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ARTICLE

IgG AND CD4+ RESPONSES POST IMMUNIZATION WITH CIDR1A-PFEMP1 RECOMBINANT PROTEIN

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ABSTRAK

Global malaria eradication need the malaria vaccine quickly. Complex Plasmodium sp life cycle and human immunity to malaria drive recombinant peptide-based malaria vaccine development. The malaria vaccine candidate CIDR1α-PfEMP1 is crucial for malaria pathogenicity because to its cysteine-rich interdomain region 1α of Plasmodium falciparum erythrocyte membrane protein 1. IgG and CD4+ responses were examined in 12 Wistar rats after CIDR1α-PfEMP1 recombinant protein immunization. Rats were split into two groups: a control group (0.9% NaCl injection) and a treatment group (150 µg pure CIDR1α-PfEMP1 recombinant protein subcutaneous injection). Injections were given three times during three weeks. After vaccination, blood was drawn every two weeks. IgG and CD4+ concentrations were assessed by ELISA. Data distribution-based independent T-test and Mann-Whitney test were used. Injection frequency increased IgG and CD4+ levels. IgG concentrations and CD4+ levels differed significantly between control and treatment groups after all injections, but only after secondary II immunization ($p<0.05$). Finally, the CIDR1α-PfEMP1 recombinant protein boosts humoral and cellular immune responses by increasing IgG and CD4+ levels, suggesting it could be a malaria vaccine candidate.

Keywords: Malaria; IgG; CD4 and Vaccine

АБСТРАКТ

Глобальное искоренение малярии требует быстрого создания малярийной вакцины. Сложный жизненный цикл Plasmodium sp и иммунитет человека к малярии стимулируют разработку малярийной вакцины на основе рекомбинантных пептидов. Кандидат в малярийные вакцины CIDR1α-PfEMP1 имеет решающее значение для патогенности малярии, поскольку он является богатым цистеином междоменным участком 1α мембранного белка 1 эритроцитов Plasmodium falciparum. IgG и CD4+ ответы были исследованы у 12 крыс Вистар после иммунизации рекомбинантным белком CIDR1α-PfEMP1. Крысы были разделены на две группы: контрольную (инъекция 0,9% NaCl) и лечебную (подкожная инъекция 150 мкг чистого рекомбинантного белка CIDR1α-PfEMP1). Инъекции проводились три раза в течение трех недель. После вакцинации кровь брали каждые две недели. Концентрацию IgG и CD4+ определяли методом ИФА. При распределении данных использовали независимый Т-тест и тест Манна-Уитни. Частота инъекций повышала уровень IgG и CD4+. Концентрация IgG и уровень CD4+ значительно отличались между контрольной и лечебной группами после всех инъекций, но только после вторичной иммунизации II ($p<0,05$). Наконец, рекомбинантный белок CIDR1α-PfEMP1 усиливает гуморальный и клеточный иммунный ответ, повышая уровень IgG и CD4+, что позволяет предположить, что он может стать кандидатом в малярийные вакцины.

Ключевые слова: Малярия; IgG; CD4 и вакцина

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INTRODUCTION

Malaria is one of the biggest health problems in the world. Globally in 2021, there were an estimated 247 million malaria cases in 84 malaria endemic countries, an increase from 245 million in 2020, one year after COVID-19. WHO Southeast Asia Region accounted for about 2% of malaria cases globally, with 5 million cases in 9 malaria-endemic countries, and Indonesia reported an increased number of deaths due to malaria in 2020.[1] The Indonesian government strives to eradicate malaria through several approaches, including early diagnosis, prompt treatment, vector control, active surveillance, and advocacy and information. However, malaria mortality and morbidity have not completely fallen. Therefore, an alternative approach, such as the malaria vaccine, is needed.[2]

