reducing the risks of off-target effects associated with double-strand breaks in the DNA.[5,9]

Main Limitation: Base editing remains limited to specific changes at the level of a single base or a few bases within the activity window; it does not allow for the insertion of new genetic sequences or the execution of wide-scale deletions or substitutions, which limits its use in some complex therapeutic scenarios[9]

1.3 Prime Editing

Basic Components: Cas9 Nickase (H840A): A modified form of Cas9 that creates a cut in only one strand of the DNA. Reverse Transcriptase enzyme: An enzyme responsible for synthesizing new DNA based on an RNA template. Prime Editing Guide RNA (pegRNA): A specially designed molecule that combines the guiding function (similar to sgRNA) and the Editing Template required to copy the desired sequence to be inserted via the reverse transcriptase enzyme.[6,10,11]

The pegRNA represents the most unique element in this platform, as the integrated template allows for the insertion, deletion, or substitution of new genetic information directly at the target site.

Mechanism of Action: The pegRNA guides the Prime Editor complex (consisting of Cas9 Nickase fused with Reverse Transcriptase) to the genetic site to be modified. After recognizing the target sequence and binding to it, the Cas9 Nickase creates a cut in one strand of the DNA, providing a starting point for the reverse transcriptase enzyme. This enzyme uses the template part of the pegRNA to copy the new sequence directly onto the cut strand, forming a "flap" carrying the modified genetic information. Cellular repair mechanisms then take over to integrate this sequence into the genome and fix it permanently.[6,10]

Editing Advantage: Prime editing is characterized by high flexibility, as it allows for the insertion, deletion, or substitution of sequences reaching lengths of several dozen nitrogenous bases with extreme precision, without the need to create a Double Strand Break (DSB) in the DNA. This design significantly reduces reliance on the Non-Homologous End Joining **(NHEJ)** pathway and its random results, thereby improving the safety profile compared to DSB-based editing. It also surpasses Base Editing in terms of the diversity and range of possible modifications.[6]

Main Limitation: Despite these advantages, Prime Editing technology faces significant challenges in practical application, most notably the difficulty of delivering the complete complex (Prime Editor protein + pegRNA) due to its large molecular size and the complexity of its components. This is known as the Delivery Challenge, in addition to a relatively lower editing efficiency compared to Base Editing in some cellular systems, especially in sensitive primary cells.[6,9]

Recent breakthroughs in 2025 have further distinguished Prime Editing (PE) from its predecessors. While Base Editing is constrained by specific transition windows, the advent of optimized PE architectures, such as PE7, integrated with AI-driven pegRNA design, has significantly elevated editing efficiency in quiescent immune cells. Unlike the canonical CRISPR-Cas9 which induces Double-Strand Breaks (DSBs), these refined systems achieve precise genomic alterations in NK cells with a near-zero profile of p53-mediated DNA damage

responses.[12]

