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CRISPR-Cas9-Based Functional Analysis of Immune Evasion Mechanisms and Vaccine Candidate Identification in *Leishmania*

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Abstract

Background: *Leishmaniasis* continues to be a major health problem worldwide because the parasite has developed several strategies to resist the immune response, making the development of vaccines and treatment challenging.
Methods: In the present work, we have used the CRISPR-Cas9 gene-editing system to explore the mechanisms of immune evasion used by *Leishmania*, and to identify new vaccine targets. Important immune-regulation genes, such as LACK, GP63, and CPB, have been successfully deleted in *Leishmania* major and *Leishmania* donovani using PCR and Sanger sequencing.
Results: The immunological profile of gene-deleted BALB/c mice was assessed following infection. The modified parasites caused a greater infiltration of CD8+ IFN-γ+ T cells than did wild-type parasites. Cytokine protein levels showed elevated IFN-γ and TNF-α, and decreased IL-4, indicating a highly polarized Th1-type immune response. Immunization with genetically engineered antigens was used to assess vaccine efficacy. Immunization with antigens from the deleted parasites resulted in a significant rise in IgG antibody levels, the IgG1/IgG2a ratio, lymphocyte proliferation, and cytotoxic T cell activity. More importantly, survival rates of 80% in vaccinated mice were significantly higher after challenge with wild-type Leishmania than in 10% of mice immunized with wild-type antigens.
Conclusions: The results demonstrate that host-protective immunity can be boosted by inactivating genes that contribute to immune evasion using the CRISPR-Cas9 technique. These approaches yield promising antigen sources for vaccines. The genetic modification of *Leishmania* species described in this research is a promising vaccine platform for developing vaccines for future generations.

Keywords
CRISPR-Cas9, Leishmania, Immune Evasion, Promastigote, Vaccine.

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Introduction

Leishmaniasis is a major public health problem worldwide, caused by Leishmania spp. It is mainly prevalent in tropical and subtropical areas. There are different clinical manifestations of the disease,

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including cutaneous type and visceral *leishmaniasis,* which may be fatal if untreated (Hussein *et al*., 2018). Although much has been learned about the biology of *Leishmania* organisms and host immune responses to them, *Leishmania*l immune evasion strategies remain complex and hinder the development of effective treatments and vaccines (Zhang and Matlashewski, 2015).

The CRISPR-Cas9 system has enabled studies of *Leishmania* genetic material and its ability to evade host defenses, as well as ideas that may guide the search for new drug targets (Rahim *et al*., 2025; Mohammed *et al.,* 2024). Knocking out specific genes and analyzing the immune response in *Leishmania*-infected hosts can provide insight into the interaction between the parasite and the immune system (Darzi *et al.,* 2025).

CRISPR-Cas9 is of great importance for identifying novel vaccine candidates in *Leishmania* research. The key features of immune evasion and the parasite's antigenic composition can be used to identify novel targets for the development of more effective vaccines (Falih *et al.,* 2021). These vaccines aim to provide an additional boost in the host's resistance to *Leishmania* infection by stimulating both humoral and cellular immune responses.

The purpose of this study is to understand the roles of key immune evasion genes in *Leishmania* through genome-wide CRISPR-Cas9 genome engineering and to determine their importance as vaccine target genes. The LACK, GP63, and CPB genes were selected because they have been previously identified in the fields of immunology, parasite survival, and virulence. The study examines how these deletions affect the host's immune response and protection against Leishmania major and *Leishmania* donovani.

Although considerable progress has been made in elucidating the causes of *leishmaniasis*, the search for effective vaccine targets has been difficult to date. Although the use of CRISPR-Cas9 technology has become a powerful tool for functional genomics research, its application to study immune evasion mechanisms and identify vaccine targets in *Leishmania* has been limited. The novelty of the present study lies in combining gene editing (CRISPR-Cas9) with immune/vaccine analyses to evaluate the significance of specific genes in relation to the parasite's pathogenicity and host immunological responses. This strategy will provide new insights into the molecular mechanisms of immune evasion. It could help in the design of more efficient vaccine strategies against *leishmaniasis*.

