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Acquisition of naturally acquired antibody response to *Plasmodium falciparum* erythrocyte membrane protein 1-DBLα and differential regulation of IgG subclasses in severe and uncomplicated malaria

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ABSTRACT

Objectives: To explore whether individuals infected with *Plasmodium falciparum* (*P. falciparum*) develop antibodies directed against *Pf*EMP1-DBLα, and to assess their IgG subclass distribution in severe and uncomplicated malaria.

Methods: The anti-*Pf*DBLα IgG and their IgG subclass distributions in plasma of severe (SM) and uncomplicated malaria (UCM) were assessed by enzyme-linked immunoabsorbent assay. The antibody profiles to *P. falciparum* blood stage antigens were evaluated. CD36 binding ability was determined by static receptor-binding assays. Rosette formation was performed by staining with acridine orange.

Results: Significantly higher number of UCM (86.48%) than SM (57.78%) plasma contained total acquisition of specific IgG to *P. falciparum* antigens ($P = 0.000$). Similar manners were seen in response to *P. falciparum* DBLα with significant difference (UCM, 59.46% *vs* SM, 40.00%; $P = 0.014$). Anti-*Pf*DBLα-IgG1 and -IgG3 were the predominant subclasses. Similar percentage of UCM (31.82%) and SM (33.33%) plasma contained only IgG1, while 13.64% of UCM and 27.78% of SM plasma contained only IgG3. Anti-*Pf*DBLα-IgG1 coexpressed with more than one subclass was noted (UCM, 27.27%; SM, 16.67%). Obviously, IgG1 coexpressed with IgG3 (9.09%) was observed in only UCM plasma. IgG1 was coexpressed with IgG2 in UCM (9.09%) and SM (11.11%) plasma, while IgG1 was coexpressed with IgG4 only in UCM plasma (4.55%). IgG subclasses to *P. falciparum* antigens were distributed in a similar manner. Only the levels of IgG1, but not IgG3 were significantly higher in UCM than in SM.

Conclusions: These data suggest that individuals infected with *P. falciparum* can develop the anti-*Pf*EMP1 antibodies with the major contribution of specific IgG subclasses. The balance and the levels of anti-*Pf*DBLα IgG subclasses play a crucial role in antibody mediated protection against severe malaria.

1. Introduction

In malaria endemic area, individuals eventually develop naturally acquired immunity after repeated malaria exposure as age dependent manner [1]. In general, immune response to mild malaria is acquired while being adults, while in severe malaria, immune protection is acquired after a few infections in stable

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transmission malaria areas [2]. Immunoglobulin G (IgG) from Africans adult was passively transferred to Gambian children [3] or to adult Thai patients [4] can protect *Plasmodium falciparum* (*P. falciparum*) asexual blood stages infection, suggesting that this immunity is mediated partly by antibody. Acquired antibodies which predominantly target variant-specific epitopes on erythrocytes infected with *P. falciparum* potentially related to protecting clinical malaria [5,6] and might protect severe malaria by low parasitemia [3]. Cytophilic IgG1 and IgG3 subclasses have been shown to play a crucial role in protection against falciparum malaria. In contrast, IgG2 and IgM classes are evidenced to block protective mechanism [7].

P. falciparum cause severe malaria due to its ability to adhere to vascular endothelial cells (cytoadherence) and the ability to form rosette by binding to uninfected red blood cells (rosetting). *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) is the dominant antigen and adhesin on the surface of iRBCs which is a major component of protective immunity. *Pf*EMP1 encoded by *var* genes is strain-specific and highly polymorphic polypeptide of high molecular mass (200–350 kDa) [8,9]. This polymorphic protein composes of variable number of adhesion domain in which Duffy binding-like domains (α, β, γ, δ, ε and X) and cysteine-rich interdomain regions (CIDR) were included. The DBLα domain involving in rosette formation is associated with cerebral malaria [10]; while CIDR domain is able to bind with CD36 associated with non-severe malaria [11]. The switching in *var* gene expression allows parasites to escape host immune response. Reversing sequestration has been investigated as an attractive contribution to the management of severe malaria.