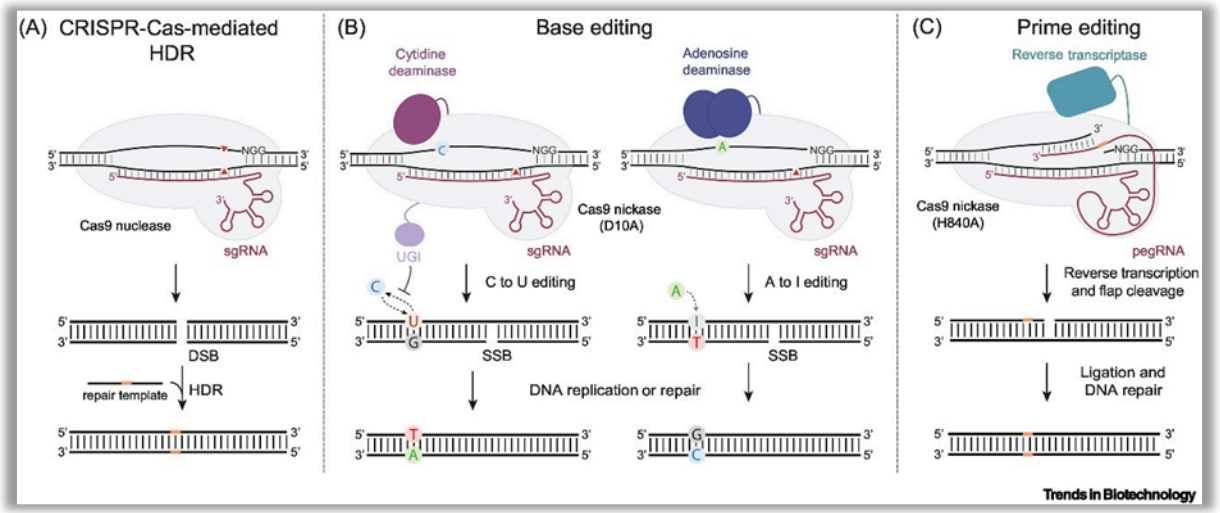

Figure 1: Comparison of genome-editing mechanisms. (A) Traditional CRISPR-Cas9 induces a DNA double-strand break (DSB), followed by cellular repair through homology-directed repair (HDR) or non-homologous end joining (NHEJ). (B) Base Editing uses a Cas nickase–deaminase complex to install targeted nucleotide conversions without generating a DSB. (C) Prime Editing uses a Cas9 nickase–reverse transcriptase fusion and a prime-editing guide RNA (pegRNA) to introduce targeted substitutions, insertions, or deletions without requiring a DSB.

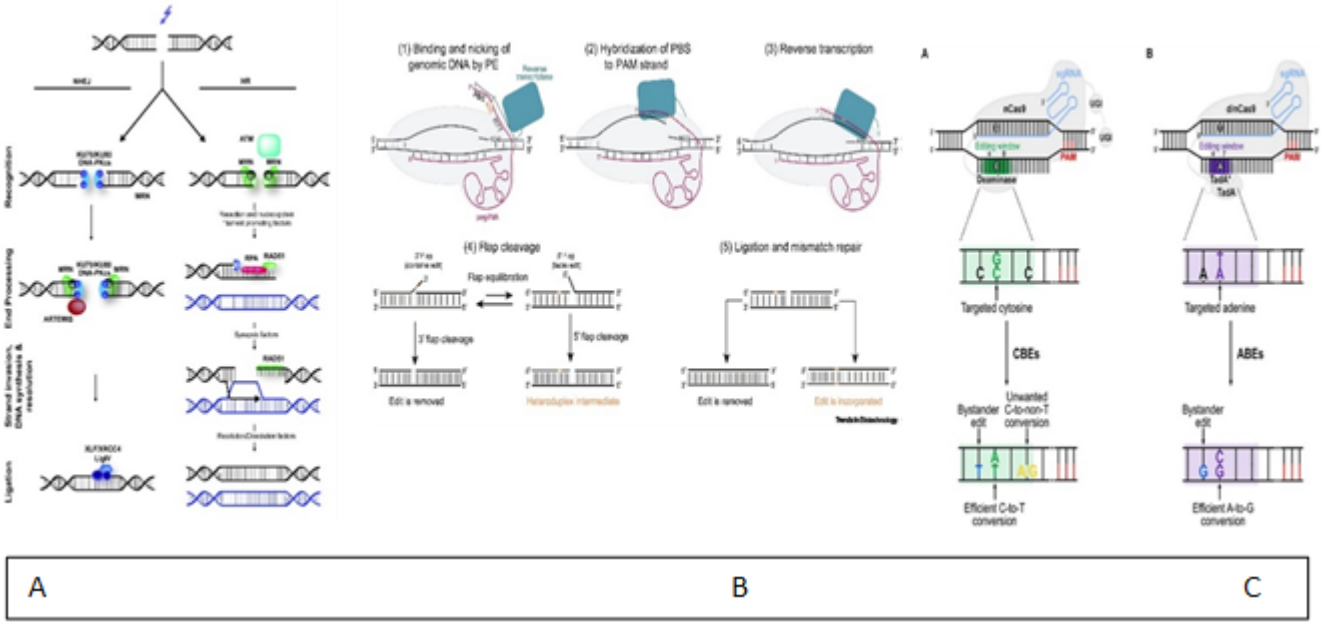

Figure 2: Schematic representation of the major mechanistic limitations of genome-editing platforms. (A) CRISPR-Cas9 induces a DNA double-strand break followed predominantly by NHEJ or, less efficiently, HDR, with the potential for unintended indels and genomic rearrangements. (B) Prime Editing avoids a double-strand break but requires a comparatively large editing complex, creating important delivery challenges. (C) Base Editing enables targeted nucleotide conversion without a double-strand break but may generate unintended bystander edits when multiple editable bases are present within the activity window.

2. Transition from Mechanical Foundations to Pathological Applications: Autoimmunity and Cancer as Two Complementary Models

A precise understanding of the mechanical mechanisms of genome editing technologies (**CRISPR-Cas9, Base Editing, Prime Editing**) is only complete by linking them to clinical pathological contexts, where a functional paradox emerges between autoimmune diseases and cancer as two complementary models of immune balance disruption. While autoimmune diseases are characterized by excessive immune system activity—where it mistakenly attacks the body's healthy tissues—cancer is characterized by an inability of the immune response to recognize and eliminate tumor cells due to sophisticated **Immune Evasion** mechanisms. This functional contrast in immune cells—particularly **Natural Killer (NK) cells** —makes them an ideal target for applying precision gene-editing techniques.[7,8] By utilizing the same molecular tools, NK cells can be reprogrammed to achieve two opposite goals: suppressing inflammatory responses in autoimmune diseases, or enhancing cytotoxic activity against tumor cells within the **Tumor Microenvironment (TME)** .

