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ANALYSIS OF MICROGLIA MORPHOLOGY AND NUMBER IN WISTAR RATS BRAIN AFTER CIDR1α-PfEMP1 RECOMBINANT PROTEIN INJECTION

ANALISIS MORFOLOGI DAN JUMLAH MIKROGLIA PADA OTAK TIKUS WISTAR PASCAINJEKSI PROTEIN REKOMBINAN CIDR1α-PfEMP1

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Abstract

One malaria vaccine candidate is Cysteine-rich Interdomain Region 1α (CIDR1α) of *Plasmodium falciparum Erythrocyte Membrane Protein 1* (PfEMP1), an essential protein involved in the pathogenesis of cerebral malaria. Microglia in the brain act as the first line of defense against brain pathological changes. The study aimed to evaluate the response of brain microglia to the CIDR1α-PfEMP1 recombinant protein injection by observing microglia morphology and number in rat's cerebral cortex. 12 Wistar rats were divided into the control group, which was injected with normal saline solution, and the treatment group, which was injected with 150 µg CIDR1α-PfEMP1 recombinant protein combined with adjuvants. Injection was conducted thrice within three-week intervals (day 1, 21, and 42). Wistar rats were euthanized on day 56, and histological slides were prepared with Hematoxylin-Eosin staining. Examination using a microscope, 400x, and Fiji Image J software showed microglia morphology of ramified and rod cells in both the control and treatment groups. The microglia number in the control group was 93.00 ± 5.77, and the treatment group was 105.75 ± 15.62. Statistical analysis using an independent t-test showed no significant differences between groups (p= 0.15). The result indicated that the injection of CIDR1α-PfEMP1 recombinant protein did not provoke pathological changes in brain tissue, which induced a microglia response. This study strengthens the potential of the CIDR1α-PfEMP1 recombinant protein as a peptide-based malaria vaccine candidate.

Keywords: CIDR1α; Malaria; Microglia; PfEMP1; *Plasmodium falciparum*; Vaccine

Abstrak

Salah satu kandidat vaksin malaria adalah Cysteine-rich Interdomain Region 1α (CIDR1α) dari Plasmodium falciparum Erythrocyte Membrane Protein 1 (PfEMP1), protein penting dalam patogenesis malaria serebral. Mikroglia di otak berperan sebagai pertahanan lini pertama terhadap perubahan di otak. Penelitian ini bertujuan mengevaluasi respon mikroglia otak terhadap pemberian protein rekombinan CIDR1α-PfEMP1 dengan mengamati morfologi dan jumlah mikrolia pada korteks serebri otak tikus. 12 tikus Wistar dibagi dalam kelompok kontrol yang diinjeksi normal saline dan kelompok perlakuan diinjeksi 150 µg protein rekombinan CIDR1α-PfEMP1 yang dikombinasikan dengan adjuvant. Injeksi dilakukan tiga kali dengan interval tiga minggu (hari 1, 21, dan 42). Tikus dieuthanasia pada hari ke-56 dan preparat histologi otak disiapkan dengan pengecatan Hematoxyline-Eosin. Pengamatan menggunakan mikroskop 400x dan Fiji Image J software menunjukkan morfologi ramified dan rod cell pada kelompok kontrol maupun perlakuan. Jumlah mikroglia pada kelompok kontrol 93,00 ± 5,7 sedangkan kelompok perlakuan 105,75 ± 15,62). Analisis statistik menggunakan independent-t test menunjukkan tidak terdapat perbedaan yang bermakna antara 2 kelompok (p= 0,15). Hasil ini mengindikasikan bahwa pemberian protein rekombinan CIDR1α-PfEMP1 tidak menimbulkan patologi pada jaringan otak yang memicu respon mikroglia. Hal ini menguatkan potensi protein rekombinan CIDR1α-PfEMP1 sebagai kandidat vaksin malaria berbasis peptida.