Immunity to mild malaria takes many years, while severe malaria develops after a relatively low number of infections, suggesting the restricted heterogeneity of some important targets for immunity [12]. *Pf*EMP1 variant-specific epitopes on infected erythrocytes surface are thought to be a major antibody target. Immunization with *Pf*EMP1-DBLα generates the specific antibody that can block the infected red blood cell adhesion *in vivo* [13]. Cytoadherence to *P. falciparum* infected erythrocytes can be inhibited by human monoclonal antibody *in vitro* [14]. *Pf*EMP1-DBLα generates antibodies that block the rosette formation and protect *in vivo* sequestration of *P. falciparum*-infected erythrocytes [13,15]. Moreover, antibodies against subsets of *Pf*EMP1 are associated with severe malaria in children [5]. In Gabon, sera from semi-immune children have significantly different levels in anti-*P. falciparum*-DBLα antibodies from non-immune children [16]. Recently, the study in India showed that seroprevalence and antibody levels to recombinant DBLα increased in high endemic areas, but low in areas of low endemicity, suggesting that DBLα had epitopes that exposed naturally to immune system [17].

Recently, the findings in Thailand on the distribution of semi-conserved features in the rosetting domain of *Pf*EMP1-DBLα showed closely similar among *P. falciparum* isolates causing severe and uncomplicated malaria [18] were different from those demonstrated in African [19]. The possible explanation is due to clinical isolates originated from different geographical areas which may mediate different levels of cytoadherences and rosetting. Moreover, vaccination with *Pf*EMP1 domain(s) could induce protective immunity against severe disease. In animal models, immunization of Aotus monkeys with CD36-binding domain indicated that immunity to homologous parasites could be induced by immunization with functional *Pf*EMP1 domains [20]. In another study, the specific antibodies generated after *Pf*EMP1-DBLα immunization disrupts rosette formation and protect the sequestration erythrocytes infected with *P. falciparum* [13]. Therefore, the information regarding the naturally acquired antibody response against *Pf*EMP1-DBLα specific domains in individuals infected with *P. falciparum* malaria in Thailand with different transmission is essential. The aim of the study is to investigate whether individuals with malaria develop antibodies direct against *Pf*EMP1-DBLα protein. The IgG subclass antibody profiles to *Pf*EMP1-DBLα protein, the binding ability to CD36 and rosette formation in severe and uncomplicated malaria were also evaluated.

2. Materials and methods

2.1. Samples

A total of 82 anonymous plasma from 45 patients with severe malaria (SM) and 37 patients with uncomplicated malaria (UCM) stored at −20 °C were studied. Twenty stored plasma from healthy individuals who had no history of malaria were used as controls.

This retrospective study received the approval from the Ethical Committee of Faculty of Tropical Medicine, Mahidol University.

2.2. Parasite isolates

Fifty two anonymous stored *P. falciparum* isolates kept in liquid nitrogen at Malaria Research Laboratory, Department of Microbiology and Immunology, Faculty of Tropical Medicine were evaluated. These comprised of 29 and 23 isolates causing severe and uncomplicated malaria. The definition of severe and uncomplicated malaria was followed by World Health Organization criteria [21].

2.3. Cultivation of *P. falciparum* erythrocytic stages

The parasites were thawed and immediately cultivated *in vitro* [22] in medium containing RPMI 1640 (Gibco Life Technologies, New York), HEPES, 10% human serum, 2 mM L-glutamine, 25 mM sodium bicarbonate and 2.5 μg/mL gentamycin, in CO_2 incubator. Growth of parasite was examined daily by staining the thin blood films with Giemsa until the trophozoite stages. Parasite binding assay and rosette formation were performed when the culture reached more than 70% trophozoite stages. The overall *in vitro* cultivation of the parasite isolates was 4–5 cycles. *P. falciparum* laboratory lines A4 and ItG derived from the laboratory line IT4/25/5 [23] were used as controls.

2.4. *P. falciparum* blood stage antigens

The *P. falciparum* laboratory line A4 was cultured *in vitro* and harvested when parasite density reached 5%–10% hematocrit. After washing with in phosphate buffered saline (PBS) pH 7.2, the parasite infected red blood cells (iRBCs) were suspended in PBS at 10% hematocrit. The parasites were pooled, sonicated (Sonics Vibra cells, Newtown, USA) and stored at −20 °C until use. The concentration of protein was determined by NanoDrop (Thermo scientific NanoDrop 2000c spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA).

