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SPLEEN-CXCL10-EXPRESSION OF *Plasmodium berghei* ANKA-INFECTED-SWISS MICE TREATED WITH Annona muricata

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Abstract: Severe malaria (SM) including cerebral malaria (CM) contributes in high-number of fatal-cases. Brain-T-helper-(Th)1-cells contribute in developing CM, while spleen-Th1-cells control the acute erythrocytic stage of *Plasmodium*-infection. Th1-cells is attracted by chemokines such as CXCL10. Experimental CM study using *Plasmodium berghei* ANKA (PbA) showed that treatment which reduces CXCL10 production associated with reducing systemic and brain inflammation, and subsequent increase survival. Aim of this study was to determine the effect of the ethanolic *Annona muricata* leaf extract (AME) toward spleen-CXCL10-expression of PbA-infected-Swiss-mice during SM. A randomized post-test-only-control-group-design was performed in this study. Thirty-six Swiss-mice were divided into 6 groups. C(-) group was control healthy mice, and C (+) was inoculated with 10^7 PbA. X1 and X2 group were healthy mice and received AME 100 and 150 mg/kg BW/day, respectively. X3 and X4 group were inoculated with PbA and treated with one of dose studied. The mice were all terminated at day 7 infection when SM occurred. Parasitemia percentage were not different among the infected groups. Results of Kruskal-Wallis-test showed a significant difference among the studied groups ($p = 0.022$). Mann-Whitney-U-test showed that the expression of the C(+) group had no significant difference than the C (-) group ($p=0.054$), however those of the PbA-infected X3 and X4-groups were significantly higher than the C(-) group ($p = 0.014$ and $p = 0.009$, respectively). The C(+) group expresses CXCL10 comparable to X3 ($p = 0.391$) and X4 ($p = 0.461$). The comparable expression was also found between the X3 and X4 groups ($p = 0.537$). The conclusion is that the use of ethanolic extracted *Annona muricata* leaves increases spleen-CXCL10-expression above normal during SM. The boosting of splenic CXCL10 expression of the *Annona muricata* intervention, has no contribution at the severity of malaria infection.

Key word: CXCL10, *Annona muricata, Plasmodium berghei* ANKA

INTRODUCTION

Malaria is an important infectious diseases because it is estimated that 619000 death occurred out of 247 million malaria cases in 2021[1]. Immune response develop during malaria determines the outcome of the disease. *Plasmodium berghei* ANKA (PbA) infected animal model, including Swiss mice, remains used recently for studying the immune response of severe malaria (SM) particularly cerebral malaria (CM)[2]. Serum CXCL10 produced by immune response is use as biomarker for CM[3]. CXCL10 is produced by innate and adaptive cellular immune response[4]. CXCL10 is a chemokine attracting cells expressing CXCR3 including Natural killer (NK) cells, NKT cells, Th1 cells, cytotoxic T lymphocytes (CTL) which reciprocally increase CXCL10 production due to interferon-gamma (IFN-γ) released by those type of cells[5]. CXCL10 and IFN-γ contribute in the development of experimental (E)-CM[6]. CXCL10-deficient or knockout- mice were protected from ECM and capable of reducing parasitemia. These related to the reduce inflammation in the brain and the better of immune responses in the spleen which were important for controlling PbA-infection[7]. CXCL10-knockout mice prevented from ECM showed higher plasma IL-10 levels and higher T-regulatory-cell IL-10-production than those of wild type mice[8]. The brain-CXCL10-expressions were modulated in SM-PbA-infected Swiss mice treated with ethanolic leaf extract of *Annona muricata* (AME)[9]. These mice also showed an increase splenocyte-IL-10 production[10, 11]. Because of the importance role of spleen-CXCL10 in determining the ability of the host in controlling PbA-infection as mentioned before, a study warrant to be performed in order to observed whether AME influence spleen-CXCL10 expression during SM in PbA-infected swiss mice.

RESEARCH METHODOLOGY

This was continuing experimental study from previous study which used 36 Swiss-mice and group them into six. Negative control group, C(-), was healthy mice, while X_1 and X_2 were groups of healthy mice received *Annona muricata*-leaves extracted by using

ethanol (AME) in doses of 100 and 150 mg/kg BW, respectively. Other control group, C-(+), was inoculated by intraperitoneal injection with 10^7 PbA, whereas PbA-infected-X_3 and X_4 groups were administered by similar AME dosage mentioned before. All mice were anesthetized and terminated by neck dislocation at day 7 of the infection. The infected mice of C-(+), X3 and X4-groups, had severe PbA-infection with no different parasitemia percentage among those groups. The detail parasitemia analyzes, material and method had been mentioned in previous AME publication[10, 11]. CXCL10 in the spleen was stained by immunohistochemistry (IHC) method. The CXCL10 was then observed by using light microscope. Allred score was used for evaluating CXCL10 expression. The CXCL10 analysis done by two medical doctors specialized in pathology anatomy. Kappa analysis was done to evaluate CXCL10 results determined by the two experts. Statistical analysis used to determined different CXCL10 expressed among six groups was Kruskal-Wallis test which then followed by Mann Whitney U test.