Currently, the malaria vaccine that has been approved by WHO to control malaria in endemic areas is RTS,S/AS01, but the efficacy against severe malaria only reaches 36.3%. [3] The complexity of the life cycle and antigenic variation of *Plasmodium* antigens is the major hurdle in malaria vaccine development, so it is essential to develop vaccines with biomolecular and genetic engineering approaches and different protein targets.[4] One of the target proteins that are widely studied for vaccine development is *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1).[5] The PfEMP1 is a complex antigenic protein that can bind to endothelial receptors, which leads to sequestration and adhesion in malaria pathogenicity. PfEMP1 consists of several domains, including cysteine-rich interdomain region (CIDR).[6] The CIDR1α is able to interact with the Endothelial Protein C-receptor (EPCR) and scavenger receptor Cluster of Differentiation 36 (CD36) that play a crucial role in the pathological mechanism of malaria. Binding to EPCR has been implicated in severe malaria due to rosetting formation, while CD36 binding has been associated with uncomplicated malaria.[7]

The host response to malaria infection is a complex reaction involving humoral and cellular immune responses. Humoral immunity is mediated by antibodies, while cellular immunity is played by helper CD4+ T cells, cytotoxic CD8+ cells, and γδ T cells. The study reported that PfEMP1 Immunoglobulin G (IgG) antibodies have a protective response and shed light on which PfEMP1 domain is the main target of protective antibodies. IgG response against EPCR-binding CIDRα domains was found to develop earlier and have a protective effect against clinical disease. Furthermore, blood-stage *Plasmodium sp.* is capable of inducing CD4+ and T lymphocyte cells that produce various cytokines and can stimulate polyclonal B cell proliferation.[8] The PfEMP1, as a surface antigen on infected erythrocytes, can induce the formation of specific antibodies against this antigen, thereby disrupting its adhesion to host receptors.[9] This result is the basis for the recombinant proteins approach to designing peptide-based malaria vaccines. Knowledge of the mechanisms of malaria immunity is important to find a safe and protective vaccine.[10] In addition, *in silico* studies showed that the CIDR1α-PfEMP1 domain can bind to B cell epitopes, which further can induce antibodies to fight malaria. This indicated the potential immunogenicity of CIDR1α-PfEMP1.[2] Previous studies on DBL2β-PfEMP1 domain membrane protein showed its ability to stimulate cellular and humoral immune responses by increasing CD4+ dan IgG levels.[11] This study analyzed the cellular and humoral immune responses by measuring CD4+ and IgG levels after injection of CIDR1α-PfEMP1 recombinant protein from Indonesian isolate to investigate its potency as a malaria vaccine candidate.

MATERIAL AND METHODS

This study was carried out at the Biochemistry Laboratory, Medical Faculty, Experimental Animal Laboratory, Dentistry Faculty, University of Jember, Unit of Central Laboratory and Innovation Technology, Center for Development of Advanced Science and Technology (CDAST) University of Jember from April 2021 to March 2022. This study has received ethical approval from the Ethics Commission of Health Research, Medical Faculty, University of Jember, with number 1502/H25.1.11/KE/2020**.** The laboratory equipment used in this study was a microplate reader (R-Biopharm), vertical electrophoresis (Bio-Rad Laboratories Inc., Hercules, CA, USA), incubator, autoclave, biosafety cabinet,

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spectrophotometry, shaker incubator, centrifuge, and UV transilluminator. The materials used in this study were ELISA assay kit (BT-Lab, Shanghai, China), *E.coli* BL21 (DE3), Luria-Bertani (LB) media (Liofilchem S.r.l., Teramo, Italy), kanamycin (Thermo Fisher Scientific Inc., Waltham, MA, USA), isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promega Co., Madison, WI, USA), lysozyme (VWR International LLC, Radnor, PA, USA), Ni-NTA resin (Qiagen, Hilden, Germany), imidazole (Sigma-Aldrich Co., St. Louis, MO, USA), Bradford protein assay (HiMedia Laboratories, Maharashtra, India), and complete and incomplete Freund's adjuvant (Santa Cruz Biotechnology Inc., Dallas, TX, USA).

