Acquisition of IgG to ICAM-1-Binding DBL Domains in the Plasmodium falciparum erythrocyte membrane protein 1 antigen family varies between Groups A, B, and C

Olsen, Rebecca W; Ecklu-Mensah, Gertrude; Bengtsson, Anja; Ofori, Michael F; Kusi, Kwadwo A; Koram, Kwadwo A; Hviid, Lars; Adams, Yvonne; Jensen, Anja T R

Published in:
Infection and Immunity

DOI:
10.1128/IAI.00224-19

Publication date:
2019

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
Olsen, R. W., Ecklu-Mensah, G., Bengtsson, A., Ofori, M. F., Kusi, K. A., Koram, K. A., ... Jensen, A. T. R. (2019). Acquisition of IgG to ICAM-1-Binding DBL Domains in the Plasmodium falciparum erythrocyte membrane protein 1 antigen family varies between Groups A, B, and C. Infection and Immunity, 87(10), [e00224-19]. https://doi.org/10.1128/IAI.00224-19
Acquisition of IgG to ICAM-1-Binding DBLβ Domains in the Plasmodium falciparum Erythrocyte Membrane Protein 1 Antigen Family Varies between Groups A, B, and C

Rebecca W. Olsen, Gertrude Ecklu-Mensah, Anja Bengtsson, Michael F. Ofori, Kwadwo A. Kusi, Kwadwo A. Koram, Lars Hviid, Yvonne Adams, Anja T. R. Jensen

Centre for Medical Parasitology at Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
Department of Immunology, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana
Department of Epidemiology, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana
Department of Infectious Diseases, Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark

ABSTRACT Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) is an important malaria virulence factor. The protein family can be divided into clinically relevant subfamilies. ICAM-1-binding group A PfEMP1 proteins also bind endothelial protein C receptor and have been associated with cerebral malaria in children. IgG to these PfEMP1 proteins is acquired later in life than that to group A PfEMP1 not binding ICAM-1. The kinetics of acquisition of IgG to group B and C PfEMP1 proteins binding ICAM-1 is unclear and was studied here. Gene sequences encoding group B and C PfEMP1 with DBLβ domains known to bind ICAM-1 were used to identify additional binders. Levels of IgG specific for DBLβ domains from group A, B, and C PfEMP1 binding or not binding ICAM-1 were measured in plasma from Ghanaian children with or without malaria. Seven new ICAM-1-binding DBLβ domains from group B and C PfEMP1 were identified. Healthy children had higher levels of IgG specific for ICAM-1-binding DBLβ domains from group A than from groups B and C. However, the opposite pattern was found in children with malaria, particularly among young patients. Acquisition of IgG specific for DBLβ domains binding ICAM-1 differs between PfEMP1 groups.

KEYWORDS PfEMP1, Plasmodium falciparum, antibodies, immunity, malaria
cysteine-rich interdomain region (CIDR) domains (2–4, 14). Particular subtypes of DBLβ and CIDRα domains have been associated with binding to endothelial receptors such as intercellular adhesion molecule 1 (ICAM-1), endothelial protein C receptor (EPCR), and CD36 (15–17). More recently, we identified particular group A PfEMP1 proteins that can bind both ICAM-1 and EPCR (18). The ICAM-1-binding DBLβ domains of such group A PfEMP1 proteins are characterized by a specific sequence motif, and IgG specific to them is acquired later in life than IgG specific for group A DBLβ domains that do not bind ICAM-1 (18, 19).

The acquisition pattern of ICAM-1-binding group B and C DBLβ-specific IgG is unknown. Therefore, the current study was designed to provide such data and to compare IgG reactivity to that of different subtypes of DBLβ domains in Ghanaian children with or without P. falciparum malaria. The aim was to provide increased understanding of how antibody-mediated immunity to PfEMP1 is acquired following natural exposure to P. falciparum.

RESULTS

Identification of ICAM-1-binding DBLβ domains. We have previously identified a novel family of group A ICAM-1-binding DBLβ domains associated with cerebral malaria (18). Here, we used BLASTP searches and amino acid sequences encoding ICAM-1-binding group B and group C DBLβ domains from P. falciparum IT4 (20) to search for additional DBLβ domains predicted to bind ICAM-1. Seven new sequences were identified by this approach. The encoded domains were a DBLβ3-type domain (GenBank accession no. KOB58843/HB3VAR34) and a DBLβ5-type domain (KOB63129/HB3VAR21) from HB3, two DBLβ5-type domains from Dd2 (AAA75396/Dd2VAR01A and KOB84711/Dd2VAR21), one DBLβ5-type domain from 3D7 (PFL0020w), and one DBLβ5-type domain from each of two field isolates (ERS009963 and ERS010653). Dd2VAR21/KOB84711 was identical to the previously published IT4VAR13, except for one residue (E instead of V) in DBLα and one residue (C instead of R) in the ATS region. All seven new domains bound ICAM-1 as predicted (Fig. 1A) and clustered together with other ICAM-1-binding DBLβ domains from groups B and C (Fig. 1B). The average sequence similarity of the new group B and C ICAM-1-binding DBLβ domains was 50%, which is comparable to that of previously identified ICAM-1-binding group A domains (58%) (18). Domains downstream of the ICAM-1-binding DBLβ domains belonged to groups and subgroups similar to those in the previously identified ICAM-1-binding group B and C PfEMP1 proteins (Fig. 1C). To validate these findings further, we immunized rats with one of the domains (HB3VAR21-DBLβ5_D4) and used the antiserum to select P. falciparum HB3 to express VAR21 on the surface of IEs (Fig. 1D). HB3VAR21+ IEs bound ICAM-1 at high levels (Fig. 1E), confirming the ability to predict the IE adhesion phenotype from var gene sequences.