RESULTS AND DISCUSSION

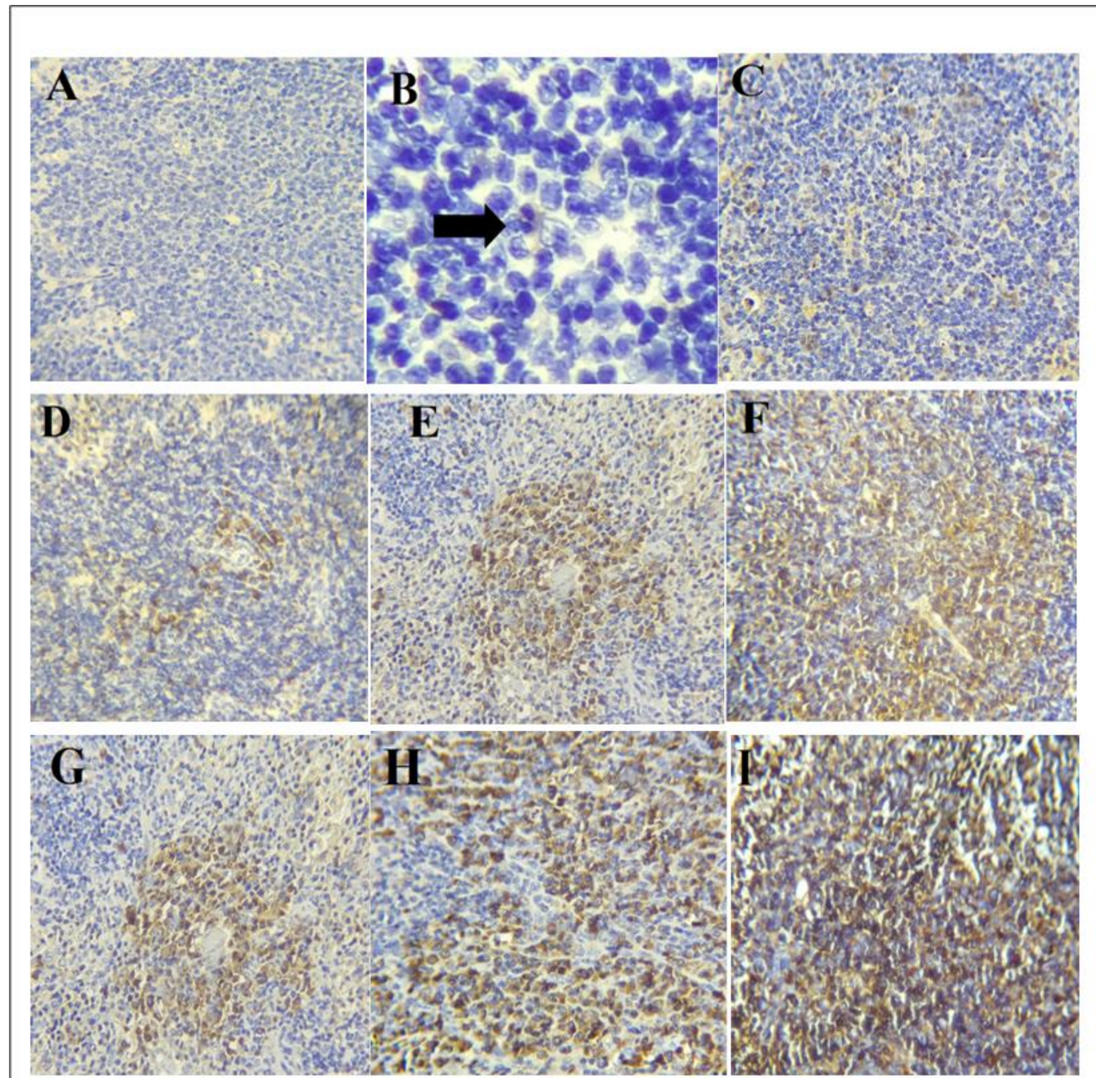

Figure 1. IHC of spleen-CXCL10 used to determine Allred score.
Allred score = proportion score + intensity score.(A: Proportion 0% , score = 0; B: Proportion 1% , score = 1; C: Proportion 2-10%, score = 2; D: Proportion 11-33% , score = 3; E: Proportion 34-66% , score = 4; F: Proportion >66% , score = 5; G: Weak Intensity , score = 1; H: Moderate Intensity , score = 2; I: Strong Intensity , score = 3).

