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The Increase of White Pulp Diameter of Rat Spleen After CIDR1α-PfEMP1 Recombinant Protein Injection

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Abstract

Exploring new protein targets for malaria vaccine development is essential in supporting malaria control strategies. One such candidate is the cysteine-rich interdomain region of *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (CIDR1α-PfEMP1). The spleen, as a lymphoid organ, plays a critical role in immune responses, where activation is indicated by increased white pulp diameter. This study aimed to evaluate the immune response in rats following CIDR1α-PfEMP1 recombinant protein injection by measuring the spleen's white pulp diameter. Twelve male rats were divided into two groups: a control group receiving 0.9% NaCl and a treatment group receiving 150 μg of CIDR1α-PfEMP1 protein. The recombinant protein was administered subcutaneously on days 0, 21, and 42. On day 56, the rats were euthanized, and their spleens were collected, processed, and stained with hematoxylin-eosin (HE). White pulp diameters were measured microscopically (100×) using ImageJ software. The mean white pulp diameter was 22.590 ± 3.986 μm in the control group and 36.607 ± 6.739 μm in the treatment group. Statistical analysis using the independent t-test showed a significant difference ($p = 0.003$). These results suggest that CIDR1α-PfEMP1 recombinant protein stimulates immune activity, as indicated by increased white pulp diameter in rat spleens.

Keywords: CIDR1α-PfEMP1; recombinant protein; spleen; white pulp.

Abbreviation: CIDR1α-PfEMP1: cysteine-rich interdomain region of Plasmodium falciparum Erythrocyte Membrane Protein-1

INTRODUCTION

Malaria is a deadly disease caused by Plasmodium species. Nearly half of the world's population remains threatened by this disease, especially children and pregnant women. Beyond its impact on health, the high mortality rate among children due to severe malaria causes a crippling socioeconomic burden on the population of Sub-Saharan Africa (Miura et al., 2024).

Despite the continuous efforts to prevent and treat malaria, World Malaria Report 2024 showed an increase of 11 million new cases compared to the previous year, bringing the global number to 263 million cases in 2023. This figure also indicates stagnation in malaria control since 2015 (World Health Organization, 2024). Malaria control is increasingly threatened by drug-resistant *P. falciparum* and *P. vivax* strains. To make matters worse, the main therapies for malaria, chloroquine and artemisinin, are reported to be no longer effective in treating malaria caused by *P. falciparum* and *P. vivax*, especially in Asia and Africa. Mosquito resistance to insecticide-treated nets and changes in mosquito behavior towards biting humans further complicate this disease's control (Duffy et al., 2024; Miura et al., 2024).

In the midst of these challenges, the first and second licensed malaria vaccines, RTS,S and R21, have been administered to children in 17 malaria high-risk countries. These vaccines were designed based on the circumsporozoite protein (CSP) and are a new front line in malaria control. The success of the first malaria vaccines proves that malaria vaccines can strengthen the lineup of malaria control, and finding the new antigen target for new vaccines is now pivotal (Duffy et al., 2024).

One manifestation of severe malaria caused by *P. falciparum* is the result of cytoadherence and rosetting mechanisms, mediated by the *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) antigen. This process causes microvascular obstruction, ischemia, hypoxia, and multi-organ failure, which underpins the high morbidity and mortality of *P. falciparum* malaria. One domain of PfEMP1, known as the cysteine-rich interdomain region (CIDR1α), binds to the EPCR

receptor on the endothelial surface. Inhibiting this interaction is believed to prevent cytoadhesion, thereby reducing the risk of multiple organ failure caused by *P. falciparum*. Therefore, this antigenic protein is one of the targets for the development of severe malaria vaccines (Tuikue Ndam et al., 2017; Turner et al., 2018).