2.5. Expression of P. falciparum DBLα protein

The *P. falciparum* DBLα (*Pf*DBLα) protein was expressed and purified. In brief, *P. falciparum* A4 was cultured as described above. Total RNA were extracted from malaria parasites when reaching at least 5% parasitemia [24]. RNA was treated with deoxyribonuclease (DNAse Free, Ambion, UK). Complementary DNA (cDNA) was synthesized by cDNA synthesis kits (Bioline, UK) with hexamers randomly based on the recommendation of manufacturer. Negative control reaction was done using identical amounts of template without reverse transcriptase. PCR amplification was also completed. The fragments of 1 200 base pairs of *Pf*DBLα domains were amplified using degenerate primers forward 5′-GAATTCATGCATGGTAGGGAGCATCCT-3′ and reverse 5′- AAGCTTTTAGCGATATTCCGTATGAGAAAATG-3′ which contained the restriction site of *Eco*RI and *Hin*d III. The PCR conditions were established using hot start (95 °C, 5 min) followed by 30 cycles of 95 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min and extension at 72 °C, 5 min. The PCR products were checked by 2% agarose gel. *Pf*DBLα was confirmed by DNA sequencing (Macrogen, South Korea) and the PCR products were ligated into pGEMT easy vector. Then, the blue white colonies were selected and ligated into pMAL-p5X vector including the restriction site of *Eco*RI and *Hin*d III. For protein expression, *Escherichia coli* strain BL21 was used. The transformed cells were cultured at 37 °C in Luria–Bertani medium supplemented with 0.2% glucose, 50 μg/mL ampicillin and 50 μg/mL chloramphenicol until an optical density (OD) at 600 nm reached 0.3 to 0.5. Then, cells were induced to express the *Pf*DBLα fusion proteins by isopropyl-β-D-thiogalactoside. The samples were spun and the pellet re-suspended in lysis buffer containing 50 mM Tris–HCl pH 7.5, 300 mM NaCl, 1 mM MPMSF and 0.5% triton-X100. The cells were disrupted by sonication for 10 s ON and 10 s OFF pulses for 30 min on ice, followed by spinning. Purification of each fusion protein was done by amylose resin chromatography based on the recommendation of manufacturer (New England Biolabs, UK). The protein was stored in 10 mM Tris–HCl buffer, pH 7.4 containing, 0.2 M NaCl, 10 mM β-mercaptoethanol and 1 mM EDTA at −20 °C. Determination of the protein purity was done by electrophoresis on sodium dodecyl sulfate-10% polyacrylamide gel. The quantity of the protein was determined based on the method of Bradford by Bio-Rad protein assay kit (Bio-Rad, Munich, Germany). The *Pf*DBLα protein was successfully expressed in *Escherichia coli* with molecular weight of 46.66 kDa.

2.6. CD36 binding ability under static conditions

The static receptor-binding assays were used for analysis of purified CD36 (R&D Systems, UK) [24]. In brief, 50 μg/mL purified CD36 were spotted in triplicate onto 35 mm petri dishes (Nunc, Roskilde, Denmark) and coated at 37 °C in a humid atmosphere for 2 h. Petri dishes were blocked overnight at 4 °C with 1% bovine serum albumin in PBS, and incubated at 37 °C for 30 min after washing. *P. falciparum* iRBCs were centrifuged and resuspended in RPMI 1640 medium-HEPES, pH 6.8 at 3% parasitemia and 1% hematocrit, then 1.25 mL was added to each dish. The dishes were incubated at 37 °C for 1 h with gentle swirling every 15 min. Dishes were gently washed until non adherent RBCs were removed. The binding RBCs were fixed for 20 min with 2% glutaraldehyde (Sigma, St Louis, MO) in PBS. Then, dishes were stained with Giemsa (Merck, Poole, UK). Bound iRBCs (per mm^2) to the surface of CD36 were counted under light microscope. For controls, the A4 and ItG, *P. falciparum* laboratory lines served as controls. All assays were done in duplicate.

