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Differential Leukocyte Count Responses Post Injection of Duffy-binding-like Domain-2β of PfEMP1 Recombinant Protein in Wistar Rat

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Abstract: Malaria due to *Plasmodium falciparum* causes a high mortality rate, and vaccination is a valuable approach to control it. One malaria vaccine candidate is *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1), specifically Duffy binding-like 2β (DBL2β) domain (DBL2β-PfEMP1), which has a vital role in severe malaria pathogenesis. The DBL2β-PfEMP1 recombinant protein is immunogenic. This study aimed to investigate the immune response of DBL2β-PfEMP1 protein by analyzing the differential leukocyte count. Twenty-three rats were randomly divided into control and five treatment groups. Rats were injected on days 0, 21, and 42 with a physiological solution of 0.9% NaCl, adjuvant, DBL2β-PfEMP1 protein, and each mixture of DBL2β-PfEMP1 protein with doses of 150, 300, and 450 µg/200gBW and adjuvant. Blood was collected on day 56 and prepared for differential leukocyte count examination with a visual microscopic examination by two expert observers. The results showed that DBL2β-PfEMP1 recombinant protein and adjuvant increased the eosinophils and neutrophils but decreased monocytes and lymphocytes and did not affect the basophils. Statistical analysis showed significant differences between groups for eosinophils (between control and DBL groups; Adj and DBL groups; DBL and other groups except DBL150+adj) and monocytes (between control and all doşe groups with adjuvant; DBL and all doşe groups with aduvant), but not for basophils, neutrophils, and lymphocytes. In conclusion, the serial injection of DBL2β-PfEMP1 recombinant protein showed different responses in each leukocyte cell type. Further analysis by time-series differential leukocyte count examination will be essential to determine the responses of each type of leukocyte to support the research on malaria vaccine development.
Keywords: Differential leukocyte; malaria vaccine; *Plasmodium falciparum*; recombinant protein.

INTRODUCTION

Plasmodium falciparum is the leading cause of severe malaria, with a high mortality rate (Indrayana et al., 2023; Wiser, 2023); it causes more than 600,000 deaths each year globally, most of which occur in children (Walker & Rogerson, 2023). Current malaria control strategies by spraying mosquitoes are no longer effective due to the emergence of both mosquito and parasite resistance to the chemical compounds used (Savi, 2022). Therefore, developing a highly effective and durable vaccine against human malaria parasites, specifically *Plasmodium falciparum*, remains a top priority (Draper et al., 2018).

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Plasmodium falciparum exhibits immense pathogenicity compared to other human *Plasmodium* parasites. The main factor that plays a role in this increased pathogenicity is the cytoadherence of infected erythrocytes to endothelial cells of the capillaries in vital organs and tissues. Cytoadherence is primarily mediated by the parasite's protein deposited on the surface of infected erythrocytes called *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1). The protein consists of extracellular and intracellular parts. The extracellular part varies in sequence and length. However, it is composed of two domains, i.e., Duffy binding-like (DBL) domain which is classified into DBLα, DBLβ, DBLγ, DBLδ, DBLε, DBLξ, and DBLχ, and cysteine-rich interdomain region (CIDR) which is classified into CIDRα, CIDRβ, and CIDRγ (Plewes et al., 2019; Wiser, 2023). The Duffy binding-like-2β (DBL2β) domain mediates binding to Intercellular Adhesion Molecule 1 (ICAM-1) (Klein et al., 2008; Lennartz et al., 2019), and the binding is associated with an increased risk of severe malaria, especially cerebral malaria (Lennartz et al., 2017). The study found that PfEMP1 is the essential target of antibodies associated with a reduced incidence of severe malaria (Chan et al., 2019). Therefore, DBL2β of PfEMP1 could be a potential malaria vaccine candidate. The DBL2β-PfEMP1 can act as an antigen that can stimulate B cells to produce antibodies, as shown by a previous study (Rachmania et al., 2021).

