Effect of Annona Muricata on CD8 and Granzyme-b Expression in Severe Plasmodium berghei Anka Infected Swiss Mice

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***Abstract*- Several studies have reported the role of T cells in Plasmodium berghei ANKA (PbA) that related with the blood and liver patology. Granzyme B (GzmB) is a serine protease released by CD8+ T cells and natural killer cells during cellular immune response, The purpose of these study to determine the effect of Annona muricata leaf-ethanolic exract (AME) on GzmB and CD8-expression-score in spleens of PbA-infected-Swiss-mice. This experimental study used randomized post test only control group design. Thirty six female Swiss mice were divided into 6 groups. C1-group was control-healthy mice, G1 and G2-groups were healthy-mice treated with AME dosage 100 and 150 mg/kg BW/day for 14-day-period. C2-group was control-PbA-infected-group. G3 and G4-groups received AME dosage 100 and 150 mg/kg BW, respectively for 7-day-pre and post-PbA-infection. GzmB and CD8 in the spleen stained by immunohistochemistry-method, then two-pathology-anatomy-experts determined Allred-score by using light microscope. Statistical analyzes used were One-way-ANOVA-test followed by post-hoc-test, and. Pearson-correlation-test. G4 and C1-groups had no significant different GzmB-expression-score (5.60 ± 0.46 and 4.10 ± 1.27; p = 0.134), while those of G3 and C2-groups (6.03 ± 0.48 and 6.70 ± 0.99) were significantly higher than C1-group (p = 0.015 and p = 0.012). AME (150mg/kg BW/day)-treatment associated with GzmB-expression-normal score during severe-PbA-infection. G3, G4 and C2-groups (5.03 ± 0.79; 6.20 ± 1.46 and 6.60 ± 2.77) had no significant different CD8-expression-score compare to C1-group (3.80 ± 2.60; p = 0.829; p = 0.284 and p = 0.150). The correlation score of GzmB and CD8 expression was not found in healthy Swiss mice infected with PbA (r = -0.248; p = 0.158). AME in dose of 150 mg/kg BW/day-treatment normalize GzmB-expression-score of severe-PbA-infected-Swiss-mice, however AME at any dose studied have no influence on CD8- expression-score of those mice.**

Keywords: Annona muricata, P. berghei Anka, granzyme B, CD8.

I. INTRODUCTION

Malaria is undoubtedly still regarded as the most serious tropical disease affecting mankind worldwide with the greatest impact in sub-Saharan Africa. During 2010, 106 countries around the world were perceived as endemic to malaria with an estimated 3.3 billion people at risk of developing the disease. In 2010 it is estimated to reach 216 million cases with 655,000 estimated deaths worldwide, of which, 86% in children under 5 years.[1]This disease is caused by the protozoan parasite of the genus Plasmodium with the female Anopheles mosquito that acts as a vector. Four species of Plasmodium are known to infect humans, namely P. ovale, P. vivax, P. malariae and P. falciparum, with the latest being the most violent and deadly [1]. The Plasmodium berghei ANKA (PbA) strain has long been used as a model for experimental cerebral malaria (ECM) due to its high reproducibility rate and the development of histopathologic and neurologic symptoms similar to human cerebral malaria (CM) [2]. Previous research by Bopp et al. shown that different mouse strains that are infected with PbA are susceptible or resistant to ECM in varying degrees [3]. This infection is deadly in about a week [4], it has been argued that the disease shows many characteristics that align serious illness in humans; thus, the disease has been used to study the cellular

mechanisms that mediate pathology [5]. For malaria, pathology is entirely linked to the blood stage of the life cycle of the parasite and, in the case of PbA infection, is a mediated immune. Many types of cells play a negative or positive role in the progression towards fatal illness, $CD8^+$ and $CD4^+$ T cells, $CD25^+$ Foxp31 regulatory T cells, natural killer cells, natural killer T cells, and CD T cells. Among these cell types, $CD8^+$ T cells play an important role in the effector phase of PbA-triggered cerebral malaria (ECM), although the magnitude and specificity of these responses are largely undocumented [6].