IgG specific for ICAM-1-binding group A DBLβ domains dominates in healthy children. Group A PfEMP1-specific IgG is acquired earlier in life than antibodies targeting group B and C PfEMP1 antigens, and IgG to group A PfEMP1 therefore tends to dominate among healthy individuals living in areas with natural transmission of P. falciparum parasites (12, 21, 22). This was also the case here, when we compared IgG reactivity to ICAM-1-binding DBLβ domains in group A and group B PfEMP1 proteins, employing plasma from a cohort of healthy Ghanaian children. Because only small sample volumes were available for this testing, we selected five of the ICAM-1-binding group B DBLβ proteins identified above and five previously identified corresponding domains from group A (19). The IgG reactivity to each of the ICAM-1-binding DBLβ domains from group A was higher than the reactivity to any of the domains from group B. This was consistently the case with samples from the same donors but collected at six different time points over a 1-year period (Fig. 2; also see the data set in the supplemental material). This finding extends the earlier reports by demonstrating that the dominance of group A PfEMP1-specific IgG among healthy individuals remains when the comparison is restricted to ICAM-1-binding DBLβ domains only.
FIG 1 ICAM-1-binding DBLβ domains. (A) ICAM-1 binding (ELISA optical density at 450 nm [OD450 nm]; means ± SD from three independent experiments) of 11 DBLβ domains, seven of which were predicted to bind ICAM-1 (6 group B and 1 group C). PFD1235w_D4 (group A) and IgG to ICAM-1-Binding DBLβ Domains Infection and Immunity October 2019 Volume 87 Issue 10 e00224-19 iai.asm.org (Continued on next page)
However, when we used plasma from children with acute *P. falciparum* malaria instead of plasma from healthy children, the pattern was the opposite, as levels of IgG specific for group B DBL\(/\)/H9252 domains were higher than those for group A domains (Fig. 2). This suggests that acute malaria episodes markedly perturb the steady-state ("healthy") hierarchy of IgG reactivity to group A and group B PfEMP1 proteins, leading to a transient inversion of the group-specific IgG ratio. To examine this possibility further, we proceeded with a more detailed analysis of DBL\(/\)/H9252-specific antibody responses, including kinetics and a larger panel of domains.

The PfEMP1 group hierarchy of DBL\(/\)/H9252-specific IgG is influenced by malaria episodes. Plasma levels of IgG to *P. falciparum* antigens, including PfEMP1, tend to increase in relation to malaria episodes among individuals with natural exposure to these parasites but decline again shortly after resolution of the infection (23, 24). The IgG responses to a large panel of DBL\(/\)/H9252 domains in Ghanaian children monitored over 6 weeks after acute *P. falciparum* malaria episodes showed a similar pattern. Responses were highly variable, and marked but mostly transient IgG responses to all the different types of DBL\(/\)/H9252 domains were observed in some but not all children (Fig. 3; also see the data set in the supplemental material). Nevertheless, the most prominent overall increase in DBL\(/\)/H9252-specific IgG reactivity associated with malaria episodes was to group B and C antigens (Fig. 3C). This finding was underpinned by analysis of IgG responses to the individual DBL\(/\)/H9252 domains 2 weeks after admission, where responses generally peaked compared to the levels on admission and at week six (Fig. 3 and 4; also see the data set in the supplemental material). At that time, IgG reactivity to all but one of the ICAM-1-binding DBL\(/\)/H9252 antigens from group B and C PfEMP1 was higher than that to ICAM-1-binding group A domains (Fig. 4B). The difference between the two groups of PfEMP1 antigens was due to low IgG reactivity against group A DBL\(/\)/H9252 domains in children younger than 7 years (Fig. 4C). Furthermore, the IgG reactivity to group B and C domains did not differ between age groups (*P* = 0.5) (Fig. 4C), and significantly more
of the children were seropositive for group B DBLβ/H9252-specific IgG (62 to 85%) than for corresponding domains from group A (30 to 60%) (P < 0.05) (Table S3). Overall, it appears that most of the clinical episodes involved parasite populations expressing a mixture of PfEMP1 variants, including proteins containing different types of DBLβ/H9252 domains (Table S3), although parasites expressing group A ICAM-1-binding DBLβ/H9252 domains seemed underrepresented among the younger children. Furthermore, our data suggest that prominent responses to group B and C DBLβ/H9252 domains can cause a transient inversion of the ratio of IgG specific for ICAM-1-binding group A as well as group B and C DBLβ.

IgG reactivity to ICAM-1-binding DBLβ/H9252 domains in group A and groups B and C is similar in children with uncomplicated and those with severe, noncerebral malaria. Previous studies have shown that IEs obtained from young children with severe malaria primarily express PfEMP1 encoded by group A var genes, while expression of PfEMP1 encoded by group B and group C var genes appears associated with uncomplicated disease in slightly older children (8, 25–27). We therefore proceeded to compare the DBLβ-specific IgG responses in children with severe malaria to those in children with uncomplicated malaria. IgG reactivity to ICAM-1-binding group B and C DBLβ domains was higher than that to corresponding group A domains when each group of patients was considered separately (Fig. 5; also see the data set in the supplemental material). However, no statistically significant differences were noted when IgG reactivity to ICAM-1-binding DBLβ domains from either group A or groups B and C was compared between children with severe or uncomplicated malaria (Fig. 5). While this may seem at variance with the well-documented relationship between expression of group A PfEMP1 and severe malaria, it should be noted that the subset of group A dual-receptor-binding PfEMP1 containing ICAM-1-binding DBLβ domains has been associated specifically with cerebral malaria and not severe malaria in general (18), and that only 2 of the 124 children studied fulfilled the criteria for such a diagnosis.

