Role of hypoxia-inducible factor (HIF)-1α and CD11b in pathogenesis of experimental cerebral malaria in mice

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Summary

Malaria is one of transmittable diseases caused by *Plasmodium* spp. infection. Severe malaria, including cerebral malaria can lead to deaths. Involvement of cytokines and other molecular markers have been shown in development of cerebral malaria signs.
The aims of the present study is to determine the effect of *Plasmodium berghei* ANKA to the HIF-1α and CD11b expressions, and the correlation between HIF-1α CD11b with cerebral malaria symptoms in mice model. C57BL/6 mice were divided into two groups, control group and group that was infected by *Plasmodium berghei* ANKA. Parasitemia and clinical score were observed until 7th day, then brains were collected and stained by immunohistochemistry to determine HIF-1α and CD11b expression in both groups. The HIF-1α and CD11b expressions were observed under 1000x magnification in light microscope. There was a significant differences between each group (p=0.001) of HIF-1α expression in response to *Plasmodium berghei*

ANKA infection. In contrast, there were no differences on CD11b expression in response to *P. berghei* ANKA infection (p=0.096). There was no correlation between the expression of HIF-1α and CD11b (r=0.220) in mice with cerebral malaria.
In conclusion, *Plasmodium berghei* ANKA infection enhanced HIF-1α expression but not CD11b due to the variability of microglia in the brain.

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1. Introduction

Malaria is one of public health problems that has been included as one of the important issues in the Millennium Development Goal (MDG).[1] In 2010, it was estimated there were 132.8 million people (57.1% of the total population) at risk of *P. falciparum* infection in Indonesia. There were about 400 deaths due to malaria in 2010 which started to decrease every year.[2]

Plasmodium falciparum expressed *P. falciparum* erythrocyte membrane protein-*1* (PfEMP-1) which can modify the infected erythrocyte's cytoskeleton and the host cell membrane. This protein binds to various endothelial receptors to allow parasite sequestration that can lead to inflammation, obstruction of circulation and tissue damage that underlie the pathogenesis of severe malaria.[3] The species which can cause severe malaria are *P. falciparum, P. vivax* and *P. knowlesii*.[4]

In cerebral malaria (CM), hypoxia occurs due to the sequestration of *P. falciparum*- infected erythrocyte in vascular endothelial cells in the brain. The sequestration of this infected red cells can cause obstruction in blood circulation.[5] Hypoxia-inducible factor (HIF)-1α is a transcription factor that is activated as result of the lack of oxygen supply to the cells. HIF-1α will activate the gene transcription of the cells including the reactive oxygen species (ROS) and proinflammatory mediators genes.[6]

In the brain, there is monocyte-derived phagocyte cell known as microglia. Despite the report on the presence of microglia being activated in the brain with cerebral malaria,[7] the protective role or its pathological function of this cell remains unclear. Microglia activation, like the other phagocyte cells, is marked by the expression CD11b and other molecules. CD11b is an integrin molecule which is mainly owned by leukocytes that have important roles in the immune process.[8] It has been found in one study that showed the correlation between the expression of HIF-1α and CD11b in lung tissue of acute lung injury in the mouse model of severe malaria.[9] Therefore, we sought to examine if there were association between the HIF-1α and CD11b expression in the experimental cerebral malaria in mice.

2. Materials and methods

2.1 Animals

Sixteen to twenty weeks-old C57BL/6 mice with the body weight range of 20-25g from the Eijkman Institute for Molecular Biology were used. The study was conducted in Parasitology Laboratory and Biomedical Laboratory, the Faculty of Medicine, Universitas Brawijaya, from April 2014 until September 2015.

2.2 Ethics Statement

Ethical clearance has been approved by Ethical Commission of Faculty of Medicine, Universitas Brawijaya, Malang (No. 410/EC/KEPK/07/2014 date 10 July 2014).

The experiment was performed according to Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and under regulations of animal Care and Use of Universitas Brawijaya In Indonesia.