The pathogenesis of the disease is not clearly understood, the research data shows the important role of hypoxia, immune system, effector T cell infiltrationand toxins released by parasites [7]. The role of activated T cells entering CNS and causing damage has attracted much attention [8]. Several studies have reported the role of T cells in PbA, special parasites for C57BL / 6 mice, pathology induced in blood and liver stages malaria [9,10]. $CD8^+$ T cells are protective during the liver stage but are pathogenic in the brain during the blood stage [11], whereas $CD4^+$ T cells are protective both during the liver and blood stages [12], as well as in the brain [13]. The function of $CD8^+$ T cells depends on the location or network in which they are present. Evidence is accumulating on the infiltration of peripheral immune cells to the brain [7]. During ECM pathology, a series of events occur that cause coma and death that can not be prevented. The initiation of this event occurs due to expression of increased chemokine receptor and adhesion molecule in the brain that facilitates T cell infiltration [14] and parasitic red blood cell blockage (pRBCs) in the blood vessels. These events can lead to insufficient blood supply, oxidative stress, edema vascular permeability [8] and blood brain disorders [15, 16].

Cell death during ECM was associated with elevated levels of necrotic (cathepsin b and calpain1) and active apoptotic (caspase-3) cell death proteases, suggesting apo-necrotic mode of death. We have previously shown that this protease induces nerve cell death through cytoskelet destruction [17]. In addition, inhibition of caspase-3 and Bcl-2 over expression, an anti-apoptotic protein, prevents ECM induced cell death [18]. This may be due to stress rather than pathogen-induced signaling cascades because some reports suggest no CTL role in pathology. CTL kills the resident cell with direct cytotoxicity via Granzyme-B/perforin. Due to the presence of T cells in mortem samples and T cell pathogens in ECM. Granzyme B (GzmB) is a serine protease released by $CD8^+$ T cells and natural killer cells during cellular immune response, and is one of two dominant mechanisms in which T cells mediate cell death [19].

Annona muricata, a member of the Annonaceae family, is a known plant that widespread throughout the tropical and subtropical regions of the world such as South and North America, Africa, Asia, and Southeast Asia including Thailand [20]. Extract of A. muricata presents many beneficial effect including antioxidants, anti-inflammatory, and antiparasitic [20-25]. Extensive phytochemical evaluation of A. muricata extract has demonstrated the presence of various compounds, including alkaloids, polyphenols, flavonoids, essential oils, cyclopeptides, and kaempferol [26]. In addition, A. muricata has been shown to be a rich source of commonly acetogenin compounds that are active compounds in this plant extract [27]. Furthermore, A. muricata leaf extract was tested against P. falciparum and showed promising antimalarial effects [23, 27].

Aims and Scope

The aim of the study is to investigate whether AME decreasesCD8 and granzyme-B expressionsof severe PbA infected Swiss mice. This study about the effectivity of A. muricata –leaf-ethanolic-extract to decrease CD8 and GzmB expressions during severe parasitemia of Swiss albino mice inoculated with PbA.

II. MATHERIALS AND METHODS

This experimental study used randomized post test only control group design. Thirty six female Swiss mice were divided into 6 groups. C1-group was control-healthy mice, G1 and G2-groups were healthy-mice treated with AME dosage 100 and 150 mg/kg BW/day for 14-day-period. C2-group was control-PbA-infected-group. G3 and G4-groups received AME dosage 100 and 150 mg/kg BW, respectively for 7-day-pre and post-PbA-infection. GzmB and CD8 in the spleen stained by immunohistochemistry-method, then two-pathology-anatomy-experts determined Allred-score by using light microscope. Statistical analyzes used were One-way-ANOVA-test followed by post-hoc-test, and. Pearson-correlation-test.

III. RESULTS

Level of Granzyme

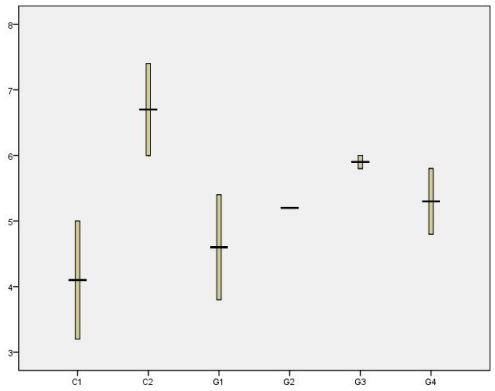

Figure 1. Boxplot Graph of Granzyme expresssed in the mice with and without treatment of A. Muricata

Healthy mice in G1 and G2 groups which were treated with A. muricata in dose of 100 and 150 mg/ Kg BW/ day, respectively, had higher granzyme levels than C1 which were healthy mice without A. muricata treatment. G2 group showed the highest granzyme levels among those healthy groups (figure 1). C2-group which was PbA-infected mice without A. muricata treatment had higher GzmB score than C1-group. PbA-infected mice in G3 and G4 groups which were treated with A. muricata in dose of 100 and 150 mg/ Kg BW/ day, respectively, had lower GzmB-expression than C2. G4 group showed the lowest granzyme levels among those of PbA-infected group (figure 1). By comparing GzmB-expression in treated groups with and without PbA inoculation, it was found that GzmB-expression of G3-group (PbA inoculated and 100 mg/Kg BW/day A. muricata) was lower than G1 group (100 mg/Kg BW/day A. muricata). GzmB-expression of G4-group (PbA inoculated and 150 mg/Kg BW/day A. muricata) was similar with G2-group (150 mg/Kg BW/day A. muricata).