2.7. Rosette formation

For performing the rosette formation, one hundred microliters of fresh mixed suspension of iRBCs and 2 μL of 0.01% acridine orange solution were used [24]. The suspension of 10 μL was dropped under a 22 mm × 22 mm cover slip. The assays were determined under fluorescent microscope by counting two hundreds or more of trophozoite stages in duplicate. The rosette formation was counted if two or more uninfected RBCs were bound to a single iRBCs. The rosetting rate was determined by the ratio of number of rosette to total number of iRBCs.

2.8. Enzyme-linked immunoabsorbent assay (ELISA)

The anti-*Pf*DBLα IgG and their IgG subclasses in plasma were determined by ELISA. The checkerboard titration was done to determine the optimal conditions. Plasmas from healthy individuals were used as controls. All tests were done in duplicate. Briefly, wells of microtiter plates were coated with 50 μL of 200 ng/mL *Pf*DBLα antigen suspended in 0.1 M carbonate-bicarbonate buffer pH 9.6. Plates were incubated at 4 °C overnight and washed three times with PBS plus 0.05% Tween-20 (PBS-T). The unbound sites were blocked for 2 h at 37 °C with blocking buffer containing 2.5% powdered milk in PBS-T. After being washed, plates were added with 50 μL of diluted plasma (1:100) in 1.25% powdered-milk in PBS-T and incubated for 1 h at 37 °C. Plates were then washed again and incubated with peroxidase-conjugated rabbit anti-human IgG (Dako A/S, Denmark) in 1.25% powdered-milk in PBS-T for 1 h at 37 °C for IgG antibody detection (1:8 000). After washing, the color reaction was developed by using o-phenylenediamine substrate (Dako A/S, Denmark). The OD was measured at a wavelength of 492 nm with reference wavelength of 620 nm by an ELISA reader (Sunrise™, TECAN, Switzerland). Total IgG higher than the mean OD of control plus one standard deviation (OD + 1SD) was considered positive. For determination of IgG subclass, after washing the plasma, the appropriate dilution of mouse anti-human IgG1 (1:1 000), IgG2 (1:5 000), IgG3 (1:7 000) and IgG4 (1:2 000) (Sigma, USA) were added. The plates were incubated for 1 h at 37 °C, washed as above and then react with peroxidase-conjugated rabbit anti mouse IgG (1:2 000) (Dako A/S, Denmark) at 37 °C for 1 h. After washing, the color reaction was developed by using o-phenylenediamine substrate (Dako A/S, Denmark). The OD was measured at a wavelength of 492 nm with reference wavelength of 620 nm by an ELISA reader (Sunrise™, TECAN, Switzerland). The IgG subclasses greater than the mean OD of control plasma plus one standard error (OD + 1SE) were considered as positive.

The specific IgG and IgG subclasses to *P. falciparum* blood stage antigens in plasma were also determined by ELISA as described above, except the coating concentration in which 50 μg/mL *P. falciparum* A4 antigen was used.

2.9. Data analyses

The data were analyzed using SPSS software version 18. The antibody profile to *P. falciparum* blood-stage antigens and *Pf*DBLα protein in plasma from severe and uncomplicated malaria were compared by Mann–Whitney *U* test. $P < 0.05$ was considered as statistically significant difference.

3. Results

3.1. Sample characteristics

Plasmas from patients with severe malaria were hyperparasitaemia except one was cerebral malaria. The median parasite count (median and interquartile range) in patients with SM [187 300 parasites/μL, interquartile range (IQR) = 115 162.50–440 000.50 parasites/μL] was significantly higher than those with UCM (72 590 parasites/μL, IQR = 43 345.50–111 922.50 parasites/μL) ($P = 0.000$).

3.2. Antibody response to PfDBLα protein and P. falciparum blood stage antigens

The antibody responses to *Pf*DBLα protein were determined in plasma from 45 SM to 37 UCM by ELISA. The percentage of IgG responders to *Pf*DBLα protein was lower than those responded to *P. falciparum* blood stage antigens (Figure 1). About 18 out of 45 SM (40.00%) and 22 out of 37 UCM (59.46%) plasma contained IgG to *Pf*DBLα protein with significant difference ($P = 0.014$). Whereas 26 SM (57.78%) and 32 UCM (86.48%) plasma contained IgG to *P. falciparum* blood stage antigens with significant difference ($P = 0.000$).

