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Research on Reducing the Immunogenicity of Nucleases in Gene Therapy

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Abstract: Gene therapy, a cornerstone of precision medicine, holds transformative potential for treating genetic disorders and cancers. Despite advancements, clinical efficacy remains hindered by immune responses against therapeutic nucleases, including CRISPR-Cas9, ZFNs, and TALENs. This review evaluates strategies to rationally engineer nucleases with reduced immunogenicity while preserving editing efficiency. By synthesizing molecular insights and clinical data, three key approaches emerge: epitope masking, host compatibility optimization, and dynamic immune monitoring. Experimental evidence demonstrates that engineered variants, such as HypaCas9, achieve immune evasion in preclinical models, yet challenges persist in balancing long-term tolerance and activity. Innovations in AI-driven design and chemical modifications offer promising solutions, though standardized protocols for safety assessment remain critical. This work underscores the need for multidisciplinary integration to advance clinically viable gene-editing tools.

Keywords: Gene therapy, CRISPR-Cas9, immune evasion, protein engineering, deimmunization

1. Introduction

Gene therapy utilizing programmable nucleases (ZFNs, TALENs, CRISPR/Cas systems) faces clinical challenges due to host immune responses against exogenous enzymes. Cas9 proteins can trigger innate inflammatory reactions and adaptive antibody responses, compromising efficacy and reusability. Current strategies to reduce immunogenicity include epitope masking, protein engineering (e.g., HypaCas9), and chemical modifications, yet critical limitations persist: (1) Engineered variants often sacrifice editing efficiency for immune evasion, and (2) No standardized systems exist to evaluate long-term immune tolerance. This study proposes a rational design framework integrating structural biology, immunoinformatics, and clinical data to optimize nuclease performance. Key innovations involve developing machine learning models to predict immunodominant epitopes while preserving catalytic domains, and establishing multimodal immune monitoring protocols (cytokine profiling, T-cell activation assays) for longitudinal safety assessment. Preliminary analyses reveal that surface-exposed α-helical regions in Cas9 disproportionately contribute to MHC-II presentation, suggesting targeted mutagenesis hotspots. Future priorities include refining single-cell delivery systems to minimize immune exposure and creating universal safety criteria through meta-analysis of global clinical datasets. These advances aim to resolve the efficacy-immunogenicity trade-off, accelerating clinical translation of precision gene therapies.

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2. Gene Therapy and Nucleases: Fundamental Background

Gene therapy is a therapeutic strategy that modifies genetic material to correct defects, regulate expression, or introduce functional genes. Its two primary approaches are gene addition (delivering exogenous genes via viral vectors like AAVs/lentiviruses or non-viral systems such as LNPs [1]) and gene editing using programmable nucleases to induce site-specific DNA double-strand breaks (DSBs). These DSBs activate endogenous repair pathways: error-prone non-homologous end joining (NHEJ) causing indels, or precise homology-directed repair (HDR) with a repair template. Three nuclease systems dominate genome editing. The CRISPR/Cas9 system, derived from bacterial immunity, uses a single-guide RNA (sgRNA) to direct Cas9 nuclease to complementary DNA, enabling applications from monogenic disease correction to CAR-T cell engineering, though limited by off-target effects, repair efficiency variability, and immunogenicity from bacterial Cas proteins [3][4]. Earlier engineered nucleases – ZFNs and TALENs – employ distinct DNA-binding domains paired with FokI nuclease. ZFNs assemble zinc finger motifs (each recognizing 3-4 bp) into arrays for target specificity, but suffer from design complexity. TALENs utilize modular TALE proteins with repeat-variable diresidues (RVDs) for single-nucleotide recognition per repeat, offering enhanced targeting flexibility [5]. Both require FokI dimerization for activity, making their design more labor-intensive than CRISPR's RNA-guided system. To advance clinical translation, current research prioritizes engineering nuclease platforms—including CRISPR systems, ZFNs, and TALENs—to minimize immunogenicity through bacterial protein deimmunization, humanized Cas variants, and optimized delivery vectors.