Table No. 1. Statistical analysis of granzyme expressed in the spleen

Groups (Mean ± SD) Allred score	C2	G1	G2	G3	G4
C1 (4.10 ± 1.27)	0.012*	0.426	0.122	0.015*	0.134
C2 (6.70 ± 0.99)		0.134	0.406	0.820	0.426
G1 (5.20 ± 0.99)			0.910	0.210	0.922
G2 (5.60 ± 0.77)				0.793	1.000
G3 (6.03 ± 0.48)					0.824
G4 (5.60 ± 0.46)					

Tukey HSD test with significant difference ($p<0.05$)

The GzmB-expression-score was normally distributed in all studied group. Analysis of variance, however, showed that data in the studied groups were homogen ($p=0.339$). Therefore, One-way ANOVA test was performed and showed significant difference among the studied groups ($p = 0.007$). The GzmB-expression-score of either C2 or G3-group was significantly higher than C1-groups ($p = 0.012$ and $p = 0.015$, respectively), while those of G4-group was not different than C1-group ($p = 0.134$) (Table 1). This indicated that PbA-infection associated with increase GzmB-expression-score and that AME in dose of 100 mg/kg BW/day was not normalized GzmB-expression-score. Interestingly, AME in dose of 150 mg/kg

BW/day was normalized GzmB-expression-score. By comparing PbA-infected-treated-groups and control-PbA-infected-group, it was found that GzmB-expression-score of G3 and G4-groups were not significantly different than C2-group (p = 0.820 and p = 0.426, respectively). Similar finding was observed by comparing GzmB-expression-score between healthy-treated-groups and control-healthy-group. This was based on table 1. that GzmB-expression-score of G1 and G2-groups were not significantly different than C1-group (p = 0.426 and p = 0.122, respectively). The two leather analyzes were suggested that AME was not associated with GzmB- expression-score in both healthy and PbA-infected-Swiss-mice.

Level of CD8

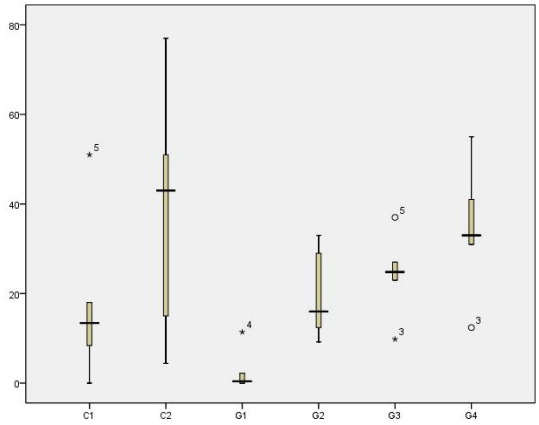

Figure 2. Boxplot Graph of CD8 expresssed in the mice with and without treatment of A.Muricata

The highest CD8-expression among healthy groups was observed in G2 group treated with A. muricata in dose of 150 mg/kg BW/day (Figure 2). Meanwhile among groups inoculated with PbA showed that the highest CD8-expression was found in the G4-group treated with A. muricata in dose150 mg/ Kg BW/ day. This followed by C2 groups without treated A. muricata, and G3 groups which were treated with A. muricata in dose of 100 (figure 2). By comparing CD8-expression in treated groups with and without PbA inoculation, it was found that CD8-expression of G3 (PbA inoculated and 100 mg/Kg BW/day A. muricata) group were higher than G1-group (100 mg/Kg BW/day A. muricata). CD8 levels of G4 (PbA inoculated and 150 mg/Kg BW/day A. muricata) group was higher G2 (150 mg/Kg BW/day A. muricata).

The CD8 expression was normally distributed in all studied group. Analysis of variance, however, showed that data in the studied groups were homogen (p=0.073). Therefore, One-way ANOVA test was performed and showed significant difference among the studied groups (p = 0.001). Tukey HSD test showed no significant different of CD8 expression between C1 and G1 or G2 group. No significant difference of CD8 expression levels were found between C2 and G3 (p = 0.686) or G4 (p = 0.999) groups. CD8 levels of G3 and G4 groups were not significantly different (p = 0.879). All of these pointed out that AME-treatment at any dose studied was not associated with CD8-expression score in either healthy or PbA-infected-Swiss-mice.

