RESEARCH ARTICLE

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Annona muricata Associated with Increase Phytohemaglutinin Induced Spleen IL-10 Production of Swiss Mice During Cerebral Malaria Phase

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Background: Cerebral malaria (CM) prompts patient death. *A. muricata*-leaf extract (AME) having anti-plasmodial and immunomodulatory properties may provide advantages for malaria-endemic communities. Anemia of CM-patients is due to reduce interleukine (IL)-10 and increase macrophage migration inhibitory factor (MIF) production which interfere erythropoiesis. The objective of the study was to determine whether AME influenced spleen-IL-10 and MIF production and Hb or erythrocyte counts during CM-phase. *Method*: A post test only control group design study was done by using 30 swiss mice which were randomly divided into 6 groups. The C(+) and C(−) groups were *Plasmodium berghei* ANKA (PbA)-inoculated and healthy-mice. Healthy-X_1 and X_2 groups received ethanolic-AME 100 and 150 mg/Kg BW/day. PbA-inoculated X_3 and X_4 groups received either AME dose. IL-10 and MIF produced by splenocytes stimulated with phytohemaglutinin (PHA) or lipopolysacharide (LPS) *ex-vivo*, were measured by Elisa. Hb and erythrocyte counts were measured by automatic haematology analyzer. *Results*: IL-10 was tested by Kruskal-Wallis ($p < 0.0001$), then Mann-Whitney showed a significantly higher IL-10 of X_3, X_4 than C(+) group ($p = 0.003$ and $p = 0.017$). PHA and LPS-induced splenocyte MIF production were tested by One-way ANOVA and Kruskal-Wallis ($p = 0.176$ and $p = 0.413$). Mann-Whitney test showed a lower Hb of C(+), X_3 and X_4 than C(−) groups ($p = 0.045$, $p = 0.038$, and $p = 0.016$). Erythrocyte counts were not different among 6 groups studied ($p = 0.072$). IL-10 and MIF had no correlation with Hb or erythrocyte counts during CM-phase. The conclusion is that AME associates with the increase spleen-IL-10 production during CM-phase. The spleen-IL-10 and MIF might not influence Hb and erythrocyte counts during CM-phase. *Conclusion*: AME associates with the increase spleen-IL-10 production during CM-phase. The spleen-IL-10 and MIF might not influence Hb and erythrocyte counts during CM-phase.

Keywords: *Annona muricata*, Cerebral Malaria, MIF, IL-10.

1. INTRODUCTION

Introduction Severe malaria, including sever malaria anemia (SMA) and cerebral malaria (CM), causes fatal malaria cases. Decline malaria incident and fatal malaria cases from 2000 to 2015 has been reported by WHO.[1] Fatal malaria cases, however, remain occur. SMA is major cause of morbidity and mortality in areas classified as holoendemic *P. falciparum*.[2] SMA is affected by the imbalance between IL-10 and tumor necrosis factor-alpha (TNF-α). The lower ratio of IL-10/TNF-α was related with more severe degree of anemia.[3] The increase ratio of IL-10/TNF-α was found in children with uncomplicated malaria. IL-10 prevents the pathologic effect of TNF-α on hematopoiesis. IL-10 is protective toward SMA mediated by inhibition of pro-inflammatory cytokine productions.[4] Other studies showed that macrophage-migration inhibitory factor (MIF) might contribute to immunopathologic of severe malaria. The increase of MIF level was associated with fatal outcome of cerebral malaria (CM) cases.[5] MIF was produced by lymphocytes and macrophage responding to Plasmodium infection, and had been associated with malaria anemia.[6,7] MIF was released by macrophages after they phagocyte *Plasmodium*–infected the red blood cells and the parasitized-red blood cells (pRBCs). This also occurs when macrophages phagocyte haemozoin, *Plasmodium* pigment. MIF interferes erythropoiesis, therefore MIF might involve in the

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pathogenesis of malaria anemia.[7] Increase of circulating MIF levels were found during *Plasmodium* infection, and this support the hypothesis that *Plasmodium* infection is potent inducer of circulating MIF which might contribute to the pathogenesis of malaria anemia.[8] Interestingly, MIF supports the development of Th2 type cells which release IL-4 and IL-10 in malaria mouse model.[9]

Traditional plants are used for malaria treatment in several area untill now.[10] Studies showed that those plants have both anti-malaria effect and immunomodulatory effect.[11,12] Recent study showed that ethanolic-*Annona muricata* leaf extract (AME) was associated with the reduce of TNF-α and the increase of nitric oxide (NO) produced by spleen of Swiss mice during CM phase.[13] Since experimental (E) CM mice model is also used for other severe malaria syndromes.[14] By utilizing ECM Swiss mice inoculated with *P. berghei* ANKA (PbA), this recent study was aimed to determine whether AME affected spleen IL-10 and MIF production during CM phase of Swiss mice. Additionally, whether any correlation was found between IL-10 and MIF production in this study. Third, whether AME made better hemoglobin (Hb) level or erythrocyte counts during CM phase of Swiss mice. Fourth, whether better hematologic parameter measured associated with either IL-10 or MIF production in AME-treated mice during CM phase.