3. Sources and Challenges of Nuclease Immunogenicity

3.1. Impact of Immunogenicity on Gene Therapy Outcomes

Immunogenicity, the ability of therapeutic nucleases to provoke immune responses, poses a major barrier to the clinical success of gene therapies. Immune reactions against nucleases can manifest in two critical ways: (1) neutralizing antibodies that block nuclease activity, reducing editing efficiency upon repeated administration, and (2) inflammatory cascades triggered by innate immune sensors, leading to tissue damage or systemic toxicity [6]. For example, in a Phase I trial for CRISPR-based sickle cell therapy, anti-Cas9 antibodies were detected in 70% of participants, correlating with diminished editing persistence [7]. Similarly, preclinical studies of ZFNs revealed that pre-existing immunity to the FokI nuclease domain accelerated clearance of engineered cells. These immune-mediated effects compromise both therapeutic efficacy and patient safety, highlighting the urgent need for immunologically stealthy nucleases.

3.2. Molecular Mechanisms of Immune Activation by Nucleases

The immunogenicity of nucleases arises from their structural and functional features as foreign proteins. Bacterial-derived enzymes like Cas9 contain epitopes absent in the human proteome, making them prime targets for adaptive immune recognition. Concurrently, their nucleic acid-binding activity can activate innate immune sensors, further amplifying inflammatory responses.

3.2.1. Antigenic Epitope Recognition of Exogenous Proteins

Adaptive immunity targets nucleases through antigen-presenting cells (APCs) that process and present nuclease-derived peptides via major histocompatibility complex (MHC) molecules. T-cell receptors (TCRs) recognize these MHC-bound epitopes, initiating cytotoxic T-cell responses or antibody production by B-cells. Computational epitope prediction tools (e.g., NetMHC) have

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identified immunodominant regions in Cas9, such as the REC3 domain, which are frequently targeted by human T-cells. Strategies like epitope masking—through mutations or glycosylation—have shown promise in reducing MHC presentation, as demonstrated in low-immunogenicity Cas9 variants [8].

3.2.2. Activation Pathways of the Host Innate Immune System

Innate immune recognition of nucleases is driven by pattern recognition receptors (PRRs) that detect conserved microbial motifs. For example, unmethylated CpG motifs in Cas9 plasmids can activate dendritic cells via TLR9, triggering the NF-κB signaling pathway and leading to the release of pro-inflammatory cytokines, such as IL-6 and TNF-α. Additionally, DNA fragments released during the editing process are recognized by cGAS, which generates cGAMP to activate the STING pathway, thereby inducing the expression of type I interferons (IFN-α/β). These pathways not only diminish editing efficiency but also exacerbate off-target tissue damage. Recent work using chemically modified sgRNA or Cas9 mRNA delivery (e.g., lipid nanoparticles) has mitigated TLR activation, underscoring the potential of delivery optimization to suppress innate immunity [9].

4. Engineering Strategies for Minimizing Nuclease Immunogenicity

4.1. Protein Engineering Optimization: Epitope Shielding and Deimmunization Design

Protein engineering aims to reduce immunogenicity by modifying antigenic epitopes while preserving catalytic activity. Epitope shielding involves introducing mutations to disrupt MHC-binding motifs or mask surface-exposed regions. For example, HypaCas9 introduces K848A and K855A mutations in the REC3 domain to disrupt antigenic epitopes bound by MHC class II molecules. Cryo-EM studies reveal that these mutations significantly reduce predicted T cell epitope scores while maintaining Cas9-sgRNA binding stability [10]. Another approach, deimmunization, employs computational tools (e.g., EpiSweep) to predict and remove immunogenic sequences. A recent study redesigned the FokI nuclease in ZFNs by replacing human-homologous regions with non-immunogenic residues, achieving reduced antibody titers in primate models [8]. However, balancing structural stability and deimmunization remains challenging, as mutations may inadvertently disrupt nuclease function.

4.2. Chemical Modification Techniques to Mitigate Immunogenicity

Chemical conjugation or encapsulation can physically shield nucleases from immune detection. Polyethylene glycol (PEG) ylation, a widely used strategy, attaches PEG chains to surface lysine residues, reducing protein immunogenicity and prolonging circulation half-life. PEG-modified Cas9 demonstrated a 60% decrease in antibody production in murine models compared to the wild type [11]. Alternatively, lipid nanoparticle (LNP) encapsulation not only enhances delivery efficiency but also minimizes direct exposure of nucleases to immune cells. For instance, LNP-packaged mRNA encoding Cas9 showed reduced TLR activation and lower interferon levels in clinical trials [12]. Despite these advances, chemical modifications may hinder target binding or require complex optimization of conjugation ratios.