Materials and Methods

The culture conditions of Leishmania strains

In each experiment, *Leishmania major* and *Leishmania donovani* strains were used. RPMI 1640 medium (Gibco, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin was used to culture these strains. Cultures were placed every three to four days and maintained in a humidified incubator at 25°C (Oliveira *et al.,* 2025; Ajah *et al.,* 2021).

Gene editing by CRISPR-Cas9 system

1. Design and synthesis of the CRISPR-Cas9 architecture

a. Using the Benchling electronic tool, guide RNAs (gRNAs) were generated to target genes associated with immune evasion, such as *LACK, GP63*, and *CPB*.(gRNAs were designed using Benchling based on high scores for target sites, low potential for off-targets, and selection of the appropriate PAM site).

b. A pCas9 expression vector (Addgene #62988) containing the *Cas9* gene for genome editing was generated by synthesizing and cloning guide RNAs(The CRISPR-Cas9 expression vector (pCas9, Addgene #62988) was constructed via Gibson assembly by inserting synthetic gRNA oligonucleotides into the plasmid backbone to enable targeted genome editing in *Leishmania*).

c. Sequencing and restriction digestion were used to validate the constructs (The recombinant constructs were confirmed by specific enzymatic digestion and Sanger sequencing to verify correct assembly and sequence integrity before transferring the parasite.

2. *Leishmania* promastigote transfection

a. Electrophoresis was used to transfect *Leishmania* promastigote parasites. Briefly, 10 µg of plasmid DNA was combined with 10^7 promastigote parasites and resuspended in a cold electrophoresis buffer. A Bio-Rad Gene Pulser Xcell operating at 1200 V with a 10 ms pulse was used for electrophoresis(Kushwaha *et al.,* 2025).

b. After Electrophoresis, the cells were incubated at 25°C for 24–48 hours to allow expression of *Cas9* protein and gRNAs.

3. Selection and Clonal Expansion

a. Phleomycin (5 μg/mL) was used for seven days to select transformed cells. The remaining clones were then isolated and cultured. Successful removal of the target genes was confirmed by polymerase chain reaction (PCR) and Sanger sequencing, as described by(Daoui *et al.,* 2025).

Immune Evasion Mechanism Analysis

1. Mouse Model Infection

a. BALB/c mice (males and females, 6–8 weeks old, purchased) were injected subcutaneously with 10^6 mast cells in 100 μL of phosphate-buffered saline (PBS).

b. The number of parasites in the liver and spleen was measured using a limited dilution assay, and the mice were monitored for signs of infection.

1. Immune response analysis

a. Flow Cytometry: Samples from the spleen and liver were collected at different times post-infection to assess immune cell infiltration. Single-cell suspensions were prepared and stained with fluorochrome-conjugated antibodies against CD4, CD8, IFN-γ, and IL-4. The samples were analyzed using a BD flow cytometer. Data were analyzed using a standard gating strategy based on forward and side scatter to identify lymphocyte populations, followed by filtering for $CD4^+$ and $CD8^+$ T cells and cytokine-secreting cells. At least three biological replicates were included for each experimental group**.**

b. Cytokine Analysis: Serum samples were collected on days 7, 14, and 21 post-infection, and ELISA kits (BT LAB) were used to measure cytokine levels (IFN-γ, TNF-α, and IL-4).

Identifying candidates for new vaccines

1. Antigen preparation:

a. *Leishmania* antigens were prepared using ultrasound (Branson Sonifier 250) for wild-type and genetically modified parasites. The supernatant was collected after centrifugation at 12,000 g for 10 minutes and used for antigen analysis.

b. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher).

2. Immunization of Mice:

a. Mice were immunized with 100 micrograms of *Leishmania* antigens emulsified in complete Freund's adjuvant (CFA) for the first dose, and with incomplete Freund's adjuvant (IFA) for subsequent doses. The immunization schedule consisted of three injections spaced two weeks apart.

b. Control mice were immunized with the adjuvant alone(de Oliveira *et al.,* 2025) .

