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Natural and vaccine-induced acquisition of cross-reactive IgG inhibiting ICAM-1-specific binding of a PfEMP1 subtype associated specifically with cerebral malaria

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ABSTRACT

Cerebral malaria (CM) is a potentially deadly outcome of *Plasmodium falciparum* malaria that is precipitated by sequestration of infected erythrocytes (IEs) in the brain. The adhesion of IEs to brain endothelial cells is mediated by a subtype of parasite-encoded PfEMP1 that facilitate dual binding to host ICAM-1 and EPCR. The PfEMP1 subtype is characterized by the presence of a particular motif (DBLβ_motif) in the constituent ICAM-1-binding DBLβ domain. The rate of natural acquisition of DBLβ_motif-specific IgG and the ability to induce such antibodies by vaccination are unknown, and the aim of this study was to provide such data.

We used ELISA to measure DBLβ-specific IgG in plasma from Ghanaian children with malaria. The ability of human immune plasma and DBLβ-specific rat anti-sera to inhibit the interaction between ICAM-1 and DBLβ was assessed using ELISA and *in vitro* assays of IE adhesion under flow.

Acquisition of DBLβ_motif-specific IgG coincided with age-specific susceptibility to CM. Broadly cross-reactive antibodies inhibiting the interaction between ICAM-1 and DBLβ_motif domains were detectable in immune plasma and in sera of rats immunized with specific DBLβ_motif antigens. Importantly, antibodies against the DBLβ_motif inhibited ICAM-1-specific *in vitro* adhesion of erythrocytes infected by four of five *P. falciparum* isolates from cerebral malaria patients. We conclude that natural exposure to *P. falciparum* as well as immunization with specific DBLβ_motif antigens can induce cross-reactive antibodies that inhibit the interaction between ICAM-1 and a broad range of DBLβ_motif domains. These findings raise hope that a vaccine designed specifically to prevent CM is feasible.

Keywords. *Plasmodium falciparum;* PfEMP1; DBLβ cross-reactive antibodies; ICAM-1 binding motif; adhesion inhibition.

INTRODUCTION

Plasmodium falciparum is the major cause of the estimated 430,000 deaths due to malaria reported annually (1). The pathogenesis of *P. falciparum* is linked to sequestration of IEs in various tissues, which can lead to tissue-specific inflammation, circulatory obstruction, and organ dysfunction (reviewed in ref. 2). IE sequestration is mediated by members of the erythrocyte membrane protein 1 (PfEMP1) family. These proteins are encoded by approximately 60 *var* genes per *P. falciparum* genome, and are expressed on the IE surface where they bind to a range of host receptors (reviewed in ref. 3).

Despite extensive inter- and intra-clonal diversity, the PfEMP1 proteins can be classified into three major groups (A, B and C), based on *var* gene sequence and chromosomal context (4, 5). Group A is less diverse than the other groups, and expression of Group A PfEMP1 proteins on the IE surface has repeatedly been linked to the development of severe malaria (6, 7). This is consistent with the restricted serological diversity of *P. falciparum* parasites from patients with severe malaria (8, 9). It also fits the observation that acquisition of immunity to complicated disease often precedes development of protection from uncomplicated malaria and asymptomatic parasitemia, and that PfEMP1 expression is modulated by PfEMP1-specific immunity (10-12). More recently, the PfEMP1 Groups have been further sub-divided according to their constituent Duffy-binding-like (DBL) and cysteine-rich inter-domain region (CIDR) domains, and a number of multi-domain blocks, known as domain cassettes (DCs), have been identified (13-16). Three of these, (DC4, DC8, and DC13) have been linked to severe malaria in children (6, 14, 17, 18). DC4 consists of three domains (DBL$\alpha_{1.1/1.4}$-CIDR$\alpha_{1.6}$-DBLβ_3) and defines a subfamily of Group A PfEMP1 proteins that mediates binding to intercellular adhesion molecule 1 (ICAM-1) (15). IE adhesion to ICAM-1 appears associated with severe malaria, implicating DC4-specific antibodies in clinical protection as they are acquired early in life by children living in malaria endemic areas and are associated with

clinical protection from malaria (6, 15, 19). However, until recently the role of IE adhesion to ICAM-1 specifically in CM was unclear (20-24).

DC8 consists of four domains (DBLα_2-CIDR$\alpha_{1.1}$-DBLβ_{12}-DBL$\gamma_{4/6}$) and is found among group B/A genes, while the two-domain (DBL$\alpha_{1.7}$-CIDR$\alpha_{1.4}$) DC13 is found in some group A PfEMP1 proteins (14). Endothelial protein receptor C (EPCR) is the cognate receptor for DC8- and DC13-containing PfEMP1 (25). Some studies have reported high transcript levels of *var* genes encoding EPCR-binding PfEMP1 variants in parasites from children with severe malaria, including CM, and perturbed EPCR expression in brain tissue of CM patients (26-28). While these findings point to a role for EPCR in severe malaria in general, and CM in particular, available evidence overall remains equivocal (29-31).

We have previously proposed that the above ambiguities may be reflecting that the pathogenesis of CM involves *P. falciparum* parasites expressing PfEMP1 capable of mediating IE adhesion to both ICAM-1 (via DBLβ) and EPCR (via CIDRα_1) (3). A few such dual receptor-binding PfEMP1 proteins were identified shortly after, although the study did not link them to CM specifically, and did not document concomitant binding to both receptors (32). However, those gaps were recently closed by our demonstration of a link between CM and Group A PfEMP1 proteins capable of binding ICAM-1 and EPCR simultaneously (33). This dual receptor-binding sub-group of PfEMP1 proteins is characterized by an EPCR-binding CIDRα_1 domain followed immediately by a DBLβ domain featuring a specific ICAM-1-binding motif (DBLβ_motif domain) (33).

