

EFFECT ON ROSETTE FORMATION OF ANTIBODIES TO DUFFY BINDING-LIKE 1 ALPHA DOMAIN OF *PLASMODIUM FALCIPARUM* ERYTHROCYTE MEMBRANE PROTEIN 1

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Abstract. The highly conserved Duffy binding-like domain 1 α (DBL1 α) of *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1) is central to cytoadherence properties of infected erythrocytes. Antibodies against DBL1 α in plasma from African children in high malaria transmission settings disrupt rosettes (clumping of infected to uninfected erythrocytes) and may possibly protect against severe malaria. This phenomenon has not been established in low transmission settings in Asia. Using ELISA reactivity towards a recombinant DBL1 α was examined in 53 plasma samples from patients with uncomplicated falciparum malaria in Thailand compared to 13 negative plasma controls and related to disruption of rosette formation. Plasma reactivity to DBL1 α was stronger in patient samples compared to non-immune controls ($p < 0.0001$). Overall, antibody concentrations against DBL1 α did not correlate with rosette disruption of *P. falciparum* strain FCR3S1.2 ($r = -0.06$; $p = 0.72$), but plasma containing antibodies to DBL1 α did disrupt rosette formation by $> 15\%$ in 52% and $\geq 50\%$ in 10% of samples. There was no correlation between presence of antibodies and patient's age. In conclusion, patients with uncomplicated falciparum malaria produce antibodies against DBL1 α domain of PfEMP1, resulting in partial disruption of rosette formation, similar to the results obtained in African children. This might have a protective effect on disease severity.

Keywords: antibody, DBL1 α domain, PfEMP1, rosette formation, uncomplicated falciparum malaria

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INTRODUCTION

Malaria remains a major cause of morbidity and mortality. The vast majority of malaria deaths are caused by *Plasmodium falciparum* (Greenwood *et al*, 2005; Snow

et al, 2005; WHO, 2016). It is known that repeated multiple exposures to *P. falciparum* parasites induce after several years immunity-mediated protection against severe disease. This protective immunity is thought to be mediated partly by antibodies against variable surface proteins expressed by the parasite blood stages, of which *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) is the major antigen (Miller *et al*, 2002; Mackinnon and Marsh, 2010).

Against PfEMP1 (200-350 kDa) is encoded by a *var* family and contains several Duffy antigen binding-like ectodomains (DBL1–5), including one to two cysteine-rich inter-domain regions (CIDRs) (Su *et al*, 1995; Smith *et al*, 2000). PfEMP1 binds to a variety of host cells receptors involved in cytoadherence to host endothelium and in rosette formation whereby infected red blood cells (iRBCs) bind to clusters of uninfected RBCs (MacPherson *et al*, 1985; Kraemer and Smith, 2006). Antibodies to PfEMP1 can disrupt rosette formation and protect against cytoadherence of iRBCs (Carlson and Wahlgren, 1992; Baruch *et al*, 1995; Smith *et al*, 1995). Duffy binding-like domain- α (DBL1 α) has the most conserved sequence of all PfEMP1 domains and mediates the rosetting and cytoadhesive phenotypes of *P. falciparum* iRBCs (Chen *et al*, 1998a; Flick and Chen, 2004; Kraemer *et al*, 2007; Albrecht *et al*, 2011). In addition, heparin or heparan-sulfate can bind directly to DBL1 α domain and thereby disrupt rosetting and cytoadhesive properties of PfEMP1 (Barragan *et al*, 2000; Vogt *et al*, 2003). Thus, it has been proposed that DBL1 α has a central role in parasite rosette formation and sequestration in the microvasculature, central to the pathogenesis of severe malaria (Treutiger *et al*, 1992; Rowe *et al*,

1995; Chen *et al*, 2000; Hedding *et al*, 2001; Pathirana *et al*, 2005).

