A CRISPR-based approach to targeting a pathogenic *TLR7* gene variant in systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease where the body's immune system becomes highly sensitive to non-pathogenic ssRNA, triggering an immune attack on healthy tissue. This incites inflammation, tissue damage, and life-long, adverse side effects. Research has linked over 50 genes to SLE, including the *TLR7* gene, which is often overexpressed in individuals with SLE. Recently, a pathogenic variant of the *TLR7* gene, known as *TLR7*-Y264H was demonstrated to oversensitize the toll-like receptors in the cell, resulting in the symptoms of SLE. There is a significant gap in research for SLE treatments, as current options often fail to provide long-term disease control with minimal toxicity. Therefore, this study explores a CRISPR-based approach to rectify this pathogenic variant of TLR7. In light of recent research highlighting the role of *TLR7*-Y264H mutation's overexpression in SLE patients, this study investigates whether a CRISPR-based therapeutic approach can effectively dampen SLE symptoms.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease affecting over 3 million individuals worldwide.[1] SLE is characterized by aberrant immune system activation causing inflammation and damage across multiple organ systems. SLE is influenced by genetic and environmental factors and has variations in clinical presentation and severity among different populations.[1,2] For instance, women are 10 times more likely to develop SLE than men are; this may be partly due to the number of SLE-associated genes found on the X chromosome.[3] Additionally, the frequency of SLE varies by ancestry, with those of African descent having the highest frequency, followed by Asian, and then European.[4,5]

The most common symptoms of SLE include joint pain, arthritis, skin rash, fatigue, hair loss, photosensitivity, impaired memory, and chest pain.[6] Patients are often presented with blood deficits like anemia, leucopenia, lymphopenia, and low platelet count.[6] SLE can also lead to organ-specific damage, such as heart, liver, and kidney inflammation.[6]

Current treatment options for SLE aim to prevent or mitigate resulting tissue damage but often come with unwanted side effects requiring frequent doctor visits.[7–9] For instance, non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed to patients with SLE to treat joint pain, but long-term use of NSAIDs can cause additional gastrointestinal and kidney problems.[8,9] Antimalarial drugs, such as chloroquine, are also commonly prescribed to prevent organ damage, but risk retinal damage.[8,9] Corticosteroids can rapidly decrease inflammation, however, long-term use of corticosteroids increases the risk of infection, osteoporosis, high blood pressure, and weight gain.[8,9] Immune-suppressive antibodies, such as B cell targeting belimumab, also reduce flare-ups but risk increased susceptibility to infection by dampening overall immunity.[9] Thus, there is a need to explore novel, targeted, and safer options for patients.

Gene editing technology, such as CRISPR/Cas9, can offer a more targeted approach to treating SLE with its ability to precisely alter specific sequences of DNA.[10,11] Originally discovered as part of a viral defense mechanism in bacteria, CRISPR/Cas9 works to direct the Cas9 enzyme using a specific guide RNA, directing the enzyme to the desired genomic location to cut out, introduce, or repair changes in a target sequence. Over the past decade, this system has been refined to enable more precise, advanced targeting with fewer off-target effects.[12–14] For example, a gene editing technology called prime editing is a next-generation CRISPR/Cas9 that allows single-nucleotide base edits in DNA.[14] Here, I explore a way to safely and effectively treat SLE using prime editing technology. The goal of this work is to explore the potential of prime editing in SLE, offering a strategy to target a specific mutation in patient cells to mitigate symptoms of disease and control disease-led autoimmunity.

Immune dysfunction in SLE: implications for gene editing

SLE is a complex, polygenic autoimmune disease associated with over 50 genes.[15–17] These genes often result in the malfunction of key immune cells, such as B cells, T cells, and dendritic cells, and their activity.[18] One gene implicated in this process is *TLR7*, which is located on the X chromosome. This gene encodes an intracellular receptor protein, called toll-like receptor 7 (TLR7), that acts as a sensor for atypical nucleic acids within the cell, such as viral-derived single-stranded RNA (ssRNA). Upon identifying ssRNA, TLR7 triggers downstream immune activation, including the production of inflammatory cytokines such as type I interferons (IFNs). In B cells, TLR7 is activated when the B cell-surface receptor internalizes an antigen containing ssRNA, which then triggers a signaling cascade within the B cell. This leads to activation and differentiation into antibody-producing plasma cells and the B cells become hyper-responsive to self-RNA, resulting in the production of autoantibodies. As such, aberrant TLR7 activation in cells such as B cells can lead to autoimmunity. Indeed, TLR7 protein expression was significantly higher in the blood of SLE patients than in healthy individuals.[19,20]