The Indonesian isolate of CIDR1α-PfEMP1 recombinant protein was produced from pET30a-CIDR1α-PfEMP1 clone and expressed in *E.coli* BL21 (DE3) according to previous research procedures.[12] Competent cells *E.coli* BL21 (DE3) were cultured using LB media and added 50 µg/mL kanamycin, then incubated at 37°C in a shaker incubator to reach an optical density of 600 nm (OD600) 0.6-0.8. Cultures were induced with 0.3 mM IPTG and incubated for 8 hours at room temperature. Then, the culture was harvested, and pellets were dissolved with extraction buffers (300 mM NaCl, 50 mM NaH2PO4, and 5 mM imidazole), then incubated with 1 mg/mL lysozyme for 30 min.

According to the manufacturer's protocol, the soluble fraction is purified using Ni-NTA agarose based on affinity chromatography. The soluble fraction is inserted into a column containing 1 mL of Ni-NTA resin and washed with 1 mL wash buffer I, which contains 300 mM NaCl, 50 mM NaH2PO4, and 20 mM imidazole twice, and wash buffer II twice. Recombinant proteins were eluted gradually using 0.5 mL elution buffer (300 mM NaCl, 50 mM NaH2PO4 with pH 8.0) containing a serial imidazole concentration.[13] Purified recombinant proteins were visualized using SDS-PAGE (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the gel was stained with Coomassie brilliant blue. CIDR1α-PfEMP1 recombinant protein levels were measured according to the protocol Bradford assay using a spectrophotometer with a wavelength of 595 nm.

During the course of the research, a total of twelve male Wistar rats (Rattus norvegicus) weighing between 200 and 250 grams were utilized. Four rats were assigned to the control group, and eight rats were assigned to the treatment group. After being placed in a plastic box with a wire lid, rats were placed in an environment that was optimal in terms of light and nutrition. After a period of two weeks during which they were acclimatized, they were then randomly assigned to either a control group or a treatment group. In order to administer the CIDR1α-PfEMP1 recombinant protein, the rats in the control group were administered a 0.9% NaCl solution. On the other hand, the rats in the treatment group were administered a 0.6–0.75 µg/BB injection. This was accomplished by dissolving 150 µg of recombinant protein in 200 µL of PBS and then emulsifying it with Freund's adjuvant at a ratio of 1:1. While Complete Freund's Adjuvant was utilized for the primary immunization process, Incomplete Freund's Adjuvant was utilized for the secondary immunization process. The immunization was administered three times, specifically on days 0, 21, and 42 of the study.[14] Blood samples were collected from an orbital vein every two weeks after immunization. Termination of experimental animals was carried out with ketamine and xylazine on day 56.

The procedures for measuring IgG and CD4+ were carried out using ELISA in accordance with the methodology provided by the manufacturer (BT-Lab, Shanghai, China). To microplates that had been coated with IgG antibodies, 40 microliters of mouse sera, 10 microliters of antibodies, and 50 microliters of streptavidin-HRP were added. Following a one-hour incubation period at 37 degrees Celsius, the microplate was washed with wash buffer five times. It was then incubated in the dark at 37 degrees Celsius for ten minutes after the substrate was introduced. One last step was the addition of a stop solution, after which

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the absorbance was measured using a microplate reader at a wavelength of 450 nm.

In order to evaluate whether or not the data were normal, we utilized the Saphiro-Wilk test. Additionally, in order to ascertain whether or not the data were homogenous, we utilized the Levene test method. For the purpose of determining whether or not the data were distributed normally, an independent T-test was utilized, and a Mann-Whitney test was utilized for the purpose of determining whether or not the data were normally distributed. When it came time to examine the difference between the control group and the treatment group, a confidence interval with a 95% level of certainty was utilized.