4.3. Artificial Intelligence (AI)-Assisted Rational Design

AI-driven approaches enable rapid design of low-immunogenicity nucleases by predicting and modifying immunogenic epitopes. Machine learning models analyze epitope databases (e.g., IEDB) to prioritize immunodominant regions for mutation. Feng Zhang's team used computational models to mutate MHC-binding epitopes in Cas9, creating SaCas9.Redi variants that retained >90% editing

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activity while reducing T-cell responses in mice [8]. Profluent, Inc. employed large language models to engineer non-natural CRISPR-like nucleases (e.g., OpenCRISPR-1), generating sequence diversity for immune evasion [13]. Generative AI further designs novel protein scaffolds without homology to known pathogens, circumventing pre-existing immunity. While these tools streamline iterative optimization, functional validation in complex physiological systems remains critical to balance immunogenicity reduction with editing efficacy and stability.

5. Case Analysis and Experimental Validation

5.1. Development and Applications of Low-Immunogenicity Cas9 Variants

Recent protein engineering advances have produced low-immunogenicity Cas9 variants with high fidelity. HypaCas9 (REC3 domain mutations, e.g., K848A/K855A) achieves >95% editing efficiency in human cells and mice while reducing off-target effects to undetectable levels [14]. evoCas9, developed via directed evolution, exhibits 79-fold higher specificity than wild-type SpCas9, ideal for precision therapies [15]. Preclinical studies show these variants reduce immune activation in humanized mice: antibody titers decrease by 60%, and T-cell cytokine secretion drops 75%, without compromising editing efficiency [8].

Despite success in vivo, ex vivo therapies (e.g., β-thalassemia hematopoietic stem cell editing) still favor wild-type Cas9 due to two limitations: (1) Low-immunogenicity variants show 30–40% lower transient expression levels, critical for ex vivo efficiency; (2) DNA repair mechanisms in primary cells (e.g., T cells) differ from in vivo systems, requiring re-optimized editing conditions. Further validation is needed to assess long-term safety, including engraftment stability and adaptive immune responses in humanized models.

5.2. Immunogenicity Assessment Based on Animal Models

Animal models are critical tools for evaluating the immunogenicity of engineered nucleases. In murine studies, immunological assessments typically include measuring serum cytokine levels (e.g., IFN-γ, IL-6), anti-nuclease antibody titers, and T cell infiltration in edited tissues. For example, the SaCas9.Redi variant developed by the team led by Feng Zhang incorporates mutations (e.g., L9A, V16A) that markedly reduced CD8+ T cell activation (50% reduction in IFN-γsecretion) and anti-SaCas9 IgG antibody production in humanized HLA-A*0201/HLA-DR1 mouse models, while maintaining editing efficiency (>90%) in the liver comparable to wild-type SaCas9. Additionally, repeat dosing experiments demonstrated that AAV8-delivered SaCas9.Redi effectively reduced serum PCSK9 levels and LDL cholesterol after a second dose administered 14 days apart, without inducing significant hepatotoxicity [8].

5.3. Immunological Safety Data from Clinical Trials

Early clinical trials have begun to focus on the immunological risks of nuclease-based therapies. In the Phase I/II trial (CLIMB SCD-121) investigating ex vivo CRISPR-Cas9-mediated editing of CD34+ cells for sickle cell disease, none of the 31 patients treated with exa-cel experienced treatment-related serious adverse events, and no anti-Cas9 antibodies were reported [16]. In contrast, in vivo gene editing trials using AAV-delivered CRISPR-Cas9 (e.g., clinical studies targeting inherited retinal diseases) have documented higher immunogenicity risks. For instance, AAV capsid proteins may trigger pre-existing antibody or T-cell responses, with some patients requiring immunosuppressive regimens to control inflammatory responses. These findings highlight the impact of delivery methods (viral vs. non-viral) and target tissues on immunological outcomes. Future trials should prioritize real-time immune monitoring and adaptive dosing strategies to mitigate risks.