How immunity to the highly variable PfEMP1 antigens develops is not completely understood. In culture, parasite populations switch to a new variant of PfEMP1 at an average rate of about 2% per asexual cycle, although this can be considerably higher in patients (Roberts *et al*, 1992). Interestingly, the particular *var* expressed is dependent in part on the variant produced in previous cycles, likely the result of selection through the host immune response (Horrocks *et al*, 2004; Frank *et al*, 2007; Mok *et al*, 2008; Duffy *et al*, 2009). Recently it has been shown that plasma samples from African children in a high malaria transmission setting in Cameroon contain antibodies to recombinant DBL1 α (NTS-DBL1 α of IT4var60 gene) expressed by *P. falciparum* strain FCR3S1.2. There was a correlation between these antibody levels and disruption of rosette formation of *P. falciparum* laboratory strain FCR3S1.2 (Albrecht *et al*, 2014). In addition, immunization of rats and macaques against the NTS-DBL1 α domain from *P. falciparum* FCR3S1.2 has been shown to diminish sequestration of this strain (Moll *et al*, 2007). It is not known if patients in low transmission settings, and thus with much less exposure to these antigens, develop a similar response to NTS-DBL1 α and whether plasma obtained from patients in this setting can disrupt rosette formation.

We assessed the levels of naturally circulating antibodies against NTS-DBL1 α domain in plasma from patients with uncomplicated falciparum malaria obtained in low transmission settings in Thailand. The ability of these antibodies to disrupt rosette formation might protect against the severity of falciparum malaria.

MATERIALS AND METHODS

Study subjects and sample collection

Plasma samples were obtained from patients with uncomplicated *P. falciparum* infections admitted to Mae Sot and Mae Ramat Hospital, Tak Province, Thailand (Thai-immune), and from non-infected individuals from non-endemic regions in Bangkok, Thailand (Thai-non immune) and in Stockholm, Sweden (Swedish-non immune). As a positive control, purified IgG obtained from pooled Malawian hyper-immune plasma was used (Taylor *et al*, 1992). Citrate anticoagulated blood samples were collected prior to start of antimalarial drugs treatment. Five ml of *P. falciparum* blood samples ($n = 53$) were collected in acid citrate dextrose tube. Blood samples were prepared for microscopic malaria parasite identification of thick and thin blood smears and blood group assessment. Samples were then centrifuged at 800g for 5 minutes at 4°C and plasmas were stored at -80°C until further assessments.

Written informed consents were obtained from all patients prior to blood sampling. Ethical approvals were obtained from the Ethics Committee of Faculty of Tropical Medicine, Mahidol University, Bangkok (registration no. NCT01442168) and from the ethical committee at Karolinska Institutet (registration no. 2009/3:5).

Heterologous production of recombinant N-terminal sequence of Duffy binding-like α domain (NTS-DBL1 α) in *Escherichia coli*

E. coli SG13009 (Qiagen, Venlo, Netherlands) harboring pQE60 or pQE70 vector (Qiagen, Düsseldorf, Germany) was cultured in LB broth medium at 37°C until $A_{600\text{nm}}$ of 0.8-1.0 was reached, then was incubated for an additional 3 hours at room

temperature after addition of 100 nM isopropyl-beta-D-thiogalactopyranoside. The culture was centrifuged at 990g for 20 minutes and the pellet was suspended in washing buffer (20 mM HEPES, 30 mM imidazole and 500 mM NaCl) and incubated with 1 mg/ml lysozyme (BioSite, San Diego, CA) on ice for 30 minutes, then 1X Complete Cocktail Protease Inhibitor solution (Roche, Basel, Switzerland) was added. The suspension was sonicated for 30 seconds on ice using a Branson Digital Sonifier (Danbury, CT) at 30% amplitude for five times and subsequently centrifuged at 13,250g for 20 minutes. Purification of His-tag NTS-DBL1 α was performed using a His GraviTrap column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. In short, the supernatant was applied onto the column and the flow through solution applied once more to the column, which then was washed extensively with washing buffer and recombinant NTS-DBL1 α eluted with 20 mM HEPES buffer containing 500 mM imidazole and 500 mM NaCl. Elution was monitored at $A_{280\text{nm}}$ and the peak fractions were collected and analyzed by SDS-PAGE. Fractions containing the highest concentration of pure protein were pooled and concentrated. The identity of the purified protein was confirmed by western blot using PfEMP1-variant (ITvar60)-specific antibodies (Angeletti *et al*, 2012).