3. Assessment of Immune Responses:

a. Antibody Response: An ELISA assay was performed to measure IgG and IgG1/IgG2a subclass antibodies in serum, using 96-well plates coated with *Leishmania* antigens(Farhan *et al.,* 2024).

b. Proliferation Test: Splenocytes from mice immunized with 10 μg/ml *Leishmania* antigens were cultured. [3H] thymidine concentration was measured after 72 hours to assess T cell proliferation(Mohammed *et al.,* 2025).

c. Cytotoxic T Cell Test: Splenocytes were examined for cytotoxicity using the chromium^51 release assay.

4. Protection Challenge:

a. Immunized mice were injected subcutaneously with a lethal dose (10^6 promastigote) of wild *Leishmania* parasite. The mice were monitored for clinical signs of disease, and their survival rates were recorded. Thirty days after infection, quantitative polymerase chain reaction (qPCR) was used to quantify parasite load in spleen and liver tissues.

5. Statistical Analysis

- All data are mean ± SD. GraphPad Prism version 9.0 was used to determine statistical significance.
- The Student's t-test was used for comparisons between two groups, and the one-way ANOVA was used for comparisons among more than two groups. The Kaplan-Meier method was used for survival analysis, and the log-rank test was used to determine statistical significance.
- Pg values less than 0.05 were considered statistically significant.

Result

Confirmation of CRISPR-Cas9 Gene Editing in Leishmania Gene Editing Efficiency

The efficiency of the gene editing was validated by PCR and Sanger sequencing. Guide RNAs (gRNAs) were created to target genes involved in immune evasion, including LACK, GP63, and CPB. The cells were then incubated for 48 hours, after which the DNA was isolated and the target gene was PCR-amplified. The outcome demonstrated a lack of expression of the targeted gene in the gene-edited cells compared to wild-type cells.

Table 1: PCR Confirmation of Gene Knockout Efficiency

Gene Targeted	PCR Product Size (bp)	Wild-type (WT)	Knockout (KO)
LACK	750	750	No Band
GP63	1000	1000	No Band
CPB	500	500	No Band

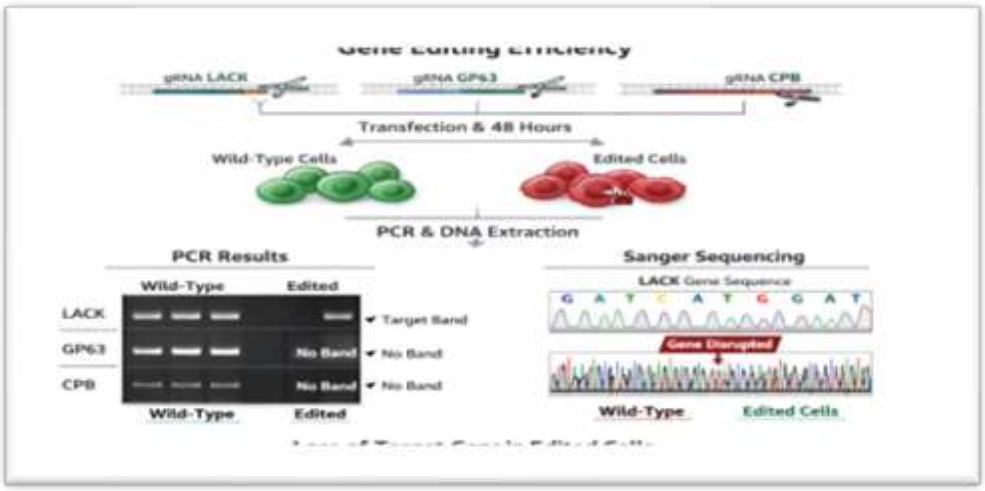

Figure 1: PCR Amplification of Gene Targets in *Leishmania* Promastigotes

2. Immune Evasion Mechanism Analysis

Immune Cell Infiltration in Infected Mice

Flow Cytometry was used to analyze immune cell infiltration in the spleen and liver following injection of genetically modified *Leishmania*. The data showed that knockout-infected mice had significantly higher infiltration of CD8+ IFN-γ+ cells than wild-type-infected mice.