2.3 Experimental Design

The C57BL/6. mice were divided into 2 groups consist of 10 mice each that were assigned as infected group which infected with *P. berghei* ANKA to be served as a model for experimental cerebral malaria and non infected group with no malaria infection. Mice was inoculated intraperitoneally with $1x10^7$ *P. berghei* ANKA-infected erythrocyte resuspended in 0.2 mL blood. Parasitemia was examined daily for 7 days using thin blood smear stained with Giemsa. The parasitemia was counted in 1.000 erythrocyte under 1000x magnification with light microscope and the counting wasconverted into percentage of infected red cells over the non-infected ones

Clinical signs were scored daily for 7 day by Waknine-Grinberg *et al* scoring system with some modifications (Table 1).[10]

At day 7, mice were sacrificed using chloroform and the brain was collected and preserved in 10% formalin.

Table 1 Modified Cerebral Malaria Clinical score

Domain	Clinical feature	Score
Appearance	Normal	0
	Matted fur	1
Behaviour	Normal	0
	Bent back, stagger	1
	Partial paralysis, Immobilize	2
	Convulsion,Coma	3
Body Temperature	Normal	0
	Shivering	1

2.4 Brain Histopathology and Immunohistochemistry

Brain histopathology specimen and Hematoxylin-Eosin (HE) staining was conducted in the Pathology Anatomy Laboratory dr. Soetomo Hospital, Surabaya.

Immunostains were performed in the Biomedical Laboratory, the Faculty of Medicine, Universitas Brawijaya, Malang. Deparaffinized sections (5 μm) were incubated in 60°C and subsequently serially immersed in xylene, absolute ethanol, 90% ethanol, 80% ethanol, 70% ethanol, and sterile aqua distillata. For antigen retrieval sections were immersed in a citric buffer solution (pH 6.0) and then heated in 95°C waterbath for 20 minute and cooled at room temperature before finally washed with PBS. Three percent H_2O_2 were added into methanol and the tissue was incubated for 15 min (in order to block endogenous peroxidase activity) before washing for 3 times with PBS . Blocking of non-specific protein was done using a mixture of 0.25% Triton and PBS and 5% FBS. The incubation was done overnight and subsequently washed 3 times.

HIF-1α (Santa Cruz, catalog number: sc-53546) and CD11b (Biolegend, catalog number: 101202) monoclonal antibodies were diluted with PBS buffer and 5% FBS at 1:100 and 1:200 dilutions respectively. Sections were incubated overnight at 4°C, then washed for 3 times. They were next incubated with secondary antibody for 30 min at room temperature, washed 3 times and finally incubated with SA-HRP (Star Trek Universal HRP Detection System, catalog number: STUHRP700 H, L10) for 20 min at room temperature and subsequently was rinsed with tap water. Lastly, sections were incubated with Diaminobenzidine (DAB) chromogen and DAB buffer (1:50) for 3-10 min, washed 3 times with PBS then with sterile water for 3 times. A light Mayer's Hematoxylin counterstain (5-10 minutes) was used, following which slides were washed, dried and mounted with cover glass.

Histopathological characteristics of the brain with cerebral malaria, which include cortex edema, cerebral hemorrhage, leukocyte adhesion, and erythrocyte adhesion in endothelial cells,[11] were observed in HE-stained sections. HIF-1α expression was detected as the brown color nuclei in the microglia, whereas CD11b expression was detected on the surface of the nuclei. Specimen were observed under a 1000x magnification with light microscope and counted per cells in 20 high power field and analyzed in cells/HPF scale.

2.5 Statistical Analysis

The data were analyzed using IBM SPSS 21.0 edition. The normality test was performed, variables were analyzed using Independent t-test or Mann-Whitney, Pearson or Spearman correlation test was used to examine the association between parameters tested based on the normality test.