In a recent landmark study, whole-genome sequencing identified a novel *TLR7* gain-of-function gene variant (Y264H) that causes SLE.[21] Researchers demonstrated that the Y264H mutation resulted in altered receptor binding affinity, creating an increased sensitivity to TLR7 ligands and enhanced signaling. When the Y264H variant was introduced into mice, the mice developed a lupus-like disease, evidenced by kidney inflammation, spleen enlargement, aberrant survival of autoreactive B cells, and production of autoantibodies. However, when the TLR7 gene was knocked out, inflammatory cytokine production was diminished, as was the presence of autoreactive B cells and antinuclear antibodies.[22]

With the Y264H missense mutation, TLR7 becomes hyper-sensitive to ssRNA. The Y264H mutation means that at the 264th amino acid, a tyrosine has been replaced with a histidine. These self-RNA are harmless and are native to the host. However, due to the extrafollicular B cell response, the B cells will identify the dead matter and signal to the surrounding immune system to attack any organic material that resembles that composition. The arrival of BCR is internalized, then identifies an antigen to be delivered to the intracellular compartments which activates TLR7 among other TLRs. This results in inflammation and flaring in SLE patients, with the immune system attacking key tissues and organs such as the skin, heart, lungs, kidney, and brain.

While the genetic knock-out of *TLR7* results in the loss of autoantibodies, a gene-editing approach to treating SLE would not involve the deletion of *TLR7* because its functions remain

vital to the immune system. For example, TLR signaling enables the production of antibodies and the presentation of antigens to T cells causes the production of cytokines, which are important for normal immune function. However, with certain genetic variants like the Y264H missense mutation, these cytokines then facilitate the reactions seen in SLE. Thus, an effective gene editing treatment for SLE would involve modification but not deletion of the *TLR7* gene to maintain its beneficial function in the immune system while mitigating the SLE symptoms.

Advancements in gene editing: prime editing as an approach to rectifying the TLR7-Y264H variant in SLE

CRISPR/Cas9, originally an adaptive immune system in bacteria and archaea, has been harnessed as a powerful gene-editing tool in mammalian cells. This method was adapted to mammalian cells by introducing the bacterial Native *S. pyogenes* system into the cells.[23] These native *S.pyogenes* molecules are a two-part guide RNA System (crRNA:tracrRNA): cas 9 protein and the two RNAs (CRISPR RNA and tracer RNA). The system utilizes a guide RNA to direct the Cas9 nuclease to a specific DNA locus, where it creates a double-stranded break. The cell's endogenous machinery can repair this break through two main pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is often used for gene knockouts and functions by leveraging its error-prone nature that leads to random insertions or deletions that disrupt the gene's function. In contrast, HDR allows for precise gene editing, including knock-ins and point mutations by using a provided DNA template.[10,11]

In the last decade, CRISPR/Cas9 has evolved to enable more precise edits to DNA. In the first generation of CRISPR/Cas9 technology, an induced double-strand break relies on the cell’s HDR machinery to create a genetic deletion or knockout.[10,11] A double-stranded break HDR repair relies on the provided single-stranded DNA donor template encased in the Cas9 enzyme. This DNA donor template contains flanking homology arms on each side to ensure location accuracy and stabilization. Once the double-stranded break is induced, a DNA exchange is introduced to the targeted area. More specifically, the 5’ end on both strands is resected by the enzyme RecBCD until it hits a PAM site that is identified at a 5’-NGG-3’ (N being any base). Now the 3’ is sticking out and a REC A protein will drag it down to the homologous DNA to find where the base sequence is complementary to extend the 3’ end and reach the PAM site. RecA will pull 3’ down into the DNA loop and continue to pull it until it reaches a PAM site; then, mediated by enzymes, the template will be broken away and the opposing strand will spontaneously join back to the original DNA because of complementary base pairing. One strand of the DNA is repaired when the DNA polymerase’s exposed DNA strand extends in the 3’ to 5’ direction to complete the strand. The DNA ligase then repairs the DNA to create 2 complete pieces of DNA. The expected result is a successful knock-in, allowing the individual cell to have optimal function when targeting pathogens. However, possible off-target effects and unwanted base mutations may occur at the edit site, making it an undesirable pathway.