RESULT

Following the synthesis procedure, the recombinant protein CIDR1α-PfEMP1 was synthesized in a soluble fraction by utilizing Ni-NTA agarose. Subsequently, the protein was purified with the process. Figure 1 illustrates the molecular weight of the recombinant protein band of CIDR1α-PfEMP1 that has been purified. The molecular weight was determined to be 27 kilodaltons. According to the results of the BSA standard curve analysis, the amount of protein being present was 0.135 mg.

The findings of the investigation were as follows. The coefficient of determination (R2) was found to have a value of 0.09962, and the equation for the curve was computed as follows: y = 0.078x – 0.0703. For the purpose of conducting a study of a humoral immune response, it was required to ascertain the quantity of IgG antibody that was present.

In accordance with the micrograms per milliliter unit of measurement, the concentration of the medication is presented in Table 1. In order to arrive at this concentration reading, a standard formula, which can be seen below, was applied in conjunction with absorbance. The formula reads as follows: y equals 0.317 times x plus 0.0163, and R2 equals 0.9674.

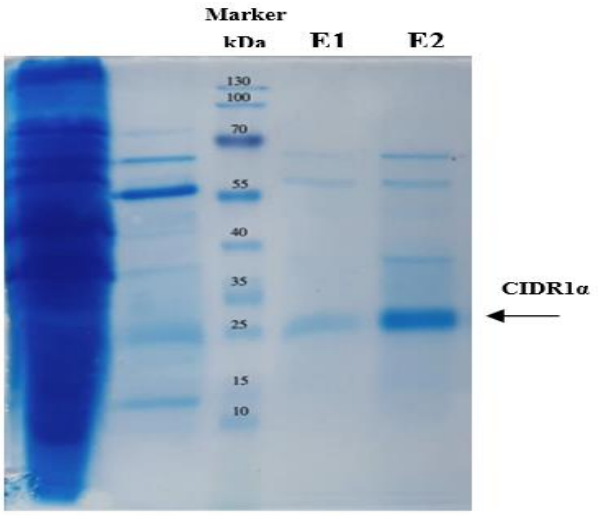

Figure 1. Visualization of the CIDR1α-PfEMP1 recombinant protein of Indonesian isolate with SDS-PAGE. It appears as a 27 kDa protein (arrow)

E1: The 1st elution of purified protein; E2: The 2nd elution of purified protein.

Figure 2 demonstrates that the levels of IgG antibodies that were found in the treatment group after both primary and secondary immunizations were significantly higher than those found in the control group. This was the case regardless of whether the immunizations were primary or secondary.

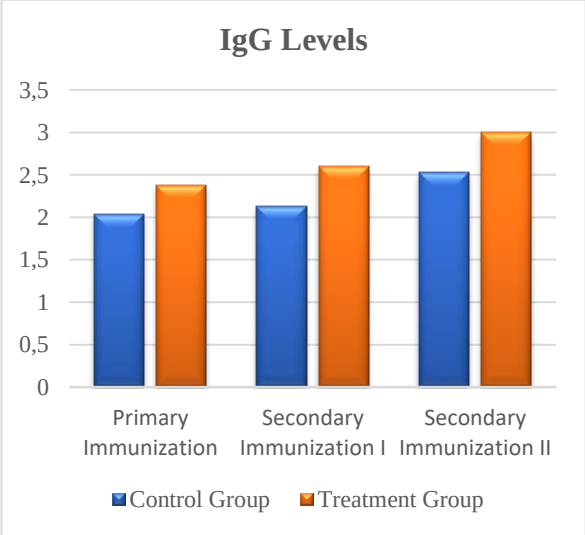

Figure 2. IgG levels after primary and secondary I and II immunizations in control and treatment groups. Blue: control group; Red: treatment group.