2. METHOD

AME used was provided and analyzed by SidoMuncul Company, Indonesia. Research design and group of Swiss mouse were mention elsewhere.[13] This study was performed in Medical Faculty of Diponegoro University (MFDU). The ethical approval was given by ethical committee of MFDU and Karyadi Hospital Semarang. Briefly, healthy animal groups used were named as negative control (C(−)) and treated groups which were X_1 and X_2 groups, while PbA-inoculated Swiss mice was grouped as positive control (C(+)), X_3 and X_4. The difference from previous study was the AME doses which were 100 and 150 mg/kg BW/day. The X_1 and X_3 groups received 100 mg/kg BW/day, while X_2 and X_4 groups received other doses. The treatment was given for 14 days for healthy mice. The PbA-inoculated, X_3 and X_4, groups received AME in 7 days before and 7 days post inoculation. Inoculation dose of PbA was provided by Parasitology Department of UGM. PbA-inoculation dose was 10^7 pRBC. Splenocytes in concentration of 10^7 cells/well were cultured and stimulated with PHA or LPS in 24 well culture plates. Elisa method was used to measure IL-10 and MIF. IL-10 was measured in those stimulated with PHA, while MIF measured in those stimulated with either PHA or LPS. Hb and RBC were measured by hematology analyzer.

3. RESULTS

3.1. IL-10 Produced by PHA-Stimulated Splenocyte

IL-10 produced by PHA-stimulated splenocyte expressed lymphocytic-IL-10 production in the spleen (Fig. 1(A)). Shapiro Wilk normality test which revealed that lymphocytic-IL-10 produced by negative control and X_2 groups were not normally distributed ($p \leq 0.050$). Kruskal Wallis test done in studied groups showed a significant difference among six groups ($p < 0.0001$). Mann-Whitney U test between groups was done. Lymphocytic-IL-10 production of negative control group was significantly