The rate of natural acquisition of IgG against the DBLβ_motif associated specifically with CM, and the ability to induce such antibodies by vaccination, are both unknown. The current study was therefore designed to investigate if cross-reactive IgG specific for DBLβ_motif domains and/or their ICAM-1-binding motif are acquired following natural exposure to *P. falciparum* parasites, and if ICAM-1 adhesion-inhibitory antibodies can be induced by immunization with specific

DBLβ_motif proteins and with peptides representing the ICAM-1-binding motif therein. Confirmation of these hypotheses would support the feasibility of developing a vaccine designed specifically to prevent CM.

MATERIALS AND METHODS

Plasma and parasite samples

Plasma samples (n= 79) for the present study were collected in 2014 at Hohoe Municipality Hospital in the Volta Region of Ghana from children with acute malaria (Table 1) (33, 34). *P. falciparum* parasites were collected at this hospital (n= 14) as well as at the Korogwe District Hospital (n= 19) in Korogwe District in Northeastern Tanzania (35). Clinical manifestations of malaria were classified according to the definitions and associated criteria of the World Health Organization. Patients were categorized as having cerebral malaria (CM; n= 7) if they had a positive blood smear of the asexual form of *P. falciparum*, unrousable coma (Blantyre coma score, BCS ≤2) with exclusion of other causes of coma and severe illness. Patients were categorized as having severe malarial anemia (SA; n=12) if haemoglobin <5 g/dL and BCS >2. Patients were classified as having severe malaria other than SA and CM if they presented with hyperparasitaemia (>250,000 parasites/µL), multiple convulsions (>2 episodes in 24 h), respiratory distress (i.e., rapid, deep, and labored breathing) or combinations of these symptoms. Patients with uncomplicated malaria (UM; n= 48) had less than 250,000 parasites/µL.

The study was approved by the Ethical Review Committee of the Ghana Health Services (file GHS-ERC 08/05/14) and by the National Ethical Review Committee of the National Institute for Medical Research, Tanzania (NIMR/HQ/R.8a/Vol.IX/559). A pool of plasma from *P. falciparum*-exposed Tanzanian individuals (36) and 25 Danish non-exposed individuals were used as positive and negative controls, respectively. Long-term *in vitro* culture-adapted and fully sequenced parasite clones 3D7, HB3, and IT4 were also studied.

Recombinant proteins

The genes encoding the DBLβ domains used were amplified from genomic DNA or produced as synthetic genes (http://eurofins.dk) (Table 3). Amplicons were sub-cloned into a modified pET15b vector and expressed as his-tagged proteins in *E. coli* Shuffle C3030 cells (https://neb.com) as described (15). All the proteins were purified (Fig. S1) by immobilized metal ion affinity chromatography using HisTrap HP 1 mL columns (GE Healthcare), and are referred to by the codes listed in Table 3.

Recombinant Fc-tagged ICAM-1 was expressed in HEK293 cells and purified on a HiTrap Protein G HP (http://www3.gehealthcare.dk/) as described (37).

DBLβ-specific anti-sera

We generated rat antisera to recombinant proteins M1, M6, M9, M10, N27 and N33 (Table 3), and to two synthetic peptides (https://schafer-n.com/) that corresponded to the ICAM-1-binding motifs in M6 (M6pep: LYAKARIVASNGGPGYYNTEVQKKDRSVYDFLYELHLQNGGKKGPPPATHPYKSVNTRD KRDATDDTTP) and M9 (M9pep: LYKEAEIYARNGGPGYYNTEVQKEDKPVVDFLYELHLQNGGKKGPP AATHPSKSVTTRVKRDTTVDTPS). M1, M6, M9 and M10 were selected from different branches of the previously published phylogenic tree of DBLβ domains, to represent dual ICAM-1- and EPCR-binding Group A PfEMP1 proteins (33). In a similar way, N27 and N33 were chosen as random examples of ICAM-1-binding group B PfEMP1 proteins.

In each case, Wistar rats were immunized with the antigen (25 µg) in Freund's incomplete adjuvant followed by two booster vaccinations two weeks apart (15 µg/boost). Blood was collected two weeks after the last immunization. All animal procedures were approved by The Danish Animal Procedures Committee ("Dyreforsøgstilsynet") as described in permit no. 2013-15-2934-

00920, and all experiments were done according to the guidelines described in Danish act LBK 1306 (23/11/2007) and BEK 1273 (12/12/2005).

IgG purification

IgG specific for M6 and M6pep were affinity-purified from rat antisera as described (38). In brief, M6 and M6pep (1 mg/mL) were dialysed overnight against coupling buffer and coupled to Hi-trap NHS-activated HP columns, as described by the manufacturer (http://www3.gehealthcare.dk/). Anti-sera were diluted 1:1 in PBS and affinity-purified on the columns, followed by elution of bound IgG in low-pH buffer (glycine/HCl, pH 2.75) and pH adjustment by Tris/HCl (1 M, pH 9.0).

Measurements of DBLβ-specific IgG levels

MaxiSorp microtiter plates (http://www.sigmaaldrich.com/) were coated with recombinant DBLβ domains (50 µL; 5 µg/mL) as described previously (15). Plasma samples (diluted 1:100 in blocking buffer) were incubated (50 µL/well, 1 h, room temperature) in duplicate wells. The plates were washed (PBS + 1% Triton X-100), and bound antibody was detected with HRP-conjugated anti-human IgG (1:3,000 in blocking buffer) (http://www.agilent.com). After incubation (1 h) and washing as above, bound detection antibody was detected using OPD tablets, according to the manufacturer's instructions (http://www.agilent.com). The OD values were read at 490 nm using a VERSAmax microplate reader (http://www.moleculardevices.com/). Antibody reactivity was expressed in arbitrary ELISA units (EU) calculated as $(OD_{sample} - OD_{background})/(OD_{positive\ control} - OD_{background}) \times 100$ (ref. 39).