2.1 Immune Cells and Cancer: From Natural Immunosurveillance to Advanced Genetic Engineering

2.1.1 Cancer: The Global Burden and the Necessity for a Precision Immunotherapy Approach

Cancer is classified as one of the most prominent global health challenges of the twenty-first century. Approximately 20 million new cases were recorded in 2022, with this number expected to rise significantly by 2050 due to population aging, environmental factors, and lifestyle changes.[13] Cancer ranks among causes of death globally, causing about 10 million deaths annually, posing a massive economic and demographic burden on health systems, especially in developing countries.[13] The danger of cancer lies in its biological complexity, as it arises from the accumulation of genetic mutations and epigenetic disturbances that disrupt cell cycle control mechanisms, enabling tumor cells to grow uncontrollably, invade adjacent tissues, and spread to distant organs (**Metastasis**). These characteristics have demonstrated the limited effectiveness of traditional treatments (surgery, chemotherapy, radiotherapy), especially in advanced stages, which has necessitated a transition toward

precise molecular and immunological therapeutic strategies.

2.1.2 Concept of Immunosurveillance and Immune Evasion

The body relies on its innate defense against cancer through **Immunosurveillance**, a continuous system where cells monitor for, and eliminate, neoplastic transformations before clinical progression. **NK cells** serve as the primary first-line defenders alongside **Cytotoxic T cells (CD8+)**, characterized by the unique "**Missing Self-Recognition**" property.[7,8]

Healthy cells maintain high expression of **MHC-I** molecules, which bind to inhibitory receptors (such as **KIR/NKG2A**) on the surface of NK cells, keeping them in a resting state. **Transformed cells**, however, lose MHC-I and express **stress ligands (MICA/B, ULBP-1-6)**, which bind to activating receptors (**NKG2D**), triggering the NK cell to perform immediate killing via **Perforin/Granzyme B**.[12]

NK cells play a dual role, combining innate killing and immune regulation. They kill cells infected with viruses or transformed cells and regulate inflammation via **IL-10/TGF-β** to limit excessive responses, while also demonstrating adaptive-like memory in certain contexts such as chronic viral infections.[7,8]

Cancers have developed multiple immune escape strategies to evade NK cells, including the loss of MHC-I, the expression of inhibitory ligands such as PD-L1 and TIGIT, secretion of immunosuppressive cytokines such as TGF-β, recruitment of Myeloid-Derived Suppressor Cells (MDSCs), shedding of activating ligands such as MICA, and metabolic suppression within the tumor microenvironment.[12]

Scientific evidence demonstrates the importance of NK cells, as deficiencies in their function are associated with increased susceptibility to malignancies, while enhanced NK activity correlates with improved clinical outcomes in certain cancers.[7,8]

Immune surveillance relies on **NK cells** as commanders and **T-cells** as soldiers. Since cancers primarily attack NK cells. engineering NK cells via **Base/Prime Editing** becomes the strategic key for successful immunotherapy.

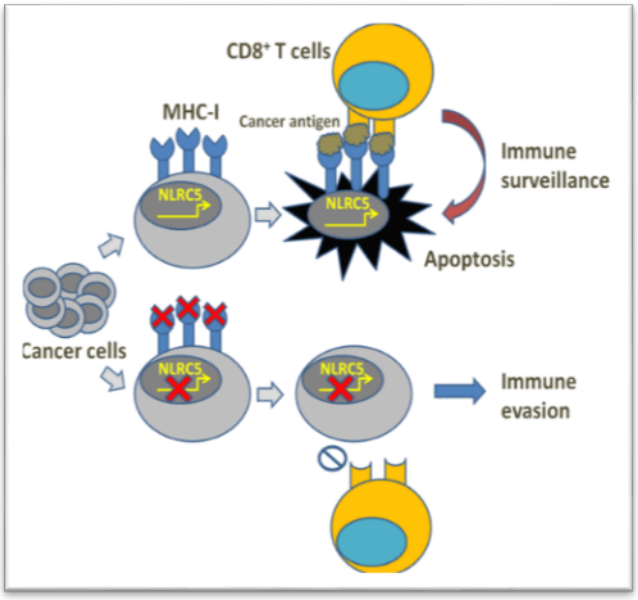

Figure 3: Role of NLRC5 in MHC class I expression and T-cell immunosurveillance. In the normal state, NLRC5 promotes MHC-I expression and antigen presentation, enabling recognition of tumor-associated antigens by CD8+ T cells. Reduced NLRC5 activity may decrease MHC-I expression and impair CD8+ T-cell recognition, thereby contributing to tumor immune evasion.