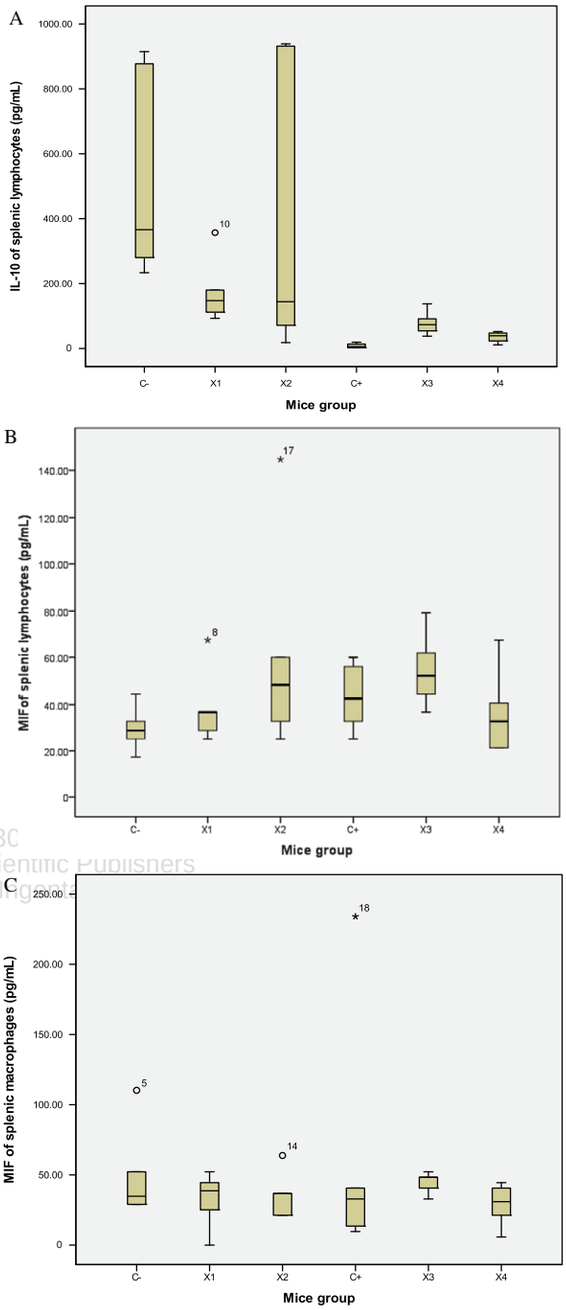

Fig. 1. (A) was box Plot of IL-10 produced by PHA-stimulated splenocyte expressed lymphocytic-IL-10 production in spleen. (B) was box Plot of MIF produced by PHA-stimulated splenocyte expressed MIF produced by lymphocytes in spleen. (C) was box Plot of MIF produced by LPS-stimulated splenocyte expressed MIF produced by macrophages in spleen. Mice group was coded as C(−) revered to untreated healthy mice as negative control, while X_1 and X_2 groups were healthy mice treated with AME dose of 100 and 150 mg/kg BW/day, respectively. C(+) was revered to untreated PbA-inoculated mice as positive control. X_3 and X_4 groups were PbA-inoculated mice treated with either AME dose mention before.

higher than positive control, X_3 and X_4 groups ($p = 0.004$, $p = 0.003$ and $p = 0.006$, respectively). Analyzes between PbA-inoculated groups, it was found that IL-10 production of positive control group was significantly lower than X_3 and X_4 group

($p = 0.003$ and $p = 0.017$, respectively). IL-10 production of X_3 group was significantly higher than X_4 group ($p = 0.042$). Lymphocytic-IL-10 production of negative control group was significantly higher than X_1 group ($p = 0.028$), meanwhile those of C-groups were not different with X_2 group ($p = 0.584$).

Additionally, IL-10 production of X_1 and X_2 groups was not different ($p = 0.917$). By comparing X_1 and X_2 groups, it was found that the increase of AME dose was not associated with lymphocytic-IL-10 production of healthy mice. Lymphocytic-IL-10 production of AME treated healthy group was compared to AME treated and PbA-inoculated group. It was a significantly higher lymphocytic-IL-10 production of X_1 group than X_3 group ($p = 0.019$). No significant different of IL-10 produced by X_2 than X_4 groups ($p = 0.076$) was found.

3.2. MIF Produced by PHA and LPS-Stimulated Splenocyte

PHA-induced splenocyte MIF production expressed lymphocytic-MIF production in the spleen (Fig. 1(B)). Shapiro-Wilk test for PHA-induced splenocyte MIF production showed that MIF levels were normally distributed ($p > 0.05$), and the data was homogen (Levene test showed $p = 0.067$). One-way ANOVA showed no significant difference among 6 groups studied ($p = 0.176$). LPS-stimulated splenocyte MIF production indicated macrophage-MIF production in the spleen (Fig. 1(C)). Kruskal Wallis test showed that MIF produced by LPS-stimulated splenocyte was not significantly different among 6 groups studied ($p = 0.413$).

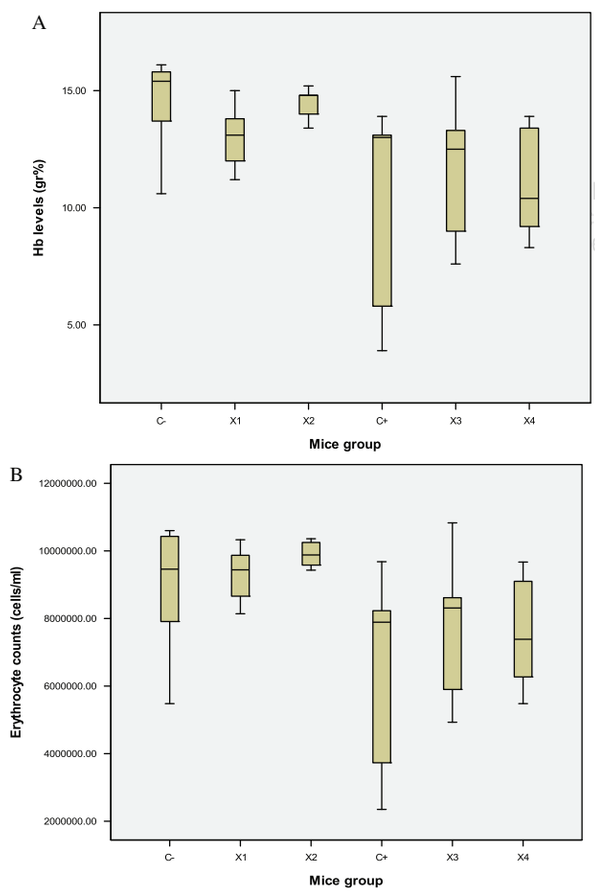

Fig. 2. A and B were box plot of Hb levels and erythrocyte count of mice groups. Mice group was coded as C(−) revered to untreated healthy mice as negative control, while X_1 and X_2 groups were healthy mice treated with AME dose of 100 and 150 mg/ kg BW/ day, respectively. C(+) was revered to untreated PbA-inoculated mice as positive control. X_3 and X_4 groups were PbA-inoculated mice treated with either AME dose mention before.