**DISCUSSION**

*P. falciparum* causes the most severe form of malaria and is responsible for the vast majority of malaria-related deaths (28). This is not least due to the presence in this species of the PfEMP1 family of adhesive proteins, which are expressed on the surface of IEs in a mutually exclusive manner (only one variant expressed at a time) (4). Different members of the PfEMP1 family enable the adhesion of IEs to a range of vascular host receptors, which facilitate IE evasion of splenic clearance (5). It furthermore promotes tissue inflammation and organ dysfunction, while parasite switching among different
FIG 4 Plasma levels of IgG specific for DBLβ domains from group A, group B, and group C. Samples were obtained from 124 Ghanaian children diagnosed with severe or nonsevere *P. falciparum* malaria 2 weeks prior to taking the blood sample. (A) Levels of IgG antibodies specific for individual ICAM-1-binding DBLβ domains (columns) from group A (left) and groups B and C (center) or for non-ICAM-1-binding domains from group A (right). Shading indicates the IgG level score: black, 4; dark gray, 3; gray, 2; light gray, 1; white, 0. The domain subtypes are indicated in Table S1. Danish controls (*n* = 20) did not react with any of the domains (data not shown). (B) Mean week 2 scores of IgG specific for individual ICAM-1-binding DBLβ domains from group A (black) and groups B and C (white). Error bars indicate 95% confidence intervals. (C) Mean IgG scores against all DBLβ domains in individual children, grouped according to age. Medians are indicated by horizontal red lines.
PfEMP1 family members (variants) frustrates the development of protective PfEMP1-specific immunity (reviewed in reference 1).

Severe *P. falciparum* malaria has repeatedly been linked to IE adhesion to particular host receptors, mediated by groups and subgroups of structurally related group A PfEMP1 (reviewed in reference 1). Group A PfEMP1 proteins mediating adhesion to EPCR appear to be of particular relevance to the pathogenesis of severe malaria in patients with or without cerebral symptoms (8, 17, 25, 26, 29, 30). Cerebral malaria, one of the most severe complications of *P. falciparum* infection (reviewed in reference 31), is associated with expression of a subgroup of group A PfEMP1 variants with a dual-receptor adhesion phenotype (18, 29). These proteins carry an ICAM-1-binding DBL/H9252 domain next to an EPCR-binding CIDR/H9251 domain. Although ICAM-1-binding DBL/H9252 domains also occur in group B and C PfEMP1, these domains are structurally distinct, do not have a neighboring EPCR-binding but a CD36-binding CIDR/H9251-6 domain, and do not appear to play a role in cerebral malaria pathogenesis (18).

Two recent studies found similar antibody reactivity against group A CIDR/H9251 in uncomplicated and severe malaria during acute disease (34, 35), while at convalescence older children with severe (likely noncerebral) malaria had higher antibody levels against such EPCR-binding CIDR/H9251 than those with uncomplicated malaria (34). The PfEMP1 reactivity between convalescent groups did not differ in the study by Kessler et al. (35), although higher seroprevalence to the conserved group A-associated ICAM-1-binding DBL/H9252 domain (18) was observed relative to that of CIDR/H9251.

In a longitudinal study assessing antibody acquisition against 32 non-ICAM-1-binding DBL/H9252 domains (three α, eight β, five γ, nine δ, six ε, and one ζ), asymptomatic Tanzanian children were shown to acquire antibodies to group A prior to group B and C domains (13). In addition, it has recently been shown that the breadth of antibodies that inhibit adhesion of IEs to ICAM-1 increases with age in Malian children (36), although the study did not investigate whether these IEs expressed group A, B, or C PfEMP1 antigens. We find that among group A PfEMP1 proteins, acquisition of IgG to DBL/H9252 domains that do not bind ICAM-1 appears to precede acquisition of IgG to those that do (19).

As the acquisition pattern of IgG specific for ICAM-1-binding DBL/H9252 domains from groups B and C is not currently known, we first used a BLASTP search to extract new potential ICAM-1-binding DBL/H9252 domains from these PfEMP1 groups. Seven such domains were identified and shown to bind ICAM-1 and to be structurally related to known ICAM-1-binding DBL/H9252 domains from group B and C PfEMP1 (Fig. 1).

In healthy children, we found that IgG reactivity to ICAM-1-binding DBL/H9252 domains...
from group A was higher than reactivity to corresponding domains from group B and C PfEMP1 (Fig. 2). Overall, these findings are in agreement with the previously reported dominance of responses to group A PfEMP1 over other PfEMP1 groups (12, 13, 37). However, when the assays were repeated with plasma obtained from children with acute malaria, we found higher overall IgG reactivity to ICAM-1-binding DBLβ domains from groups B and C rather than group A (Fig. 2). Furthermore, the most prominent change after acute malaria was a transient increase in IgG reactivity to ICAM-1-binding DBLβ domains from groups B and C (Fig. 3), and 2 weeks after the acute attack, reactivity to each of the group B and C ICAM-1-binding DBLβ domains was higher than that to the corresponding group A domains (Fig. 4). The most parsimonious explanation for the disease-related inversion of the ratio of IgG reactivity to ICAM-1-binding DBLβ domains from group A versus groups B and C is that the disease episodes in our study children were caused mainly by parasites expressing group B and C PfEMP1 or group A PfEMP1 without ICAM-1-binding DBLβ domains. This interpretation is consistent with the fact that only two of the study children were diagnosed with cerebral malaria (associated with parasites expressing group A DBLβ domains [18]). This in turn may explain why we did not observe differences in IgG reactivities between children of similar age with severe and uncomplicated malaria (Fig. 5). In agreement with this, a recent study found that children with uncomplicated and cerebral malaria had similar breadth and magnitude of responses to different P. falciparum antigens, including DBLβ domains (35). Finally, our data suggest that IgG specific for ICAM-1-binding DBLβ domains from group A and associated specifically with cerebral malaria (18) is acquired later in life than IgG specific for ICAM-1-binding DBLβ domains from group B and C PfEMP1 (Fig. 4C). We previously made a similar observation when IgG reactivity to ICAM-1-binding DBLβ domains in group A was compared to reactivity to DBLβ domains from the same group that do not bind ICAM-1 (19). Thus, the age where IgG specific for group A ICAM-1-binding DBLβ domains is acquired coincides with the age where cerebral malaria incidence peaks (38, 39). This is in striking contrast to the case for group A DBLβ domains that do not bind ICAM-1 (19) and for group B and C DBLβ domains that do (this study). However, whether a causal relationship exists remains to be investigated in a study area where the incidence of cerebral malaria is higher.