Measurements of antibody-mediated inhibition of DBLβ binding to ICAM-1

Inhibition of recombinant DBLβ domain binding to ICAM-1 by human immune plasma and rat anti-sera was measured by ELISA. In brief, wells of MaxiSorp plates were coated with

recombinant ICAM-1 (ref. 37) (50 µL/well; 2 or 4 µg/mL; 0.1M glycin/HCl buffer; pH 2.75) by incubation overnight (4°C) and blocked with blocking buffer (1 h; room temperature). His-tagged DBLβ proteins (0.5-16 µg/mL final concentration) were mixed with immune plasma or anti-sera (1:5 final concentration) or purified IgG (10 µg/mL final concentration) and added to duplicate wells (1 h; room temperature). The plates were washed and binding detected using HRP-conjugated anti-penta-his antibody (http://www.qiagen.com/) as described above. All antisera were prescreened by ELISA to verify absence of his-tag-reactive antibodies.

In vitro *culture and antibody selection of* P. falciparum *parasites*

The *P. falciparum* clones 3D7, HB3, and IT4 were maintained in long-term *in vitro* cultures, and antibody-selected for IE surface expression of specific PfEMP1 proteins as described (15, 38). In brief, we used the human monoclonal IgG antibody AB01 to select 3D7 IEs for expression of PFD1235w (40). HB3 IEs were similarly selected for surface expression of VAR03 using a rat anti-serum against M8, and IT4 IEs for expression of VAR13 by a rat anti-serum against N27 (ref. 33). In all cases, expression of the required PfEMP1 on the surface of MACS-purified mature IEs was monitored by flow cytometry using PfEMP1-specific antisera, essentially as described (38, 41). Only cultures with >60% antibody-labeled IEs were used.

In addition, primary isolates of *P. falciparum* parasites from 33 of the above-mentioned malaria patients were cultured *in vitro* for up to 28 days (median; 25 and 75% percentile [8 days; 2.5 and 13 days]) in Albumax (10%) (http://www.thermofisher.com/), supplemented with normal human serum (NHS, 2%), essentially as described (42). The genotypic identity of the isolates was routinely verified by genotyping as described (43), and *Mycoplasma* infection was regularly excluded using the MycoAlert *Mycoplasma* Detection Kit (http://www.lonza.com/) according to the manufacturer's instructions.

184 ***Adhesion of IEs to ICAM-1 under physiological flow* in vitro**

185 Microslides ($VI^{0.1}$) (http://www.ibidi.com/) were coated with recombinant ICAM-1-Fc
186 protein (37) (50 µg/mL, 4°C, overnight) and blocked using PBS plus 2% BSA. Parasite
187 suspensions, adjusted to 3% parasitemia and 1% hematocrit in RPMI-1640 supplemented with 2%
188 NHS (pH 7.2), were flowed over the coated slides (5 min) at a shear stress of 1 dyn/cm^2 as
189 described (44). Bound IE/mm^2 in five separate fields were counted, using a Leica inverted phase
190 contrast microscope (20× magnification). To assess the capacity of affinity-purified DBLβ-specific
191 IgG to inhibit adhesion, IEs selected for expression of particular PfEMP1 variants were pre-
192 incubated with the purified IgG (15 min, room temperature). The receptor specificity of the IE
193 adhesion observed was verified by pre-incubating the ICAM-1-coated flow channels with an
194 ICAM-1-specific antibody (40 µg/mL, clone 15.2, AbD Serotec).

195 Inhibition of ICAM-1-adhering (≥ 10 adherent IEs/mm^2) erythrocytes infected with primary
196 *P. falciparum* field isolates was tested using pooled rat antisera (1:100 dilution) to two peptides
197 representing the ICAM-1-binding motifs in M6 (M6pep) and M9 (M9pep). A minimum of three
198 independent experiments were completed for each of the tested laboratory clones (3D7, HB3, IT4),
199 whereas each of the field isolates was tested in one experiment with five technical replicates. All
200 assays were blinded to the operator.

201 ***Immunofluorescence microscopy of IEs labeled with PfEMP1-specific antibodies***

202 Immunofluorescence microscopy was done essentially as described (45). Briefly, aliquots (50
203 µL) of erythrocytes infected by parasites expressing PFD1235w, HB3VAR03, or IT4VAR13,
204 respectively were adjusted to 5% parasitemia and resuspended in PBS containing 1% Ig-free BSA
205 (https://www.sigmaaldrich.com). Antisera were added (1:50 dilution) and incubated on ice (1 h).
206 Following three washes, cells were resuspended and labeled with anti-rat-FITC secondary antibody
207 (1:500) and incubated as before. Cells were washed three times and thin smears made. Nuclei were

visualized by adding 5 µL ProLong Gold anti-fade (http://www.thermofisher.com/) prior to addition of coverslips. Immunofluorescence was visualized with a Nikon Eclipse TE2000 microscope equipped with an ×63 objective.

Bioinformatics

Multiple alignments of DBLβ domains known to bind ICAM-1 were made using MUSCLE v. 3.7 software (46), and sequence distance trees made with MEGA software (47). A WebLogo 3 sequence logo (48) of the ICAM-1 binding motif was generated based on alignment of the included DBLβ_motif domains (Table 3) with the consensus motif: I[V/L]x3N[E]GG[P/A]xYx27GPPx3H (15, 33).