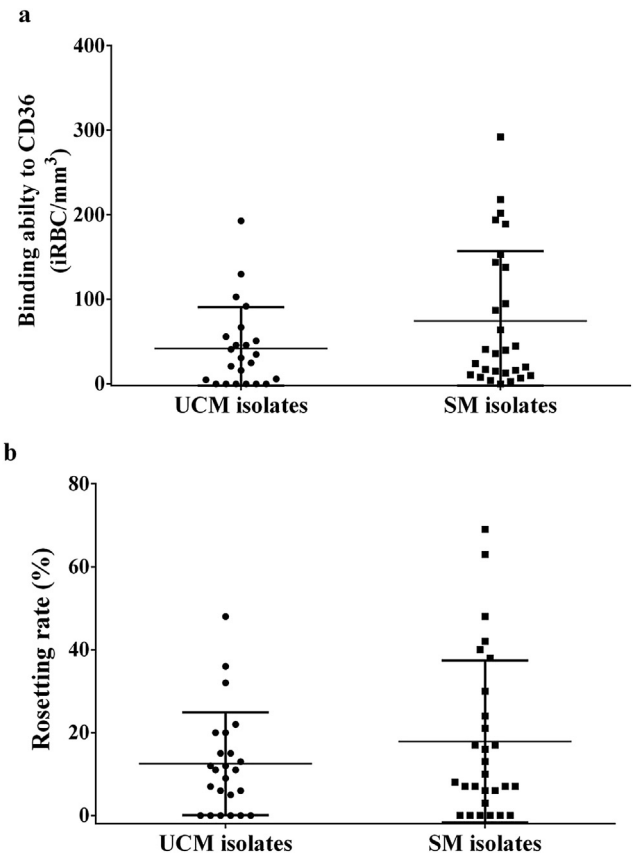

Figure 1. Binding ability to CD 36 and rosetting rate of isolates causing UCM and SM.
(a) Binding ability to CD 36; (b) rosetting rate.

When the IgG subclasses responders to *Pf*DBLα protein were taken into account, higher percentages of UCM (72.73%) than SM (44.44%) plasma contained IgG1. The same trends were found in IgG2 (UCM, 36.36% *vs* SM, 27.78%) and IgG4 (UCM, 27.78% *vs* SM, 6.67%). In contrast, for IgG3, higher percentages of SM (38.39%) than UCM (27.78%) plasma were observed. Only one SM plasma had specific antibodies of all four subclasses. The anti-*Pf*DBLα-IgG1 and -IgG3 were the predominant subclasses, in which 31.82% of UCM and 33.33% of SM plasma contained only IgG1, while 13.64% of UCM and 27.78% of SM plasma contained only IgG3. Whereas 11.11% of SM plasma contained only IgG2, and 4.55% of UCM plasma contained only IgG4. None of UCM plasma contained only IgG2. In addition, 27.27% of UCM and 16.67% of SM plasma which contained anti-*Pf*DBLα-IgG1 coexpressed with more than one other subclass were noted, in which IgG1 coexpressed with IgG3 was found only in UCM plasma (9.09%). Anti-*Pf*DBLα-IgG1 coexpressed with -IgG2 was found in UCM (9.09%) and SM (11.11%) plasma, while IgG1 coexpressed with IgG4 was found only in UCM plasma (4.55%).

Similar patterns of IgG subclasses responders to *P. falciparum* blood stage antigens were found, which were 12.50% of UCM and 26.92% of SM plasma containing only IgG1. None of UCM plasma had only IgG2 or IgG3, but 3.84% of SM plasma had both IgG2 and IgG3. UCM (21.87%) and SM (19.23%) plasma had IgG coexpressed with other subclasses antibodies. About 18.75% of UCM and 11.38% of SM plasma contained four IgG subclasses. Plasma IgG1 coexpressed with IgG3 (UCM, 21.87% *vs* SM, 19.23%) and IgG2 antibodies (UCM, 12.50% *vs* SM, 3.84%) were found.