***Kata Kunci**: CIDR1α; Malaria; Mikroglia; PfEMP1; Plasmodium falciparum; Vaksin*

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INTRODUCTION

Malaria remains a significant global health problem with high morbidity. World Health Organization (WHO) reported an estimated 249 million malaria cases in endemic countries, resulting in a mortality rate of 14.3 per 100,000 individuals at risk in 2022. Southeast Asia region is at the second rank of the heaviest burden of malaria infections globally, after African regions, and Indonesia is at the second rank, trailing India (World Health Organization (WHO), 2023), with the highest Annual Parasite Incidence (API) is Papua, West Papua, and East Nusa Tenggara provinces (Ministry of Health of the Republic of Indonesia, 2021). Among the 5 *Plasmodium spp*-causing malaria in humans, *Plasmodium falciparum* stands as the predominant malaria-causing species in Indonesia (Ministry of Health of the Republic of Indonesia, 2018).

The clinical manifestations of falciparum malaria vary from asymptomatic malaria, uncomplicated malaria, to severe malaria with potentially life-threatening complications (Wiser, 2023). Severe anemia and cerebral malaria are the most commonly reported complications of falciparum malaria in children (Kafai & John, 2018). The fatality rate of cerebral malaria is 19%, with the majority of deaths reported to occur within the first 48 hours of a patient's hospitalization (Kessler et al., 2018; Dvorin, 2017). In addition, a large number of children who survived cerebral malaria are showing signs of neurological sequelae. At the same time, in the long term, others could develop cognitive deficits, epilepsy, and behavioral abnormalities. Some of the widely reported neurological sequelae left after cerebral malaria include ataxia, hemiplegia, blindness, and speech disorders (World Health Organization (WHO), 2014; Trivedi & Chakravarty, 2022).

The CIDR1α domain forms part of the semi-conserved head structure of PfEMP1, facilitating the attachment of IRBCs to the endothelial protein C receptor (EPCR) (Turner et al., 2013). Previous research has consistently linked the capacity of parasites expressing the CIDR1α-PfEMP1 domain to bind EPCR with an increased risk of severe malaria in children, such as cerebral malaria and severe anemia due to malaria (Jespersen et al., 2016; Mkumbaye et al., 2017; Shabani et al., 2017; Sulistyaningsih et al., 2021; Ndam et al., 2017). The role of the CIDR1α-PfEMP1 domain in malaria pathogenesis makes it a potential target for triggering immune responses against malaria infection. Currently, several efforts have been made to develop a malaria vaccine based on PfEMP1, and the findings have shown that using CIDR1α-PfEMP1 as a target antigen can trigger a wide-scale immune response, even though the antigen presented is only part of the domain structure that makes up PfEMP1 (Harmsen et al., 2020; Sulistyaningsih et al., 2022a; Turner et al., 2018).

The brain in the central nervous system (CNS) has an immune system distinct from other body regions. Microglia is the immune system component in the brain that has a vital role in modulating the immune response and maintaining brain homeostasis along with different parts of the neurovascular unit (NVU) (Verkhratsky et al., 2019). Microglia can detect damage to brain tissue or foreign objects in the brain parenchyma, triggering microglial activation. In cerebral malaria, microglial activation may contribute to neuroinflammatory pathology (Nishanth & Schlüter, 2019). Several studies have been conducted to determine the role of microglia on cerebral malaria pathogenesis, both *in vitro* and *in vivo* (Capuccini et al., 2016; Mbagwu et al., 2020). This study investigates the microglia response after CIDR1α-PfEMP1 recombinant protein injection by calculating their number and morphology in Wistar rats.

MATERIALS AND METHODS

Samples and Ethical Clearance

The samples of this study were Wistar rats (Rattus norvegicus), aged around 2–3 months and weighing between 200–250 g. The ethical approval was received from the Ethical Committee of the Faculty of Dentistry, University of Jember, with reference number No. 2139/UN25.8/KEPK/DL/2023.