Leukocytes or white blood cells are blood components involved in the body's immune response (Yazdani et al., 2023). Leukocytes contain eosinophils, basophils, neutrophils, lymphocytes, and monocytes. Neutrophils, monocytes, and lymphocytes play a vital role in malaria immunity. Neutrophils act as the first line of defense against parasites and can induce phagocytosis and release reactive oxygen species to combat parasites. Monocytes can induce cytokine production and engage in phagocytosis to reduce malaria burden. Lymphocytes, including B and T cells, are responsible for innate and adaptive immunity against malaria (Lau & Sun, 2018). Specific immune cells in the blood induced by vaccines can be an early indicator of vaccine effectiveness (Huber et al., 2020). In adaptive immunity to tackle malaria, both humoral immunity, which is mediated by B cells, and cellular immunity, which is mediated by both CD4+ T cells and CD8+ T cells, are crucial to targeting the merozoites, cell surface antigens and blood-stage parasites (Plewes et al., 2019). The DBL2β-PfEMP1 protein, which plays a vital role in the pathogenesis of severe malaria, can be a candidate for a malaria vaccine. Recombinant protein DBL2β-PfEMP1 is immunogenic in an *in vivo* study as revealed by its ability to stimulate adaptive immunity, including humoral and cellular immune responses, especially those involving Immunoglobulin G (IgG) and Cluster of Differentiation 4+ (CD4+) (Rachmania et al., 2021). Immunogenicity tests of DBL2β-PfEMP1 recombinant protein in experimental animals have been carried out. However, the immune response of this recombinant protein has not been thoroughly studied. The previous study directly measured the IgG and CD4+ levels after the DBL2β-PfEMP1 recombinant protein injection (Rachmania et al., 2021).

Furthermore, the vaccine's effectiveness can be seen from the immune response in leukocytes, as the prior study showed an increase in the total leukocyte count after DBL2β-PfEMP recombinant protein injection. However, the study did not assess each type of leukocyte cell (Putri et al., 2022). Therefore, this study analyzed the differential leukocyte count after injection of DBL2β-PfEMP1 recombinant protein in Wistar rats (*Rattus norvegicus*).

MATERIALS AND METHODS

Ethical Clearance

The study used male Wistar rats as the animal model. All study procedures have been approved by the Ethical Committee of Faculty Medicine, University of Jember, No. 3094/UN25.1.10.2/KE/2024.

Production and Purification of DBL2β-PfEMP Recombinant Protein

The DBL2β-PfEMP recombinant protein was produced in *Escherichia coli* BL21 (DE3). The recombinant cells were cultured using Luria-Bertani (LB) media mixed with 50 µg/mL kanamycin and incubated in a shaker incubator at 190 rpm for 4 hours and induced using 50 µL of 0.5 mM IPTG. The culture was harvested by centrifugation for 10 min at 4oC; the pellet was mixed with 4 mL of extraction buffer (Tris HCl 50 mM pH 8.0, NaCl 500 µM, imidazole five µM, NaH2PO4 50 µM) and lysozyme, and incubated for 30 minutes at 4℃.

Protein purification was performed based on affinity chromatography using Ni-NTA GenScript agarose. The soluble fraction was added to a column containing 1 mL Resin Ni-NTA slurry and washed with 1×2 mL of wash buffer 1 (NaCl 500 mM, NaH2PO4 50 mM, and imidazole 50 mM with pH 7.0), 1×2 mL wash buffer 2 (NaCl 500 mM, NaH2PO4 50 mM, and imidazole 50 mM with pH 6.5), 1×2 mL wash buffer 3 (NaCl 500 mM, NaH2PO4 50 mM, and imidazole 50 mM with pH 6.0), and 1×2 mL wash buffer 4 (NaCl 500 mM, NaH2PO4 50 mM, and imidazole 80 mM with pH 5.5). The recombinant protein was eluted using 3×500 µL of elution buffer 1 (500 mM NaCl, 50 mM NaH2PO4, and 100 mM imidazole with pH 8.0) and 3×500 µL of elution buffer 2 (500 mM NaCl, 50 mM NaH2PO4, and 150 mM imidazole with pH 8.0). The purified protein was then visualized using SDS-PAGE, and the concentration was measured using Bradford protein assay.