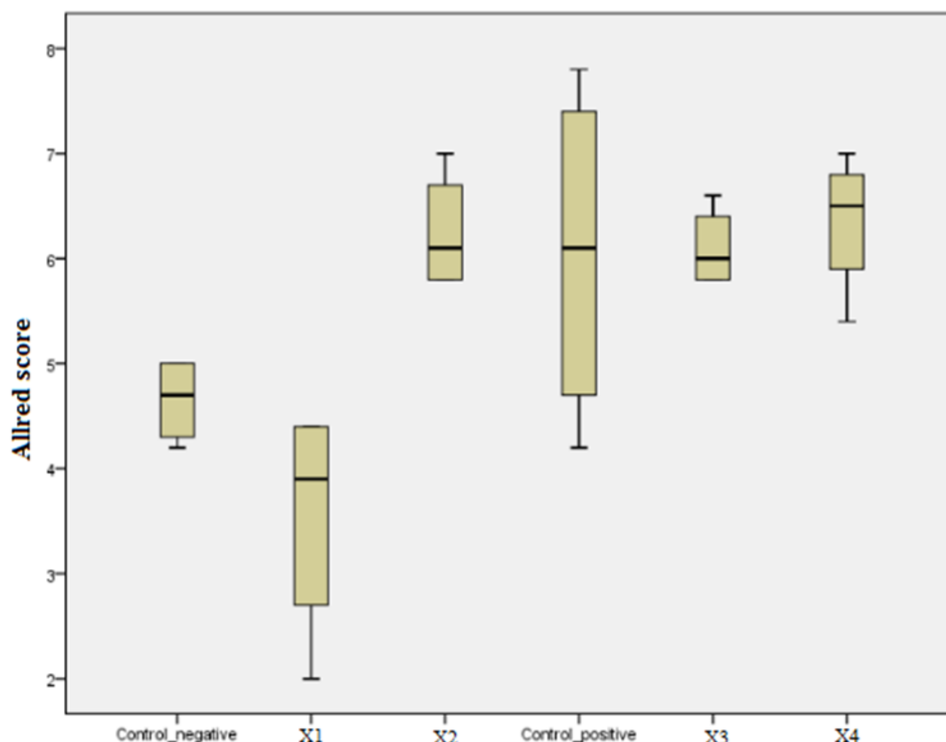

Figure 2. Graph Box plot of CXCL10 expresssed in the healty or PbA-inoculatrd swiss mice with or without AME-treatment. Control_negative, C- (-): healthy mice; X1: healthy mice with 100 mg/kg BW/day *A.muricata* treatment; X2: healthy mice with 150 mg/kg BW/day *A.muricata* treatment; Control_Positive, C-(+): PbA-inoculated mice; X3: PbA-inoculated mice with 100mg/kg BW/day *A.muricata* treatment; X4: PbA-inoculated mice with 150mg/kg BW/day *A.muricata* treatment

Table 1. Statistical analysis of CXCL 10 expressed in the spleen of healthy of PbA-inoculated mice with or without *A.muricata* treatment

Groups	Control (-)	X1	X2	Control (+)	X3	X4
Control (-)	(-)	(p=0.340)	(p=0.015)*	(p=0.054)	(p=0.014)*	(p=0.009)*
X1		(-)	(p=0.016)*	(p=0.044)*	(p=0.014)*	(p=0.016)*
X2			(-)	(p=0.356)	(p=0.798)	(p=0.461)
Control (+)				(-)	(p=0.391)	(p=0.461)
X3					(-)	(p=0.537)
X4						(-)

Mann-Whitney U test with significant difference ($p<0.05$)

Kappa test was done to measure the agreement between the two medical doctors specialized in anatomical pathology who observed the CXCL10, and the test result was 0.931 indicated that the two observers had very good agreement. The normality test showed that CXCL10 expressions was normally distributed in all studied group. Analysis of variance, however, showed that data in the studied groups were not homogen (p=0.024). Therefore, Kruskal Wallis Test, a nonparametic test, was performed, and the test showed significant difference among the studied groups (p = 0.022). The C-(+)-PbA-infected- control group was significantly expressed higher CXCL10 than those of the healthy-AME treated-X1-group (p = 0.044), and no significant different than healthy-C(-) group (p = 0.054). Interestingly, the PbA-infected X3 and X4 groups which received AME treatment, were expressed significantly higher CXCL10 expression than the C-(-)-healthy control group (p = 0.014 and p = 0.009, respectively). Those of X3 and X4 groups however were not different than C-(+)-PbA-infected-control-group (p = 0.391 and p = 0.461, respectively). Additionally, X3 and X4 group showed no difference of CXCL10 expression (p = 0.537). Interestingly all PbA infected groups including C-(+), X3 and X4 groups showed no different CXCL10 expression than healthy-X2 group which received AME-treatment.