Expression and Purification of CIDRα1-PfEMP1 Recombinant Protein

The CIDR1α-PfEMP1 recombinant protein was expressed in *Escherichia coli* BL21 (DE3) cells containing pET-30a-CIDRα1 (Dewi et al., 2018). Recombinant protein production was initiated by inoculating 50 µL of *E. coli* BL21 (DE3) transformants into 5 mL of Luria Bertani Broth,

supplemented with kanamycin and incubating in an incubator shaker at 160 rpm and 37 °C for 16 hours. The starter culture was then transferred into 250 mL of LB liquid medium containing kanamycin and incubated at 190 rpm for 3–4 hours at room temperature until it reached an optical density at 600 nm (OD_{600}) of 0.6–0.8. Protein expression was induced by adding 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by continued incubation for 8 hours. The culture was harvested by centrifugating at 6,000 rpm and 4 °C for 10 minutes (Setyoadji et al., 2021).

The recombinant protein was extracted using extraction buffer (composed of 50 mM NaH_2PO_4, 300 mM NaCl, and 5 mM Imidazole, pH 8.0) in a 1:2 ratio along with lysozyme (1 mg/mL) and incubated for 30 minutes at 4 °C. Pellets were sonicated at 4 °C for 18–20 cycles and further centrifuged at 10,000 rpm for 30 minutes at 4 °C (Setyoadji et al., 2021).

Recombinant protein purification was conducted based on affinity chromatography using Ni-NTA resin as the manufacturer's procedure. A serial elution buffer was applied, which contained 80, 100, and 120 mM imidazole concentrations. The purified protein was measured for its concentration using Bradford protein assay; the absorbance was read at a wavelength of 595 nm. The concentration was calculated using the formula y= 0,0078x–0,0703 (R2= 0,09962). The purified protein was visualized using the Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 45% polyacrylamide gel concentration.

Injection of CIDR1α-PfEMP1 Recombinant Protein in Wistar Rats

The rats were acclimatized for 14 days with standard nutrition and *ad libitum*. 12 rats were randomly divided into a control group, which consisted of 4 rats and a treatment group, which consisted of 8 rats. The control group received 0.368 mL of 0.9% normal saline, and the treatment group was injected with 150 µg of CIDR1α-PfEMP1 recombinant protein combined with Complete Freund's Adjuvant (day 0) and Incomplete Freund's Adjuvant (day 21 and 42). All rats in the control and treatment groups were injected thrice at three-week intervals.

Euthanasia and Brain Collection

Rats were euthanized on day 56 using a combination of ketamine (150 mg/kg) and xylazine (15 mg/kg) by the expert, i.e., a laboratory technician, and were supervised by a medical doctor. The rats' brains were collected by surgery (Aboghazleh et al., 2024), washed using 0.9% normal saline, and placed in a tissue pot containing 10% Neutral Buffered Formalin (NBF) for 24–48 hours with a 1:15 volume ratio, prepared into slides and stained using Hematoxylin and Eosin for the histological examination using a light microscope with 400x magnification (Widayat, 2018). The microglial examination includes the morphology and number of microglia.

Microglia Morphology and Measurement of Microglia Number

Histological observations of microglia were carried out by observing the morphology and number of microglia in the cerebral cortex using a light microscope (Olympus CX21) with 400x magnification. The number of microglia was calculated in ten consecutive visual fields for each slide. Two observers performed the examination using a "blind" approach to minimize bias.

Microglia identification was performed based on morphology and documented using the AmScope FMA050 Digital Microscope Camera. The images were then processed using Fiji Image J software to calculate the number of microglia and further analyzed for their morphological changes.