Statistics

We used Pearson product moment correlation (r) or Spearman rank-order correlation (r_s) to evaluate parameter association, and one-way analysis of variance (F), Kruskal-Wallis one-way analysis of variance on ranks (T), and Mann-Witney test (U) to test for inter-group differences.

RESULTS

Delayed acquisition of IgG to ICAM-1-binding Group A-type DBLβ domains

Group A PfEMP1 proteins that contain a DBLβ domain with the motif (I[V/L]x3N[E]GG[P/A]xYx27GPPx3H; DBLβ_motif domains) can bind to the host endothelial receptor ICAM-1 and always feature a neighboring CIDRα1 domain that enables concomitant binding to another endothelial receptor, EPCR (33). Expression of these dual receptor-binding PfEMP1 is associated with CM, which is a major cause of mortality and severe morbidity among African children. The age at which most CM cases occurs varies with transmission intensity, but generally falls later than the peak prevalence of parasitemia and malaria-related severe anemia. DBLβ domains present in Group A PfEMP1 proteins, but without the above motif (DBLβ_non-motif domains), do not bind ICAM-1 and are less conserved in the C-terminus (33).

We first used ELISA to measure levels of IgG with specificity for 14 recombinant DBLβ_motif domains (M1-M12, M14-M15; Fig. 1) and 13 non-motif DBLβ domains (N20-N32) in plasma from 79 Ghanaian children with different clinical presentations of *P. falciparum* malaria (Table 1). The antibody reactivity to all these Group A PfEMP1 proteins varied substantially among the children (Fig. 2A) and also among the different DBLβ domains (Fig. 2B). Overall, the plasma levels of IgG increased with age ($P(r)<0.001$), with levels of IgG specific for DBLβ_motif proteins being generally lower than DBLβ_non-motif-specific IgG ($P(T)<0.001$). However, this latter difference was mainly due to low IgG recognition of DBLβ_motif among the younger age groups (≤4 years-of-age) (Fig. 2C). Thus, levels of IgG specific for DBLβ_motif were significantly lower than DBLβ_non-motif-specific IgG levels among children aged 1-2 years and 3-4 years ($P(T)\leq0.001$), but not in the two older age classes considered (5-6 years and >6 years; $P(T)\geq0.21$).

Overall plasma levels of IgG specific for DBLβ_motif as well as DBLβ_non-motif domains were similar in patients with severe and uncomplicated malaria (P(U)=0.4 in both cases), while levels of DBLβ_non-motif domain-specific IgG were lower in the children with severe malaria than in the patients with uncomplicated disease (scores 7 and 12.5; P(U)=0. 02, respectively).

Taken together, these results indicate that DBLβ_motif and non-motif proteins are similarly immunogenic, but that acquisition of DBLβ_motif-specific IgG is acquired later in life than DBLβ_non-motif-specific IgG.

Immunization with DBLβ_motif antigens induces cross-reactive, neutralizing IgG

Plasma from clinically immune individuals living in areas of stable and intense transmission of *P. falciparum* parasites can inhibit the interaction between ICAM-1 and a range of DBLβ_motif domains (15, 33). In this study, a pool of plasma from 10 of the children (selected for reactivity with DBLβ_motif domains (Fig. 2A and Table 1) and plasma availability) inhibited ICAM-1 binding to DBLβ_motif protein M9 (Fig. 3A). These data suggest the presence of neutralizing IgG capable of recognizing multiple DBLβ_motif-containing PfEMP1 variants (cross-reactive IgG). If such antibodies could be induced by vaccination, it would increase the feasibility of developing a broadly protective PfEMP1-based vaccine against cerebral malaria. However, our data could also reflect the presence of many different variant-specific IgG specificities, where each antibody specificity is capable of inhibiting the binding of ICAM-1 to only a particular DBLβ_motif domain variant (a broad repertoire of IgG with narrow specificity). It is inherently difficult to distinguish between these two alternatives in naturally acquired immunity. Nevertheless, truly cross-reactive PfEMP1-specific human antibodies have previously been demonstrated in a study employing naturally acquired monoclonal IgG specific for the VAR2CSA-type PfEMP1 involved in the pathogenesis of placental malaria (49).

To investigate the relative importance of the above non-exclusive alternatives, to further assess the functional significance of DBLβ_motif in acquired immunity, and as a first step towards PfEMP1-based vaccination specifically against cerebral malaria, we immunized four rats with DBLβ_motif proteins M1, M6, M9, and M10, respectively (Table 3 and Fig. 1S). We used ELISA to test the ability of the anti-sera to inhibit binding of ICAM-1 to 14 DBLβ_motif proteins (M1-M7 and M9-M15) and two ICAM-1-binding DBLβ_non-motif proteins (N27 and N33). Each of the four DBLβ_motif-specific anti-sera inhibited binding of ICAM-1 to most of the DBLβ_motif proteins by more than 50%, but had little effect on ICAM-1-binding to DBLβ_non-motif domains (Fig. 3B). When pooled, the DBLβ_motif-specific anti-sera strongly inhibited (>75%) binding of ICAM-1 to all DBLβ_motif domains, with much less effect (<50%) on ICAM-1-binding to the DBLβ_non-motif domains (Fig. 3B). We next affinity-purified IgG from three of the rat anti-sera, using M6pep, to evaluate the involvement of IgG directly targeting the ICAM-1 binding region in the above inhibition. The purified M6pep-specific IgG generally inhibited ICAM-1 binding to the same degree as the anti-sera (Fig. 3B), with strong correlation between the anti-serum and motif-specific IgG data for M6 (r_s=0.78; P<0.001).