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Table 1. Average levels of IgG and CD4+ in control and treatment groups after CIDR1α-PfEMP1 recombinant protein injection and its statistical analysis

Types of Immunizations	Groups (*Mean* ± SD) (µg/mL)		*p-value*
	Control	Treatment	
IgG concentration			
Primary	2.03 ± 0.15	2.38 ± 0.08	0,002*
Secondary I	2.13 ± 0.15	2.60 ± 0.29	0,038*
Secondary II	2.53 ± 0.21	3.00 ± 0.28	0,041*
CD4+ concentration			
Primary	2.45 ± 0.10	2.55 ± 0.12	0,254
Secondary I	2.51 ± 0.26	2.60 ± 0.13	0,604**
Secondary II	2.73 ± 0.05	2.99 ± 0.12	0,009*

Notes *= significantly different between groups ($p<0.05$)
**= analysis using Mann-Whitney test due to not normally distributed data

The normality test using the Saphiro-Wilk test and the homogeneity test using the Levene test showed that the data were normally distributed and homogenous ($p>0.05$). Further statistical analysis to determine the difference between control and treatment groups using the independent T-Test showed $p<0.05$ for all immunizations, indicating a significant difference in IgG concentration between the control and treatment groups in primary, secondary I, and secondary II immunization.

The CD4+ concentration was measured to explore a cellular immune response. **Table 1** presented the CD4+ concentration in control and treatment groups after primary, secondary I, and II injection**s.** It was slightly different between the control and treatment groups after primary and secondary I injections of CIDR1α-PfEMP1 recombinant protein. However, there was a significant difference between the control and treatment groups after the secondary II injection (**Figure 3**), as statistical analysis ($p= 0.009$) in Table 1.

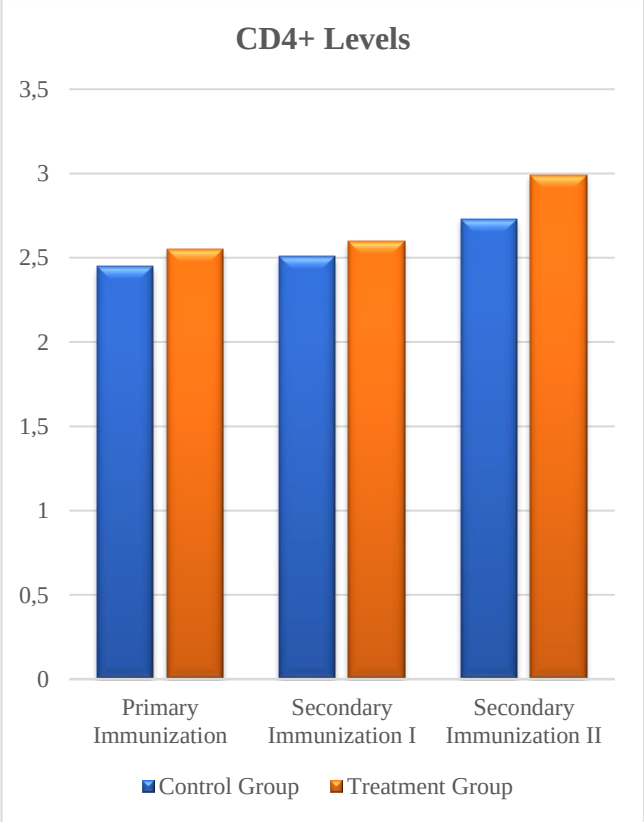

Figure 3. CD4+ levels after primary and secondary I and II immunization in the control and treatment group. Blue: control group; Red: treatment group.

The normality test using the Saphiro-Wilk test showed that data were normally distributed, except for the secondary I immunization group. The homogeneity test using the Levene test showed all data groups were homogenous. Further statistical analysis using the independent T-test for primary and secondary II immunization and Mann-Whitney test for secondary II immunization showed no difference between control and treatment groups ($p> 0.05$), except for secondary II immunization ($p=0.009$) (Table 1).