The median plasma levels (median and interquartile range) of IgG and IgG subclasses to *Pf*DBLα protein in UCM and SM plasma were shown (Table 1). The median IgG levels were higher in SM than in UCM plasma ($P = 0.018$). However, the median IgG1 levels in SM were significantly lower than those in UCM plasma ($P = 0.012$). For IgG subclasses, the median levels of IgG2, IgG3 and IgG4 to *Pf*DBLα protein in SM were slightly lower than those in UCM plasma with non-significant differences (Table 1). These IgG and IgG1 in responses to *Pf*DBLα protein showed similar manner to those in response to anti-*P. falciparum* blood stage antigens, in which the levels in UCM were higher than those in SM plasma (IgG, $P = 0.000$; IgG1, $P = 0.025$). Likewise, the median IgG2, IgG3 and IgG4 levels to *P. falciparum* blood stage antigens in SM were slightly lower than those in UCM plasma with non-significant differences (Table 2).

Similar distribution of IgG subclasses in response to *P. falciparum* blood stage antigens were noted. The median

Table 1
Antibody response to *Pf*DBLα-protein in UCM ($n = 37$) and SM ($n = 45$).

Anti-*Pf*DBLα antibodies	Median OD (IQR)		*P*
	UCM	SM	
IgG	0.65 (0.22–1.11)	0.22 (0.07–0.43)	0.018*
IgG1	0.54 (0.10–0.94)	0.15 (0.00–0.32)	0.012*
IgG2	0.17 (0.00–0.23)	0.09 (0.00–0.29)	0.765
IgG3	0.15 (0.03–0.18)	0.07 (0.00–0.25)	0.901
IgG4	0.13 (0.00–0.20)	0.00 (0.00–0.06)	0.302

*Significantly different of the median IgG level and IgG1 level against *Pf*DBLα in UCM and in SM plasma ($P < 0.05$).

Table 2
Antibody response to *P. falciparum* blood stage antigens in UCM (n = 37) and SM (n = 45).

Anti-*P. falciparum* antibodies	Median OD (IQR)		*P*
	UCM	SM	
IgG	1.11 (0.77–1.77)	0.39 (0.16–0.68)	0.000*
IgG1	0.57 (0.35–0.85)	0.45 (0.29–0.67)	0.025*
IgG2	0.25 (0.13–0.43)	0.16 (0.09–0.26)	0.120
IgG3	0.21 (0.08–0.45)	0.27 (0.19–0.36)	0.669
IgG4	0.20 (0.11–0.31)	0.19 (0.05–0.41)	0.712

*Significantly different of the median IgG level and IgG1 level against *P. falciparum* blood stage antigens in UCM and in SM plasma ($P < 0.05$).

levels of IgG and IgG1 to *P. falciparum* blood stage antigens were significantly higher in UCM than in SM plasma.

3.3. Cytoadherence to CD36 and rosetting rate of P. falciparum isolates causing severe and uncomplicated malaria

The binding ability to CD36 and rosette formation among severe and uncomplicated malaria isolates were shown in Figure 1. The parasite line, *P. falciparum* clone ItG (originated from Brazilian IT lineage) was used as a positive control. The numbers of iRBCs (>5 per mm^2) which adhered to purified CD36 were considered positive. As the results, 93% and 75% of SM and UCM isolates bound to purified CD36 receptor, respectively. When the numbers of iRBCs from SM and UCM isolates which adhered to CD36 were compared, no significant differences were found (P = 0.321). However, the median number of iRBCs adhered to CD36 in SM isolates (median = 38.00, IQR = 11.50–142.50) was higher than that in UCM isolates (median = 33.00, IQR = 1.25–64.25) (Figure 1a). There was a correlation between parasite density and the binding ability to CD36 (P = 0.020). For the rosetting rate, 80.76% of isolates could form rosettes (23 SM and 19 UCM isolates). The median rosetting rate in SM (median = 9.00, IQR = 3.75–28.50) was lower than that in UCM isolates (median = 11.00, IQR = 1.25–18.50) with non-significant difference (Figure 1b). The correlation between parasite density and rosetting rate (P = 0.028) was noted.