Opsonizing antibodies against PfEMP1 have been suggested to play a role in immunity to P. falciparum malaria (40), while other mechanisms, such as recruitment of complement (41), interaction with immune cells (42), and inhibition of vascular adhesion, might also play a role. Sequestration of P. falciparum IEs to the microvascular endothelium contributes to the pathogenesis of severe malaria in children, and broadly, cross-reactive antibodies inhibiting the interaction between ICAM-1 and DBLβ domains are detectable in immune plasma (18, 19, 29). We were unable to test these potential effector functions due to the limited plasma volumes available, but these are aspects that should be investigated in future studies.

In conclusion, our study demonstrates significant differences in the acquisition of IgG to ICAM-1-binding DBLβ domains from group A as well as group B and C PfEMP1. These differences are likely to be of significance in the development of PfEMP1-based vaccines to prevent severe P. falciparum malaria in general and cerebral malaria in particular (43).
| Characteristic                  | Severe (n = 50) | Uncomplicated (n = 74) |
|--------------------------------|----------------|------------------------|
|                                | 1–2 yr (n = 16) | 3–4 yr (n = 16)        | 5–6 yr (n = 13) | >6 yr (n = 5) | 1–2 yr (n = 20) | 3–4 yr (n = 25) | 5–6 yr (n = 14) | >6 yr (n = 15) |
| Age (yr)                       | 2.1 (1.7; 2.4)  | 4.2 (3.2; 4.5)         | 5.5 (5.3; 5.6) | 7.8 (7.7; 8.3) | 2.3 (1.8; 2.7)  | 4.1 (3.5; 4.3)  | 6.5 (5.4; 6.8)  | 9.0 (8.0; 10.7) |
| Blantyre coma score            | 5.0 (5.0; 5.0)  | 5.0 (3.3; 5.0)         | 5.0 (5.0; 5.0) | 5.0 (3.0; 5.0) | 5.0 (5.0; 5.0)  | 5.0 (5.0; 5.0)  | 5.0 (5.0; 5.0)  | 5.0 (5.0; 5.0)  |
| Hemoglobin (g/dl)              | 8.6 (6.1; 10.0) | 8.2 (7.4; 8.6)         | 9.1 (8.0; 10.6) | 9.8 (7.9; 11.8) | 10.0 (9.4; 10.6) | 9.8 (8.1; 11.2) | 10.9 (9.5; 11.2) | 11.2 (10.4; 11.8) |
| Parasitemia                   | 87.8 (24.5; 152.8) | 101.7 (8.8; 161.0) | 52.2 (5.9; 148.7) | 77.5 (49.2; 151.5) | 42.5 (31.3; 85.3) | 34.7 (12.3; 62.0) | 23.5 (11.8; 82.8) | 30.9 (13.0; 62.3) |
| Parasitemia                   | 87.8 (24.5; 152.8) | 101.7 (8.8; 161.0) | 52.2 (5.9; 148.7) | 77.5 (49.2; 151.5) | 42.5 (31.3; 85.3) | 34.7 (12.3; 62.0) | 23.5 (11.8; 82.8) | 30.9 (13.0; 62.3) |
| Temperature (°C)              | 38.9 (38.1; 39.7) | 38.1 (36.8; 39.2) | 38.5 (37.5; 39.2) | 38.4 (36.7; 38.6) | 38.6 (37.5; 39.3) | 38.4 (36.8; 39.5) | 38.8 (38.2; 39.2) | 38.6 (37.4; 39.6) |

*Values are medians (25th; 75th percentile). Two study participants were diagnosed with cerebral malaria.
admission and 2 and 6 weeks later. Patients receiving blood transfusion prior to follow-up were excluded from the study. Plasma collected from healthy children (n = 91) (Table 2) was also included in the study (46). A minority of those children had occasional, asymptomatic parasitemia. The study was approved by the Ethical Review Committee of the Ghana Health Services (file GHS-ERC 08/2015).

### Identification of group B and group C DBLβ domains

*P. falciparum* IT4 amino acid sequences encoding published ICAM-1-binding group B and group C DBLβ domains, var01 (GenBank accession no. AAO67411), var13 (ABM88750), var14 (AAD03351), var16 (AAS89259), var27 (ABM88759), var31 (AAF18980), and var41 (ABM88768) (20), were used to search new potential ICAM-1-binding DBLβ domains from GenBank or from assemblies of Illumina whole-genome sequencing data as described previously (47).

### Production of recombinant proteins

His-tagged DBLβ domains (see Table S1 in the supplemental material) were expressed in Escherichia coli Shuffle C3030 cells (New England Biolabs) from synthetic genes (https://eurofins.dk) or from DNA constructs generated by PCR from genomic DNA using specific primers (Table S2). The domains were purified by immobilized metal affinity chromatography (18, 19, 48). Recombinant ICAM-1-Fc (D1-D5 with a human Fc tag) was expressed in HEK293 cells and purified on a HiTrap protein G HP column (GE Healthcare) as described previously (48).