In contrast, base editing, and more recently, prime editing uses a deactivated Cas9 (dCas9) as a base-modifying enzyme. Rather than creating a double-stranded break, the dCas9 chemically alters a specific nucleotide base. Prime editing, however, creates a single-strand break in the DNA and can search and replace genetic sequences in a genome. It has high precision due to its guide RNA, and it has high efficiency because of the single-stranded break imposed as opposed to a double-stranded break.

This approach is unique because the pegRNA extension allows for precise genome location. For example, because it is a Cas9 nickase instead of a nucleus, only the top (positive) strand will be nicked, rather than creating a double-stranded break. When the stand is cut, there will be a genomic flap that will be chemically attracted to the 3' peg extension. The end of the extension is known as a primer binding site (PBS) which is coded to be complementary to the genomic flap. Next to it on the 5' side is the reverse transcription template (RTT). The reverse transcriptase (RT) reverse transcripts the RTT transcript to synthesize the desired bases onto the end of the genome. The edits for the genome will be expressed in the RTT section of the pegRNA. There will also be downstream homology in the RT template to allow the flap of the edit to create flap equilibration (Figure 4). This can result in a change in the DNA or maintenance of the original sequence. Together, this is known as the PE2 system.

To increase the likelihood of the edit being installed, a normal CRISPR guide RNA can be added to the system. This second guide RNA will direct the prime editor protein to nick the opposite side of the strand. This is known as a PE3 system. This treatment should only have to be administered a few times over the patient's lifetime due to the ability of the edited cells to multiply.

Overall, SLE is a complex autoimmune disorder that currently has limited treatment options. The genetic complexity and heterogeneity of SLE present significant challenges for diagnosis and management, as there is currently no single effective treatment. Next-generation gene editing technology offers the potential for more precise targeting and editing of DNA, which could lead to safer, more effective therapies. This work focuses on targeting the recently discovered causal *TLR7* variant in B cells and its role in SLE.

Methods

To target the *TLR7*-Y264H mutation implicated in SLE, public data was collected and analyzed to confirm that TLR7 should be the targeted gene for the CRISPR-Cas9 therapy in B cells. The NIH human genome and UCSC Genome Browser collections were utilized to identify the TLR7 CRISPR pegRNA sequence, with a specific focus on the codon at amino acid position 264. The pathogenic variant involves a cytosine in the mutated sequence that is typically a thymine.[22] This substitution was introduced and confirmed through the seminal Brown et. al publication.[22] Crystal structures of both wildtype and mutant TLR7 were created via AlphaFold (https://alphafold.ebi.ac.uk/entry/Q9NYK1].T).

To facilitate precise gene correction, PegRNA sequences were designed through PegIt software.[24] The pegRNA was constructed with the following components: a spacer sequence, RT template sequence, Primer binding site, and extension sequence. The computational software validated the legitimacy of the sequence.

To validate the role of B cells in SLE pathology, the experimental approach confirmed the concentration of TLR7 and the role the protein has in the B cell. A cell line selection was facilitated in which human peripheral blood mononuclear cells (which are the source of primary B cells) were isolated as chosen through the CellxGene software. Then, the CellxGene software was used to compile and visualize the TLR7 cell concentration data (Figure 1). It found that out of all the immune-driven cells, B cells had the highest concentration of TLR7 of all immune cells.

Results

Previous work demonstrates a higher level of TLR7 expression in SLE blood compared to healthy individuals (Figure 1A). [25] To further refine our approach, we explored a viable cell type for CRISPR-targeting. Using the CellxGene portal, we evaluated the levels of TLR7 mRNA expression in various blood cell types (Figure 1B). B cells express TLR7 at the highest level.