4. Discussion

Naturally acquired immunity targeting blood stages *P. falciparum* is important for protection against clinical malaria. Antibody mediated immunity to *P. falciparum* is short-lived and maintains for only few weeks to months. However, *P. falciparum* induces and maintains long-lasting specific memory B cells in human, which is observed in other study [25]. Specific IgG1 and IgG3 antibodies to *P. falciparum* have been shown to associate with protection [5].

For naturally acquired protective immunity, *Pf*EMP1 is so far the most plausible target and well characterized parasite-induced proteins expressing on the erythrocyte surface [12]. Although *Pf*EMP1 is composed of a highly conserved intracellular domain and highly polymorphic extracellular domain, *Pf*EMP1 variants share an overall common structure. Antibody specific to *Pf*EMP1 is predicted to play important role in protective immunity in individuals infected with *P. falciparum* that takes years to develop the antibody due to its polymorphic characteristic and the changed expressions among different variants. It is clearly demonstrated that the acquisition of antibodies against specific domains and the time over are different in which such immune responses are acquired between areas as well as between domains. Previous studies had recently demonstrated that the antibodies increased against DBL1α, DBL2β and CIDR1α, but not to DBL4γ or DBL5β. In the present study, through the use of recombinant protein technology, the naturally acquired IgG response as well as IgG subclasses distribution and patterns of *P. falciparum* specific antibodies against *Pf*DBLα protein in plasma of individuals with severe and uncomplicated malaria were evaluated along with the anti-*P. falciparum* antibody to blood stage antigens. It seems that anti-*P. falciparum* antibodies play a crucial role in immune protection against asexual blood stages of the parasite. The findings indicated that the majority of uncomplicated malaria and only half of severe malaria had total acquisition of specific IgG to *P. falciparum* blood stage antigens. Similar results were obtained with regard to responders to *Pf*DBLα protein, but the number of responders was lower, with significance higher in UCM than in SM. These results indicated that naturally acquired antibody to blood stage *P. falciparum* also contains anti-*Pf*DBLα antibody which associates with severity of malaria. The lower number of responders to *Pf*DBLα protein than blood stage antigens may be due to the reasons as follows: a) each infection is accompanied by different *Pf*EMP1-DBLα variants based on large repertoire *Pf*EMP1-DBLα variants; b) antibody is raised against the more conserved or cross-reactive epitope maintaining within the expressed *Pf*DBLα [16]; c) individual immune status and number of malaria episodes. Therefore, the antibody which responses to either variable or conserved region of *Pf*EMP1 is potentially important to be further explored.

Measurement of total IgG response alone might not be adequate to assess immunity. Therefore, the ability of the host to mount an appropriate subclass response to *P. falciparum* may be crucial in protection against infection. Therefore, the levels of antibodies and the balance of antibody subclasses are of great importance in antibody-mediated protection against malaria. The antibody protection will be successfully achieved if there are sufficient levels of antibodies with the correct specificity and appropriate subclass. The possible mechanism of protection is that the potential antibody can induce phagocytic clearance, reduce cytoadherence, inflammation and parasite burden [26]. The results on the expression of *P. falciparum* specific IgG subclass either to *Pf*DBLα or *P. falciparum* blood stage antigen in individual plasma showed that the majority contained IgG1 and IgG3 antibodies indicating the predominant subclasses. In general, most individuals had IgG4 antibodies together with other three IgG subclasses. Individuals who had IgG2 antibodies frequently had antibodies of IgG1 and IgG3. The distributions of specific antibodies subclasses to *Pf*DBLα and *P. falciparum* blood stage antigens in SM and UCM in our study were essentially similar to those reported previously against blood stage antigens in African and Asian adults [22], indicating that the first natural antigenic stimulations trigger B cells to produce IgG1 or IgG3. While subsequent antigenic stimulation will trigger B cells to produce IgG2 and IgG4. Such results suggested that, upon repeated antigenic stimulations, the expression of isotype reflected the sequential activation of specific B cells [27]. Recently, the cytophilic antibodies IgG1 and IgG3 against *P. falciparum* crude blood stage antigen has been reported to