Preliminary studies demonstrated the potential of recombinant CIDR1α- PfEMP1 protein to induce IgG and CD4+ responses following immunization in Wistar rats (Istinaroh et al., 2024; Turner et al., 2018). CD4+ development and maturation occur in the spleen, the largest lymphoid organ, which plays a key role in initiating and modulating immune responses, as well as stimulating and activating T and B lymphocytes. This process occurs in the germinal center located within the lymphoid nodules, which are part of the spleen white pulp (Wang et al., 2023). Activation in the germinal center indicates the priming of naïve T lymphocytes, which can serve as an indicator of the body's immune response cascade being activated to build immunity against an antigen. Thus, it is essential to examine the effects of the CIDR1α- PfEMP1 antigen protein as a malaria vaccine candidate on germinal center activation in the spleen. This study aimed to observe the immune response of Wistar rats injected with CIDR1α-PfEMP1 recombinant protein by measuring the diameter of the spleen white pulp (Al-Salem, 2023).

MATERIALS AND METHODS

Study design

This research is a true-experimental study designed as a post-test only control group design. The study was conducted at the Molecular Biology and Biotechnology Laboratory, Center for Development of Advanced Science and Technology (CDAST), the Biochemistry Laboratory and the Histology Laboratory, Faculty of Medicine, and the Animal Laboratory, Faculty of Dentistry, University of Jember. This study has been approved by the Ethical Committee of the Faculty of Dentistry, University of Jember with No. 1898/UN25.8/KEPK/DL/2023.

Subjects

The study used male Wistar strain rats (*Rattus norvegicus*), aged 2-3 months, weighing 150-250 grams, with healthy physical characteristics. The sample size was determined using the resource equation method. An 'E' value, which represents the degrees of freedom for analysis of variance, was calculated, with an E-value of 10-20 considered an adequate sample size. If the E-value is under 10, increasing the number of animals can enhance the likelihood of obtaining significant results; conversely, if the E-value exceeds 20, adding more animals will not improve the chances of achieving significant results (Rachmania et al., 2021). This study utilized 2 groups with 8 rats in each group. We calculated the E-value using the following formula:

E = (number of groups x number of experimental animals) - number of groups
E = (2 x 8) - 2
E = 16 - 2
E = 14

The E-value was 14, indicating an acceptable range. However, due to the ethical considerations, research efficiency, and adherence to the principle of reduction, where the control group should be halved from the treatment group, the study used 4 rats for the control group (Kramer & Font, 2017; Yurista et al., 2016).

Procedures

Immunization

Twelve rats were allocated into two groups: a treatment group comprising 8 rats that were injected with CIDR1α-PfEMP1 recombinant protein at a dose of 150 µg, and a control group consisting of 4 rats that were injected subcutaneously with a 0.9% NaCl solution on days 0, 21, and 42 (Viola et al., 2018). Since the recombinant protein has a molecular weight higher than 20 kDa, it was combined with Complete Freund's Adjuvant (Santa Cruz, sc-3727) for the initial injection and Incomplete Freund's Adjuvant (Santa Cruz, sc-24019) for the subsequent injections at a 1:1 ratio (Bittner et al., 2018; Noh et al., 2022; Ruiz et al., 2017).

Microscopic Examination of the Spleen and White Pulp Diameter

On day 56, the rats were euthanized using ketamine and xylazine intramuscularly, followed by organ necropsy, and the spleen was prepared for histological examination with hematoxylin and eosin (H.E.) staining. Two observers performed the examination, and the results were presented as the average results from the two observers. It was conducted using an Olympus CX21LED microscope at 100X magnification, and images were captured with an AmScope FMA050 (Fixed Microscope Adapter). The 10-white pulp diameters of the spleen was determined view using ImageJ software using the formula (Ayu et al., 2016; Hidayat, 2015):

White pulp diameter = (Maximum transverse diameter + Maximum perpendicular diameter)/2

Data analysis

The data reliability among observers was assessed using the Cronbach's alpha test, with $\alpha>0.7$ considered reliable (Bonasia et al., 2015; Singla et al., 2017). The homogeneity data was assessed using Levene's test, while the normality data was checked using the Shapiro-Wilk test. To analyse the difference between the control and treatment groups, a statistical analysis using an

independent t-test was performed with a significant level of 0.05.