Injection of DBL2β-PfEMP Recombinant Protein and Blood Extraction on Wistar Rats

This study used twenty-three male Wistar rats aged 2-3 months, weighing 150-250 g, and physically healthy. The rats were randomly divided into five groups: Control, Adjuvant (Adj), DBL, DBL150+adj, DBL300+adj, and DBL450+adj. Each rat was placed in a 40×30×13 cm (L×W× H) cage with wood shavings as the floor. The rats were fed standard pellets and water. The cage was washed weekly to maintain hygiene in the rat's living area.

Each rat in each group was injected subcutaneously on days 0, 21, and 42. The control group was injected with a physiological solution of 0.9% NaCl. The Adj group was injected with Freund's complete adjuvant in the primary injection and Freund's incomplete adjuvant in the subsequent injection. The DBL group was injected with 150µg/200gBW DBL2β-PfEMP recombinant protein. The DBL150+adj group was injected with a 150µg/200 DBL2β-PfEMP recombinant protein and adjuvant mixture. The DBL300+adj group was injected with a 300µg/200gBW DBL2β-PfEMP recombinant protein and adjuvant mixture. The DBL450+adj group was injected with a 450µg/200gBW DBL2β-PfEMP recombinant protein and adjuvant mixture. On day 56, rats were terminated using a ketamine-xylazine, and blood was collected for differential leukocyte count examination.

Preparation and Examination of Leukocyte Differential Count

The blood smears were made by preparing a clean, dry, and labeled slide. A total of ± 3 µl of blood sample was dropped on the end of the slide. Another glass object will be used as a spreader by placing it in front of the droplet at an angle of 30-40º. Then, the spreader is pulled back until the blood sample droplets spread to cover

the side of the spreader slide and pushed quickly using sufficient pressure. The blood smear was fixed using methanol and stained using Giemsa.

The differential leukocyte count was examined by counting each type of leukocyte using a light microscope with 1,000x magnification in the reading zone or counting area (Khasanah et al., 2023). Each leukocyte cell was differentiated, classified, and counted until 100 leukocyte cells, and the percentage of each leukocyte was calculated (Smith et al., 2016). The examination was performed twice by two different observers. The final results were obtained by calculating the average percentage from two observers.

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 28.0 software. The data distribution was analyzed using the Saphiro-Wilk test, and the data homogeneity was analyzed using Levene's test. The data with normal distribution were analyzed using the One-way ANOVA test followed by the post-hoc Bonferroni test. In contrast, the abnormal data distribution was analyzed using the Kruskal-Wallis test, followed by the post-hoc Kruskal-Wallis test. Statistical analysis was performed with a confidence interval of 95%.

RESULTS AND DISCUSSION

The DBL2β-PfEMP recombinant protein was visualized as a ~72kDa band, as reported by a previous study. It resulted from the elution buffer 2 with 150 mM imidazole (E2.1; E2.2; E2.3) (Figure 1)

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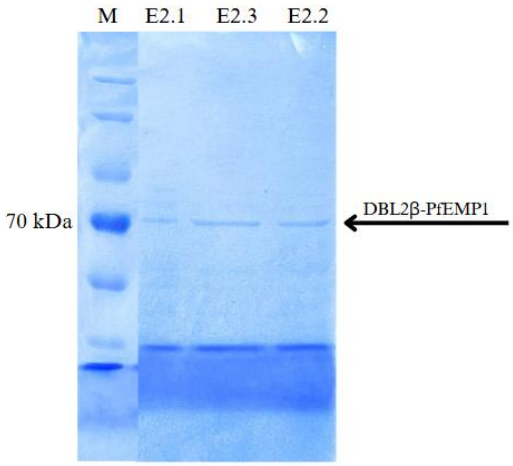

Figure 1. Visualization of DBL2β-PfEMP Recombinant Protein using SDS-PAGE. The protein was a ~72kDa. M: Protein marker; E2.1: Elution 1 from elution buffer 2; E2.3: Elution 3 from elution buffer 2; E.2.2: Elution 2 from elution buffer 2