Statistical Analysis

The data were presented as mean ± standard deviation (SD) and analyzed using SPSS version 24 software. The data distribution was evaluated using the Shapiro-Wilk test, while data homogeneity was assessed using Levene's test. The statistical analysis was performed to differentiate the microglia numbers in control and treatment groups using an independent test with a confidence interval of 95%.

RESULTS

Visualization of CIDRα1-PfEMP1 Recombinant Protein

Visualization of purified CIDR1α-PfEMP1 recombinant protein showed a 27 kDa protein, as presented in Figure 1 (Sulistyaningsih, et al., 2022b). The thickest protein band was yielded from the second elution with 80 mM imidazole concentration (E1.2).

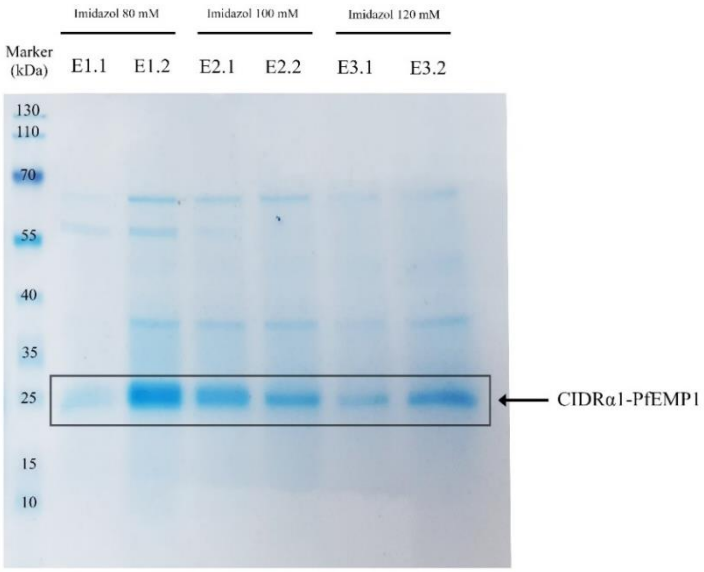

Figure 1. Visualization of purified CIDR1α-PfEMP1 recombinant proteins using SDS-PAGE. The targeted protein is 27 kDa. The purified protein was eluted using an elution buffer with different imidazole concentrations (E.1.1–E3.2). The thickest band was observed from the second elution of elution buffer with 80 mM imidazole concentration (E1.2)

Morphology of Microglia

The microglial examination was performed on the microscopic slide of the brain on day 56 post-treatment. Microscopic observation of microglia showed the elongated flat and small irregular nuclei with invisible cytoplasm, as shown in Figure 2. Based on the nuclei structure, microglial cells showed ramified and rod cell morphology both in the control and treatment groups.