In the context of solid tumors, the application of precision editing has shifted toward multi-loci orchestration. Strategic co-editing of inhibitory checkpoints, specifically NKG2A and FAS, using Base Editing has been shown to not only prevent exhaustion but also fundamentally reprogram the metabolic fitness of NK cells. This ensures sustained cytotoxic persistence within the immunosuppressive and hypoxic tumor microenvironment, a critical factor for the success of 'off-the-shelf' CAR-NK therapies.[15]

2.1.3 The Immunotherapy Revolution and the Emergence of Modified T-cells (CAR-T Cells)

The deep understanding of the complex interactions between the tumor and the immune system has led to a radical shift in cancer treatment, manifested in **Cancer Immunotherapy** . This field aims to restore or enhance the innate ability of the immune system to eliminate tumors. A pioneering achievement in this field is **Chimeric Antigen Receptor T-cells (CAR-T cells)**, where T-cells are extracted from the patient and then genetically modified to express artificial receptors designed specifically for the direct recognition of specific antigens on the surface of cancer cells. This technology has shown high biological response rates (reaching **80-90%** in some types of blood cancers), establishing the concept of cell therapy as a fundamental pillar in modern anti-cancer treatments.[16,17]

2.1.4 From CAR-T Cells to NK Cell Engineering: Toward a Safer and More Flexible Therapeutic Generation

Despite the significant achievements made by **CAR-T** cells, their use is accompanied by core challenges that have limited their clinical generalization. Most notable among these are the very high costs associated with custom manufacturing for each individual patient (**Autologous Therapy**) and serious immune risks such as **Cytokine Release Syndrome (CRS)**, in addition to limited effectiveness in solid tumors. These constraints have pushed research toward safer and more flexible alternatives, bringing **Natural Killer (NK) cells** into the spotlight as a promising therapeutic option.

NK cells are distinguished by their innate ability to kill tumor cells without the need for prior sensitization or antigen presentation via MHC molecules. They also have a lower tendency to cause excessive immune reactions, which opens the field for developing **"Off-the-shelf"** treatments derived from healthy donors. This

has led to a strategic shift in immunotherapy, represented by the move from T-cell engineering to the **precision genetic engineering of NK cells** . With the development of advanced genome editing tools, such as **Base Editing** and **Prime Editing**, it has become possible to reprogram NK cells with high precision to enhance their anti-tumor efficacy, improve their survival, and reduce their inhibition within the tumor environment—all while minimizing the genetic risks associated with double-strand DNA breaks.

This represents the next generation of immunotherapies, which combines a deep understanding of cancer biology with the latest genetic engineering techniques to provide safer and more efficient therapeutic solutions for both cancer and autoimmune disorders.[18]