Assessment of anti-NTS-DBL1 α antibodies

ELISA was used to assess plasma anti-NTS-DBL1 α antibody levels. In brief, Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 10 $\mu\text{g/ml}$ NTS-DBL1 α in coating buffer (15 mM Na_2CO_3 and 35 mM NaHCO_3 , pH 9.6), followed by incubation with 3% (w/v) bovine serum albumin (Sigma-Aldrich) in

phosphate-buffered saline (PBS) for 1 hour at room temperature and then plates were washed three times with PBS-T (0.05% Tween 20 in PBS). Plasma samples [1:500 and 1:1,000 dilution in phosphate-buffered saline (PBS)] were incubated for 1 hour at room temperature. Plates were washed as described above and bound and IgG was detected by incubating for 1 hour at room temperature with alkaline phosphatase-conjugated goat anti-human IgG (Sigma, St Louis, MO) diluted 1:1,000 in PBS, followed by incubation with p-nitrophenyl phosphate solution (Sigma) for 45 minutes. $A_{405\text{ nm}}$ then was measured in an ELISA plate reader (Multiskan EX Version 1.2, LabSystems, Stockholm, Sweden). Cut-off threshold for seropositivity is defined as mean plus standard deviation (SD) value of negative control samples (Albrecht *et al*, 2014). All assays were performed in duplicate.

Rosette disruption assay

Rosetting formation and disruption by plasma were assessed using *P. falciparum* strain FCR3S1 with high rosetting capability and the ability to bind non-immune IgM (Fernandez *et al*, 1998). Cut-off point for rosette disruption is defined as > 15% disruption compared to control (Carlson *et al*, 1990). Parasites were cultured using standard protocol (Moll *et al*, 2008). In brief, parasites were cultivated at 5% hematocrit in malaria culture medium supplemented with 10% Swedish non-immune AB+ serum and synchronized at ring stage by treating with 5% (w/v) sorbitol (Lambros and Vanderberg, 1979). Rosetting rate was determined by calculating the number of trophozoite-infected red blood cells (iRBCs) forming rosettes relative to total number of trophozoite iRBCs present in culture. A rosette is defined as at least two or more uninfected RBCs bound to one iRBC (Moll *et al*, 2008).

The ability of a patient's plasma to disrupt rosettes was assayed as described previously (Treatiger *et al*, 1992). In brief, plasma (1:5 dilution) was added to 20 ml of parasite culture (2% hematocrit and 5-10% parasitemia and incubated at 37°C for 60 minutes. Parasites were stained with acridine orange and rosetting rate was measured. For each sample at least 100 iRBC were counted. As a positive control, Malawian IgG from hyper-immune plasma pool was used (Taylor *et al*, 1992) and plasma samples from non-immune Thai and Swedish blood donors were employed as negative controls.

Statistical analysis

Data were analyzed using SPSS version 18 statistical software (IBM, Armonk, NY) and GraphPad Prism version 5 (GraphPad Software, La Jolla, CA). Correlation between antibody levels and rosette disruption were assessed by Pearson's method. Relationship between antibody levels, rosette disruption and admission parasite density were assessed by Spearman's method. A *p*-value of < 0.05 is considered significant.

RESULTS

Plasma anti-NTS-DBL1 α immune status

Of a total of 53 Thai patients with uncomplicated *P. falciparum*, mean (SD) age was 30 (9) years, hematocrit 38% (5%), and median (range) parasite density 26,777 (10,034-99,590)/ μ l. Using recombinant NTS-DBL1 α as antigen in an ELISA, $A_{405\text{ nm}}$ (mean \pm SD) of non-immune Thai and Swedish plasma negative samples was 0.30 ± 0.30 (Fig 1), and $A_{405\text{ nm}}$ (mean \pm SD) of Malawian IgG from hyper-immune plasma pool (positive control) was 3.26 ± 0.13 . Using a cut-off value of $A_{405\text{ nm}}$ 0.60, 41/53 (77%) Thai-immune plasma samples were positive for presence of anti-NTS-

