Original Article

CRISPR/Cas9-Mediated Targeting of the Integrin Beta-2 Gene as a Gene-Editing Strategy for Leukocyte Adhesion Deficiency Type I

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Abstract

Background: Leukocyte adhesion deficiency (LAD) is a genetic immunodeficiency that severely impairs the leukocyte adhesion cascade and migration to the sites of infection and inflammation. As a result, the main consequence is recurrent and severe infections with bacteria and fungi, which can lead to death. The most common variant, LAD-I, is due to mutations in the integrin beta 2 (*ITGB2)* gene responsible for the β2 integrin (CD18) subunit. Corrective strategies based on gene therapy and genome editing are the most promising methods for treating genetic defects.

Methods: In the present experimental *in vitro* study conducted at Tarbiat Modares University, Tehran, Iran, in 2024, we designed a single-guide RNA (sgRNA) for the CRISPR/Cas9 system to target exon 6 of *ITGB2* and performed validation experiments. We also investigated peptide nanocarriers as a potential strategy for delivering the CRISPR components. The PX458 plasmid encoding Cas9 and the designed sgRNA was delivered into HEK293T cells, followed by genome-editing analysis.

Results: Genome-editing analysis confirmed a measurable level of indel formation at the target locus. Furthermore, the results of MPG nanopeptide production and purification demonstrate the potential of peptide nanocarriers for genome-editing systems.

Conclusion: Overall, this work establishes a foundation for subsequent knock-in and gene-correction studies in clinically relevant immune cell models, paving the way for potential LAD-I gene therapy applications.

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Keywords ● Leukocyte adhesion deficiency Type I ● ITGB2 protein ● CRISPR-Cas systems ● Gene editing ● Cell-penetrating peptide

What's Known

- Gene editing of the integrin beta 2 gene (*ITGB2)*, which encodes the β2 integrin protein (CD18*)*, has been widely explored as a therapeutic strategy for Leukocyte Adhesion Deficiency type I (LAD-I).

What's New

- This study demonstrates the functional activity of a newly designed guide RNA targeting exon 6 of *ITGB2*, providing proof-of-concept for its ability to induce site-specific genome editing.
- Additionally, the study demonstrates the ability of MPG peptide to form nanocomplexes with PX458-sgITGB2, supporting its further evaluation as a non-viral CRISPR delivery platform.

Introduction

Leukocyte adhesion deficiency type I (LAD-I) is an inherited autosomal recessive disorder of the immune system that results from mutations in the β2-integrin gene (*CD18*).[1] Leukocytes, and more specifically neutrophils, fail to execute their main function of attaching to the blood vessel lining and reaching the site of infection due to the lack of functional CD11/CD18 integrins. Integrins are classified as cell adhesion molecules (CAMs), a

group of cell-surface proteins. They mediate key cell attachment to the extracellular matrix (ECM) and transmit signals between the ECM and the cell. They are decisive factors in the processes of cell division, migration, death, and tissue repair.[2] Current therapies mostly depend on hematopoietic stem cell transplantation, which, besides being effective, comes with major risks and limitations.[3, 4] Although transplantation of donor-derived stem cells might enhance the immune system of the patients suffering from LAD, it remains a very risky procedure with potentially fatal outcomes, especially in patients with active infections, since it entails complete suppression of the immune system by high-dose chemotherapy and radiation therapy.[5] Moreover, donor T-cells might provoke a situation known as graft-versus-host disease (GvHD), during which the donor's cells recognize the patient's cells as foreign and activate an immune response to annihilate them.[6] On the other hand, gene therapy is slowly becoming a viable option for treatment in many cases, but some conditions will still require more studies to confirm the safety and effectiveness of this technique.[7] Gene therapy has become an established therapeutic modality for several inherited and acquired disorders, although many applications remain under clinical investigation.[8] One of the newest approaches in gene therapy is genome-editing techniques.[9] Gene editing can be defined as a group of technologies that enable scientists to modify DNA.[10] These techniques provide a possibility to remove, insert, or modify genetic material at precise spots in the genome. In fact, clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9)-assisted genome editing has turned out to be a powerful tool for precise repair of single-gene disorders.[9] Several proof-of-concept studies have demonstrated the feasibility of using CRISPR for editing immune-cell genes, but efficient delivery systems remain a major challenge.[11] Peptide-based delivery systems are regarded safe, efficient, and versatile non-viral methods for performing gene and genome editing because they are compatible with the human body, exhibit diverse structural features, and are capable of allowing cells to take in large biomolecules.[12] Among them, MPG and MiRGD stand out as two of the most common cell-penetrating peptides (CPPs) discussed in scientific literature, showing excellent nucleic-acid delivery performance with very little cytotoxicity.[13, 14] MPG, which is a product of combining HIV gp41 and the nuclear localization sequence of the SV40 T antigen, can create stable non-covalent complexes with nucleic acids and can facilitate their rapid entry through the plasma membrane.[15, 16] In this study, we designed a sgRNA that targets exon 6 of *ITGB2*, a region associated with LAD-I mutations in Iran. Using HEK293T cells as a model system, we aimed to validate the function of the sgRNA and establish a basis for future knock-in and therapeutic studies in relevant immune cells. We also investigated the capacity of the MPG peptide nanocarrier for CRISPR delivery.