RESULTS AND DISCUSSION

The recombinant CIDR1α-PfEMP1 was expressed as a single band of approximately 27 kDa, as reported in a previous study (Sulistyaningsih et al., 2022). The histological appearance of the spleen is presented in Figure 1.

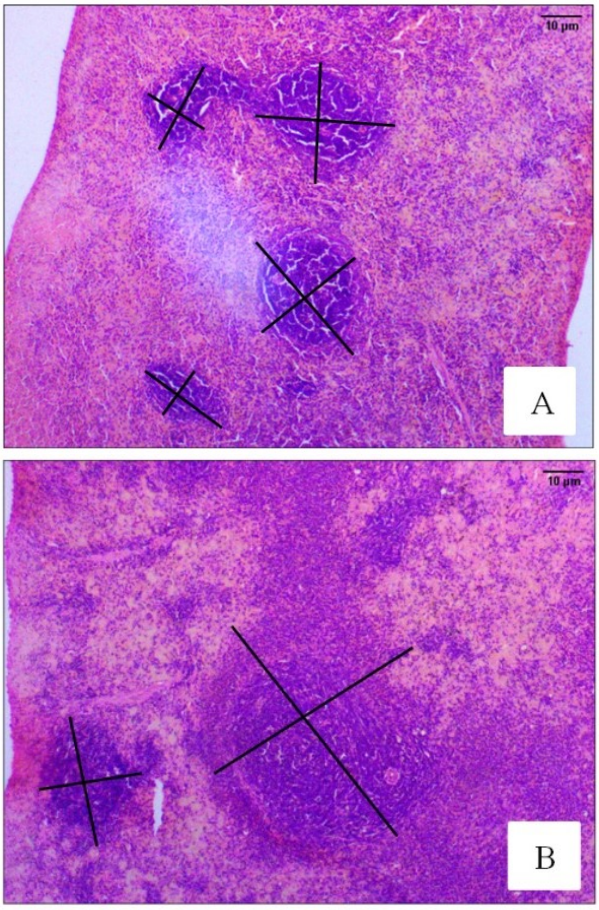

Figure 1. Histological appearance of the spleen stained with HE at 100X magnification. A: control group, B: treatment group. The black lines indicate the transverse diameter (longer line) and the perpendicular diameter (shorter line).

The examination results were presented as the average from the two observers, and the Cronbach's alpha test showed an α=0.963 (α>0.7), indicating reliable data. The Levene's test results indicated that the data were homogeneous (p>0.05), while the Shapiro-Wilk test results showed that the data were normally distributed (p>0.05). The independent t-test results revealed a significance value of 0.003 (p<0.05), indicating a significant difference between the control group and the treatment group. The mean white pulp diameter for the control and treatment groups, and the statistical analysis are presented in Table 1.

Table 1. The diameter of white pulp.

Group	Average diameter of white pulp ± SD (µm)	p-value
Control	22.590±3.986	0.003*
Treatment	36.607±6.739	

(*): a significant difference (p<0.05)

Discussion

The spleen is the largest lymphoid organ with a rich blood supply. It plays a vital role in filtering blood and serves as a site for immune responses to antigens in the bloodstream. The spleen comprises red pulp and white pulp. The red pulp features a dense network of reticular fibers filled with various cells, including erythrocytes, lymphocytes, plasma cells, macrophages, and other granulocytes. Its primary function is to filter blood, removing antigens, microorganisms, platelets, and old or abnormal erythrocytes. In contrast, the white pulp is the immune part of the spleen, primarily composed of clusters of lymphocytes found in lymphatic nodules surrounding the central artery. The lymphocytes near the central arteries in the white pulp are mainly T cells, which form periarteriolar lymphatic sheaths (PALS), while the lymphatic nodules are predominantly made up of B cells. Antigen-presenting cells (APCs) and macrophages are also present in the white pulp. These cells identify trapped antigens and trigger immune responses against them. Consequently, T cells and B cells interact, become activated, proliferate, and carry out their immune functions (Lewis et al., 2019).