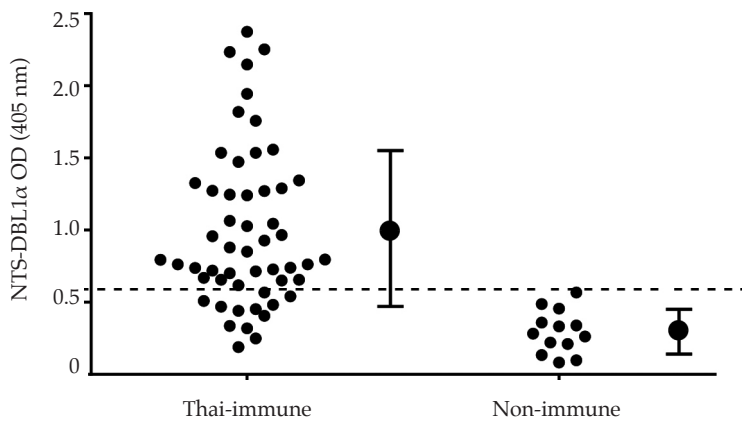


Fig 1—Naturally acquired antibody level to NTS-DBL1 α in 53 plasma samples from Thai patients with uncomplicated falciparum malaria. Antibody level to affinity purified recombinant NTS-DBL1 α was measured by ELISA and expressed as OD_{405 nm}. Negative controls were 13 non-immune Thai and Swedish plasma samples. Dash horizontal line indicates cut-off value (OD_{405 nm} = 6.0). The long and short bar indicates mean and \pm SD, respectively.

DBL1 α antibodies (Fig 1). The presence of antibodies against NTS-DBL1 α was negatively correlated with admission parasite density (Spearman $r = -0.296$, $p = 0.033$) and admission hematocrit (Pearson $r = -0.357$, $p = 0.009$), but not with age (Pearson $r = -0.112$, $p = 0.425$) (Fig 2).

Plasma rosette disrupting activity

Thai plasma samples ($n = 42$) were tested for activity against rosette formation. Mean (SD) age and hematocrit of the patients was 30.5 (8.9) years and 39% (4%), respectively. Median (range) parasite density was 25,153 (10,034-99,590)/ μ l. A total of 22/42 (52%) plasma samples were able to disrupt rosette formation of *P. falciparum* FCR3S1.2 iRBCs (Fig 3). In 4/42 (10%) samples rosette disruption was $\geq 50\%$, and 18 (43%) plasma samples with positive anti-NTS-DBL1 α antibodies showed rosette disruption activity ($>15\%$

of rosettes were disrupted). Only 4 (9%) plasma samples with negative anti-NTS-DBL1 α antibodies showed rosette disruption activity. However, the proportion of rosette disruption is not significantly correlated with the antibody level against NTS-DBL1 α (Pearson $r = -0.058$, $p = 0.716$) (Fig 3) or with patient's age (Pearson $r = 0.199$, $p = 0.206$), admission parasite density (Spearman $r = 0.059$, $p = 0.712$) or admission hematocrit (Pearson $r = -0.044$, $p = 0.787$) (data not shown).

DISCUSSION

Adherence of *P. falciparum* iRBCs to endothelial cells and to uninfected RBCs

(rosette formation) contribute to microcirculatory flow obstruction, which forms a central feature in the pathophysiology of falciparum malaria (MacPherson *et al*, 1985; Wahlgren *et al*, 1994; Cooke and Coppel *et al*, 1995; Miller *et al*, 2002; Kirchgatter and Del Portillo 2005; Dondorp *et al*, 2008a). Both adherence phenotypes are conferred by PfEMP1; of which those involved in rosette formation are encoded by group A *var* genes (Chen *et al*, 1998b; Jensen *et al*, 2004). The NTS-DBL1 α domain of PfEMP1 encoded by IT4*var*60 gene is implicated in rosette formation and is associated with severe disease (Albrecht *et al*, 2014).

In the current study, plasma from patients with uncomplicated falciparum malaria contained antibodies reactive towards recombinant NTS-DBL1 α . Antibody levels were negatively correlated