Materials and Methods

Study Design and Ethical Approval

This study protocol was reviewed and approved by the Ethics Committee of Tarbiat Modares University (approval code: IR.MODARES.REC.1403.018).

sgRNA Design and Synthesis

Epidemiological data on *ITGB2* gene mutations and their concentration in the region of exons 5 to 9, especially the c.533C>T mutation in exon 6, clearly demonstrate the importance of target selection in the present study and can be considered a strong basis for the development of future therapeutic strategies in LAD-I. To achieve this goal, one sgRNA aimed at *ITGB2* exon 6 (5′-TCAGGGTGCGTGTTCACGAA-3′) was designed using the CRISPOR and Benchling tools, considering the least possible off-target effects. The secondary structure of the designed sgRNA was checked using online RNA-folding tools to ensure proper folding of the sgRNA. The sgRNA sequence was produced and inserted into a CRISPR-Cas9 expression vector controlled by the U6 promoter (PX458). The PX458 vector was digested with BbsI, and the annealed sgRNA sequence was inserted into the linearized plasmid using Golden Gate assembly with T4 DNA ligase. The recombinant plasmids were transformed into competent *Escherichia coli* DH5α cells, and positive clones were confirmed by PCR and Sanger sequencing.

Cell Culture

Human embryonic kidney cells (HEK293T) were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics at a temperature of 37 °C under a humidified atmosphere containing 5% CO□. The selection of these cells was based on their high transfection efficiency, which allowed for the first stage of the CRISPR construct validation.

The pSpCas9(BB)-2A-GFP vector (PX458; Addgene, Watertown, MA, USA) was used for the expression of CRISPR-associated protein 9 (Cas9) and the designed single-guide RNA (sgRNA). The plasmid was complexed with Polyethyleneimine (PEI; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Genomic DNA Extraction and Editing Analysis

Genomic DNA was isolated 48 h post-transfection. The target region was PCR-amplified. The primers used were (5′-CTGCTAGGAGAGAGGAAACAGG-3′). Raw chromatogram files and indel frequencies were quantified using Sanger sequencing followed by TIDE (Tracking of Indels by Decomposition) analysis. Editing efficiency was calculated as the proportion of alleles with insertions or deletions at the sgRNA cut site.