DISCUSSION

The study analyzed the humoral and cellular immune responses by measuring IgG antibody and CD4+ concentration after CIDR1α-PfEMP1 recombinant protein injection in Wistar rats. Recombinant proteins are proteins expressed by a particular host cell that carry recombinant DNA that has been transformed into the cell by

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a specific method. It involves the insertion of foreign DNA fragments in an organism's genome to enhance or decrease or block or combine the expression of an endogenous gene. The use of recombinant proteins has specific purposes, including increasing protein production, modifying gene sequences, and further producing commercial products.[15] [16] The CIDR1α-PfEMP1 recombinant protein in this study is a recombinant protein expressed from a clone of CIDR1α-*var* gen of *Plasmodium falciparum* Indonesian isolate in *E.coli* cells[12]

The immune response to malaria is complex, involving naturally acquired immunity. Regarding acquired immunity, malaria infection could induce humoral and cellular immune responses. The humoral immune response consists of the formation of antibodies targeting various antigens expressed by different phases of the parasite. Antibodies act against sporozoites that attack liver cells and block merozoites from infecting erythrocytes and opsonizing merozoites.[17,18] The study results suggest a protective association for antibodies targeting merozoites and suggest a specific antibody role in protection against malaria. IgG plays a protective role in blocking the invasion of merozoites into erythrocytes and is persistent protective of children and adults.[18–20] This study showed that the CIDR1α-PfEMP1 recombinant protein induces a humoral immune response characterized by increased IgG levels in the treatment group, including after primary, secondary I, and II immunizations. Although there was also an increase in IgG concentration in the control group after serial injection, the increase was not higher than that in the treatment group, and it was confirmed by the statistical analysis that there were significant differences between the control and treatment groups in serial immunizations. Overall, there is an upward trend in the average IgG levels along with the administration frequency, where the highest levels are obtained after secondary immunization II.

A study on the relationship of immunity to antibodies targeting merozoites provides evidence supporting the role of specific antibodies in protecting against increased malaria symptoms. In previous studies, as in this study, there was an increase in anti-merozoite IgG levels after malaria infection, both naturally induced and experimental. While the study reported a persistent rise in IgG in both children and adults, IgG plays a protective role by blocking the invasion of merozoites into erythrocytes by involving complement to the immune system.[18,19] IgG levels in malaria exposure have not yet reached predictive clinical immunity. Therefore, IgG has the potential to be a biomarker of malaria risk because it can be used to identify individuals exposed to infection *P. falciparum.*[21] IgG antibodies to merozoite antigens have been used for many years as biomarkers of immunity to monitor changes in the transmission *of P. falciparum.*[18]

IgG-specific antibody levels after primary immunization in the treatment group were higher than those of the control group, and this first immunization resulted in the lowest increase in IgG concentration compared to other immunizations. This result could be explained by the molecular weight theory of immunogenic protein. Proteins with a molecular mass of more than 2000 will be immunogenic, as the synthetic peptides as small as 6-20 residues have produced antibody responses. However, the CIDR1α-PfEMP1 recombinant protein has ~27 kDa. Another factor is the protein dose. Immunogens from soluble or membrane proteins need a concentration of 10-100 µg per injection for mice and 50-250 µg for rabbits. In this study, a dose of 150 µg was given to Wistar rats, while this dose is in the middle of the recommended dose, i.e., 100–200 µg.[14] The study showed that the given dose of CIDR1α-PfEMP1 recombinant protein in a serial administration increased the IgG concentration.

Overall, antibodies to blood-stage *P. falciparum* always appear during acute malaria episodes, and titers are rapidly increasing. This significant change in IgG titer becomes an approach to acute malaria screening as an indication of an increased immune response to

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malaria, and a decrease in titer is considered an indication of reduced immunity to malaria.[20] IgG levels became higher after secondary immunization I and II in line with studies conducted on monkeys (*Rhesus macaques*), which test the immunogenicity of a vaccine candidate by injecting three times, namely primary, secondary I, and II immunizations. The study results showed the highest antibody levels were in post-secondary II immunization.[22] Various studies on the role of specific antibodies, such as IgG on *P. falciparum*, provide insight into how vaccines work based on specific antigens, enabling the identification of essential endpoints for measuring vaccine efficacy in clinical trials.[18,20]

The cellular immune response in this study was measured by CD4+ concentration. The CD4+ concentration increased along with injection frequency, and the highest concentration was observed after the secondary II immunization. The statistical analysis confirmed these results, showing significant differences between control and treatment groups only in these injection groups (Table 1).