Table 2: Flow Cytometry Analysis of Immune Cell Populations

Immune Marker	Wild-type (WT)	Knockout (KO)	p-value
CD4+ T	30%	32%	0.24
CD8+ T	28%	45%	<0.01*
IFN-γ	15%	27%	<0.01*
IL-4	5%	4%	0.53

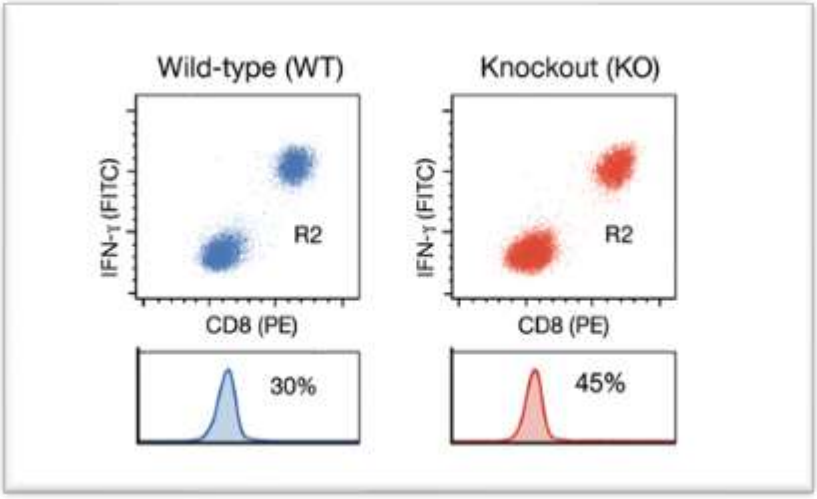

Figure 2: Flow Cytometry Analysis of CD8+ T Cells in Spleen and Liver

3. Cytokine Profiling

Cytokine Production in Infected Mice

Serum samples were collected from infected mice at days 7, 14, and 21 post-infection to measure **IFN-γ**, **IL-4**, and **TNF-α** levels using ELISA. The data showed that knockout-infected mice had higher **IFN-γ** levels than wild-type-infected mice, indicating a stronger **Th1** immune response.

Table 3: Cytokine Levels in Serum Samples

Cytokine	Wild-type (WT)	Knockout (KO)	Day 7
IFN-γ	100 pg/mL	250 pg/mL	100
IL-4	75 pg/mL	50 pg/mL	75
TNF-α	120 pg/mL	180 pg/mL	120

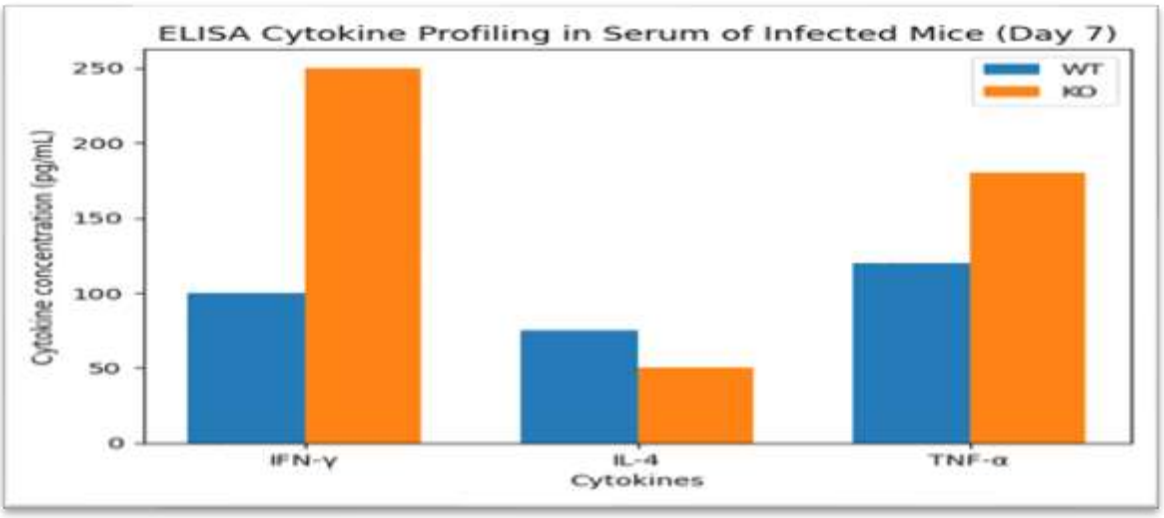

Figure 3: ELISA Cytokine Profiling in Serum of Infected Mice

4. Identification of Novel Vaccine Candidates

Antigen Preparation and Immunization

Antigens were prepared from wild-type and genetically modified *Leishmania* parasites. Mice were immunized with **100 µg** of *Leishmania* antigens emulsified in **CFA** for the first dose and **IFA** for subsequent doses. Antibody responses were assessed post-immunization using ELISA.