3. Result

This study demonstrated increased in parasitemia started from day 2-4 after infected with *P. berghei*

ANKA. The experimental mice with malaria showed increased parasitemia until Day 6 or 7.

The histopathology observation using HE staining showed that the brain of infected mice had edema cortex, hemorrhage, and leucocyte adhesion in the brain endothelial cells. The significance differences of HIF-1α expression between the control group and group with malaria infection (p=0.001) shown in Figures 1 and 3a. However, no differences in CD11b expression were observed however between the two groups of mice (p=0.096) (Figures 2 and 3b). There were no correlations found between HIF-1α expression and CD11b expression (r=0.220, p=0.351) in this study.

The correlation between parasitemia and HIF-1α expression was moderate (r=0.622; p=0.001), whereas the correlation between parasitemia and CD11b expression (r=0.469, p=0.037) was weak. Strong correlation was found between the clinical score of cerebral malaria and HIF-1α expression (r=0.855, $p\leq0.001$), but found no correlation between clinical score and CD11b (r=0.362, p=0.117).

4. Discussion

Cerebral malaria is marked with the sequestration of infected erythrocyte as shown in the autopsy of the human brain. Previous observation showed the occurrence of endothelial dysregulation in CM as a result of upregulation of ICAM-1, V-CAM, and E-Selectin in the brain endothelial cells.[12] Although there were differences in the brain histopathology between human cerebral malaria and in the mouse model of cerebral malaria in which the infected erythrocyte sequestration was absent, but the significant leucocyte accumulation in the brain of the CM C57BL/6 mice was similar to the one found in the human brain with CM.[4,13]

In this study, the experimental CM mice infected with malaria were sacrificed on Day 6 or 7 as they demonstrated the CM signs. According to Jennings *et al*, the mice died on day 7 were usually due to the encephalitis process caused by the increase of proinflammatory cytokines.[14]

Plasmodium cytoadhesion is mediated by the parasite ligand namely *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) that is expressed on the surface of infected red blood cells. endothelial protein C receptor (EPCR).[15] Rosetting of infected red blood cells and the non-infected ones was also observed in CM.[16]

In CM, cytoadherence and rosetting process could lead to the hypoxia state in brain.[5] Hypoxia is a state where oxygen availability (O_2) is declined. In order to make into a homeostatic state this will require a regulator protein called HIF-1. HIF-1 is a transcription factor consisted of HIF-1α subunit which is regulated by O_2 and HIF-1β subunit. HIF-1 regulates hundreds of genes expression through many mechanisms. HIF-1 binds directly to hypoxia response elements (HRE) located in the target gene.[6]

In this study, the expression of HIF-1α was observed on day 7. We found that HIF-1α expression was higher in the mice infected with *P. berghei* ANKA rather than non infected group (p=0.001). HIF-1α can activate the genes that lead to the increase of cell metabolisms. The hypoxic cells need more energy and glucose.[8] Furthermore, HIF-1α played a role in macrophage polarization depending on its metabolism state. M1 polarization is marked by the increase of glycolysis, pentose phosphate pathway, and oxidative phosphorylation activation, whereas M2 is marked by the oxidative activity and the decrease of glycolytic activity.[17]

There were moderate correlation between parasitemia and HIF-1α expression (r=0.662, p=0.001), whereas strong correlation was observed between of HIF-1α expression with clinical score (r=0.855, $p\leq0.001$). These are possible because the more parasites circulating in the blood the more sequestration could occur. The cytoadherence/sequestration process could activate cellular and humoral inflammatory mediators resulting in cerebrovascular occlusion and hemorrhage.[18] The site of infection site has the hypoxic microenvironment with the low level of O^2 pressure below 1% due to the cells destruction and activation of Inflammatory cells. This state would induce myeloid cells to secrete NO (nitric oxide), protease granule and antimicrobial peptide.[19] This mechanism may explain why the clinical score of the CM mice become more severe if there are increase of HIF-1α expressions.