In this study, the white pulp diameter of the rat's spleen increases after the CIDR1α-PfEMP1 recombinant protein injection, as shown in the treatment group. The increase is statistically significant compared to the control group (p=0.003) (Table 1). As the foreign antigen, the protein injection will cause a spleen reaction, including the induction of an immune response, resulting in an increase in the white pulp. Exposure to the recombinant protein can enhance cellularity in the B cell area and increase secondary follicles with prominent germinal centers. Immature B cells, or immunoblasts, will proliferate in response to antigenic stimulation. The proliferation of B cells increases the diameter of the white pulp. These cells will mature into plasma cells that produce immunoglobulin and migrate to the red pulp. The antigen protein activates central memory T cells and T-dependent B cell germinal centers, leading to antibody production (Mota & Madden, 2022).

This study utilized a dose of 150 µg, which falls within the 100-200 µg of antigen protein administered to rats to elicit a strong immune response (Greenfield, 2020). Previous research indicated that injection of the CIDR1α-PfEMP1 recombinant protein at a dose of 150 µg induces leukocyte, IgM, IgG, and CD4+ responses (Istinaroh et al., 2024; Sulistyaningsih et al., 2022). Additionally, another study on injection of a different recombinant protein domain (DBL2β-PfEMP1) found

that a dose of 100 µg of DBL2β-PfEMP1 recombinant protein produced the most effective response in increasing total leukocyte counts (Ari Santi Putri, 2022; Sulistyaningsih et al., 2022).

Antigen-presenting cells (APCs) in the PALS can recognize the protein antigen that enters the body. APCs release various pro-inflammatory and anti-inflammatory cytokines that affect several organs. APCs present antigens through major histocompatibility class I (MHC I) and major histocompatibility class II (MHC II) molecules, stimulating cytokine production to recruit other immune cells. Th1 cells produce cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which activate macrophages, monocytes, and natural killer (NK) cells in the cellular immune response. Th2 cells, on the other hand, produce cytokines like IL-4, IL-5, IL-6, and IL-10, which activate the humoral immune response. IL-4 and IL-5 promote the proliferation and differentiation of B cells into plasma cells that generate antibodies specific to the PfEMP1 antigen (Lewis et al., 2019; Mota & Madden, 2022).

Antibodies against the CIDR1α-PfEMP1 recombinant protein play a crucial role in preventing cytoadherence, contributing to severe malaria. A previous study demonstrated that the CIDR1α-PfEMP1 recombinant protein possesses immunogenic properties that can stimulate the production of specific IgG antibodies and CD4+ T lymphocytes in Wistar rats (Istinaroh et al., 2024). Another study indicated that antibodies generated by DBL2β-PfEMP1 decreased the risk of severe malaria by 37% by blocking cytoadherence and disrupting rosette formation (Tessema et al., 2018).

The results of this study represent a preclinical trial that supports the immunogenicity testing of a protein candidate for a malaria vaccine. The development of a malaria vaccine undoubtedly requires significant effort, particularly due to the complexity of the life cycle of P. falciparum, the causative species. To enhance the understanding of the immune response complexity following the injection of the CIDR1α-PfEMP1 recombinant protein, further research is needed that incorporates additional organ structure parameters, more comprehensive biochemical markers, such as cytokine levels, and the capacity of generated antibodies to block the cytoadherence and rosette formation.

CONCLUSIONS

Injection of the CIDR1α-PfEMP1 recombinant protein increases the diameter of the spleen white pulp of rats (*Rattus norvegicus*), indicating the presence of immune response activity. It supports the CIDR1α-PfEMP1 recombinant protein as a malaria vaccine candidate. Further study on other immunological and biochemical markers is required to emphasize the potency of the recombinant protein.

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Competing Interests: The authors declare that there are no competing interests.

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