Immunization of the CIDR1α-PfEMP1 recombinant protein in experimental animals induces cellular immune response with increased CD4+ concentrations, especially after secondary immunization II. Previous studies conducted in mice showed that T cells are essential for controlling and resolving blood-stage infection, and CD4+ plays a role in the immune response to *P. falciparum* infection on erythrocytes. Mice without CD4+ cells showed significantly higher parasitemia after infection compared to the control group and were unable to reduce parasitemia during infection. In humans, CD4+ cells play a major role in regulating the immune response to *P. falciparum* by inhibiting the parasite's growth. CD4+ response is needed to control the first phase of infection by limiting parasitemia. In this study, the CIDR1α-PfEMP1 protein is part of an erythrocytic stage protein of *P. falciparum*.

In vitro stimulation of CD4+ cells with *P. falciparum* antigens results in the proliferation of CD4+ cells from the peripheral blood of individuals who have never had a prior malarial infection and IFN-γ secretion. Both did not correlate with serum antibodies against antigens.[18] Other studies reported that *P. falciparum* antigen stimulates IL-4 secretion from CD4+ cells that correlate with serum antibody concentrations but did not correlate with lymphocyte proliferation or IFN-γ release. These studies' results indicated that human immune response is controlled by distinct subsets of CD4+ cells that correlated with Th1 and Th2 cells and that both helper and effector functions of CD4+ cells contribute to malaria immunity.[18] CD4+ are needed to assist B lymphocyte cells in producing antibodies to kill parasites and also produce cytokines that strengthen the phagocytic and parasiticidal responses of the innate immune system and regulate later immune responses to limit immunopathology.[18,23]

A new subset of CD4+ cells, i.e., T follicular helper cells (Tfh), plays a vital role in malaria infection. Tfh cell is a potent inducer of antibody production, which activates B cells in spleen germinal centers to generate antibodies and memory B cells response. A new subset of Tfh, i.e., circulating Tfh (cTfh), has been detected. A study conducted in mice with *P. yoelii* and *P. berghei* indicates that severe malaria infection inhibits the germinal center's establishment in the spleen and induces high frequencies of Tfh cell precursors but reduces Tfh cell differentiation. However, inhibition of TNF and IFNγ resulted in the restoration of the Tfh cell differentiation.[24]

In general, this study showed an increase of CD4+ concentrations in the treatment group after the CIDR1α-PfEMP1 recombinant protein injection, although a significant difference was observed only after the secondary II injection. As the Tfh cells, a subset of CD4+ cells, play an important role in antibody production and are observed during acute disease, this subset should be targeted as a potential way to improve malaria vaccine efficacy.[2518,26] However, studies showed a major target for immunoregulatory pathways during malaria infection are CD4+ cells that play critical roles

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in priming phagocytic cells to capture and kill malaria parasites and helping B cells produce anti-parasitic antibodies. Furthermore, CD4+ cell functions could improve vaccine responses and increase the development of natural immunity.

CONCLUSION

The increase in IgG and CD4+ levels post immunization of CIDR1α-PfEMP1 recombinant protein indicated the immunogenicity of this protein. A trend of increasing IgG and CD4+ levels along with the immunization frequency suggested the potential of CIDR1α-PfEMP1 recombinant protein as a malaria vaccine candidate. Further studies with dose variations can be conducted to determine the best dose of this recombinant protein to induce an immune response.

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DECLARATIONS

All authors represent no conflict of interest with this study.

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