Although CD8-expression-score of those with PbA-infection (C2, G3 and G4 groups) were higher than those of C1-group (figure 2), however no significant different of CD8-expression-score were observed between C1 and C2 groups (p = 0.150; Table 2) Additionally, CD8-expression-score of C1 group was not significantly different either with G3 (p = 0.829) or G4 (p = 0.284) groups. These indicated that PbA-infectiondid not associate with CD8-expression-score above normal value. AME treatment at any dose studied was not modulate CD8-expression-score in PbA-infected-mice. No significant CD8-expression difference was observed between C2 and G3 or G4-group. This again indicated that AME at any dose studied was not influence CD8-expression in those with PbA-infection. Tukey HSD test showed significant different of CD8 levels between G1 and G3 groups (p = 0.021) and not significant different between G2 and G4 groups (p = 0.845) (Table 2). By comparing CD8-expression of G1 and G3 group, PbA-infection associated with increase CD8-expression. This last finding however, indicated that PbA-infection did not influence CD8-expression. The last finding was also consistent with previous analyses which compare C1 and C2-group.

Table 2. Statistical analysis of CD8 expressed in the spleen

Groups (Mean ± SD) Allred Score	C2	G1	G2	G3	G4
C1 (3.80 ± 2.60)	0.150	0.267	0.899	0.829	0.284
C2 (6.60 ± 2.77)		0.001*	0.645	0.686	0.999
G1 (1.36 ± 1.83)			0.036*	0.021*	0.003*
G2 (4.90 ± 0.81)				1.000	0.845
G3 (5.03 ± 0.79)					0.879
G4 (6.20 ± 1.46)					

Tukey HSD test with significant difference ($p<0.05$)

Pearson correlation test showed that no correaltion was found between granzyme and CD8 expression ($r = -0.248$; $p = 0.158$).

IV. DISCUSSION

AME has effect on GzmB-expression-score of those with severe-PbA-infection but not in those healthy. AME in dose of 150 mg/kgBW/day normalizes GzmB-expression-score in spleen-T-zone of Swiss mice during severe PbA-infection. C2-group with severe-PbA-infection had a significantly increase GzmB-expression-score (6.70 ± 0.99) above normal value of C1-group (4.10 ± 1.27; $p = 0.012$) (Table 1). AME in dose of 100 mg/kgBW/day was not normalize GzmB-expression-score of G3-group with severe-PbA-infection (6.03 ± 0.48; $p = 0.015$). Higher AME dose however, successfully reduced GzmB-expression-score of G4-group with severe-PbA-infection, as shown by no significant different than healthy control as mentioned above. This AME study has to be further confirmed because no different was found between severe-PbA-infection-control group (C2-group) and those severe-PbA-infection-AME-treated with either studied dose (G3 and G4-groups). No different of GzmB-expression between healthy-control (C1-group) and those healthy groups receiving of either AME dose (G1 and G2-groups). AME in two studied doses therefore, do not influence GzmB-expression during healthy condition. GzmB express by many type-cells including NK-cells, CTLs, CD4+Tcells and activated-CD4+FoxP3+Treg-cells [28,29]. Those type-cells modulate the immune respon during in vitro and in vivo pathologic condition [30,31]. Further study on cell-type-expressing-GzmB and its effect on immune responses in either the healthy or severe-PbA-infected with AME-treatment would complete the present study.

CD8+T-cells might not have any contribution in GzmB-expression in this AME-study. This based on the finding showed that no significant correlation was observed between granzyme-B and CD8-expressions (Pearson correlation test; $r= - 0.248$; $p = 0.158$). This might link to the fact that AME was not associated with CD8+expression in PbA-infected Swiss-mice (Table 2). Severe-PbA-infected-mice treated with AME (G3 and G4-groups) showed no significant different of CD8+expression than those of healthy-control (C1-groups) and severe-PbA-infected-control (C2-group). This therefore, other cell-types as the main source GzmB warrant to be further studied.

Severe-PbA-infection was not associated with increase CD8-expression in spleen-T-zone of Swiss-mice. The present study was not in line with previous study. Previous study use PbA-infected-B6-mice and treated them with chloroquine on day 4 infection [32]. High proportion of CD8+gzmB+-cells in previous study was observed on day 5 PbA-infection. Additionaly, the peak number of those cells occure on day 5 untill 7 infection, and the decrease number of those cells were observed by day 14 infection when parasite clearance occur. This was different than our present study which used different mouse-strain and did not use anti-malaria therapy. Our present study used IHC read by experts to determine CD8 and gzmB-expression, while previous study use flow cytometry and ex-vivo intracellular cytokine staining (ICS). Additionally, CTL specificity and CTL assay done, showed that specific-CTLs had ability to lyses target-

cells. By performing more advance laboratory method therefore would complete present AME-study in PbA-infected Swiss-mice.