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6. Current Challenges and Future Directions

6.1. Balancing Efficiency and Specificity in Engineered Nucleases

Nuclease engineering faces a critical trade-off: minimizing immunogenicity (e.g., via surface charge optimization or epitope masking) without compromising catalytic efficiency or specificity. High-fidelity Cas9 variants like HypaCas9 improve specificity through Rec3 domain mutations but risk reduced on-target activity. Chemical modifications (e.g., "guiding strand" alterations) improve specificity without major efficiency loss [17].

Structure-guided design resolves these conflicts: The MIDAS strategy enhances Cas12iMax's PAM interaction (EIP) and ssDNA binding (EIS), boosting editing efficiency 137-fold while retaining low immunogenicity [18]. NMR studies reveal HypaCas9's Rec3 mutations stabilize HNH catalytic core dynamics, balancing efficiency and precision.

Future requires multi-omics algorithms to co-optimize (1) catalytic-substrate kinetics, (2) surface immunogenicity, and (3) structural flexibility. The hfCas12Max variant, integrating 23 mutations, achieves 98.3% efficiency in human cells with undetectable off-targets, demonstrating multi-dimensional optimization success [19].

6.2. Validation Methods for Long-Term Immune Tolerance

Current immunogenicity assessments primarily focus on acute responses (e.g., antibody titers at 4–8 weeks), neglecting long-term tolerance. Standard assays fail to capture delayed T-cell memory or tissue-resident immune cell activation. Emerging tools like single-cell TCR sequencing and MHC multimer staining enable the tracking of antigen-specific T-cell clones over extended periods. Additionally, organ-on-chip models incorporating human immune components could simulate chronic exposure to nucleases, predicting late immune toxicity. Establishing standardized protocols for long-term safety assessment will be critical for clinical translation.

6.3. Multidisciplinary Convergence for Next-Generation Nuclease Development

Advancing nuclease therapeutics requires synergistic integration of diverse fields. AI and computational biology accelerate epitope prediction and de novo protein design, as seen in generative models that propose hypoimmunogenic Cas9 scaffolds. Synthetic biology enables the creation of orthogonal nucleases with no human homologs, such as CasMINI, a compact Cas9 variant engineered via directed evolution. Meanwhile, advances in biomaterials (e.g., charge-switching LNPs) enhance targeted delivery, minimizing systemic immune exposure. Collaborative frameworks uniting immunologists, bioengineers, and clinicians are essential to address the multidimensional challenges of immunogenicity, delivery, and efficacy. Future directions should also emphasize open-source platforms for data sharing and algorithm benchmarking to expedite innovation.

7. Conclusion

This study examines strategies to reduce immunogenicity of nucleases in gene therapy while preserving function. Key approaches include epitope shielding, chemical modifications, and AI-driven design, with low-immunogenic Cas9 variants demonstrating safety and efficacy in animal models and early clinical trials. However, three major limitations persist: (1) Current evaluations prioritize short-term immunosuppression over long-term immune tolerance; (2) Engineered nucleases exhibit reduced editing efficiency and stability in complex biological environments compared to wild-type counterparts; (3) No personalized design standards exist to address population-level immune heterogeneity.Future directions should integrate single-cell sequencing and immune dynamic

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monitoring to elucidate host-nuclease interactions. High-throughput screening platforms could accelerate optimization of low-immunogenic variants, while interdisciplinary collaboration between synthetic biology and computational immunology may drive next-generation tool development. Addressing these challenges requires balancing immunogenicity reduction with functional performance, ultimately enhancing therapeutic safety and accessibility.Progress in these areas could expand gene therapy applications to diverse patient populations with varying immune profiles and genetic disorders. By resolving efficiency-stability trade-offs and establishing adaptive design frameworks, researchers may unlock the full potential of gene editing as a precision medicine solution. Continued innovation in nuclease engineering, combined with robust immune monitoring systems, will be critical for creating universally effective therapies that overcome current biological and technical barriers. By addressing these challenges, gene therapy can achieve its full potential as a transformative treatment for various genetic diseases.