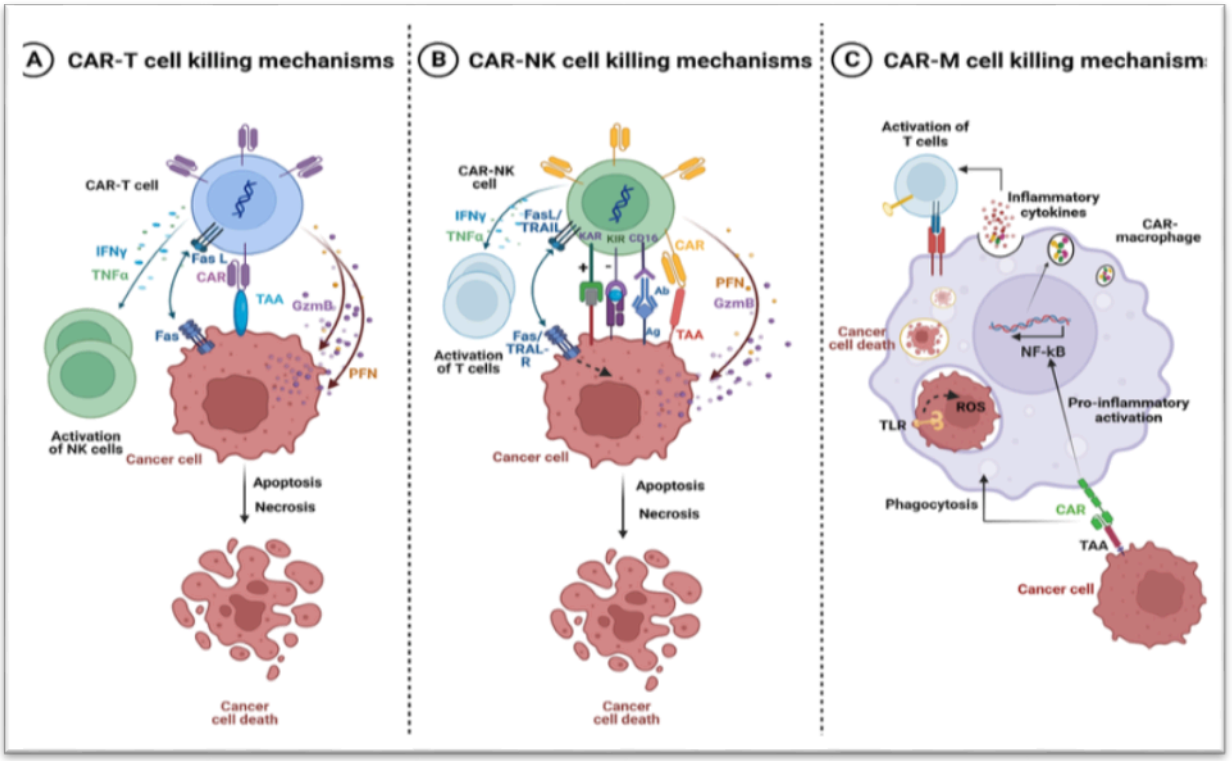

Figure 4: Comparison of CAR-engineered immune-cell killing mechanisms. (A) CAR-T cells primarily mediate direct cytotoxicity through the release of perforin (PFN), granzyme B (GzmB), FasL, and inflammatory cytokines (IFNγ and TNFα), leading to cancer cell death. (B) CAR-NK cells combine CAR-dependent recognition with innate cytotoxic mechanisms, including the release of IFNγ, TNFα, and FasL/TRAIL, and can also contribute to the activation of T cells. (C) CAR-engineered macrophages (CAR-M) exert anti-tumor effects through phagocytosis, as well as pro-inflammatory activation via NF-κB, ROS, and TLR pathways, leading to tumor cell elimination.

3. Applications of Precision Genome Editing in Cell Engineering for the Treatment of Autoimmune Diseases

Next-generation genome editing tools, specifically **Base Editing** and **Prime Editing**, have revolutionized immunotherapy by providing the precision necessary to reprogram immune cells in an effective and safe manner. While primary cellular therapies, such as **CAR-T** cell therapy, relied essentially on the **CRISPR-Cas9** system to perform **Gene Knockouts**, the latest technologies have enabled the possibility of introducing point genetic modifications or small substitutions with minimal unwanted genetic damage. This radical development is directed toward improving the response against cancer and controlling diseases resulting from immune system dysfunction, particularly autoimmune diseases.

3.1 Modification of Natural Killer (NK) Cells and Autoimmune Therapy

Natural Killer (NK) cells are an ideal candidate for genetic engineering, not only for their innate ability to kill cancer cells but also for their decisive role in regulating the immune response and controlling chronic inflammation and autoimmune diseases. A defect in NK cell function can lead to the exacerbation of diseases such as **Systemic Lupus Erythematosus (SLE)** or **Multiple Sclerosis (MS)**.[19,20]

3.1.1 Engineering for Immunosuppression

Precision editing tools (**Base Editing** and **Prime Editing**) are used to perform genetic modifications in NK cells that do not target killing, but rather aim to change the **Cytokine Profile** .

NK cells can be modified to enhance the secretion of immunosuppressive cytokines (such as **TGF-beta** or certain types of **ILs**) instead of pro-inflammatory cytokines, which helps suppress excessive autoimmune responses without causing broad-spectrum immunosuppression.[18,20]

3.1.2 Correction of Disease-Specific Mutations

Base Editing technology is specifically utilized to perform point genetic corrections in the genes of NK cells or other immune cells that carry known pathogenic genetic mutations characterized by weakening immune regulation or leading to excessive self-destructive activity for killer cells.[4,21]

3.1.3 Disabling Inhibitory Receptors:

Prime Editing can be used to insert or replace precise genetic sequences in NK cells to disable inhibitory receptors that reduce their efficiency in controlling inflammation, thereby enhancing their regulatory role in the disease microenvironment.[18,22]

In a transformative step recorded in **2025**, the first applications of **Multiplex Base Editing** in primary human NK cells were documented. This allowed for the achievement of contrasting mutations (**loss-of-function and gain-of-function**) within the same cell. This development enables the design of NK cells to target specific mechanisms in autoimmunity. For example, Base Editing can be used to correct point mutations in genes associated with diseases like **Systemic Lupus Erythematosus (SLE)**, or to engineer NK cells expressing **Chimeric Autoantigen Receptors (CAARs)** . These do not target cellular killing, but rather reprogram overactive B-cells or T-cells causing the disease. This advanced strategy represents a bridge between **CAR** applications successful in tumors and the increasing need for precise and sustainable treatments for autoimmune diseases.[18]