Overall, these data indicate that immunization with single DBLβ_motif antigens can induce cross-reactive IgG that inhibits binding of ICAM-1 to the homologous as well as a broad range of heterologous DBLβ_motif domains.

DBLβ_motif-specific IgG is broadly inhibitory of IE adhesion to ICAM-1 under physiologic flow

The ability of neutralizing IgG to interfere with receptor-specific IE sequestration *in vivo* likely depends on the characteristics of the involved PfEMP1 *per se*, on their expression on the IE surface, as well as on the shear forces at the anatomical location of the interaction of IEs with host endothelium. We have previously shown that naturally acquired IgG can inhibit ICAM-1-specific

adhesion of erythrocytes infected by *P. falciparum* expressing DBLβ_motif-containing PfEMP1 (33). To test if such antibodies could also be elicited by immunization with recombinant PfEMP1 proteins containing DBLβ_motif, we tested the ability of DBLβ_motif-specific IgG to inhibit adhesion of IEs to ICAM-1 in an *in vitro* assay simulating physiologic flow conditions (44). M6-specific IgG significantly inhibited ICAM-1-specific adhesion of IEs expressing the homologous PfEMP1 (PFD1235w; P(F)<0.001; Fig. 4A) or a heterologous PfEMP1 (HB3VAR03; P(F)<0.001; Fig. 4B). This was also the case for IgG specific for M9 and M10 (Table 3) and IgG purified on the ICAM-1-binding motif in M6 (M6pep; P(F)<0.001). The M9-specific antibodies also affected ICAM-1-specific adhesion of IEs expressing IT4VAR13, a Group B PfEMP1 protein that binds ICAM-1 but does not contain DBLβ_motif (ref. 33) (Fig. 4C). Conversely, an anti-serum to the ICAM-1-binding domain in IT4VAR13 (N27) inhibited ICAM-1-specific adhesion of the homologous IEs (Fig. 4C), but had no effect on ICAM-1-specific adhesion of IEs expressing the DBLβ_motif-containing PfEMP1 proteins PFD1235w (Fig. 4A) or HB3VAR03 (Fig. 4B).

We conclude from these experiments that immunization with DBLβ_motif antigens induce cross-reactive IgG that inhibit ICAM-1-specific adhesion of IEs that express a variety of PfEMP1 proteins containing DBLβ_motif domains. The inhibition of native PfEMP1 protein to ICAM-1 under conditions of flow thus mirrors that observed with recombinant proteins in ELISA.

Immunization with peptides representing the ICAM-1-binding region induces antibodies broadly inhibiting the binding of recombinant and native DBLβ_motif domains to ICAM-1

The results above suggested that IgG targeting the ICAM-1-binding region in DBLβ_motif domains is of particular importance for inhibiting the binding of Group A dual receptor-binding PfEMP1 to ICAM-1. This interpretation is further supported by our recent data showing that DBLβ_motif-purified antibodies from naturally infected humans and experimentally vaccinated

312 animals inhibit ICAM-1-specific adhesion of IEs expressing the DBLβ_motif-containing PfEMP1
313 protein PFD1235w (33). To assess directly if inhibitory and cross-reactive antibodies could be
314 elicited by peptide immunization, we immunized rats with peptides representing the ICAM-1-
315 binding region in DBLβ_motif domains (M6pep and M9pep) and tested their ability to inhibit
316 binding of ICAM-1 to DBLβ_motif domains. Antisera from rats immunized with M6pep only, or
317 with M6pep and M9pep, were broadly inhibitory of the binding of ICAM-1 to 10 DBLβ_motif
318 domains (M2-M7, M9, M11-M13). The peptide antisera did not affect binding to two ICAM-1-
319 binding DBLβ_non-motif domains (N27 and N33) (Fig. 5A). Experiments assessing the ability of
320 the antisera to inhibit adhesion of IEs to ICAM-1 under flow corroborated these findings. Thus,
321 both the above antisera (M6pep and M6pep/M9pep) significantly inhibited adhesion of IEs
322 expressing the DBLβ_motif-containing PfEMP1 proteins PFD1235w (Fig. 5B; expressing M6
323 native protein) and HB3VAR03 (Fig. 5C; expressing M8 native protein), but had no effect on IEs
324 expressing IT4VAR13 (expressing N27 native protein), which does not contain a DBLβ_motif
325 domain (Fig. 5D). Furthermore, the single- and dual-peptide antisera yielded immunofluorescence
326 patterns typical of IgG reacting with IE surface-expressed PfEMP1 when tested against IEs
327 expressing either HB3VAR03 or PFD1235w, but did not label IEs expressing IT4VAR13 (Fig. 5E).
328 In contrast, the IEs expressing IT4VAR13were labeled by an IT4VAR13-specific antiserum, but not
329 by the single- and dual-peptide antisera (Fig. 5E).

330 Finally, we assessed the ability of erythrocytes infected by 33 primary *P. falciparum* isolates
331 from Ghana (N=14) and Tanzania (N=19) to adhere to ICAM-1 under flow. We also tested the
332 ability of pooled rat anti-serum to M6pep and M9pep to inhibit adhesion of ICAM-1-adhering
333 isolates. Twenty-two of the isolates (three from children with uncomplicated malaria, 14 from
334 patients with severe malaria, and five from children with cerebral malaria) showed adhesion of IEs
335 to ICAM-1 (Fig. 6A). Adhesion of eleven of these isolates (one from a child with uncomplicated

malaria, six from children with severe malaria, and four from children with cerebral malaria) was inhibited (>25%) by the anti-peptide serum pool (Fig. 6B).