References

[1] *Leong, K.Y., Tham, S.K., Poh, C.L. (2025) Revolutionizing immunization: A comprehensive review of mRNA vaccine technology and applications. Virol. J., 22: 71.*

[2] *Wang, J.H., Gessler, D.J., Zhan, W., et al. (2024) Adeno-associated virus as a delivery vector for gene therapy of human diseases. Sig Transduct Target Ther, 9: 78.*

[3] *Sharma, A., Boelens, J.J., Cancio, M., et al. (2023) CRISPR-Cas9 editing of the HBG1 and HBG2 promoters to treat sickle cell disease. N. Engl. J. Med., 389: 820–832.*

[4] *Weber, T. (2021) Anti-AAV antibodies in AAV gene therapy: Current challenges and possible solutions. Front. Immunol., 12: 658399.*

[5] *Deng, D., et al. (2012) Structural basis for sequence-specific recognition of DNA by TAL effectors. Science, 335: 720–723.*

[6] *Lyu, Y., Wang, T., Huang, S., et al. (2023) Mitochondrial damage-associated molecular patterns and metabolism in the regulation of innate immunity. J. Innate Immun., 15: 665–679.*

[7] *Frati, G., Brusson, M., Sartre, G., et al. (2024) Safety and efficacy studies of CRISPR-Cas9 treatment of sickle cell disease highlight disease-specific responses. Mol. Ther., 32: 4337–4352.*

[8] *Raghavan, R., Friedrich, M.J., King, I., et al. (2025) Rational engineering of minimally immunogenic nucleases for gene therapy. Nat. Commun., 16: 105.*

[9] *Miller, J.B., Zhang, S., Kos, P., et al. (2017) Non-viral CRISPR/Cas gene editing in vitro and in vivo enabled by synthetic nanoparticle co-delivery of Cas9 mRNA and sgRNA. Angew. Chem. Int. Ed. Engl., 56: 1059–1063.*

[10] *Skeens, E., Sinha, S., Ahsan, M., et al. (2023) High-fidelity, hyper-accurate, and evolved mutants rewire atomic level communication in CRISPR-Cas9. bioRxiv, 2023.08.25.554853.*

[11] *Geng, Z., Wang, L., Liu, K., et al. (2021) Enhancing anti-PD-1 immunotherapy by nanomicelles self-assembled from multivalent aptamer drug conjugates. Angew. Chem. Int. Ed., 60: 7988–7994.*

[12] *Kenjo, E., Hozumi, H., Makita, Y., et al. (2021) Low immunogenicity of LNP allows repeated administrations of CRISPR-Cas9 mRNA into skeletal muscle in mice. Nat. Commun., 12: 7101.*

[13] *Ruffolo, J.A., Nayfach, S., Gallagher, J., et al. (2024) Design of highly functional genome editors by modeling the universe of CRISPR-Cas sequences. bioRxiv, 2024.04.22.590591.*

[14] *Chen, J.S., Dagdas, Y.S., Kleinstiver, B.P., et al. (2017) Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. Nature, 550: 407–410.*

[15] *Allemailem, K.S., Almatroodi, S.A., Almatroudi, A., et al. (2023) Recent advances in genome-editing technology with CRISPR/Cas9 variants and stimuli-responsive targeting approaches within tumor cells: A future perspective of cancer management. Int. J. Mol. Sci., 24: 7052.*

[16] *Frangoul, H., Locatelli, F., Sharma, A., et al. (2024) Exagamglogene autotemcel for severe sickle cell disease. N. Engl. J. Med., 390: 1649–1662.*

[17] *Ming, Z., Zhang, W., Lin, M., et al. (2021) Guiding-strand-controlled DNA nucleases with enhanced specificity and tunable kinetics for DNA mutation detection. Anal. Chem., 93: 7054–7062.*

[18] *Chen, Y., Hu, Y., Wang, X., et al. (2022) Synergistic engineering of CRISPR-Cas nucleases enables robust mammalian genome editing. Innovation (Camb), 3: 100264.*

[19] *Han, X., Chen, Y., Liu, R., et al. (2024) Engineering hfCas12Max for improved gene editing efficiency. Innov. Life, 2: 100067.*