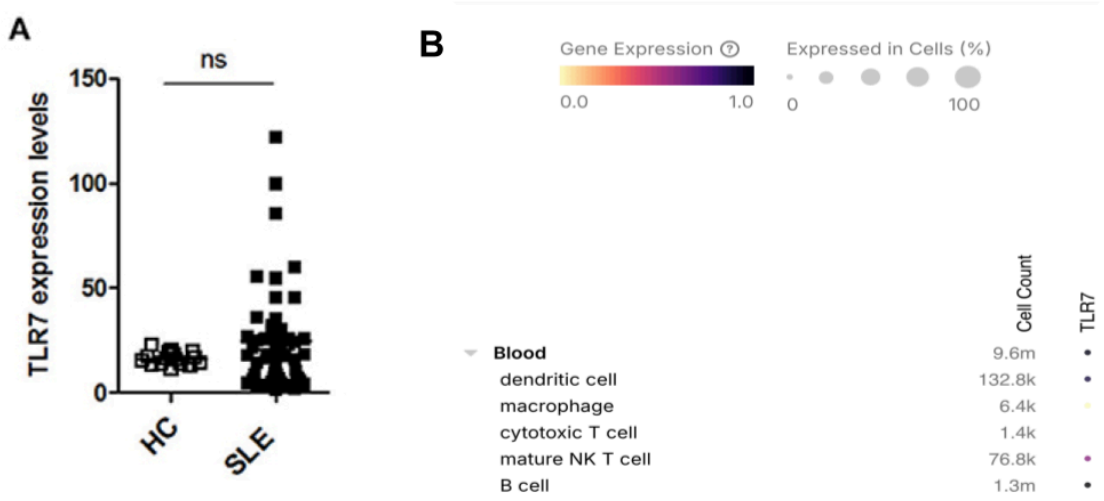

Figure 1: Rationale for CRISPR target and high-level immune differences in SLE.
A) High TLR7 expression in a subset of SLE patients. A) Relative TLR7 expression in peripheral blood mononuclear cells (PBMCs) was compared between healthy controls (HC) and SLE patients. The expression levels of TLR7 mRNA were measured by RT-PCR. PCR data were normalized to the expression of housekeeping genes and are presented here as $2^{\Delta CT}x10^3$*. Meaning, that the numerical scale analyzing the expression of TLR7 was set as a ratio to the typical expression rate in healthy controls. (A) TLR7 expression in PBMCs from HCs (n = 16) and SLE patients (n = 47). There is a greater concentration of TLR7 toll-like receptors in SLE patients than in HC. Adapted from Wang et. al.[3] B)* B cells have the highest concentration of TLR7 of all immune cells. *B) The dot plot illustrates values through color and size. The color of the dot approximates average gene expression. The size of the dot is the percentage of cells within each cell type that expresses the gene. The values are normalized through a log transformation of scaled pseudocounts (ln(CPTT+1)) and then averaged. Recorded unscaled TLR7 gene expression is as follows: dendritic cell (1.71), macrophage (1.61), mature NK T cell (1.67), B cell (1.73).*

Gene expression and protein levels in cells are provided on a scaled model (Figure 1B). The provided cell types are all involved in the immune response. The B cell holds the highest value for TLR7 gene expression on a modified point scale at 1.73 as opposed to the second highest, dendritic cell, at 1.71. Hence, TLR7 has a greater role in B cells than that of any other immune response cells. Blood was the chosen tissue due to the accessibility of extraction for the provided cell types.

5'-ACCTCAACCAATTACAAATTCTTGACCTAAGTGGAAATTGCCCTCGTTGTTATAATGCCCCTTTCCTTGTGCGCCGTGTAAAAATAATTCTCCCCTACAG-3'
[Healthy DNA sequence]

5'-ACCTCAACCAATTACAAATTCTTGACCTAAGTGGAAATTGCCCTCGTTGTCATAATGCCCCATTTCCTTGTGCGCCGTGTAAAAATAATTCTCCCCTACAG-3'
[Pathogenic variant DNA sequence in SLE]

Figure 2: TLR7 sequence in patients without and with SLE. *This is the positive strand of TLR7 comparing the sequence of more common in individuals without SLE compared to that more common in those with SLE The 264th codon (underlined) contains a mutated cytosine in the SLE as opposed to the typical sequence. The blue and red nucleotides correspond to the desired edit site to correct the pathogenic variant. In making the pegRNA sequence, the TRR will be complementary to the provided positive strand where in which the nick will be made at the PAM site upstream, and within close proximity to the intended edit site. The pegRNA will be complementary to the positive strand, while the guide RNA will be complementary to the negative strand (not shown).*