### ELISA

Binding of ICAM-1-Fc to immobilized DBLβ domains and levels of antigen-specific IgG in human plasma (diluted 1:100) were assessed by enzyme-linked immunosorbent assay (ELISA) using MaxiSorp plates (Sigma-Aldrich). The plates were coated with 22 group A and nine group B and C recombinant DBLβ proteins (2 μg/ml overnight at 4°C). Sixteen domains were already known to bind ICAM-1 (18, 20), eight were already known not to bind ICAM-1 (19), and the remaining seven DBLβ proteins (groups B and C) were predicted to bind ICAM-1 based on the sequence analysis done in this study.

Binding of ICAM-1-Fc and human IgG antibodies to the immobilized DBLβ domains was detected using horseradish peroxidase-conjugated rabbit anti-human IgG (1:3,000; Dako) (18). Plates were developed using TMB PLUS2 (Kem-En-Tec) according to the manufacturer’s instructions. Optical density (OD) values were read at 450 nm using a VersaMax microplate reader (Molecular Devices). Plasma antibody reactivity was expressed in arbitrary ELISA units (EU) calculated by the equation

\[\text{OD}_{\text{sample}} - \text{OD}_{\text{background}}/\left(\text{OD}_{\text{positive control}} - \text{OD}_{\text{background}}\right) \times 100 (49)\]

and translated into IgG level scores 0 (0 to 25 EU), 1 (26 to 100 EU), 2 (101 to 250 EU), 3 (251 to 750 EU), and 4 (>750 EU). A pool of *P. falciparum*-exposed Tanzanian individuals and 26 nonexposed Danish individuals were used as positive and negative controls, respectively. The mean (plus two standard deviation (SD)) value among the negative-control plasma samples was used as a cutoff value to define seropositivity.

### Generation of antiserum

Rats were immunized with recombinant HB3VAR21-DBLβ_D4 as described previously (48). All experimental animal procedures were approved by The Danish Animal Procedures Committee (Dyreforsøgstilsynet) as described in permit no. 2013-15-2934-00920 and according to the guidelines described in Danish acts LKB 1306 (23/11/2007) and BEK 1273 (12/12/2005).

### Plasmodium falciparum parasite culture

*P. falciparum* HB3 was maintained in vitro and selected with rat anti-HB3VAR21-DBLβ_D4 as described previously (48, 50). IE surface expression of PIMEP1 was regularly monitored by flow cytometry, and only cultures with more than 60% HB3VAR21+ IES were used. The identity and clonality of the parasites used were routinely verified by genotyping as described previously (51). Mycoplasma infection was excluded regularly using the MycoAlert mycoplasma detection kit (Lonza) according to the manufacturer’s instructions.

### Flow cytometry

*P. falciparum* IES were DNA labeled with ethidium bromide and surface labeled with rat anti-HB3VAR21-DBLβ_D4 (1:20) and fluorescein isothiocyanate (FITC)-conjugated secondary rabbit anti-rat IgG (1:150; Vector Labs) as described previously (50). Fluorescence data from ethidium bromide-positive cells were collected on an FC500 MPL flow cytometer (Beckman Coulter) and analyzed using WinList, version 9.0 (Verity Software House).

### Parasite adhesion spot assay

Falcon 1007 petri dishes were coated overnight (4°C) with recombinant CD36 (0.05 μg/spot), ICAM-1-Fc (0.3 μg/spot), or EPCR (0.1 μg/spot) in triplicates (48). Dishes were blocked (1 h) in phosphate-buffered saline (PBS) with 3% bovine serum albumin (BSA). Mature IES were adjusted to 1% parasitemia and 1% hematocrit in RPMI 1640 supplemented with 2% normal human serum, added to the dishes, and incubated (37°C, 30 min) as described previously (52). After removal of nonadherent IES by sequential washing using prewarmed RPMI wash buffer, the remaining cells were fixed in 1.5% glutaraldehyde (15 min) and stained with Giemsa. After rinsing with water, the dishes were

---

**TABLE 2** Characteristics of healthy Ghanaian study participants

| Characteristic | Value for year: |  |
|---------------|-----------------|---|
|               | May (n = 78)    | Sept (n = 86) | Nov (n = 80) | Jan (n = 78) | March (n = 73) | May (n = 63) |
| Age (yr)      | 4 (3; 4.5)      | 4 (3; 4.5)    | 4 (3; 4.5)   | 4 (3; 4.5)   | 4 (2.5; 4.5)  | 4 (2.75; 4.5) |
| Hemoglobin (g/dl) | 10.7 (9.6; 11.3) | 10.1 (8.9; 10.9) | 10.5 (9.2; 11.3) | 10.8 (9.9; 11.7) | 10.3 (9.3; 11.5) | 11.0 (10.3; 11.9) |
| Parasitemia (no. of parasites/μl) | 1,240 (560; 3,610) | 2,400 (1,060; 7,930) | 1,500 (510; 3,850) | 2,080 (670; 4,970) | 920 (360; 2,560) | 1,000 (600; 2,320) |
| Temperature (°C) | 37.1 (36.8; 37.2) | 37.1 (36.8; 37.2) | 37.0 (36.8; 37.1) | 37.0 (36.7; 37.2) | 37.1 (36.9; 37.3) | 37.1 (36.9; 37.2) |

*Values are medians (25th; 75th percentile).*
air dried overnight before the number of adherent IEs per square millimeter was quantified using ImageJ software (http://rsb.info.nih.gov/ij/). A minimum of three independent experiments in triplicate were done. All assays were blinded.

**PIEMP1 sequence similarity and phylogenetic trees.** The Praline multiple-sequence alignment tool (http://www.ibi.vu.nl/programs/praline/) was used to calculate the average amino acid similarity of DBLβ domains. Multiple alignments of DBLβ domains were made using Muscle (https://www.ebi.ac.uk/Tools/msa/muscle/) and analyzed using Mega 5.0 software (53) to create cladograms.