The present AME study is supported by previous study which observed spleen-IFN-γ-production of the mice used in this present study [33]. PbA-infection was associated with reduce-IFN-γ-production in the spleen of Swiss-mice. A significant reduce-IFN-γ-production was also obeserved in those PbA-infected which treated with AME at any dose studied. No different of IFN-γ-production was observed between PbA-infected group and group of PbA-infected treated with AME at any dose. It is known that IFN-γ is critically support CTL activity [34].

Clinical malaria is known to be accompanied by an increase in the concentration of pro-inflammatory cytokines in plasma, which often correlates with the severity of the disease [35]. Little is known about the dynamics and kinetics of this mediator in the initial phase of infection including the liver phase when some cytokines are considered to play an important role in the development of the disease. Furthermore, cyclic parasitic proliferation begins in red blood cells which is immediately followed by the occurrence of clinical symptoms.

IFN-γ, is produced in large quantities by active natural killer (NK) cells and $CD8^+$ T cells, and IFN-γ is one of the pro-inflammatory cytokines that may play a key role in the initial phase of malaria, namely during the stage liver as has been shown in several murine models [36]. Murine malaria shows that NK cells contribute in limiting the initial phase of asexual parasite replication [37]. Additionally, human malaria shows that IFN-γ concentration increases and this correlates with NK cell activation during blood stage infections [38].

CTL activity depends on antigen presentation in conjunction with a major-histocompatibility-class I (MHC-I) complex molecule, while NK cells recognize their target by MHC method unrestricted and in the absence of antibodies. Both CTL and NK cells can destroy their target cells indirectly by producing IFN-γ or directly using a Fas-dependent mechanism, or by exocytosis of cytotoxic granules [39]. These granules contain perforin and a group of serine proteinases called granzymes [40]. Granzymes (Gzms) are mainly, produced by CTL and NK cells [41]. Gzms can be released during cytotoxicity and their levels in biological fluids are soluble markers for CTL and NK cell activation and increased concentrations are thought to reflect CTL and NK cell involvement in various disease states. Gzm levels that dissolve in the circulation increase in viral infections [42]. Although the biological function of most Gzms remains to be solved Granzyme A (GzmA), which is a trypsine-like enzyme, is less efficient at DNA fragmentation compared to GzmB which has activity against the procaspase substrate [43]. GzmB and CD8-expression-score of G3-group were lower than C2-group, however those expression-scores were not significantly different (Table 1. and 2.). CTL assay therefore would necessary be performed to confirm this AME-study.

IFN-γ is not only a key factor in protecting against Plasmodium infection, but also the pathogenicity factor in severe malaria symptoms [44]. It is well known that PbA-infection leads to the development of ECM, and P. berghei NK65 infection induces liver injury. Mice lacking IFN-γR on the background of C57BL/6 or 129 did not develop ECM. ECM also does not develop in mice infected with PbA after reduced $CD4^+$ T cells or $CD8^+$ T cells [45]. $CD8^+$ αβ cytotoxic T cells, which express perforin or GzmB, have an important role in the development of ECM [46]. Both $CD4^+$ αβ and $CD8^+$ αβ T cells have the same potential to produce IFN-γ, however IFN-γ-producing-$CD8^+$ αβ T cells are not important for ECM development [47]. IFN-γ promote $CD4^+$ α T cells which support the recruitment of $CD8^+$ αβ cytotoxic T cells to the brain. Surprisingly, the $CD4^+$ αβ T cells does not accumulate significantly in the brain [48]. NK-cells might play a critical role for developing ECM. Reduce-NK cell number can prevent ECM after PbA-infection, and NK cells are also associated with the IFN-γ in mice develop ECM. NK cells activate $CD8\alpha^+$ DC which prime $CD8^+$ αβ T cells after PbA-infection [49]. Furthermore, reduce number of CD8+ γδ T cells can also prevent ECM after infection with PbA [50]. It is therefore, important to determine whether AME-treatment influence the relation between IFN-γ-producing-NK-cells, CD8+ DCs, both CD8+ αβ T-cells and CD8+ γδ T-cells.

V. CONCLUSION

AME in dose of 150 mg/kg BW/day-treatment normalize GzmB-expression-score of severe-PbA-infected-Swiss-mice, however AME at any dose studied have no influence on CD8- expression-score of those mice.

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