We conclude that immunization with linear peptides that represent only the ICAM-1-binding region of specific DBLβ_motif domains can induce cross-reactive antibodies that are capable of inhibiting the binding of ICAM-1 to a range of recombinant and native, IE-expressed DBLβ_motif domains. Importantly, the motif antibody inhibits binding of four of five *P. falciparum* isolates from cerebral malaria patients.

DISCUSSION

Cerebral malaria (CM) is one of the most severe complications of *P. falciparum* malaria, and a leading cause of mortality (reviewed in ref. 50). PfEMP1-mediated adhesion of IEs to the endothelial receptors ICAM-1 and EPCR have both repeatedly been implicated in the pathogenesis of severe malaria (6, 25, 33). However, a specific and direct link to the development of CM has been missing until recently, when we identified a sequence motif in PfEMP1 proteins associated specifically with the development of CM (33). This ICAM-1-binding motif (DBLβ_motif; Fig. 1) is found in some Group A PfEMP1 proteins (15), immediately downstream of an EPCR-binding CIDRα domain (25). In the present study, we set out to study the acquisition of DBLβ_motif-specific IgG following natural exposure, and whether DBLβ_motif-specific antibodies induced by vaccination are cross-reactive and inhibit adhesion to ICAM-1.

In areas with stable transmission of these parasites, substantial protective immunity to malaria is acquired during childhood, first to severe complications and later to clinical disease. As a consequence, adults are largely protected from malaria in such areas, although sterile immunity is rarely, if ever achieved. This sequence appears to be the consequence of an ordered acquisition of antibodies to a relatively conserved set of PfEMP1 proteins associated with severe disease, followed by antibodies to a large and diverse set of PfEMP1 proteins associated with uncomplicated malaria and asymptomatic parasitemia. Where transmission is very intense, serious and fatal malaria episodes are markedly concentrated during the first few years of life, mainly as severe malarial anemia. CM, in contrast, is rare (51). Where endemicity is lower, CM tends to be seen more often, but mainly among children some years older than those that succumb to severe malarial anemia. Together these findings suggest that discrete PfEMP1 subsets are involved in severe malaria with and without cerebral involvement, and perhaps even that toddlers are relatively resistant to CM for non-immunologic reasons. This fits our demonstration here that although DBLβ domains are

367 generally immunogenic following natural exposure, acquisition of DBLβ_motif-specific IgG occurs
368 later than DBLβ_non-motif-specific IgG (Fig. 2) and coincides with the age bracket where the
369 incidence of CM peaks under transmission intensities comparable to our study area (52, 53).

370 The clinical significance of acquisition of PfEMP1-specific antibodies is thought to involve
371 their ability to interfere with sequestration of IEs in various tissues (15, 54). A particularly
372 thoroughly investigated example is the role of anti-adhesion antibodies in acquisition of protective
373 immunity to placental *P. falciparum* malaria, caused by accumulation of IEs in the intervillous
374 spaces (55, 56). Placental IE sequestration is mediated by a particular group of PfEMP1
375 (VAR2CSA) binding to oncofetal chondroitin sulfate A (57, 58), and clinical trials of vaccines
376 based on the VAR2CSA adhesive epitope and aimed to protect against this important cause of
377 prenatal and infant morbidity and mortality are currently under way. It appears that DBLβ_motif-
378 specific IgG can inhibit IE adhesion to ICAM-1 in a similar way (Fig. 3A and (15, 33)). Here, we
379 demonstrate that this inhibition can be mediated by genuinely cross-reactive antibodies, as opposed
380 to a broad repertoire of IgG species each with narrow specificity for a single or very few
381 DBLβ_motif sequences (Fig. 3). This finding is of significance, since naturally acquired protection
382 from malaria is generally believed to be the consequence of accumulation of a broad repertoire of a
383 large number of antibody specificities (9, 12, 59). Such broadly reactive IgG can inhibit adhesion of
384 erythrocytes infected with parasites isolated from patients with severe malaria (six of 13 isolates)
385 and cerebral malaria (four of five isolates) under physiologic flow conditions (Fig. 6). Furthermore,
386 such IgG can be induced by peptides (M6pep and M9pep) representing just the core element of the
387 DBLβ_motif that mediates the binding to ICAM-1 (Fig. 5 and Fig. 6).

388 Approximately half the children with acute *P. falciparum* malaria in our study had severe
389 disease, according to the WHO criteria (60), but we did not observe any significant differences in
390 plasma levels of DBLβ_motif-specific IgG in children with and without severe disease. This may

be related to the fact that, due to the low prevalence in the area (Table 1 and Table 2). It is doubtful that a relationship between DBLβ_motif-specific IgG and clinical presentation of acutely ill malaria patients would be apparent even if we had been able to include plasma samples from CM patients, due to the unavoidable variation in time between infection and presentation to hospital. It is plausible that only very large, and preferably longitudinal, studies would have the power required to document such relationships in semi-immune, naturally infected individuals.

In conclusion, our study demonstrates that CM-related DBLβ domains are immunogenic following natural exposure, and that acquisition of DBLβ_domain-specific IgG coincides with the age where CM has its peak prevalence in areas of moderate but stable *P. falciparum* transmission. Furthermore, we show that immunization with such domains in addition to peptides representing the minimal ICAM-1 binding region can induce IgG that can inhibit PfEMP1 binding to ICAM-1 and neutralize IE adhesion under physiologic flow. Importantly, these antibodies broadly neutralized adhesion of erythrocytes infected by parasites isolated from four of five children with cerebral malaria. Together, these findings raise hopes that development of a vaccine specifically against CM may be possible, despite the notorious polymorphism and intra-clonal diversity of the PfEMP1 family (recently reviewed in refs. 3, 61).