Precision genome editing offers a paradigm shift in treating systemic autoimmune pathologies. By utilizing Base Editors to correct point mutations in NK cell activating receptors, researchers can now fine-tune the 'missing self-recognition' mechanism. This allows for the selective depletion of autoreactive B-cells in conditions like Systemic Lupus Erythematosus (SLE) without the collateral systemic inflammation typically associated with non-specific genomic fragmentation.[23]

3.2 Modification of Regulatory T-cells (Tregs) and Complementary Strategies in Immunotherapy

Despite the pivotal role of NK cells in regulating the immune response, treating autoimmune diseases requires a deeper approach aimed at restoring immune balance. Therefore, precision editing tools are also used in engineering other regulatory cells, such as **Regulatory T-cells (Tregs),** which complement the role of NK cells in controlling chronic inflammation.[19,20]

3.2.1 Engineering Regulatory T-cells (Tregs)

Work is being done to modify **Treg** cells outside the body **(Ex Vivo**) using **Prime Editing** to enhance the expression of genes associated with their immunosuppressive function, such as **FOXP3** . Additionally, their

receptors can be modified to direct them toward specific organs damaged by the autoimmune disease (**Homing/Targeting Receptors**), providing a localized and targeted therapy.[20-22,24]

3.2.2 Targeting Non-Coding Genetic Elements

Precision editing techniques allow for the study and targeting of non-coding genetic elements, such as Enhancers or Promoters, which precisely control gene expression in immune cells (such as T or B cells) to alleviate the severity of inflammation. This provides a new mechanism for controlling autoimmune disease pathways at the genetic level.[6,9]

3.2.3 Comparison of Efficiency and Safety

The use of Base Editing/Prime Editing allows for the treatment of autoimmune diseases that require precise correction (compared to Cas9), where the safety of the edited cell and the avoidance of Off-Target Effects are of paramount importance to ensure that the immune dysfunction does not worsen.[5,6,9]

3.3 Precision Editing, Correction of Immune Dysfunction, and the Role of NK Cells in Immune Tolerance

The most precise application of genome editing tools lies in correcting genetic defects or reprogramming immune cells that cause autoimmune diseases (such as **Rheumatoid Arthritis** and **Systemic Lupus Erythematosus**). These diseases, characterized by a strong genetic component, require extreme precision that only second-generation technologies (**Base/Prime Editing**) provide.

3.3.1 Precision Directed Editing Strategies for Autoimmunity

Instead of the random killing of immune cells, precision editing tools are directed toward gene correction to "retrain" the immune system, focusing on the pivotal role of NK cells in regulating inflammation:

3.3.1.1 Correction of Point Mutations and Gene Expression (Base/Prime Editing)

Mutation Correction: Base Editing can be used to correct point mutations that cause disease (such as the **PTPN22 R620W** mutation associated with Rheumatoid Arthritis) in lymphoid cells.[5]

Enhancer Modification: Most genetic variants associated with autoimmune diseases are located in non-coding regions (**Non-coding regions**) such as Enhancers . Prime Editing can be used to perform precise modifications in these areas to control the expression level of pro-inflammatory genes (such as **MYC** and **FOXO1**), instead of completely disabling the gene.[6,9]

3.3.1.2 Enhancing the Regulatory Role of NK Cells:

Modifying cytokines: Precision editing can be used in NK cells to enhance the secretion of anti-inflammatory signals or increase the expression of immunosuppressive receptors. This helps suppress chronic inflammatory responses associated with autoimmune diseases without the need for traditional immunosuppressive drugs.[18,25]

These examples indicate that progress in editing precision is the key to moving from suppressive treatments to corrective treatments based on immune tolerance, which can be implemented with high efficiency in NK cells to regulate general immune balance.[21]

4. Challenges and Critical Evaluation (Critical Evaluation)

The transition from the laboratory to the clinical application of genome editing tools requires a rigorous critical evaluation of the fundamental differences between successive generations. The challenges focus on achieving a balance between editing precision (to avoid unwanted effects) and the efficiency of delivering large molecular complexes to target cells (NK cells and Tregs), as well as managing the immune response to foreign proteins.

4.1 Accuracy, Safety, and the Cellular Repair Pathway

The induced cellular repair pathway represents the fundamental determinant of both safety and effectiveness:

The risk associated with CRISPR-Cas9:

The most significant challenge lies in the reliance of the Cas9 system on creating a Double-Strand Break (DSB) in DNA. This break primarily activates the Non-Homologous End Joining (NHEJ) pathway, which is a fast and error-prone mechanism that leads to random insertions and deletions (Indels), potentially resulting in gene disruption (knockout). However, the greater risk arises from Off-Target Effects, where unintended DSBs may occur at other genomic sites, threatening chromosomal stability and potentially inducing harmful or oncogenic mutations.[3]

Enhanced safety in Base Editing and Prime Editing:

These systems were developed to bypass the limitations of Cas9 by avoiding double-strand breaks.