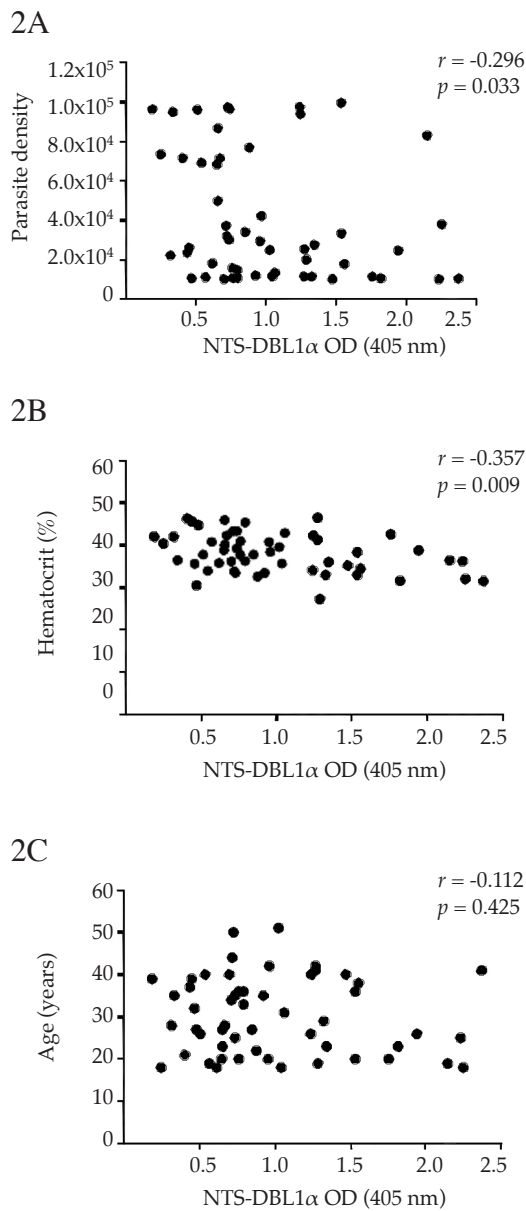


Fig 2—Correlation between naturally acquired plasma antibody levels towards recombinant NTS-DBL1α in 53 Thai patients with uncomplicated falciparum malaria and (A) admission parasite density, (B) admission hematocrit, and (C) patient’s age. Antibody level was measured as described in legend to Fig 1. r , correlation coefficient; p , p -value.

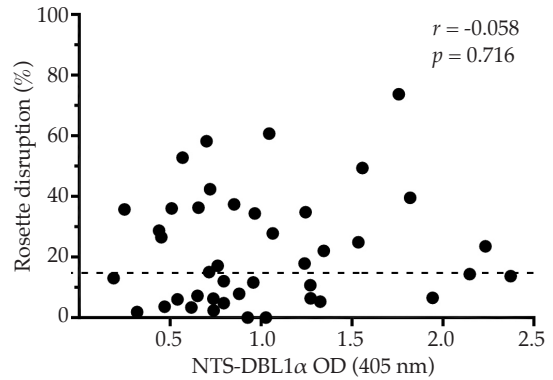


Fig 3—Correlation between naturally acquired antibody levels towards recombinant NTS- DBL1α and rosette disruption in *P. falciparum* laboratory strain FCR3S1.2, assessed in plasma from 42 Thai patients with uncomplicated falciparum malaria. Antibody level was measured as described in legend to Fig 1. A rosette is defined as at least two or more uninfected RBCs bound to one trophozoite infected (i)RBC. For each sample at least 100 iRBC were counted. Dash line indicates cut-off value (15%). r , correlation coefficient; p , p -value.

with parasite density and hematocrit. This is in agreement with findings of an African study in a high transmission setting (Albrecht *et al*, 2014). However, unlike in the African setting, multiple parasite clonal infections in Asian low transmission setting are relatively rare, (Anderson *et al*, 2011). High parasitemia and low hematocrit in low transmission settings usually denote longer duration of infection (Dondorp *et al*, 2008b; Tangpukdee *et al*, 2012; WHO, 2012), which could explain the phenomena observed.

Surface exposed malaria parasite molecules, such as PfEMP1, show high variability between strains as well as within strains during infection, thereby contributing to host immune evasion (Nielsen *et al*, 2002; Chan *et al*, 2014). However,

within the PfEMP1 sequence, the DBL1 α domain is much more conserved (Ward *et al*, 1999; Flick *et al*, 2004; Ralph and Scherf, 2005), and antibodies against recombinant NTS-DBL1 α domain have been shown to cross-react against various rosetting-associated variants (Magistrado *et al*, 2007). In this study we confirm that a majority (52%) of plasma samples containing antibodies against the conserved DBL1 α domain disrupted rosette formation of a high rosetting forming *P. falciparum* FCR3S1.2 strain. Although studies from Africa in high transmission settings showed a correlation between DBL1 α antibody level and the ability of rosette disruption (Albrecht *et al*, 2014), but this was not the case in a setting of low malaria transmission. This was not observed in the current study. A possible explanation is that DBL1 α antibodies in the current study may have higher avidity than in the African study, so that lower antibody levels are able to mediate rosette disruption.

In conclusion, PfEMP1-DBL1 α from infected erythrocytes in patients with uncomplicated malaria in Thailand induces an antibody response that is able to disrupt rosette formation. Further studies will be needed to investigate the relevance of these findings to the pathophysiology of patients with uncomplicated falciparum in low transmission settings.

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