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418 **Figure legends**

419 ***Figure 1.***

420 Sequence logo showing the ICAM-1 binding motif (as defined in (33)) of the DBLβ domains
421 1-15 used in the present study (Table 3). Residues that are critical for the direct interaction with
422 ICAM-1 (red triangles) or for the architecture of the ICAM-1 binding (white triangles) are
423 indicated. The Group A PfEMP1 ICAM-1 binding motif was identified by Lennartz *et al.* (33).

424 ***Figure 2***

425 Plasma levels of IgG with specificity for *P. falciparum* DBLβ domains. Samples were
426 obtained from 79 Ghanaian children with either severe (▲) or non-severe *P. falciparum* malaria.
427 *A*, Levels (ELISA units; EU) in plasma from individual children (columns) of IgG specific for
428 individual group A DBLβ domains (rows) containing (DBLβ_motif; M1-M12; M14-M15) or not
429 containing (DBLβ_non-motif; N20-N32; lower half) the ICAM-1-binding motif identified by
430 Lennartz *et al.* (33). Shading indicate IgG level score: Black (4: >100 EU), dark gray (3: 76-100
431 EU), gray (2: 51-75 EU), light gray (1: 26-50 EU), and white (0-25 EU). The DBLβ domain
432 numbers correspond to the numbers in Table 3. Danish controls (n= 25) did not react with any of
433 the domains (data not shown). *B*, The means of IgG level scores (defined as in *A*) of individual
434 DBLβ domains that contain (Motif; ○) or do not contain (Non-motif; ●) the ICAM-1-binding motif.
435 Error bars indicate 95% confidence intervals. DBLβ domain numbering as in *A*. *C*, The means of
436 IgG level scores (defined as in *A*) of individual children for IgG specific for DBLβ_motif (○) and
437 DBLβ_non-motif (●) domains. The statistical significance (Mann-Whitney rank-sum test) of
438 pairwise comparisons is shown along the top of the panel.

Figure 3

Ability of DBLβ_motif-specific IgG to inhibit binding of ICAM-1 to DBLβ domains. *A*, Inhibition of ICAM-1 binding by pooled immune plasma from 10 of the study children (Pool). *B*, Rat anti-sera raised against DBLβ_motif antigens tested against recombinant proteins containing (M1-M7, M9-M15) or not containing (N27, N33) DBLβ_motif. Shading indicates degree of inhibition: black (>75%), dark gray (50-75%), gray (20-50%), and white (<20%). The DBLβ domain numbers and antiserum specificities correspond to the numbers in Table 3. Data using a pool of rat-anti-sera (M1, M6, M9, M10) are also shown (Pool). Anti-sera marked with asterisks were affinity-purified on a peptide (M6pep) representing the binding motif in PFD1235w_DBLβ_D4 prior to assaying. Three independent experiments were done (three technical replicates/assay). A sequence-distance tree illustrating the relatedness of the different domains is shown along the left edge of the panel.

Figure 4

DBLβ-specific antibody-mediated inhibition of adhesion of IEs to ICAM-1 under physiologic shear stress, relative to control without antibody (None). *A*, IEs expressing PFD1235w. *B*, IEs expressing HB3VAR03. *C*, IEs expressing IT4VAR13. The specificities of the DBLβ antibodies correspond to the numbers in Table 3. Anti-serum marked with asterisk was affinity-purified on a peptide (M6pep) representing the binding motif in PFD1235w_DBLβ_D4 prior to assaying. An ICAM-1-specific neutralizing antibody (ICAM-1) and an irrelevant rat anti-IgG (https://www.sigmaaldrich.com) were included as positive and negative controls, respectively. Fewer than 0.25 IEs/mm^2 bound to uncoated channels. Means (bars) and standard deviations (error bars) of at least three independent experiments in triplicates are shown. Statistically significant reductions relative to adhesion in the absence of antibody (–) are indicated above the bars (**, P(F)<0.01; ***, P(F)<0.001). Refer to Table S1 for raw data.

Figure 5

Ability of rat antisera to the ICAM-1-binding motif in DBLβ_motif domains to inhibit binding of recombinant DBLβ domains to ICAM-1. *A,* Anti-sera from rats immunized with M6pep or with both M6pep and M9pep tested against recombinant DBLβ_motif domains (M2-M7, M9, M11-M13) and DBLβ_non-motif domains (N27, N33). Shading, DBLβ domain numbers, and antiserum specificities as in Fig. 3B. *B-D,* Inhibition by the same anti-sera of ICAM-1-specific adhesion of PFD1235w^{+} IEs (*B),* HB3VAR03^{+} IEs (*C),* and IT4VAR13^{+} IEs (*D)* under physiologic shear stress. Statistical significance of reductions is indicated as in Fig. 4. Three independent experiments were done (with three technical replicates in each). Fewer than 0.25 IEs/mm^2 were observed bound to uncoated channels (*E),* Immunofluorescence of representative IEs with surface expression of PFD1235w (top row), HB3VAR03 (center row), and IT4VAR13 (bottom row) and labeled by sera from rats immunized with M6pep only (left column) or with both M6pep and M9pep (center column), or by a rat anti-serum to N27 (right column). Refer to Table S2 for raw data.

Figure 6

ICAM-1-specific adhesion of erythrocytes infected by patient *P. falciparum* isolates, and inhibition of ICAM-1-adhering IEs by M6pep/M9pep-specific antibody. *A,* Adhesion of 33 patient isolates to ICAM-1 under physiologic flow. *B,* Antibody-mediated inhibition (>25%) of ICAM-1-specific IE adhesion among the 22 patient isolates adhering (≥ 10 adherent IEs/mm^2) to ICAM-1 under physiologic flow. The isolates were tested in one experiment with five technical replicates. Fewer than 0.25 IEs/mm^2 bound to uncoated channels. Isolates from patients with uncomplicated malaria (○), cerebral malaria (▲), and non-cerebral severe disease (●) are indicated in both panels.