The results of leukocyte differential count after injection of DBL2β-PfEMP1 recombinant protein are presented in Figure 2. Leukocyte cell types are eosinophil, basophil, neutrophil, monocyte, and lymphocyte. The average eosinophil percentage in all groups except the DBL and DBL150+adj groups was 0, and the highest eosinophil count was observed in the DBL group. For the average basophil, the highest number was observed in the Adjuvant (Adj), followed by the DBL group, and the other groups were 0. The highest percentage of neutrophils was shown in the DBL300+adj group, while the highest percentage of monocytes was observed in the DBL group. As the most significant percentage of leukocytes, lymphocytes were highest in the control group, followed by the DBL group. Statistical analysis using the

Kruskal-Wallis test (for eosinophils, basophils, and monocytes) or the One-way ANOVA test (for neutrophils and lymphocytes) showed a significant difference between groups only resulted in eosinophils and monocytes with a p-value of 0.035 and 0.006 ($p<0.05$). In contrast, basophils, neutrophils, and lymphocytes revealed no significant difference between groups with a p-value of 0.124, 0.138, and 0.500 (Figure 2). Further analysis using the Post-Hoc Kruskal-Wallis test was performed only for eosinophils and monocytes.

Figure 2 showed that for the eosinophils, the DBL group had the highest count compared to other groups, while the other groups using adjuvant had very low eosinophils. The statistical analysis using a Kruskal-Wallis test confirmed the difference between groups, which resulted in a p-value of 0.035. Eosinophils play a significant role in modulating allergic inflammation (Chusid, 2018). Adjuvants can reduce the likelihood of anaphylactic reactions by absorbing allergens and modulating allergen-specific immune responses towards a predominantly tolerogenic or Th1-biased immune response (Lin et al., 2024). Our study indicated a similar adjuvant response, which can reduce the number of eosinophils. Further analysis using the Post-Hoc Kruskal-Wallis test for eosinophils showed a significant difference was only observed between the control and the DBL group but not the other groups, including Adj, DBL150+adj, DBL300+adj, and DBL450+adj groups (Table 1). This result is similar to a previous Sulistyaningsih et al. (2022) study using CIDR1α-PfEMP1 recombinant protein. Cahyaningsih et al. (2019) also reported a similar result using *P. berghei* injection. It is known that eosinophils can induce plasma cell responses to produce and secrete specific antibodies in the early stages of the immune response (Prince et al., 2023). Another study also reported a partial role of eosinophil that occurs at the beginning of the immune response and does not have a significant role in vaccine immunity (Ledbetter et al., 2019). In this study, the examination was performed on the 56th day post the first protein injection or the 14th day post the third protein injection, which is suggested as not an early immune response. Thus, it possibly makes no significant difference between the control and treatment groups.