**Statistics.** We used Kruskal-Wallis one-way analysis of variance on ranks and Mann-Whitney test to assess intergroup differences. Data analysis was done using SigmaPlot 13.0 (Systat Software Inc., United Kingdom).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00224-19.

**SUPPLEMENTAL FILE 1**
PDF file, 0.04 MB.

**SUPPLEMENTAL FILE 2**
XLSX file, 0.3 MB.

**ACKNOWLEDGMENTS**

We acknowledge the technical support of the field team in Ghana and thank all study participants for their generous contribution of blood samples. Mette U. Madsen is thanked for excellent technical assistance.

This work was supported by Novo Nordisk Fonden (NNF16OC0022298), Lundbeckfonden (R180-2014-3098), the Danish Council for Independent Research (4004-0003), the Consultative Committee for Development Research (12-081RH), and the Division of Microbiology and Immunology Diseases, NIAID, NIH (contract number HHSN266200400016C). The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

**REFERENCES**

1. Hviid L, Jensen AT. 2015. PIEMP1—a parasite protein family of key importance in Plasmodium falciparum malaria immunity and pathogenesis. Adv Parasitol 88:51–84. https://doi.org/10.1016/bs.apar.2015.02.004.

2. Baruch DI, Pasloske BL, Singh HB, Bi X, Ma XC, Feldman M, Taraschi TF, Howard RJ. 1995. Cloning the F. falciparum gene encoding PIEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. Cell 82:77–87. https://doi.org/10.1016/0092-8674(95)90054-3.

3. Su X, Heatwole VM, Wertheimer SP, Guinet F, Herfeldt JA, Peterson DS, Ravetch JV, Wellens TE. 1995. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. Cell 82:89–100. https://doi.org/10.1016/0092-8674(95)90055-1.

4. Smith JD, Chinitis CE, Craig AG, Roberts DJ, Hudson-Taylor DE, Peterson DS, Pinches R, Newbold CI, Miller LH. 1995. Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. Cell 82:101–110. https://doi.org/10.1016/0092-8674(95)90056-x.

5. David PH, Hommel M, Miller LH, Udeinya U, Oligino LD. 1983. Parasite sequestration in Plasmodium falciparum malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. Proc Natl Acad Sci U S A 80:5075–5079. https://doi.org/10.1073/pnas.80.16.5075.

6. Lavstsen T, Salanti A, Jensen AT, Arnot DE, Theander TG. 2003. Subgrouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. Malar J 2:27. https://doi.org/10.1186/1475-2875-2-27.

7. Kraemer SM, Smith JD. 2003. Evidence for the importance of genetic structuring to the structural and functional specialization of the Plasmodium falciparum var gene family. Mol Microbiol 50:1527–1538. https://doi.org/10.1046/j.1365-2958.2003.03814.x.

8. Jensen AT, Magistrado PA, Sharp S, Joergensen L, Lavstsen T, Chiucchiuni A, Salanti A, Vestergaard LS, Lusingu JP, Hermsen R, Sauerwein R, Christensen J, Nielsen MA, Hviid L, Sutherland C, Staalsoe T, Theander TG. 2004. Plasmodium falciparum associated with severe childhood malaria preferentially expresses PIEMP1 encoded by group A var genes. J Exp Med 199:1179–1190. https://doi.org/10.1084/jem.20040274.

9. Warimwe G, Keane TM, Fegan G, Muyoskiy JW, Newton C, Pain A, Berriman M, Marsh K, Bull PC. 2009. Plasmodium falciparum var gene expression is modified by host immunity. Proc Natl Acad Sci U S A 106:21801–21806. https://doi.org/10.1073/pnas.0907590106.

10. Gupta S, Snow RW, Donnelly CA, Marsh K, Newbold C. 1999. Immunity to non-cerebral severe malaria is acquired after one or two infections. Nat Med 5:340–343. https://doi.org/10.1038/6560.

11. Goncalves BP, Huang CY, Morrison R, Holte S, Kabyemela E, Prevots DR, Fried M, Duffy PE. 2014. Parasite burden and severity of malaria in Tanzanian children. N Engl J Med 370:1799–1808. https://doi.org/10.1056/NEJMoa1303944.

12. Cham CK, Turner L, Lusingu J, Vestergaard L, Mmbando B, Kurtis JD, Jensen AT, Salanti A, Lavstsen T, Theander TG. 2009. Sequential, ordered acquisition of antibodies to Plasmodium falciparum erythrocyte membrane protein 1 domains. J Immunol 183:3356–3363. https://doi.org/10.4049/jimmunol.0901331.

13. Cham GK, Turner L, Kurtis JD, Mutabingwa T, Fried M, Jensen AT, Lavstten T, Hviid L, Duffy PE, Theander TG. 2010. Hierarchical, domain-type-specific acquisition of antibodies to Plasmodium falciparum erythrocyte membrane protein 1 in Tanzanian children. Infect Immun 78: 4653–4659. https://doi.org/10.1128/IAI.00593-10.

14. Peterson DS, Miller LH, Wellens TE. 1995. Isolation of multiple sequences from the Plasmodium falciparum genome that encode conserved do- mains homologous to those in erythrocyte-binding proteins. Proc Natl Acad Sci U S A 92:7100–7104. https://doi.org/10.1073/pnas.92.15.7100.

15. Baruch DI, Gormely JA, MA C, Howard RJ, Pasloske BL. 1996. Plasmodium falciparum erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. Proc Natl Acad Sci U S A 93:3497–3502. https://doi.org/10.1073/pnas.93.8.3497.

16. Robinson BA, Welch TL, Smith JD. 2003. Widespread functional specialization of Plasmodium falciparum erythrocyte membrane protein 1 family members to bind CD36 analysed across a parasite genome. Mol Microbiol 47:1265–1278. https://doi.org/10.1046/j.1365-2958.2003.03378.x.