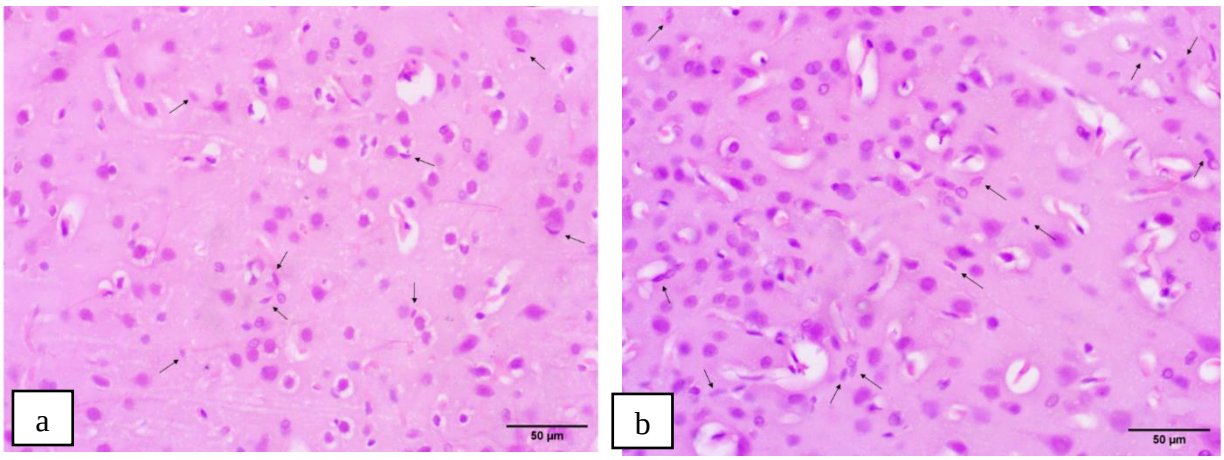

Figure 2. The histological appearance of microglial morphology (arrow) in Hematoxylin and Eosin staining was observed using a CX21 light microscope, 400x magnification, scale bar= 50 µm, in the control group (a) and treatment group (b). The slide was prepared from the brain of the animal sample, which was euthanized 56 days post-treatment

The ramified microglia appeared as cells with a small irregular nucleus and no visible cytoplasm. Meanwhile, the morphology of rod cell microglia has a flat-elongated nucleus and no visible cytoplasm. Rod cells spread sporadically. Figure 3 shows each morphology of microglia.

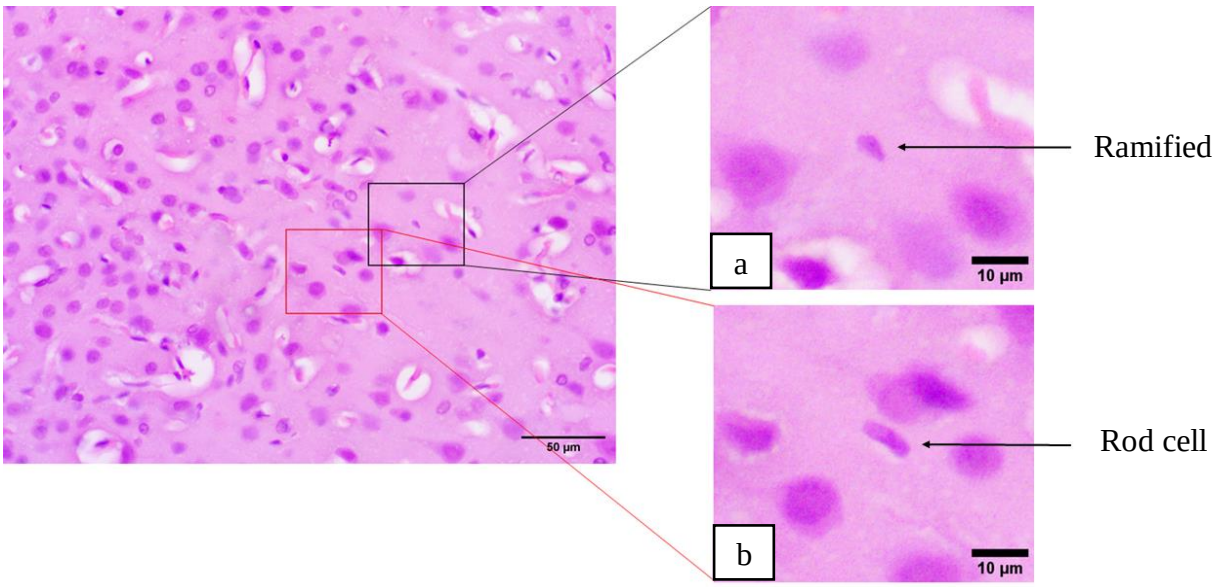

Figure 3. Various microglial morphology in the treatment group in Hematoxylin and Eosin staining, at 400x magnification, scale bar= 50 µm. Ramified microglia with a small-irregular nucleus and invisible cytoplasm (a), scale bar= 10 µm; rod cell microglia with a flat-elongated nucleus and invisible cytoplasm (b), scale bar= 10 µm. The slide was prepared from the brain of the animal sample, which was euthanized 56 days post-treatment

Analysis of Microglia Number

The average number of microglia in the control and treatment groups is presented in Table 1. The normality test using the Shapiro-Wilk test and the homogeneity test using Levene's test indicated that the data were normal distribution (P >0.05) and homogenous (P >0.05). Further analysis using an independent t-test showed P= 0.15, indicating no significant difference between the control and treatment groups. However, the microglia number in the treatment group was slightly higher than those of the control group.