Base Editing:

Allows for the correction of Point Mutations with relatively high efficiency. However, a key mechanistic limitation is the possibility of unintended modification of neighboring bases (Bystander Editing) within the defined activity window of the deaminase enzyme.[5]

Prime Editing:

Represents the most versatile generation, as reverse transcription is directed to a defined template with high accuracy, enabling complex modifications (insertion, deletion, or substitution) while maintaining genomic integrity. Nevertheless, editing efficiency in some primary cells (particularly immune cells) remains relatively lower compared to Cas9 or Base Editing.[6,10]

4.2 Critical Constraints in Cellular Delivery (Delivery Challenges)

The efficiency of delivering editing components into the nuclei of target cells (such as NK cells or Tregs) remains the primary barrier limiting widespread clinical application:

4.2.1 Molecular Size

The challenge of molecular size is most pronounced in Prime Editing systems, where the complex consists of a large fusion protein (Cas nickase combined with reverse transcriptase) along with a long pegRNA molecule. These components exceed the cargo capacity of commonly used viral vectors such as Adeno-Associated Viruses (AAVs), thereby limiting the feasibility of in vivo applications.[9]

Non-viral approaches, such as Lipid Nanoparticles (LNPs), although suitable for delivering mRNA or sgRNA, face challenges in efficiently transporting large and complex editing systems into the nuclei of sensitive immune cells such as NK cells and Tregs. This limitation reduces editing efficiency and necessitates reliance on ex vivo delivery approaches, such as electroporation, which are associated with increased complexity and cost.[18,26]

4.2.2 Reliance on Ex Vivo Modification and Critique of Electroporation

These delivery constraints impose a strong reliance on ex vivo strategies, which involve extracting cells, modifying them in controlled laboratory conditions, and subsequently reinfusing them into the patient. Electroporation is commonly used for this purpose.

Although effective for delivering editing complexes, electroporation presents significant clinical and logistical challenges. It increases manufacturing cost and time, and more importantly, induces cellular stress that negatively impacts cell viability and reduces their capacity for expansion and long-term therapeutic persistence.

NK and Treg cells are classified as primary cells that are inherently difficult to transfect.

These cells exhibit limited proliferative capacity, and the delivery of large molecular constructs into their nuclei requires high energy input, which may compromise cellular integrity and reduce their physiological functionality.[8,27]

4.3 Immune Risks and Host Resistance

The immune response to genome editing systems represents a critical factor influencing treatment safety and durability:

Immune reaction against Cas proteins:

All Cas-based systems (including those used in Base and Prime Editing) are derived from bacterial origins. Due to prior exposure, many individuals possess pre-existing immunity against Cas proteins, particularly Cas9 derived from Streptococcus pyogenes.

This immune recognition may lead to the production of antibodies or T-cell responses that neutralize the editing system before it achieves its intended function.

To address this limitation, current research focuses on exploring alternative Cas proteins from less prevalent bacterial strains (such as Cas12a) or engineering Cas variants with reduced immunogenicity. This remains an active and evolving area of investigation.[9,11]

To determine the most suitable tool for immune cell engineering, a comprehensive analysis of the mechanistic differences between CRISPR-Cas9, Base Editing, and Prime Editing is essential, as these directly influence both safety and therapeutic efficiency.[18,24]

A comparative summary of the mechanistic differences between these three platforms is provided in Table 1.

Table 1: Comparative Analysis of Genome Editing Technologies in Immune Cell Engineering.

Prime Editing	Base Editing	CRISPR-CAS9	Feature / Criteria
Single-Strand Nick in one strand	Single-Strand Nick in one strand	Double-Strand Break (DSB)	Gene Editing Mechanism
Comprehensive point correction, small insertion/deletion/substitution	Precise correction of a single base	Random Insertion/Deletion (Indel) or large replacement via HDR	Required Outcome
Reverse transcription guided by pegRNA template	Targeted repair via chemical modification	NHEJ (High) or HDR (Low efficiency)	Cellular Repair Pathway
Very high and controlled	High (Limited by activity window)	Low	Precision and Control
Large molecular size of the editing complex	Bystander Editing phenomenon	Risks of Off-Target effects and DSB	Primary Limitation
Very High	Moderate	Moderate	Delivery Challenge

CAR integration, Targeted Knock-in, and precise enhancer modification	Correcting genetic mutations	Easy gene knockout	Application in NK/Tregs

5. Conclusion and Future Perspectives

The rapid evolution of genome editing tools—starting from the traditional Cas9 system and advancing to Base Editing and Prime Editing—has substantially reshaped the field of immunotherapy. These tools have demonstrated effectiveness not only in cancer treatment through the engineering of T cells and NK cells, but also in enabling precise intervention in autoimmune disease mechanisms and the regulation of chronic inflammation.