3.3. Hemoglobin Levels and Erythrocyte Counts Among Studied Groups

Hb data in all group were normally distributed (Shapiro-Wilk test, $p > 0.05$), and the data were not homogene (Levene test, $p < 0.05$) (Fig. 2(A)). Kruskal Wallis test of Hb levels ($p < 0.050$) was then followed by Mann-Whitney U test. A significantly lower Hb level of PbA-inoculated mice including C(+), X_3 and X_4 than C(−) ($p = 0.045$, $p = 0.038$, $p = 0.016$, respectively) was observed. No significant difference was observed between C(+) and X_3 or X_4 ($p = 0.570$ or $p = 0.784$). This was further confirmed by the finding that no significant difference was observed between X_3 and X_4. No significant difference was observed between C(−) and X_1 or X_2 ($p = 0.144$ or $p = 0.313$). This further confirmed by the finding that no significant difference was observed between X_1 and X_2. Erythrocyte counts were normally distributed in every group (Shapiro-Wilk test, $p > 0.05$) and test of homogeneity of variance showed that the data were not homogeny ($p = 0.003$) (Fig. 2(B)). Kruskal Wallis test showed erythrocyte counts were not different amongs 6 group studied ($p = 0.072$).

3.4. Correlation Between Cytokines, Hb Levels and Erythrocyte Counts

Spearman test done in X_3 and X_4 groups showed that the only significant correlation was observed between IL-10 and lymphocytic MIF of X_3 group ($r = -0.883^{**}$; $p = 0.008$). No correlation was observed between cytokines and hematologic parameter (Hb and erythrocyte counts).

4. DISCUSSION

A significantly lower lymphocytic-IL-10 production found during CM phase in each group inoculated with PbA (positive control, X_3 and X_4) than healthy control, was demonstrated that lower lymphocytic-IL-10 production was associated with CM phase. This also indicated that AME treatment at any studied dose might not normalize lymphocytic-IL-10 production. Two AME studied doses were associated with increase lymphocytic-IL-10 production during CM phase. AME in dose of 100 mg/kg BW/day was associated with higher IL-10 production than dose of 150 mg/kg BW/day. AME, therefore, might contribute in up regulating lymphocytic-IL-10 production during CM phase. AME dose of 100 mg/kg BW/day might be more effective in doing such effect during CM phase.

By comparing negative control and X_1 groups, it was showed that AME dose of 100 mg/kg BW/day was associated with lower lymphocytic-IL-10 production in healthy mice. Such effect was not observed in the dose of 150 mg/kg BW/day. Although only a trend of higher IL-10 produced by X_2 than X_4 groups ($p = 0.076$) was found. A significant higher IL-10 level of X_1 than X_3 was evident. These results supported previous finding that the reduce lymphocytic-IL-10 production was associated with CM phase. All together, this study showed that AME consequence

on lymphocytic-IL-10 production might be different in healthy and CM phase. IL-10 is protective cytokine in CM.[15,16] The ability of AME in providing immunoprotection during CM phase might have limitation because the increasing lymphocytic IL-10 productions remain below normal value.

Both lymphocytic and macrophage-MIF production in the spleen was not different among 6 groups of mice in this recent study ($p = 0.176$ and $p = 0.413$). These were indicated that AME might not be interfere MIF production in both splenic-type cells during CM phase of ECM-susceptible Swiss mice. No different level of MIF produced by spleen of healthy and CM phase, suggested that MIF produced by those types of cells might not be the main cytokines contributing to the severe malaria pathogenesis of Swiss mice. The recent results were not in line with previous studies done in malaria patients and *P. chabaudi*—mouse model. High circulating level of MIF, a cytokine contributed in neuro-inflammatory diseases, associate with fatal outcome of CM patient.[5] MIF, therefore, might contribute in the pathogenesis of severe malaria. *P. chabaudi*-infected BALB/C mice, a blood stage malaria mouse model, showed a correlation between circulating MIF level and severe malaria. MIF released by macrophages occurred after ingestion of either parasitized RBC or hemozoin.[7] MIF had never been observed in CM phase of PbA-inoculated Swiss mice. The different strain of mice and *Plasmodium sp* used in those previous studies may cause the different results in the study just been done recently.