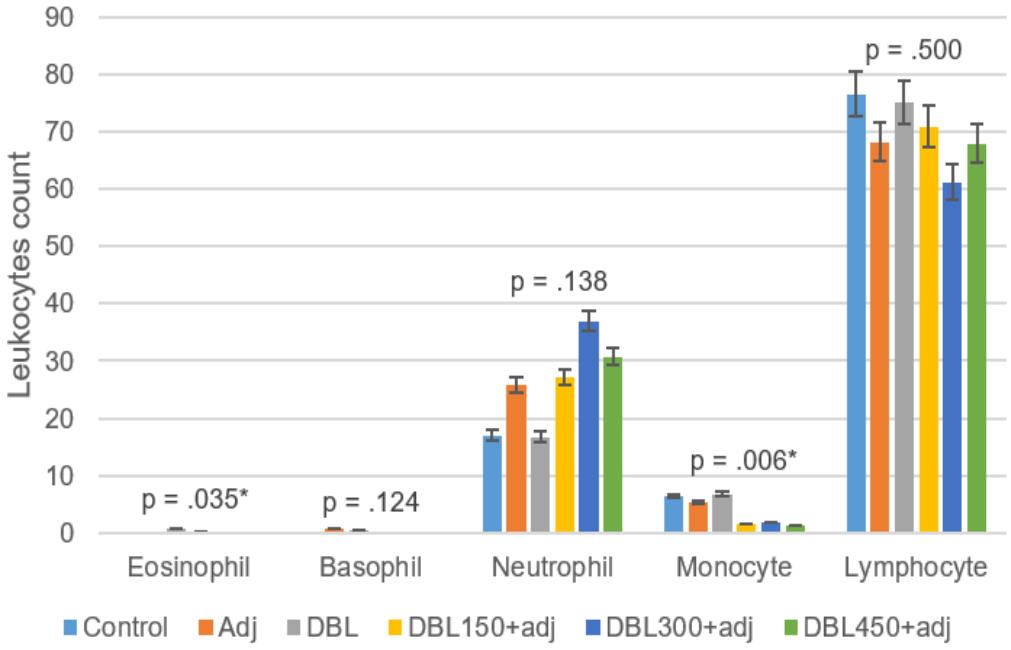

Figure 2. Differential Leukocyte Count Post-injection of DBL2β-PfEMP1 Protein Recombinant

Table 1. Results of the Kruskal-Wallis Post-hoc Test on Eosinophil

	Control	Adj	DBL	DBL_{150} +adj	DBL_{300} +adj	DBL_{450} +adj
Control		1	**.011***	.398	1	1
Adj	1		**.006***	.362	1	1
DBL	**.011***	**.006***		.068	**.006***	**.006***
DBL_{150} + adj	.398	.362	.068		.362	.362
DBL_{300} + adj	1	1	**.006***	.362		1
DBL_{450} + adj	1	1	**.006***	.362	1	

*Statistically significant

The average basophil percentage in all groups was 0, except the Adj and DBL groups. Statistical analysis using the Kruskal-Wallis test showed no significant difference between groups with a p-value of 0.14 (p>0.05) (Figure 2). These results align with the report by Sulistyaningsih et al. (2022) using CIDR1α-PfEMP1 recombinant protein and Cahyaningsih et al. (2019) using *P. berghei* injection. The number of basophils in the blood circulation is only 1% or less of all leukocytes, making it the least leukocyte (Poto et al., 2023). In malaria infection, basophil activation is enhanced by *P. falciparum* translationally controlled tumor suppressor protein (PfTCTP) (Pelleau et al., 2012). Our study used DBL2β-PfEMP1 as an antigen, suggesting no activation and affecting basophil's response.

The average percentage of neutrophils in treatment groups varies between 16.75 and 37.00, and the statistical analysis using the One Way-ANOVA test showed no significant difference between groups with a p-value of 0.138 (p>0.05). The highest neutrophil percentage was observed in the DBL300+adj group (Figure 2). The results align with the previous study by Maslachah and Sugihartuti (2017), which found that *P. berghei* injection increased the number of neutrophils in the blood circulation. Neutrophils are the first line of immune response to eradicate pathogens by phagocytizing them. Neutrophils play an essential role in the immune response by modulating cellular and humoral immunity by synthesizing and releasing immunoregulatory cytokines. Cytokines and interleukins released after neutrophil lysis will stimulate the bone marrow to release neutrophils and increase neutrophil production (Maslachah & Sugihartuti, 2017). Furthermore, Zhao et al. (2023) described the role of adjuvant as an immunostimulant that can activate the C-type lectin receptors (CLRs) pathway and further induce the recruitment of neutrophils. In this study, injection of a mixture of DBL2β-PfEMP1 recombinant protein and adjuvant was suggested to induce immune responses by increasing the neutrophil percentage. However, this response was not high enough to cause a significant difference.

The study showed that the average percentage of monocytes in treatment groups was lower than the control group, and the statistical analysis using the Kruskal-Wallis test showed a significant difference between groups with a p-value of 0.006 (p<0.05) (Figure 2). Further analysis using the Post-Hoc Kruskal-Wallis test showed a significant difference between the control group and the DBL150+adj, DBL300+adj, and DBL450+adj groups, the DBL group and the DBL150+adj, DBL300+adj, and DBL450+adj groups, and the Adj group and DBL450+adj group (Table 2). The significant difference between the control and DBL150+adj, DBL300+adj, and