Microglia] are mesodermal, fetal macrophage-derived cells, which can invade the brain and perform ramification. Ramified form was a normal form of microglia. If the infection occurred the microglia will transform and retract its rami. The change of the activation was associated with the change of its activity. This change can be due to injury, ischemic, or autoimmune change.[20]

In CM, the microglial activation forms one of the activation markers for active phagocyte known as CD11b/Mac-1/CR3.[21] CD11b is one of the integrin families when together with CD18 (CD11b/CD18) will form into Mac-1 or CR3. There air various ligands for CD11b activation including iC3b, ICAM family, fibrinogen, fibronectin, Factor X, and some protease.[22] CD11b/CD18 with its ligand harbored many functions such as adhesion, migration, phagocytosis, respiratory burst, and other functions.[8]

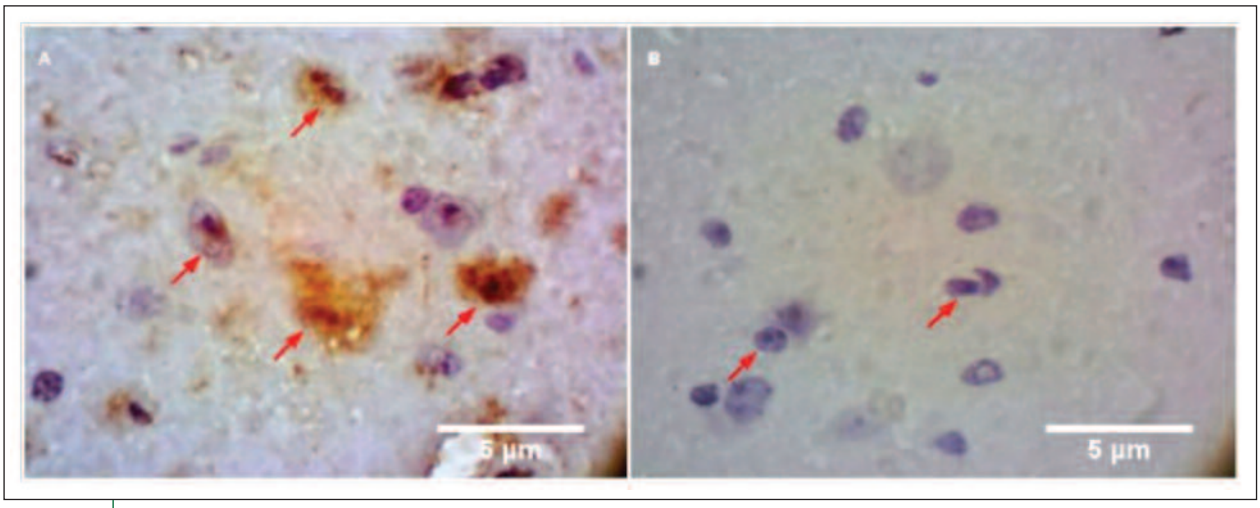

Figure 1 HIF-1α expression in (A) *Plasmodium berghei* infected group showed that positive expression in the nuclei and cytoplasm (blue arrow), (B) Non infected group no HIF-1α expression.