PbA-infected BALB/C mice, CM-resistant mice, were died later than CM-susceptible mice. PbA-infected BALB/C mice died later due to SMA. The early immune response developed in PbA-infected BALB/C mice was Th2 type cells releasing IL-4 and IL-10.[17] MIF inhibit CD4+ T cell in producing IFN-γ while it increase IL-4 and IL-10 production in malaria mice model.[9] This AME study showed that lower lymphocytic-IL-10 productions were accompanied with normal level of MIF produced in the spleen during CM phase. The correlation was observed between lymphocytic-IL-10 production and MIF produced by lymphocytes of X_3 group ($r = -0.883^{**}$; $p = 0.008$). MIF, therefore, might affect lymphocytic-IL-10 production in the spleen during CM phase of Swiss mice. This correlation had not been studied before in AME treated-PbA-inoculated Swiss mice. The inverse correlation between lymphocytic IL-10 and MIF was not supported by previous study. The difference may be due to the use of MIF-neutralized or MIF-knock out BALB/C mice infected by *P. chabaudi adami* in the previous study.[9] However other study showed that parasite MIF inhibit Th2 cytokine production.[18] This open possibility that splenic-lymphocytic MIF of AME treated mice does similar effect during CM phase. Parasite MIF may contribute in inhibiting IL-10 production during CM phase of AME treated mice. Therefore, combination of AME and treatment which reduces parasite load in PbA-inoculated Swiss mice possibly optimized protective effect of AME. This is important to be studied.

This AME study indicated that lower Hb levels associated with CM phase. Because of no association between AME treatment and Hb levels during CM phase, AME might not contribute in improving Hb level. This was further confirmed by the finding that AME at any dose studied had no association with Hb levels of CM phase mice. AME might not affect the normal Hb value in healthy Swiss mice. This further confirmed by the finding that AME at any dose studied had no association with Hb levels in healthy Swiss mice. Erythrocyte count was not different among six-studied groups ($p = 0.072$). Hb levels and erythrocyte counts might not be affected by MIF or IL-10 produced during CM phase of AME-treated groups (Table I). Together with previous finding (Fig. 1(A)), it indicated that significantly increase lymphocytic-IL-10 might not affect Hb levels and erythrocyte counts of AME-treated groups. These findings were not in line with study showing that IL-10 protects severe malaria anemia mediated by its inhibitory effect on the production of pro-inflammatory cytokine in children with malaria.[4] Other factors may be more dominant in improving Hb level and erythrocyte count during CM phase of Swiss mice used in this study. A factor which prevents high erythrocyte destruction and reduces erythrocyte generation, utilized by AME particularly in ECM is warrant to be further studied. MIF production was influenced by phagocytic activity of pRBC and parasite pigment.[7] Therefore, in order to get better protective effect, the combination of AME and treatment which reduces pRBC and parasite pigment is proposed.

Table I. Correlation between cytokines, Hb levels and erythrocyte counts.

Lymphocytic IL-10	Macrophage-MIF	Lymphocytic-MIF
X_3 group	$r = -0.225$; $p = 0.628$	$r = -0.883^{*}$; p =0.008
X_4 group	$r = 0.200$; p = 0.747	$r = 0.718$; p = 0.172
Lymphocytic IL-10	Hb levels	Erythrocyte counts
X_3 group	$r = 0.214$; $p = 0.645$	$r = 0.036$; $p = 0.939$
X_4 group	$r = -0.200$; $p = 0.747$	$r = -0.200$; $p = 0.747$
Lymphocytic MIF		
X_3 group	$r = -0.288$; $p = 0.531$	$r = -0.018$; $p = 0.969$
X_4 group	$r = -0.406$; $p = 0.425$	$r = -0.406$; $p = 0.425$
Macrophage MIF		
X_3 group	$r = 0.150$; $p = 0.749$	$r = 0.000$; $p = 1.000$
X_3 group	$r = -0.086$; $p = 0.872$	$r = -0.086$; $p = 0.872$

5. CONCLUSION

AME might increase lymphocytic IL-10 production and might not influence MIF production in the spleen of Swiss mice during CM phase. AME might not be protective for the reduce of both Hb levels and erythrocyte counts during CM phase. The increase lymphocytic IL-10 production of AME treated mice might not be beneficial for the reduce Hb levels and erythrocyte counts during CM phase.

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