DBL450+adj groups indicated the induction of immune responses that significantly affect the number of monocytes in the blood circulation. A significant decrease in the monocyte's number occurs due to a reduction in the expression of antigen-presenting molecules, which usually occurs after strong immune system activation (Bumbea et al., 2023). In terms of trained immunity, trained monocytes will migrate to peripheral organs, thereby reducing the percentage of monocytes in the blood (Netea et al., 2020). In addition, research also shows that adaptive immune cells can actively suppress the innate response, thereby reducing the number of monocytes (J. Zhao et al., 2009). These results are also conveyed by Maslachah and Sugihartuti (2017), who state that monocytes function optimally in the first week of infection, and their number decreases after chronic infection. Therefore, blood extraction on the 14th day post-injection could cause a low percentage of monocytes.

The percentage of monocytes in the Adj group was lower than that in the control and DBL groups (Figure 2), and the Adj group had a significant difference only against the DBL450+adj group (Table 2). It is known that a complete Freund's adjuvant can induce monocyte expansion to the heart and spleen, thereby reducing the number of monocytes in the blood circulation (Fontes et al., 2017), explaining the monocyte decrease in all groups using the adjuvant.

Table 2. Results of the Kruskal-Wallis Post-hoc Test on Monocyte

	Control	**Adj**	**DBL**	**DBL_{150} +adj**	**DBL_{300} +adj**	**DBL_{450} +adj**
Control		.686	.888	**.032***	**.044***	**.009***
Adj	.686		.556	.061	.082	**.017***
DBL	.888	.556		**.014***	**.020***	**.003***
DBL_{150} + adj	**.032***	.061	**.014***		.894	.611
DBL_{300} + adj	**.044***	.082	**.020***	.894		.521
DBL_{450} + adj	**.009***	**.017***	**.003***	.611	.521	

* Statistically significant

Lymphocytes count for 75.8-92.9% of leukocyte cells. The highest average percentage of lymphocytes was observed in the control group, and analysis using the One-way ANOVA test revealed no significant difference between groups with a p-value of 0.500 ($p>0.05$) (Figure 2). The results are similar to a previous study by Maslachah and Sugihartuti (2017) using *P. berghei.* At the beginning of infection, the pathogen or protein will activate antigen-presenting cells (APC), which further induces T lymphocyte activation as an immune response to eliminate the pathogen. In this study, the decrease of lymphocytes could be a sequential response after a decrease of monocytes in blood circulation. It is known that monocytes have APCs function. Furthermore, blood examination in this study was performed on the day 56 or 14 days post the third DBL2β-PfEMP1 protein injection, as the previous study showed that the increase of lymphocyte percentage occurred one week after protein injection and will decrease two weeks after protein injection (Sulistyaningsih et al. 2022), which is caused by migration of lymphocytes to the tissues (Widyastuti, 2014).

The study was designed to determine the immune response induced by the DBL2β-PfEMP1 recombinant protein as a malaria vaccine candidate. Therefore, immunological memory activation is expected. Thus, serial protein injection was performed. Immunological memory response will allow for rapid and greater response, resulting in faster, longer, and better protection than primary immune response. Those effects are needed for vaccine candidates (Actor, 2023)(Mueller & Rouse, 2008; Williams & Bevan, 2007)(Lau & Sun, 2018).

This study has limitations, including the manual examination of differential leukocyte count, which is less precise than an automatic hematology analyzer. However, to overcome this limitation, the examination was performed by two observers.

CONCLUSION

Serial injection of DBL2β-PfEMP1 recombinant protein resulted in different responses in each leukocyte cell type. Two weeks after the DBL2β-PfEMP1 recombinant protein injection would increase the eosinophils and neutrophils but decrease monocytes and lymphocytes and do not affect the basophils. However, the effect was statistically significant only for eosinophils and monocytes but not for basophils, neutrophils, and lymphocytes. The data indicated that the DBL2β-PfEMP1 recombinant protein in a doşe of DBL300+adj has optimum results in inducing the leukocyte cell type responses. Further analysis by time-series differential leukocyte count examination will be essential to determine the responses of each type of leukocyte to support the research on malaria vaccine development.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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