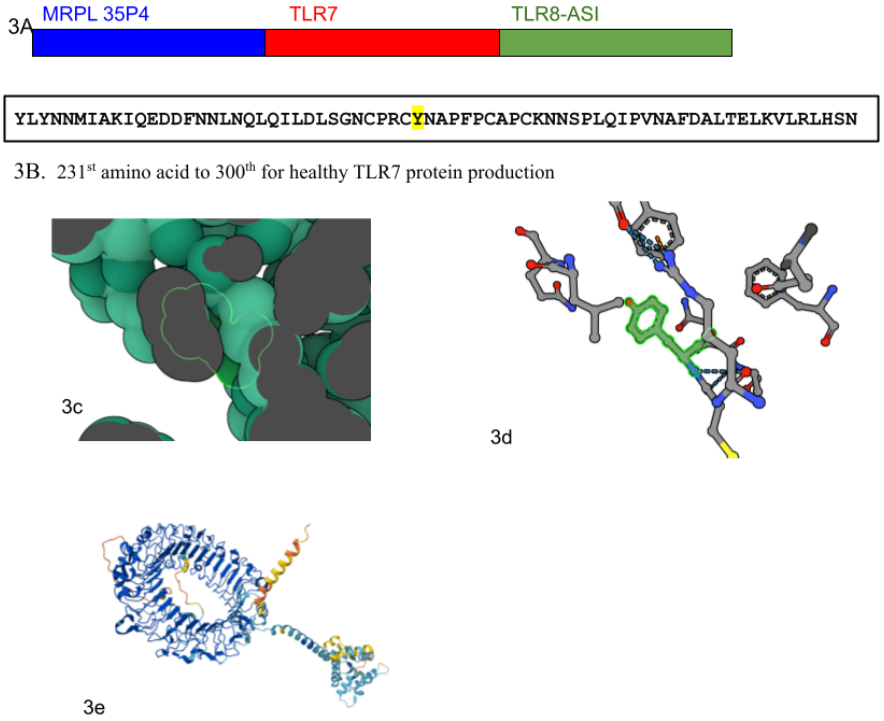

Figure 3: Components for CRISPR approach: specific section of TLR7, PAM sites, Cas9 (materials to the approach). 3*A) The location of the* TLR7 *gene on the X chromosome in relation to its neighboring genes. 3B) The amino acid chain in healthy patients from the 231st to 300th amino acid. The yellow "y" is the amino acid mutated in the TLR7 Y264H variant.*
The edit is made at the 264th amino acid codon that will revert the mutant histidine to a wild-type tyrosine in the TLR7 protein. ***Crystal structure of TLR7 mutant vs. normal.*** *3C) and 3D) 3D model of how the tyrosine is supposed to fit into the TLR7 protein at amino acid position*

264. 3E) 3D structure of the wild-type TLR7 protein. The blue regions indicate high confidence in the software's ability to replicate the structure of the protein. Crystal structure provided through AlphaFold.

Using publicly available datasets, a specific variant in the *TLR7* gene linked to SLE was selected for a gene editing approach. TLR7-Y264H is the intended target for correction by single-nucleotide conversion. Prime editing utilizes a modified CRISPR RNA, known as a pegRNA. It also includes a Cas9(H840A), a scaffold connecting the pegRNA to the Cas9 protein, a reverse transcriptase (RT) to convert the RNA into DNA, and a trimmed Evo pre-q1 to stabilize the pegRNA by preventing degradation (Figure 4 and 5). The specific CRISPR approach will be used through prime editing pegRNA, with a detailed structure included in Figure 5.