Table 1. The mean of microglia number of brain histological examination on day 56 post-treatment in control and treatment groups and its statistical analysis

Groups	Mean of microglia number (*mean* ± sd)	Independent t-test *p-value*
Control	93.00 ± 5.77	0.15*
Treatment	105.75 ± 15.62	

Note: *significance level of a *p-value*= 0.05

DISCUSSION

The CIDR1α-PfEMP1 is a prospective malaria vaccine candidate due to its essential role in severe malaria pathogenesis (Mkumbaye et al., 2017; Shabani et al., 2017), which makes it a potential target to induce an immune response against malaria infection (Jensen et al., 2020). Previous studies reported that injection of CIDR1α-PfEMP1 recombinant protein triggers antibody response characterized by elicited IgG of corresponding CIDR1α (Turner et al., 2018), and the CIDR1α-induced antibody showed the ability to impede the binding of CIDR1α and EPCR (Harmsen et al., 2020; Turner et al., 2018).

One of the challenges in developing a malaria vaccine, especially using recombinant protein-based vaccines, is finding the suitable antigenic components to induce the desired immune response (Cid & Bolívar, 2021). A previous study showed that the CIDR1α-PfEMP1 recombinant protein injection induced an increase in IgM level and leukocyte percentage on day 14 after injection (Sulistyaningsih et al., 2022b). Various factors affect the immunogenicity of an antigen, including its mass, shape, and molecular surface configuration (Bachmann & Jennings, 2010). The CIDR1α-PfEMP1 recombinant protein is 27 kDa, as in the previous study (Sulistyaningsih et al., 2022b). Studies have shown that employing antigens with low molecular weight can trigger immune

responses when combined with adjuvants (Srey et al., 2020; Sulistyaningsih et al., 2022b). Adjuvants confined the antigen in the oil deposit, increasing its exposure and extending its life span in the body, further increasing its immunogenicity (Apostólico et al., 2016).

This study investigated the microglial response after injection of CIDR1α-PfEMP1 recombinant protein through morphology analysis and number of microglial cells in the brain. Microglia is the principal immune cell of the brain and part of the blood-brain barrier (BBB), which protects the brain from any pathogens. It has been shown that microglia internalize plasmodium-infected red blood cells in an *in vitro* malaria model (Shrivastava et al., 2017), and it is activated in severe malaria, driving to neuroinflammation in cerebral malaria and leads to disease progression (Mbagwu et al., 2020). Therefore, the protein candidate vaccine should not provoke brain injury, which further activates and increases the number of microglia. It is known that microglial morphology represents its functional condition. There are several types of microglia, such as rod cell, ramified, amoeboid, phagocytic, hypertrophic, and dystrophic microglia (Savage et al., 2019). In this study, the microscopic examination of microglia showed ramified microglia with small-irregular nuclei and invisible cytoplasm and rod cell microglia with elongated-flat nuclei and invisible cytoplasm.