Expression, Purification and Preparation of PX458-sgITGB2/MPG Nanocomplex

The DNA coding sequence of the MPG peptide was previously synthesized and cloned into the plasmid pET28a (Novagen, USA). The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells (Novagen, USA). To establish a preculture, *E. coli* BL21 cells were incubated in 10 mL of Luria–Bertani (LB) medium (Merck, Germany) containing kanamycin (Sigma-Aldrich, USA) at 37 °C overnight. 300 mL of 2xYT liquid medium (Merck, Germany) containing kanamycin was inoculated with 3 mL of preculture, and after reaching an OD600 of 0.6–0.8, expression was induced with 60 µL of 1 M Isopropyl β- d-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, USA) and incubated at 37 °C. Four hours later, the MPG-expressing cells were harvested by centrifugation. The cell pellets were placed in a solution of 1 mL of lysis buffer according to previous studies.[17] The lysate was incubated at 200 rpm and 20 °C for 1 h. Then the lysate was centrifuged at 12,000 ×g for 20 min, and the supernatant was transferred to chromatography columns (Bio-Rad, USA) pre-equilibrated with lysis buffer and incubated for 1 h at room temperature. The columns were washed several times with different wash buffers according to the protocol.[16] The purity and size of the collected fractions were assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Peptides were dialyzed against cold Phosphate-buffered saline (PBS) (Gibco, USA) containing 3% glycerol. Then, the concentration of the purified peptide was checked using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA), and the isolated peptides were stored at -20 °C for subsequent experiments. A nanocomplex was formed by combining PX458-sg*ITGB2* with MPG peptide-based carriers at different N/P ratios according to the protocol.[16] The N/P ratio refers to the ratio of positive charges of the peptide to negative charges of DNA. The capacity of the peptide to bind to PX458-sg*ITGB2* was tested via agarose gel retardation assay.[17] This stage was carried out to investigate peptide nanocarriers for delivery of the CRISPR system targeting the *ITGB2* gene.

Results

sgRNA Design and Construct Validation

An sgRNA targeting exon 6 of *ITGB2* was successfully designed with high predicted on-target activity and minimal off-target risk. Cloning and Sanger sequence verification confirmed the correct insertion and integrity of the CRISPR-Cas9 construct (figure 1).

Delivery of CRISPR-Cas9

PEI successfully mediated the transfer of CRISPR plasmids into HEK293T cells, as confirmed by simultaneous GFP expression and microscopy (figure 2).

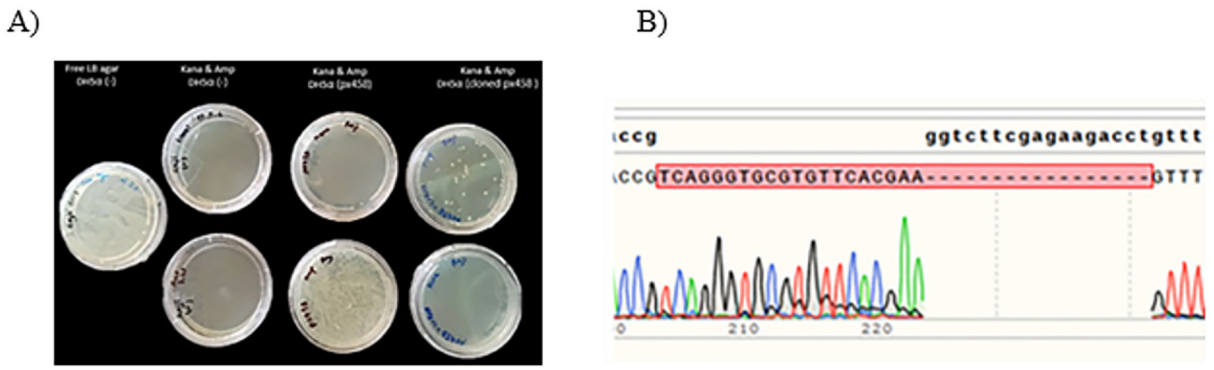

Figure 1: Verification of *ITGB2* sgRNA cloning into the PX458 vector is shown. (A) Transformation of the PX458-sg*ITGB2* plasmid into *E. coli* DH5α cells is illustrated. (B) A representative Sanger sequencing chromatogram confirms the correct insertion and integrity of the sgRNA sequence. *ITGB2:* Integrin beta 2 gene; sgRNA: Single guide RNA; PX458: PSpCas9(BB)-2A-GFP.