associate with protection, while non-cytophilic antibodies, IgG2 and IgG4 could compete and block the protective mechanism [28]. Surprisingly, only the antibody levels of IgG1 were significantly higher in UCM than in SM in this study. With the expectation that the specific IgG3 levels to *Pf*DBLα should be significantly higher in UCM than in SM since it has been shown that *P. falciparum* specific IgG3 might play a crucial role in controlling clinical manifestations during acute *P. falciparum* infection and in risk reduction of mild malaria [22]. Moreover, cytophilic IgG3 play role in antibody-dependent inhibition against *P. falciparum* blood stages [29]. In fact, IgG3 controls parasite multiplication by inducing cell-mediated lysis via complement pathways, phagocytosis and/or antibody-dependent inhibition mechanism [29]. In fact, IgG specific to *Pf*EMP1 contributed to regulate parasite densities [30]. Moreover, Gambian children with UCM have antibodies which can disrupt rosette formation. In animal model, antibodies against DBLα can prevent the *Pf*EMP1 dependent sequestration and inhibit parasite adhesion [6,13]. However, due to the limitation of plasma samples, the role of anti-*Pf*EMP1 antibody in plasma has not been further investigated for their ability to inhibit parasite adhesion or disrupt the rosette formation *in vitro*. Due to the fact that infection with *P. falciparum* is the most lethal due to its ability to cytoadherence to endothelial cells and the ability to form rosette with uninfected red blood cells, in which *Pf*EMP1 is the dominant antigen and adhesin on the surface of iRBCs considered as a major component of protective immunity. Immunization with *Pf*EMP1-DBL1α generated the specific antibodies that were able to disrupt rosettes formation and protect *P. falciparum* infected erythrocytes sequestration [13]. We have recently demonstrated in human that distributions of semi-conserved features within *Pf*EMP1-DBLα rosetting domain indicated the close similarity between isolates causing severe and uncomplicated malaria [18] which were different from the results in African [19]. Therefore, in this study, the binding phenotype to CD36 and rosette formation of isolates causing severe and uncomplicated malaria in same individuals whose plasma were tested for antibodies to *Pf*EMP1 and *P. falciparum* blood stage antigens were also evaluated. SM isolates had higher binding ability to CD36 than UCM isolates, indicating that due to the variation in *Pf*DBLα domain, *P. falciparum* could avoid from the host immune system by hiding from phagocytosis, mediating acute inflammation as well as enhancing parasite growth and survival which led to high parasitemia *in vivo* and an increase in microvascular obstruction of the blood flow [31,32]. Such results were in line with the previous study in Thailand [33].

Several studies demonstrated the association of rosette formation with severe anemia malaria and cerebral malaria in children in Africa [34] but not in Papua New Guinea [35]. In addition, the *in vitro* study showed that formation of rosettes caused by *P. falciparum* isolates in Thailand was lower than African isolates [34]. However, in this study, the rosetting rates were found to be higher in SM isolates than in UCM isolates with non-significance. Such a result together with the previous reports suggested that different geographical areas, different parasite populations and host genetic factors could affect the ability to form rosette due to their different receptors. Recently, rosetting domain has shown to be a target for malaria vaccine by the finding of monoclonal antibody specifically reacting to rosetting domain which has potently inhibited rosette formation [36].

Despite those limitations, our results suggested that individuals infected with *P. falciparum* did develop the anti-*Pf*EMP1 antibodies with the major contribution to specific IgG subclasses. The results seem to indicate that the anti-*Pf*EMP1 IgG1 and IgG3 are the predominant subclasses in SM and UCM. However, only the antibody levels of IgG1 were significantly higher in UCM than in SM, and IgG1 coexpressed with IgG3 was found only in UCM, suggesting that the balance and the levels of anti-*Pf*DBLα IgG subclass play a crucial role in antibody mediated protection against SM. Further study in large sample size is needed to make a concrete conclusion. The antibodies response to both conserved and variable part of *Pf*EMP1 and their role is potentially important to be explored for further vaccine design.

Conflict of interest statement

The authors declare that they have no competing interests.

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