Ramified microglia is a resting phase (Garman, 2011); it appears with many branching processes to monitor the surrounding area (Savage et al., 2019). However, our observation could not visualize the microglial processes well, which could be better detected using an immunostaining method targeting specific proteins expressed by microglia such as Iba-1 (Jurga et al., 2020). Microglia in the resting phase are often found in normal adult brains (Savage et al., 2019). Microglia reside within the CNS and are responsible for surveillance and immune response. Their primary function is constantly monitoring the environment, including interactions with other CNS cells such as endothelial cells, neurons, and astrocytes. Microglia express pattern recognition receptors (PRRs) on their cell surface to recognize various molecules associated with pathogens, such as pathogens-associated molecular patterns (PAMPs) and molecules released by damaged or stressed cells (damage-associated molecular patterns DAMPs), which initiate microglial activation. The ability of microglia to sense and respond to environmental changes is crucial for maintaining CNS homeostasis and protecting against infections, injuries, and diseases (Arcuri et al., 2017). When changes occur in the CNS tissue, microglia will transform from a surveillance mode to an activated state where they experience significant morphological changes, retract their processes to decrease the complexity and number of branches, and release pro-and anti-inflammatory cytokines (Morrison et al., 2017; Andoh & Gyan, 2021).

Rod cell microglia is characteristic of active microglia (Garman, 2011; Savage et al., 2019). It spreads sporadically, which is an uncommon pattern in brain injury that is arranged in a train-like appearance (Ziebell et al., 2012). Rod cell microglia could be observed in normal or pathological brain conditions and are often associated with synaptic stripping mechanisms (Savage et al., 2019), which have profound functional implications for neural circuits. In physiological conditions, synaptic pruning refines circuitry, promoting efficient signal transmission. However, rod cell microglia have been observed in a number of pathological conditions (Chen & Trapp, 2016).

A study by Kongsui et al. (2014) reported that the morphology of microglia in the brain cortex of healthy rats tends to be homogeneous. However, the study also showed different microglia sizes can be found in normal brain conditions to a certain extent (Kongsui et al., 2014). The recent research observed two variations of microglia morphology, namely ramified and rod cell, in both control and treatment groups, indicating their different functions. The ramified cell is in a resting state, and the rod cell is a form of activated microglia, which was supposed to be a neuroprotective agent. However, further confirmation is needed by an analysis of microglial activation markers.

Microglia activation can be identified by morphological and functional changes. It is characterized by increased levels of microglia activation markers and a significant increase in microglia number or size (Jurga et al., 2020). The study showed that the number of microglia in the treatment group was slightly higher than in the control group. However, the statistical analysis showed no significant difference (P= 0.15). An increase in microglia number may indicate microglial proliferation (Jurga et al., 2020). Capuccini et al. (2016), in an *in vivo* model, reported that microglia

proliferate during the onset of malaria infection, especially on days 5–7, which is accompanied by upregulation of several genes involved in the immune response, production of pro-inflammatory chemokines and cytokines, as well as chemoattractant molecules (Capuccini et al., 2016). However, an increase in microglia number does not always indicate a role for microglia as a cause of neuroinflammation. Paasila et al. (2019) reported a decrease in ramified microglia in brain regions that had suffered significant damage. Unfortunately, the genes involved in this study were not evaluated; thus, the microglia phenotype could not be confirmed. Based on this evidence, the insignificant differences in microglial numbers between control and treatment groups indicated no microglia activation causing robust proliferation.

However, this study observed microglia morphology solely on histological structure using Hematoxylin-Eosin staining, which could not be accurately differentiated due to lower specificity than another method, such as immunostaining, which targets specific markers to visualize microglia better. This could be a limitation of the study.

CONCLUSION

This study concludes that injection of CIDR1α-PfEMP1 recombinant protein does not affect the morphology and number of microglia in the cerebral cortex of rat brains. The resting phase and active state of microglia were observed in both control and treatment groups, and the number was not significantly different between groups, implying no microglia activation, which is caused by brain damage due to CIDR1α-PfEMP1 recombinant protein injection. This finding supports the development of CIDR1α-PfEMP1 recombinant protein as a malaria vaccine candidate. Further studies could be conducted using more markers to identify microglia, such as immunostaining. In addition, a study to evaluate the role of microglia in malaria immunity would be essential for supporting the development of malaria vaccines.

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