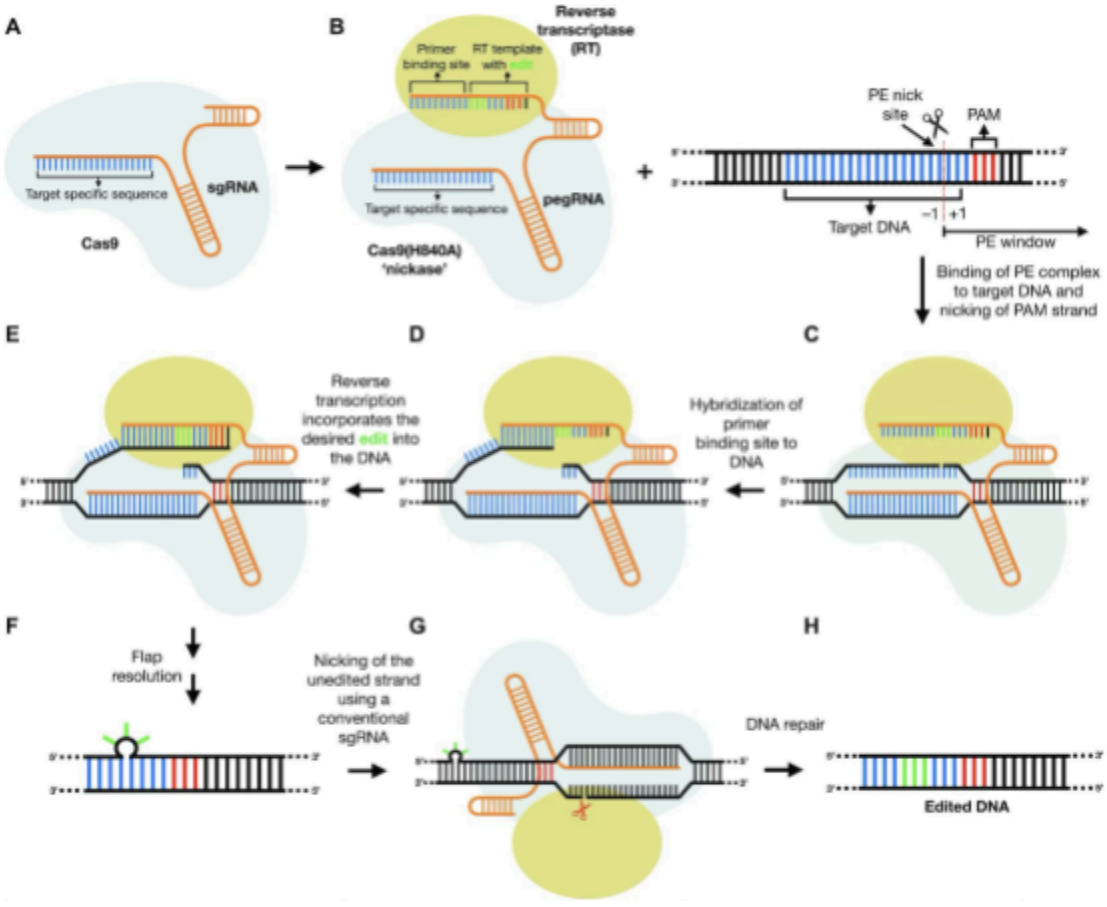

Figure 4: Proposed mechanism of CRISPR-mediated TLR7 GOF variant targeting. *Overview of the steps for how the full pegRNA (sgRNA + scaffold, protospacer, and tevopreQ$_1$ required for prime editing CRISPR/Cas9) can edit the pathogenic variant in* TLR7. *The Cas9 enzyme is depicted by the grey outline. The black 5'---3' is the pathogenic sequence (T>C variant on the positive/sense/coding strand of DNA) of the target: TLR7-Y264H. The orange sequence is the full prime editing guide RNA sequence (pegRNA), which corresponds to the orange scaffold in this Figure: the 5'--3' end of the orange sequence above.*

The target-specific sequence acts to identify the area on the genome and the primer binding site allows for the RT template to be paired with the intended region, resulting in the desired edit.