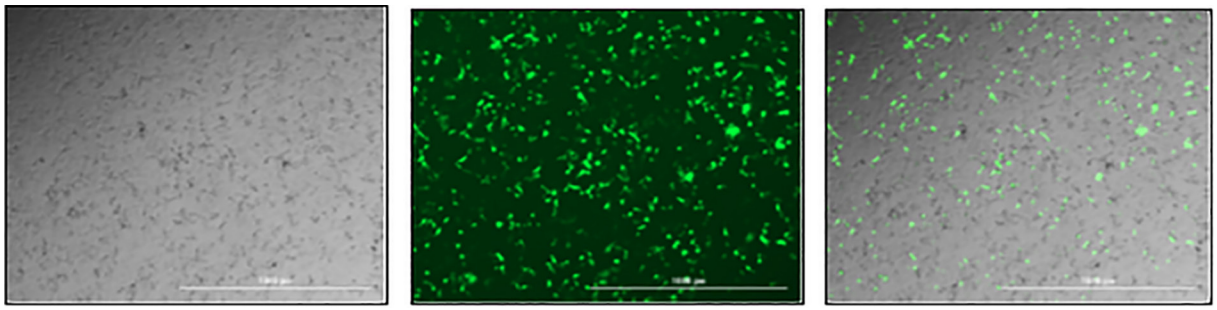

Figure 2: Representative fluorescence microscopy images show HEK293T cells transfected with the PX458-sg*ITGB2* plasmid using polyethyleneimine (PEI). PEI: Polyethyleneimine; HEK293T: Human embryonic kidney cells; PX458: PSpCas9(BB)-2A-GFP

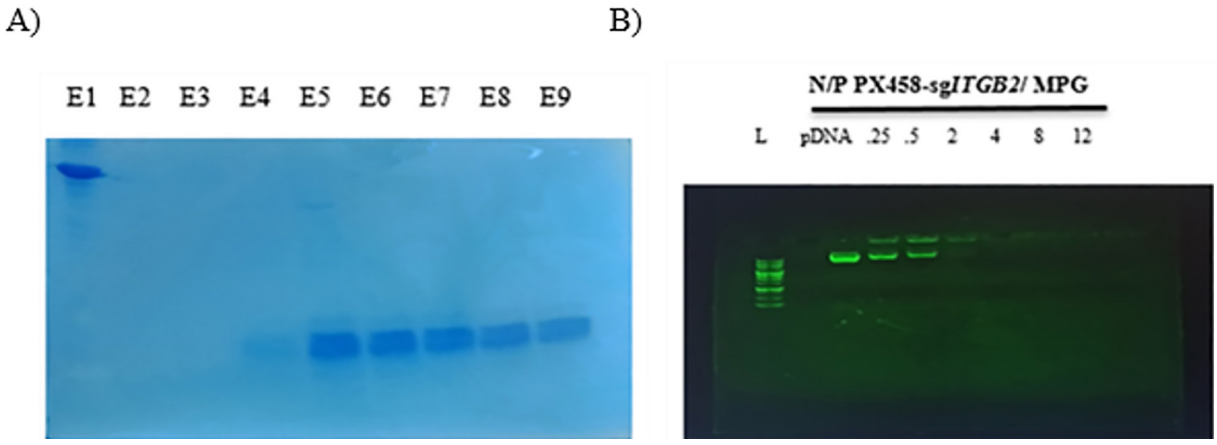

Figure 3: MPG peptide was successfully produced and purified, and its ability to form nanocomplexes with PX458-sgITGB2 was confirmed by SDS-PAGE and gel retardation assays. (A) SDS-PAGE analysis of the purified MPG peptide produced in *E. coli* BL21 using the pET28a vector is shown. The results indicate that peptide bands (E5-E9) have higher quantity and purity for optimal dialysis of the MPG peptide. (B) Gel retardation assay of PX458-sg*ITGB2*/ MPG nanocomplex at different N/P ratios is presented. "L" in the images stands for DNA Ladder, and PX458-sg*ITGB2* is used as the control. PX458-sg*ITGB2*/ MPG complexes were analyzed at N/P ratios of 0.25-12. *ITGB2:* Integrin beta 2 gene; PX458: PSpCas9(BB)-2A-GFP; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *E. coli: Escherichia coli.*

Genome Editing Efficiency

Analysis of PCR products from the target locus revealed a measurable indel frequency in transfected cells. No editing was detected in untreated control cells. These results confirm that the sgRNA is functional and capable of mediating targeted DNA cleavage at exon 6 of ITGB2.