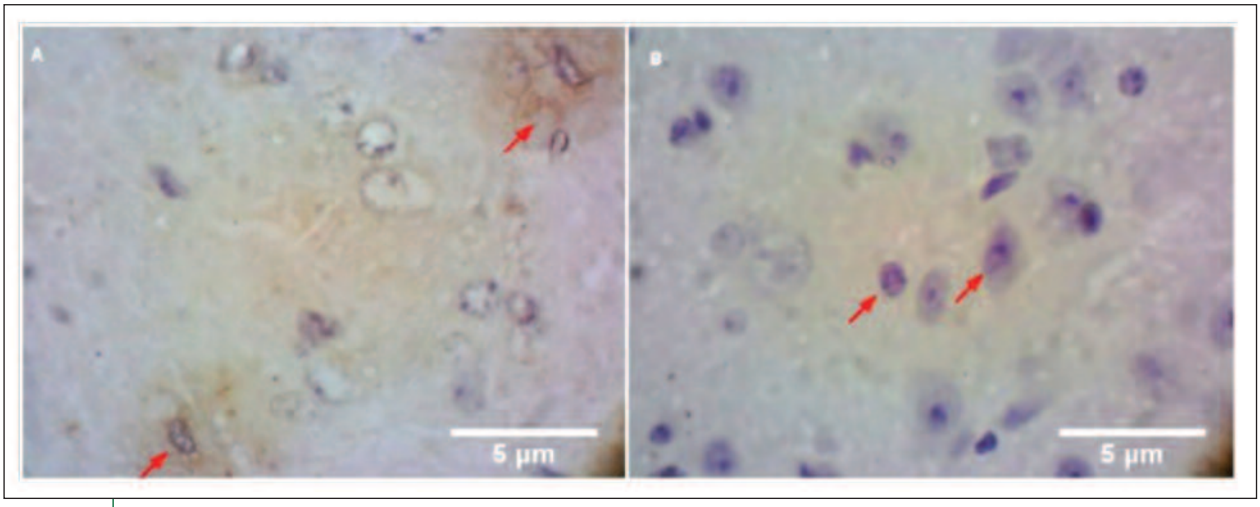

Figure 2 CD11b expression in (A) *Plasmodium berghei* infected group showed the staining was outside the nuclei (blue arrow), (B) Non infected group no CD11b expression.

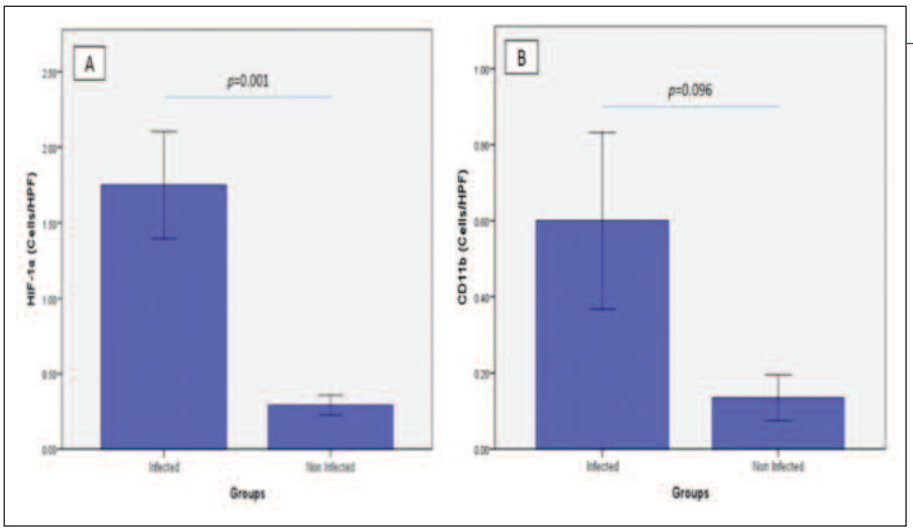

Figure 3

(A) HIF-1α expression difference graph (p=0.001) (B) CD11b expression difference graph (p=0.096).

CD11b/CD18 plays a role as Pattern Recognition Receptor (PRR) to recognize Pathogen Associated Molecular Pattern (PAMP) such as lipopolysaccharide and Damage Associated Molecular Pattern (DAMP) including damage-associated alamin High-mobility Group Box 1 (HMGB1) Protein.[23]

In this study, we found no differences between different parameters tested including parasitemia and clinical score in each group of the mice with CD11b (p=0.096). We detected weak correlation of parasitemia with CD11b expression (r=0.469, p=0.037) and no correlation between clinical score and CD11b expression (r=0.362, p=0.117).