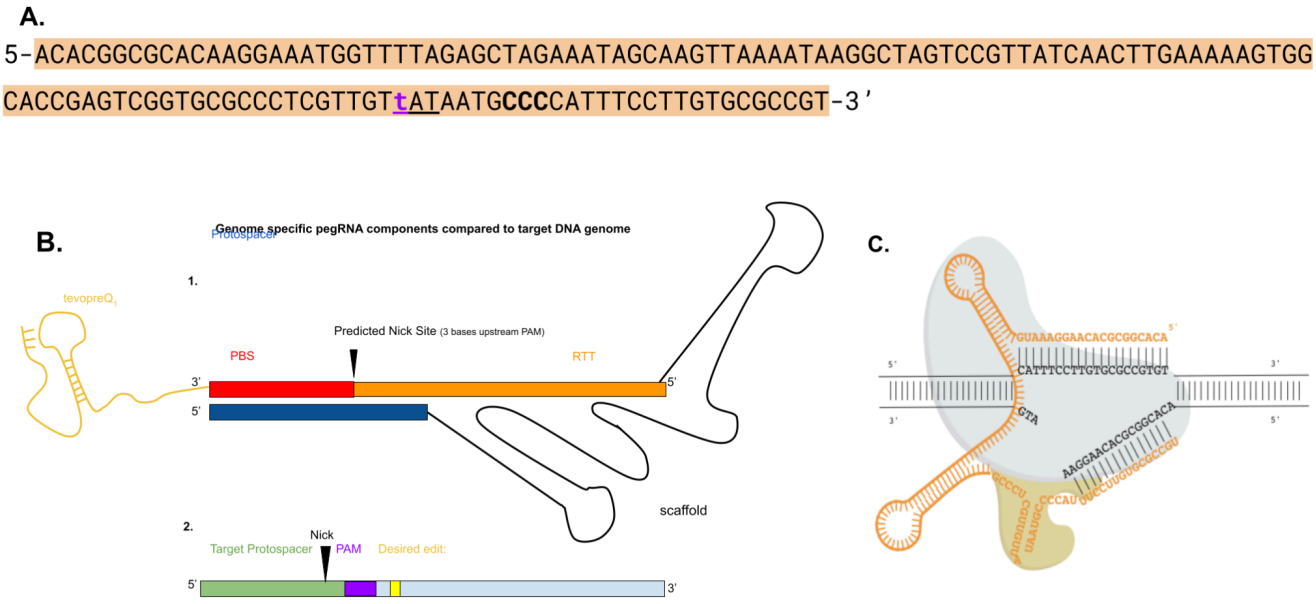

Figure 5: Full pegRNA sequence. *Targeting TLR7 p.Tyr264His (Y264H) in SLE patients, this pegRNA sequence will be used. The bolded (**CCC**) region correlates to the PAM sight on the positive strand and the underlined region correlates to the codons that create the mutated amino acid. The pink "t" is the desired change that will be inserted through the CRISPR technology; changing the histidine to tyrosine. B) The components for the pegRNA include sgRNA, scaffold, protospacer, and tevopreQ$_1$. The 5' end of the pegRNA where the protospacer is will line up with the positive strand at the target protospacer as seen in C. The 3' PBS to RTT 5' that is separated by the scaffold will line up with the negative strand and will be responsible for including the desired edit. C) Visualization of how the Protospacer is complementary to the primary strand and on the negative strand, the nick can be seen downstream of the PAM site.*

To create a full pegRNA sequence that appropriately targets and corrects the TLR7-Y264H variant, the following components are to be included: **1)** Spacer (5' - ACACGGCGCACAAGGAAATG), **2)** RT reverse transcriptase template (24 nt: GCCCTCGTTGTtATAATGCCCCAT) **3)** PBS primer binding site (15 nt: TTCCTTGTGCGCCGT) **4)** and the Extension sequence (GCCCTCGTTGTtATAATGCCCCATTTCCTTGTGCGCCGT -3') (Figure 5).

Discussion

The results of this study provide substantial evidence supporting the role of TLR7 in SLE pathogenesis and highlight the potential for CRISPR-based gene editing for therapeutic intervention. The findings confirm the hypothesis that the mutated TLR7 is a driving component of SLE, particularly in B cells. These B cells exhibit the highest expression levels of this receptor

(Figure 1B). This consistency with established papers helps bolster confidence in the experimental results.

Although the results support the feasibility of the calculated CRISPR-mediated treatment of the TLR7-Y264H mutation, several limitations must be acknowledged. One significant source of potential error is the reliance on computational models and public datasets. Although applications used for gene sequence verification and the pegRNA design do provide valuable and trustworthy insights, further validation of the application and other corresponding research could further prove the accuracy and efficiency of the proposed gene-editing approach. Future studies should focus on experimentally validating the pegRNA design through cell-based assessments.