Recent study showed that the spleen-CXCL10-expression of the PbA-infected C(+)-group was nearly significantly higher than healthy C(-)-group (p = 0.054). Interestingly, the X3 and X4-groups which were AME-treated and PbA-infected, showed the significantly higher CXCL10-expression than C (-)-group (Figure 7 and table 1). These indicated that AME-treatment made an obvious increase of CXCL10 expression above normal in the PbA-infected Swiss mice. The different-CXCL10 expression however, was not found between C-(+)-group either with X3 or X4 group. AME treatment therefore, had a limit effect in increasing the spleen-CXCL10 expression of Swiss mice during SM. The CXCL10 expressed by X3 and X4-groups was not different than those of AME-treated-healthy X2-group which was significant higher than normal value of C(-) group. This suggested that AME-treatment of PbA-infected mice was only increase spleen CXCL10 as high as those healthy who receive the intervention. This again showed that AME treatment had a restricted effect in increasing the spleen CXCL10 expression during SM. The increase of CXCL10 expression induces the survival defense mechanism of *P. falciparum* (Pf) by its growth acceleration[12]. The parasitemia

percentage of X3 and X4 groups was not different than C(+) group, suggested that the AME treatment at any studied dose did not initiate the accelerated growth of PbA. This study also showed that the increase of CXCL10 above normal in those received AME treatment did not prompt PbA survival defense mechanism. Pf inhibits CXCL10 synthesis due to the disruption of CXCL10 transcription of monocytes[12]. This explains the finding was that the spleen CXCL10 expression of healthy C(-) group was not different than PbA-infected C(+) group. The recent studies might explain that the increase CXCL10 plasma levels relate with CM patients[3]. In additionally, a treatment which reduces CXCL10 production in combination with anti-malaria medication associates with reduce systemic and brain inflammation and subsequence by increase survival. This treatment reduces apoptosis of endothelial vascular cells and microglia in the brain of ECM-mice model[13]. AME modulates CXCL10 expression in brain of Swiss mice during CM phase[14]. The AME-treatment had no apparent effect at the parasitemia levels during SM of PbA-infected Swiss mice. The AME-treatment therefore, remains need anti-malaria medication. The AME and ACT combination significantly lowers brain CXCL10 expression in the convalescence phase of Swiss mice with PbA-infection than those of PbA-infected either with AME intervention only or without any intervention[15]. The AME effect at the brain CXCL10 expression might be affected by both ACT and reduce parasitemia percentage. Whether this effect also found at the spleen CXCL10 warrants to be elucidated. CXCL10 recruits Th1-cells, major producer of IFN-γ, this spleen CXCL10 study therefore was not in accordance with previous findings that PbA-infected mice either with or without AME treatment associated with the reduce IFN-γ produced by spleen of Swiss mice[11]. Furthermore, AME-treatment associated with the increase spleen IL-10 production[10, 11]. Because of Th1 cells produce IL-10 in certain condition, the studies of any mediator involve will clarify the discrepancy. The spleen of ECM susceptible mice showed the present of NK cells needed for DCs to primed CD8+ T cell in spleen. NK cells are necessary for DC-IL-12 production which is essential to primed T cells. Additionally, DCs and IL-12 were both needed by NK cells in order to produce IFN-γ in the spleen of PbA-infected mice[16]. Whether all of these contribute in spleen CXCL10 expression remain unclear.

AME did not reduce CXCL10 in the spleen. In addition, AME does not reduce parasitemia during CM phase[17]. These findings were somehow consistent with findings in ECM observing cytokine produced by spleen[7]. The CXCL10-depletion or CXCL10-knockout gene in ECM-susceptible mice beneficials for the mice, because these mice show a reduce T cell trafficking and maintains specific T-cells in the spleen which then they expand and control the PbA-infection. AME incapable of inhibiting CXCL10 produced in the spleen, this might therefore underly the absence sufficient protective immune response which control PbA-infection.

AME increase anti-inflammatory but not pro-inflammatory cytokines. AME increase IL-10 produced by the spleen of Swiss mice during CM phase[10, 11]. AME meanwhile, reduced the spleen production of IFN-γ, a proinflammatory cytokine and no effect on the IL-12 and MIF. Lack of CXCL10 was preventing ECM, and this might be due to the increase plasma IL-10 levels and IL-10-production by T-regulatory-cells[8]. Whether AME influences other chemokines which support increase IL-10 production warrant to be further studied. The conclusion of this study is the ethanolic extracted of *Annona muricata* leaves enhances the spleen CXCL10 expression beyond normal for the period of severe malaria infection. The increase of spleen CXCL10 expression induced by the *Annona muricata* extract might not aggravate malaria infection.

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