Previous study found the activation of microglia was marked by the decrease of ramified microglia.[24] Other studies showed there were microglia activation but the data of the polarization of the microglia in CM were lacking.[25] The possible explanation is that the microglia has diverse phenotypic on the surface activation marker depending on the area of the brain. The other activation marker such as CD40, CD45, CD80, and CD86 were found to vary from each region of the brain.[26]

There were no correlation found between the HIF-1α and CD11b expression (r=0.220, p=0.351). This result was contradictory with the previous study by Medana *et al* in assessing the ramification of the microglia.[24] Our study suggested that the activation of the microglia in the CM was associated with markers other than CD11b. The variation in the CD11b expression in different region of the brain could be one of possible explanation that we failed to detect the expression of CD11b in this study.

Our study conclude that *P. berghei* ANKA infection increased the HIF-1α expression in CM mice . However, we found no significant differences in CD11b expression between different groups of mice tested. Parasitemia showed to affect HIF-1α expression with moderate correlation, but weak correlation was observed with CD11b expression. The clinical score of CM mouse showed strong correlation to affect HIF-1α but no correlation detected with CD11b expression. There was no correlation between HIF-1α and CD11b expressions in the CM mice examined in this study.

Conflict of Interest statement

We declare that we have no conflict of interest

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Περίληψη

Ο ρόλος του επαγώγιμου παράγοντα υποξίας (HIF) -1α και CD11b στην παθογένεση της πειραματικής ελονοσίας σε εγκέφαλο ποντικού

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Η ελονοσία είναι λοιμώδες νόσημα που προκαλείται από μόλυνση από *Plasmodium* spp. Η ελονοσία βαριάς κλινικής εικόνας, συμπεριλαμβανομένης και της εγκεφαλικής ελονοσίας, μπορεί να οδηγήσει στο θάνατο. Στην ανάπτυξη εγκεφαλικής ελονοσίας έχει αποδειχθεί η συμμετοχή κυτοκινών και άλλων μοριακών δεικτών. Σκοπός της παρούσας μελέτης ήταν να προσδιοριστεί η επίδραση της μόλυνσης από *Plasmodium berghei* ANKA (πειραματικό στέλεχος *Plasmodium* spp. που προκαλεί εγκεφαλική λοίμωξη) στην έκφραση των παραγόντων HIF-1α και CD11b, καθώς και η συσχέτισή τους με τα συμπτώματα της εγκεφαλικής ελονοσίας σε ποντίκια. Τα ποντίκια (C57BL/6) χωρίστηκαν στην ομάδα ελέγχου και στην ομάδα η οποία μολύνθηκε με *Plasmodium berghei* ANKA. Η παρασιταιμία και η κλινική εικόνα παρατηρήθηκαν μέχρι την 7η ημέρα και ακολούθησε ανοσοϊστοχημική χρώση των εγκεφάλων που συλλέχθηκαν, για τον προσδιορισμό της έκφρασης των HIF-1α και CD11b και στις δύο ομάδες με χρήση οπτικού μικροσκοπίου (μεγέθυνση 1000x). Παρατηρήθηκαν στατιστικά σημαντικές διαφορές μεταξύ των ομάδων ($p = 0,001$) στην έκφραση του HIF-1α κατά τη λοίμωξη *Plasmodium berghei* ANKA, ενώ αντίθετα, δεν παρατηρήθηκαν διαφορές στην έκφραση CD11b ($p = 0,096$). Τέλος, δεν σημειώθηκε συσχέτιση μεταξύ της έκφρασης των HIF-1α και CD11b ($p = 0.220$). Συμπερασματικά, η πειραματική λοίμωξη από *Plasmodium berghei* ANKA ενίσχυσε την έκφραση της HIF-1$^{\alpha}$, αλλά όχι και της CD11b, εξαιτίας πιθανά της μεταβλητότητας των μικρογλοιών στον εγκέφαλο.

Λέξεις κλειδιά
CD11b; Εγκεφαλική ελονοσία; HIF-1α; Ελονοσία; Plasmodium berghei ANKA

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