Some variability was observed in the datasets. There were a few minor discrepancies between different studies of TLR7 expression levels and how it relates to autoimmune disorders. These inconsistencies likely stem from the variety of experimental conditions, sample sizes, or data processing methods. However, the overarching conclusion is that SLE patients have a disproportionately elevated expression of TLR7 in B cells.

The TLR7 signaling pathway plays a crucial role in autoimmune diseases like SLE, where dysregulated signaling in B cells contributes to the production of autoantibodies.[25] TLR7 is an intracellular, endosomal receptor that recognizes single-stranded RNA. Its hyperactivation in B cells leads to increased production of autoantibodies and inflammatory cytokines. In the context of SLE, the goal is to modulate the hyperactive response of TLR7 in B cells. Given the importance of TLR7 signaling in healthy immune function, a complete gene knockout is undesirable. However, precise base editing to correct a recently discovered, pathogenic gain-of-function mutation in *TLR7* holds promise as a therapeutic strategy. The TLR7-Y264H mutation in SLE results in a hyperinflammatory phenotype, characterized by the overexpression and hypersensitivity of TLR7.[22] Correcting this novel, disease-causing *TLR7* gene variant aims to dampen the hyperactive immune response and alleviate symptoms in SLE. This approach benefits from restoring TLR7 signaling in SLE while preserving its vital role in healthy immunity.

The complexity and heterogeneity of SLE present noteworthy challenges for diagnosis and management, as there is currently no single effective disease-modifying treatment. Scientists have used CRISPR-based gene editing to minimize or correct symptoms of disease. Sickle cell disease, for example, has had successful treatment trials in which the patient's stem cells are removed, and the causal variant is edited with CRISPR-based gene editing.[26,27] Although it has had success in various clinical studies, scientists still experience limitations regarding the efficiency of gene editing in primary human cells.[28] When implementing a CRISPR-based approach, scientists must take into consideration means of delivery, efficiency, accuracy, and potential immunogenicity with specificity to each target gene.

While prime editing holds immense promise for SLE therapy, several limitations and considerations for future research remain. Efficient delivery of the CRISPR/Cas9 system to target cells, particularly B cells, is crucial. There are numerous aspects to consider when designing the ssDNA donor template. First, the location of the PAM cut site is vital to the efficiency of the template. The closer the PAM site is to the insertion DNA, the more efficient it will be. The optimal PAM site should be within 10 bases of the desired edit site. Another concern is the length of the homology arms. While they should include at least 50 nucleotides on either side of the edit, there may be lower efficiency with longer oligos. However, reports indicate that the functionality of the insertion remains about the same as long as at least 30 nucleotides are

included in the homology arms.[29,30] Potential off-target effects and the need for long-term efficacy require careful evaluation.

The clinical implementation of CRISPR technology is challenging beyond its chemical functionality. Treatment effectiveness can be influenced by whether the insertion is performed *in vivo* (within the living body) or *ex vivo* (outside the living body), as well as the specific location within the body where the treatment is administered. The lymph nodes, due to their concentration and production of B cells, are a potential site for treatment. One method to facilitate entry into human cells could be encapsulation of the treatment in lipid nanoparticles. Future studies should focus on optimizing delivery methods, improving editing efficiency, and assessing the safety and durability of CRISPR/Cas9-based therapies for SLE.

Gene editing holds potential for lupus research and treatment by enabling precise gene editing to study and target specific genetic factors associated with the disease. It can be used to investigate the functional impact of genetic variants linked to lupus, such as through the introduction of specific mutations in animal models to observe disease mechanisms.[31] For instance, the CRISPR-mediated introduction of the identified Y264H variant into mice aids in understanding the role of specific gene variants in disease development. Moreover, CRISPR can target additional genes like *A20 DUB* and *CXorf21*, which are implicated in SLE, to explore their roles in disease susceptibility and immune response. This technology offers a scalable approach for validating findings from genome-wide association studies and could lead to novel therapeutic targets by modifying immune cells to reduce autoimmune responses.

Conclusion

The goal of this research was to synthesize viable CRISPR pegRNA treatment from public datasets. It was predicted that B cells would be the most viable approach which was supported by the data. A CRISPR-based treatment of the TLR7-Y264H mutation is a promising strategy to mitigate the hyperinflammatory phenotype observed in SLE. Further experimental validation is necessary, but these findings lay a foundation for future research and potential clinical applications.

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