17. Turner L, Lavstten T, Berger SS, Wang CW, Petersen JE, Avril M, Brazier AJ, Freeth J, Jesspersen JS, Nielsen MA, Magistrado P, Lusingu J, Smith JD, Higgins MK, Theander TG. 2013. Severe malaria is associated with par-
asite binding to endothelial protein C receptor. Nature 498:502–505. https://doi.org/10.1038/nature12116.

18. Lennart Z, Adams Y, Bengtsson A, Olsen RW, Turner L, Ndam NT, E克卢-Mensah G, Moussiliou A, Ofori MF, Gamain B, Lusingu JP, Petersen JE, Wang CW, Nunes-Silva S, Jepsens JS, Lau CK, Theander TG, Lavstsen T, Hvidt L, Higgins MK, Jensen AT. 2017. Structure-guided identification of a family of dual receptor-binding PIEMP1 that is associated with cerebral malaria. Cell Host Microbe 21:403–414. https://doi.org/10.1016/j.chom.2017.02.009.

19. Olsen RW, E克卢-Mensah G, Bengtsson A, Ofori MF, Lusingu JPA, Castberg FC, Hvidt L, Adams Y, Jensen A. 2018. Natural and vaccine-induced acquisition of cross-reactive IgG-inhibiting ICAM-1-specific binding of a Plasmodium falciplarum PIEMP1 subtype associated specifically with cerebral malaria. Infect Immun 86:e00622-17. https://doi.org/10.1128/IAI.00622-17.

20. Janes JH, Wang CP, Levin-Edens E, Vigan-Womas I, Guillette M, Melcher M, Mercereau-Pujalona Q, Smith JD. 2011. Investigating the host binding signature on the Plasmodium falciparum PIEMP1 protein family. PLoS Pathog 7:e1002032. https://doi.org/10.1371/journal.ppat.1002032.

21. Lavstsen T, Magistrado P, Hermens CC, Salanti A, Jensen ATR, Sauerwein R, Hvidt L, Theander TG, Staalsoe T. 2005. Expression of Plasmodium falciplarum erythrocyte membrane protein 1 in experimentally infected humans. Malar J 4:211. https://doi.org/10.1186/1475-2875-4-211.

22. Rottmann M, Lavstsen T, Muggosa J, Kaestli M, Jensen ATR, Muller I, Theander T, Beck H-P. 2006. Differential expression of variant gene groups is associated with morbidity caused by Plasmodium falciparum infection in Tanzanian children. Infect Immun 74:3904–3911. https://doi.org/10.1128/IAI.02073-05.

23. Kinyanjui SM, Bejon P, Osier FH, Bull PC, Marsh K. 2009. What you see is not what you get: implications of the brevity of antibody responses to malaria antigens and transmission heterogeneity in longitudinal studies of malaria immunity. Malar J 8:242. https://doi.org/10.1186/1475-2875-8-242.

24. Ampomah P, Stevenson L, Ofori MF, Barfod L, Hvidt L. 2014. Kinetics of B cell responses to Plasmodium falciplarum erythrocyte membrane protein 1 in Ghanaian women naturally exposed to malaria parasites. J Immunol 192:5236–5244. https://doi.org/10.4049/jimmunol.1400325.

25. Jepsens JS, Wang CW, Mkumbaye SI, Minja DT, Petersen B, Turner L, Petersen JE, Lusingu JP, Theander TG, Lavstsen T. 2016. Plasmodium falciplarum var gene expression in children with severe malaria encode CDR1α domains. EMBO Mol Med 8:839–850. https://doi.org/10.15252/emmm.201606188.

26. Mkumbaye SI, Wang CW, Lyimo E, Jepsens JS, Manjurano A, Mosha J, Kavishe RA, Mwakalinga SB, Minja DT, Lusingu JP, Theander TG, Lavstsen T. 2017. The severity of Plasmodium falciplarum infection is associated with transcript levels of var genes encoding EPCR-binding PIEMP1. Infect Immun 85:e00841-16. https://doi.org/10.1128/IAI.00841-16.

27. Bengtsson A, Aparicio JP, Bonne M, Kräger SA, Avril M, Vaz M, Babar PH, Brazier AJ, Dembele AB, Keita S, Guesouss S, Issiaka D, Dicko A, Dufy PE, Fried M. 2009. Age-dependent increase in antibodies that inhibit Plasmodium falciplarum adhesion to a subset of endothelial receptors. Malar J 18:128. https://doi.org/10.1186/s12936-019-2744-7.

28. Tessema SK, Utama D, Chesnokov O, Hodder AN, Lin CS, Harrison GLA, Jepsens JS, Petersen B, Taval U, Sibia P, Kwiakowski D, Lavstsen T, Hansen DS, Oleinikov AV, Mueller I, Bara AE. 2018. Antibodies to intercellular adhesion molecule 1-binding Plasmodium falciplarum erythrocyte membrane protein 1-DBLb are biomarkers of protective immunity to malaria in a cohort of young children from Paga, New Guinea. Infect Immun 86:e00485-17. https://doi.org/10.1128/IAI.00485-17.

29. Struik SS, Riley EM. 2004. Does malaria suffer from lack of memory? Immunol Rev 268:218–260. https://doi.org/10.1111/j.0105-2896.2004.00181.x.

30. Reyburn H, Mbati R, Drakeley C, Bruce J, Carneiro I, Olomolari R, Cox J, Nya WM, Lemnge M, Greenwood BM, Riley EM. 2005. Association of transmission intensity and age with clinical manifestations and case fatality of severe Plasmodium falciplarum malaria. JAMA 293:1461–1470. https://doi.org/10.1001/jama.293.12.1461.