Production and Characterization of the PX458-sgITGB2/MPG Nanocomplex

As shown in figure 3, the analysis of the purified MPG peptide by SDS-PAGE and Coomassie blue staining and the pattern of MPG peptide bands were investigated for the optimal dialysis experiments. Our results indicate that the peptide bands have the quantity and purity for optimal dialysis of the MPG peptide. Moreover, the gel retardation assay showed that although free PX458-sg*ITGB2* showed fast electrophoretic mobility, the presence of MPG delayed its electrophoretic mobility. This result indicated that the MPG peptide neutralized the negative charge of the PX458-sg*ITGB2* molecule and delayed its mobility. Furthermore, increasing the peptide content in the PX458-sgITGB2 nanopeptide complex decreased the binding of safe stain to PX458-sgITGB2, which is consistent with the results of theoretical studies (figure 3).

Discussion

This study demonstrates the successful design and preliminary validation of a CRISPR-Cas9 sgRNA targeting exon 6 of *ITGB2*. Although editing efficiency was modest, this level of indel formation provides proof-of-concept that the sgRNA is functional.

In recent years, CRISPR/Cas9 technology has moved beyond basic research and entered clinical trials in patients with inherited blood diseases.[18] Early results from these studies have shown that the system can be an effective and safe alternative to conventional treatments.[18, 19] The clinical trial of the CRISPR-based product CTX001 stands out as a remarkable case, as it involved gene editing in hematopoietic stem cells of patients with beta-thalassemia and sickle cell disease (SCD).[19] Early clinical studies

of CTX001 demonstrated the feasibility of CRISPR/Cas9-edited autologous hematopoietic stem and progenitor cells for patients with transfusion-dependent β-thalassemia and sickle cell disease.[20] These successes indicate that inherited blood diseases are suitable targets for genome editing because hematopoietic stem cells (HSPCs) can be easily isolated, modified, and returned to the patient; the genetic mutations responsible for these diseases are usually known and limited to specific exons; and genetic modification in HSPCs results in a long-lasting and stable treatment effect. Since LAD is also caused by specific mutations in the *ITGB2* gene and directly disrupts the function of immune and blood cells, it could become an important target for CRISPR/Cas9-based gene therapy in the future.[21] Just as successful trials in thalassemia and SCD have raised great hopes, the use of similar approaches (such as precise gene editing with homology-directed repair (HDR) in hematopoietic stem cells of LAD patients) could provide an effective treatment solution for these patients in the future. The next step will be to shift this system to immune cell lines or patient-derived hematopoietic stem/progenitor cells that are more relevant, and ITGB2 function can also be evaluated in a significant manner. Furthermore, the peptide-based carriers MPG were assessed in this study for their potential as CRISPR delivery agents. At the same time, the MPG peptide was successfully expressed, purified, and characterized by SDS-PAGE and gel retardation assays, which confirmed its purity and ability to bind nucleic acids. All in all, these findings support the idea of using peptide-based nanocarriers for the safe and efficient delivery of CRISPR components in future gene-editing research. Demonstrating successful CRISPR editing using this system is an important step toward developing clinically applicable gene-correction strategies for LAD-I. Efficacy studies for this disease are ongoing by our team, and we are working to address the challenges ahead.

A key limitation of this study is the use of HEK293T cells, which exhibit low endogenous ITGB2 expression and do not fully reflect the cellular context of LAD-I. Future studies should validate the proposed genome-editing strategy in relevant hematopoietic and patient-derived cell models.

Conclusion

We designed and validated an sgRNA targeting exon 6 of *ITGB2* and demonstrated genome editing activity in HEK293T cells. This work provides a proof of concept for CRISPR-mediated *ITGB2* editing in LAD-I-associated immune cells. Furthermore, the results show that the MPG peptide was successfully produced and characterized, offering a flexible platform for future CRISPR delivery applications.

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Authors' Contribution

S.R: Conceptualization, study design, investigation, data curation, formal analysis, validation, visualization, drafting, and reviewing the manuscript; H.S: Conceptualization, study design, supervision, formal analysis, resources, drafting and reviewing the manuscript; S.H: Study design, Validation, Resources, Formal Analysis, drafting and reviewing the manuscript; AA.H: Study design, Validation, Formal Analysis, drafting and reviewing the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of AI

The authors used ChatGPT (OpenAI, USA) solely for language editing and grammatical improvement of the manuscript. The AI tool was not used for scientific content generation, study design, data analysis, interpretation of results, or formulation of conclusions. All scientific content was reviewed, verified, and approved by the authors, who take full responsibility for the integrity and accuracy of the manuscript.

Conflict of Interest: None declared.

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