Table 4: Antibody Response to *Leishmania* Antigens

Vaccine Group	IgG Titer	IgG1/IgG2a Ratio	p-value
Wild-type Antigen	1:10,000	1.2	0.05
Knockout Antigen	1:20,000	1.5	0.02*

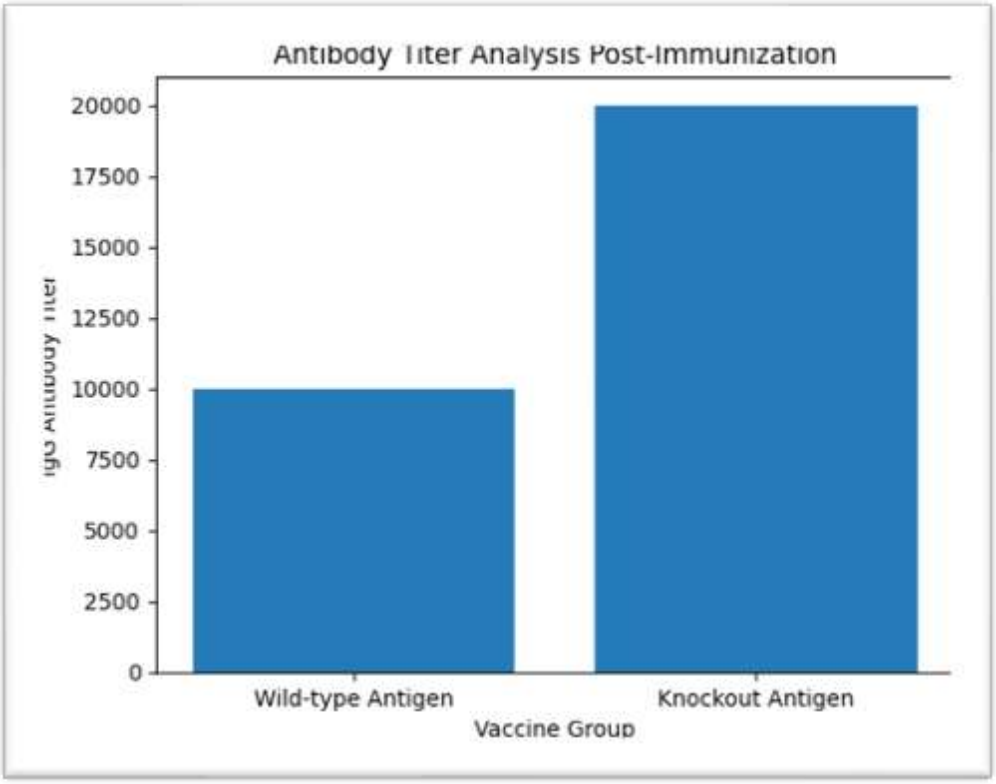

Figure 4: Antibody Titer Analysis Post-Immunization

5. Survival Rate After Injection with Wild-type *Leishmania*

Survival analysis showed that knockout antigen-immunized mice had an 80% survival rate, significantly higher than that of wild-type antigen-immunized mice (50%).

Table 5: Survival rate after injection

Group	Rate of survival (%)	p-value
Knockout Antigen	80%	<0.01*
Wild-type Antigen	50%	0.03

Discussion

This study focused on the effective use of CRISPR-Cas9 gene editing to detect immune evasion mechanisms in *Leishmania* and identify potential vaccine candidates. The results showed that the knockout of key immune evasion genes, such as LACK, GP63, and CPB, led to enhanced CD8+ IFN-γ+ T-cell infiltration in infected mice, suggesting that these genes play a significant role in suppressing effective immune responses against the parasite (Rooholamini *et al.,* 2024).