Critical Summary:

The shift from "Disruption" to "Correction":

This review highlights that the transition from the CRISPR-Cas9 system to second-generation tools reflects a qualitative shift toward more precise and safer genome correction strategies.

Prime Editing as a balanced platform:

Prime Editing emerges as a platform that offers a balance between the flexibility of Cas9 in performing diverse genetic modifications and the precision of Base Editing in avoiding double-strand breaks. It is therefore considered a highly promising system for future precision engineering of immune cells.[6,10]

Final Synthesis

Common Clinical Challenge:

Despite the improved precision of second-generation technologies, a major limitation remains the delivery efficiency of large and complex molecular systems into target cells. This challenge is further compounded by immunological risks associated with bacterial-derived proteins, which may limit their direct in vivo application.[9,11]

• Future Directions and Research Trends:

The future of genome editing in immunotherapy depends on overcoming current clinical barriers and advancing toward scalable and sustainable "off-the-shelf" therapeutic solutions:

a. Optimizing Delivery Systems:

Research efforts should focus on developing advanced nanovesicles or lipid nanoparticles (LNPs) capable of efficiently and safely delivering Base Editing and Prime Editing components to lymphoid cells at sites of inflammation in vivo. This would reduce reliance on complex and costly ex vivo modification procedures.

The development of targeted lipid nanoparticles (tLNPs) represents a promising strategy to overcome delivery challenges in primary immune cells such as NK cells. These systems are engineered by functionalizing their surface with ligands or antibodies that bind to specific receptors on NK cells, thereby enhancing cellular uptake of molecular payloads (e.g., mRNA or Prime Editor proteins) while minimizing off-target exposure and preserving cellular function. Successful implementation of this approach could enable the use of Prime Editing in an "off-the-shelf" format, particularly relevant for conditions requiring rapid therapeutic intervention.[11,18]

The trajectory of NK cell engineering is increasingly shifting toward non-viral delivery modalities. Incorporating Prime Editing systems into lipid nanoparticles may reduce risks associated with viral vectors, such as insertional mutagenesis, and facilitate scalable production of clinical-grade engineered NK cells.[26,29]

b. Engineering Less Immunogenic Cas Proteins:

To mitigate immunological risks, research is focusing on identifying alternative Cas proteins from less prevalent bacterial sources or engineering modified variants with reduced immunogenicity. These strategies aim to improve long-term safety and therapeutic durability.[9,12]

c. Multiplexed Gene Editing:

Advances in Base Editing and Prime Editing are expected to enable simultaneous modification of multiple genes within NK cells or Tregs. For instance, inhibitory receptors may be disabled while concurrently introducing genes that enhance cellular survival (such as IL-15), thereby improving the functional persistence and therapeutic efficacy of engineered cells in both cancer and autoimmune settings.[18,23]

d. Integration of Artificial Intelligence:

The integration of Artificial Intelligence is expected to play an increasingly important role in optimizing guide RNA design (sgRNA and pegRNA) and predicting off-target effects prior to experimental validation. This approach may accelerate the development and clinical translation of genome editing–based therapies.[10,15]

Conclusion

Recent evidence suggests that next-generation immunotherapies are moving toward precise and controlled reprogramming of the immune system using advanced genome editing tools. The transition from CRISPR-Cas9 to Base Editing and Prime Editing represents a significant step from gene disruption toward targeted and more controlled genetic correction.[5,7]

These technologies demonstrate the potential to enhance the functional versatility of NK cells, enabling improved anti-tumor activity while contributing to the regulation of autoimmune responses. Through targeted correction of pathogenic mutations, modulation of regulatory elements, and controlled cytokine expression, genome editing offers a promising approach for restoring immune balance with reduced systemic toxicity compared to conventional therapies.[18,26]

Prime Editing, in particular, provides a flexible platform for implementing complex and multi-layered genetic modifications in immune cells. This may support the future development of engineered NK cells with enhanced persistence, regulated activity, and improved resistance to inhibitory signals within pathological microenvironments.[6,10]

Ultimately, the successful clinical translation of these technologies will depend not only on scientific advancements, but also on overcoming manufacturing, scalability, and accessibility challenges. Collaborative efforts between academia, industry, and regulatory bodies will be essential to translate these innovations into widely available and cost-effective therapeutic solutions.

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