31. Chan JA, Boyle MJ, Moore KA, Reiling L, Lin Z, Hasan W, Avril M, Manning L, Mueller I, Laman M, Davis T, Smith JD, Rogerson SJ, Simpson JA, Fowkes FJL, Beeson JG. 2019. Antibody targets on the surface of Plasmodium falciplarum-infected erythrocytes that are associated with immunity to severe malaria in young children. J Infect Dis 219:819–828. https://doi.org/10.1093/infdis/jiy580.

32. Larsen MD, Quintana MDP, Ditte SV, Bayarri-Olmos R, Ofori MF, Hvidt L, Garred P. 2018. Evasion of classical complement pathway activation on Plasmodium falciplarum-infected erythrocytes opsonized by PIEMP1-specific IgG. Front Immunol 9:3988. https://doi.org/10.3389/fimmu.2018.003988.

33. Donati D, Zhang LP, Chen Q, Chene A, Flick K, Nystrom M, Wahlgren M, Beijarano MT. 2004. Identification of a polyclonal B-cell activator in Plasmodium falciplarum. Infect Immun 72:5412–5418. https://doi.org/10.1128/IAI.72.9.5412-5418.2004.

34. Hvidt L, Lavstsen T, Jensen AT. 2018. A vaccine targeted specifically to prevent cerebral malaria–is there hope? Exp Rev Vaccines 17:565. https://doi.org/10.1080/14728222.2018.1488891.

35. Struik SS, Laurusen E, Carvalho GS, Bandoh B, Barfod L, Cavanagh DR, Andersen GR, Hviid L. 2015. s2-Macroglubulin can crosslink multiple Plasmodium falciplarum erythrocyte membrane protein 1 (PIEMP1) molecules and may facilitate adherence of parasitized erythrocytes. PLoS Pathog 11:e1005022. https://doi.org/10.1371/journal.ppat.1005022.

36. Beales PF, Brabin B, Dormon E, Gilles HM, Loutain L, Marsh K, Molyneux ME, Olliaro P, Schapira A, Touze JE, Hien TT, Warrell DA, White N, WHO. 2008. Severe falciparum malaria. Trans R Soc Trop Med Hyg 94(S1):5–90.

37. Kusy KA, Manu EA, Manful Gwira T, Kyei-Baffour E, Dickson EK, Amponsah JA, Remarque EJ, Faber BW, Kocken CHM, Dondoo D, Gyan BA, Awandare GA, Atuguba F, Oduor AR, Koram KA. 2017. Variations in the quality of malaria-specific antibodies with transmission intensity in a seasonal malaria transmission area of Northern Ghana. PLoS One 12:e0185303. https://doi.org/10.1371/journal.pone.0185303.

38. Lavstsen T, Turner L, Jensen JE, Lowe ED, Petersen B, Wang CW, Petersen JE, Hvidt L, Theander TG, Lavstsen T, Higgins MK. 2015. Structural conservation despite huge sequence diversity allows EPCR binding by
the malaria PfEMP1 family. Cell Host Microbe 17:118–129. https://doi.org/10.1016/j.chom.2014.11.007.

48. Bengtsson A, Joergensen L, Barbati ZR, Craig A, Hviid L, Jensen AT. 2013. Transfected HEK293 cells expressing functional recombinant intercellular adhesion molecule 1 (ICAM-1)–a receptor associated with severe Plasmodium falciparum malaria. PLoS One 8:e69999. https://doi.org/10.1371/journal.pone.0069999.

49. Jensen ATR, Zornig HD, Buhmann C, Salanti A, Koram KA, Riley EM, Theander TG, Hviid L, Staalsoe T. 2003. Lack of gender-specific antibody recognition of products from domains of a var gene implicated in pregnancy-associated Plasmodium falciparum malaria. Infect Immun 71:4193–4196. https://doi.org/10.1128/IAI.71.7.4193-4196.2003.

50. Joergensen L, Bengtsson DC, Bengtsson A, Ronander E, Berger SS, Turner L, Dalgaard MB, Cham GK, Victor ME, Lavstsen T, Theander TG, Arnot DE, Jensen AT. 2010. Surface co-expression of two different PfEMP1 antigens on single Plasmodium falciparum-infected erythrocytes facilitates binding to ICAM1 and PECAM1. PLoS Pathog 6:e1001083. https://doi.org/10.1371/journal.ppat.1001083.

51. Snounou G, Zhu XP, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosol S. 1999. Biased distribution of msp1 and msp2 allelic variants in Plasmodium falciparum populations in Thailand. Trans R Soc Trop Med Hyg 93:369–374. https://doi.org/10.1016/S0035-9203(99)00120-7.

52. Roberts DJ, Craig AG, Berendt AR, Pinches R, Nash G, Marsh K, Newbold CI. 1992. Rapid switching to multiple antigenic and adhesive phenotypes in malaria. Nature 357:689–692. https://doi.org/10.1038/357689a0.

53. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096.

54. Avril M, Bernabeu M, Benjamin M, Brazier AJ, Smith JD. 2016. Interaction between endothelial protein C receptor and intercellular adhesion molecule 1 to mediate binding of Plasmodium falciparum-infected erythrocytes to endothelial cells. mBio 7:e00615-16. https://doi.org/10.1128/mBio.00615-16.

55. Berger SS, Turner L, Wang CW, Petersen JE, Kraft M, Lusingu JP, Mmbando B, Marquard AM, Bengtsson DB, Hviid L, Nielsen MA, Theander TG, Lavstsen T. 2013. Plasmodium falciparum expressing domain cassette 5 type PfEMP1 (DCS-PfEMP1) bind PECAM1. PLoS One 8:e69117. https://doi.org/10.1371/journal.pone.0069117.

56. Rask TS, Hansen DA, Theander TG, Pedersen AG, Lavstsen T. 2010. Plasmodium falciparum erythrocyte membrane protein 1 diversity in seven genomes—divide and conquer. PLoS Comput Biol 6:e1000933. https://doi.org/10.1371/journal.pcbi.1000933.