These results are consistent with previous studies showing that genes such as GP63 and LACK play critical roles in modulating immune responses, and that gene modification can restore a more effective immune response, as indicated by increased cytokine production, including IFN-γ and TNF-α. (Salim *et al.,* 2025). This suggests a Th1-biased immune response, which is considered essential for the clearance of *Leishmania* infections(Gómez-Chávez *et al.,* 2019).

This discovery is consistent with earlier research showing that Th1-type immune responses are significantly associated with protection against *Leishmania* (Moreira *et al.,* 2023; Aldali *et al.,* 2024)). Moreover,

Freund's adjuvant is effective in experimental settings but is not clinically acceptable and should be evaluated for safer alternatives.
Transcriptomic or proteomic evaluations of the effect of gene deletion on the whole organism should be performed in the future, and multiple or combination vaccines should be tested. Further studies are needed to explore long-term immunity, T cell memory, and cross-species protection.
The results presented here demonstrate that developing a *Leishmania* vaccine could be facilitated by using antigens derived from genes involved in immune evasion, and that knocking out these genes can boost host immunity. The results presented here could be useful for the development of genetically attenuated vaccines and new opportunities for specific and systematic action against *leishmaniasis*.
Genetically modified *Leishmania* vaccines are likely to promote long-term protection by stimulating long-lasting immune memory. Formation of pools of $CD4^+$ and $CD8^+$ memory T cells is particularly important for sustaining long-term Th1-mediated responses, which are characterized by rapid IFN-γ production upon re-exposure to the parasite. The present study did not directly examine long-term immune memory. However, the increases in IFN-γ and TNF-α levels suggest that long-term protection may be achieved. Additionally, conserved immune evasion targets (LACK, GP63, CPB) increase the likelihood of cross-species protection, which needs to be further confirmed in heterologous challenge models. Further research is therefore needed to assess the long-term effectiveness and efficacy of these vaccines, and to completely evaluate their potential for translation(Aghamiri *etal*.,2023)

The use of CRISPR-Cas9 technology for developing attenuated *Leishmania* strains and vaccine candidates has recently been emphasized. Targeting virulence genes and surface antigens, such as GP63, via genome-editing methods has enhanced immunity and Th1 responses, including increased production of IFN-γ and TNF-α. Likewise, GP63-based multivalent and genetically modified vaccines have shown protective efficacy in preclinical models. These results are consistent with ours, as an intermediate deletion of LACK, GP63, and CPB resulted in a dramatic increase in host protective immunity. Altogether, the existing literature is encouraging the use of CRISPR-engineered *Leishmania* as a rational platform for the development of next-generation vaccines; however, additional research is required to achieve stability, safety, and translational applicability.
Genetically attenuated *Leishmania* strains are an exciting vaccine platform with significant translational potential, as they can stimulate strong, long-lasting Th1-type immunity. Their clinical use, however, is hampered by potential attenuation issues, the organism's genetic instability, and the theoretical risk of reversion to virulence. Furthermore, regulatory and manufacturing challenges, as well as the requirement for thorough long-term safety studies in the relevant species, are important hurdles. It will be necessary to overcome these drawbacks to bring this approach to the clinic.
Although the CRISPR-Cas9 system is a robust method for studying gene function, there is a risk of off-target effects that may not be detected by careful gRNA design. In addition, genome editing efficiency in *Leishmania* is not uniform, repair mechanisms are atypical, and there may be functional redundancy, leading to unexpected results from gene disruption.

Conclusion

The Student's t-test was used for comparisons between two groups, and the one-way ANOVA was used for comparisons among more than two groups. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to assess statistical significance. Furthermore, progress toward clinical application will require compliance with regulations for genetically modified organisms and standardized production protocols. Overall, these findings provide a strong experimental foundation for targeting immune evasion genes in vaccine development against *Leishmania*.

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Ethical approval

All experimental procedures in this study were reviewed and approved by the Institutional Ethics Committee of **[Applied Science College]** under approval number **[UOF.MED.2025.001/2025]**. The study was conducted in full compliance with relevant institutional guidelines, regulations, and ethical standards.

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