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700 **Table 1: Clinical characteristic of Ghanaian study participants contributing plasma**

	Severe malaria (N=35)				Uncomplicated malaria (N=44)			
Age group (number)	1-2 (n=16)	3-4 (n=9)	5-6 (n=6)	>6 (n=4)	1-2 (n=8)	3-4 (n=15)	5-6 (n=8)	>6 (n=13)
Age years[1]	2.2 (1.9;2.7)	3.4 (3.2;4.3)	5.5 (5.3;6.6)	7.8 (7.5;8.2)	2.5 (1.8;2.7)	3.9 (3.4;4.4)	5.9 (5.3;6.9)	9.2 (8.1;10.8)
Blantyre coma score[1]	5.0 (5.0;5.0)	5.0 (4.3;5.0)	5.0 (4.3;5.0)	4,0 (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)
Haemoglobin (g/dl)[1]	9.6 (5.6;10.8)	6.3 (3.1;9.6)	10.3 (8.8;10.9)	10.8 (7.0;12.0)	9.6 (8.9;10.3)	9.6 (6.9;10.8)	9.5 (8,4;11.9)	11.4 (10.6;11.7)
Parasites per µl (x1000)[1]	41.5 (14.3;171.0)	147.3 (4.3; 210.8)	123.2 (2.9; 204.9)	114.5 (56.3; 152.6)	8.3 (0.4;26.0)	18.0 (6.3; 62.0)	19.7 (3.2;71.2)	14.7 (1.6;109.9)

701 [1]Median (25%;75%). None of the participants donating plasma was diagnosed with cerebral malaria.

Table 2: Clinical characteristics of Ghanaian and Tanzanian study participants contributing *P. falciparum* parasite isolates

	Severe malaria (N=24)				Uncomplicated malaria (N=9)			
Age group (number)	<1 (n=3)	1-2 (n=11)	3-4 (n=7)	≥5 (n=3)	<1 (n=1)	1-2 (n=2)	3-4 (n=4)	≥5 (n=2)
Age years[1]	0.9 (0.89; 0.95)	2.0 (1.67;2.53)	4.01 (3.47; 4.78)	7.4 (5.56;7.68)	0.36	2.50 2.76	3.6 (3.07; 4.58)	6.4 11.3
Blantyre coma score[1]	1.0 (0.0;5.0)	5.0 (2.0;5.0)	5.0 (2.0;5.0)	3.0 (2.0;3.0)	5.0	5.0	5.0 (5.0;5.0)	5.0 5.0
Hemoglobin (g/dl)[1]	7.8 (4.2;8.2)	4.7 (4.4;6.1)	8.6 (3.8;10.8)	10.5 (6.1;11.8)	11.8	12.0 12.3	8.6 (6.9;10.2)	12.5 11.7
Parasites per µl (x1000)[1]	64.8 (4.0; 165.4)	74.8 (33.2; 194.5)	182.9 (42.2; 456.2)	63.3 (49.2;77.5)	91.0	77.5 70.5	58.9 (18.7;133.4)	87.4 13.7

[1]Median (25%;75%). The severe malaria includes patients with cerebral malaria (N=7), severe malarial anemia (N=11) and hyperparasitaemia, multiple convulsions and/or respiratory distress (N=9).

705 **Table 3. Recombinant proteins used in the study**

	ID	Genome	PfEMP1	Domain sub-type [1]	Binds ICAM-1 [2]	Group [3]	Gene source [9]
DBLβ_motif domains[13]	M1	3D7	PF11_0521[4]	DBLβ3_D4	Yes[10]	A	
	M2	BM048	JF712902	DBLβ3_D4	Yes[10]	A	
	M3	BM066	JF712903	DBLβ3_D4	Yes[10]	A	
	M4	BM021	JF712900	DBLβ3_D4	Yes[10]	A	
	M5	BM057	JN037695	DBLβ3_D4	Yes[10]	A	
	M6	3D7	PFD1235w[5]	DBLβ3_D4	Yes[10]	A	
	M7	MN35	KJ866957	DBLβ3_D4	Yes[10]	A	
	M8	HB3	VAR03	DBLβ3_D4	Yes[10]	A	
	M9	Dd2	VAR32[6]	DBLβ1_D4	Yes[10]	A	
	M10	MN56	KM364031	DBLβ1_D4	Yes[10]	A	
	M11	A4395	KJ866958	DBLβ3	Yes[10]	A	
	M12	1914	AFJ66668	DBLβ1_D4	Yes[10]	A	
	M13	BM028	JF712901	DBLβ3_D4	Yes[10]	A	
	M14	-	KM364033	DBLβ3	Yes[10]	A	
	M15	MN062	KF984156	DBLβ1_D4	Yes[10]	A	
DBLβ_non-motif domains[13]	N20		CDO62031		No[11]	A	Synthetic gene (https://www.eurofinsgenomics.eu/)
	N21		CDO61797		No[11]	A	Synthetic gene (https://www.eurofinsgenomics.eu/)
	N22		CDO63496		No[11]	A	Synthetic gene (https://www.eurofinsgenomics.eu/)
	N23	Dd2	VAR25	DBLβ11_D4	No[10]	A	
	N24	HB3	VAR1CSA	DBLβ11_D4	No[10]	A	
	N25	3D7	PF13_0003	DBLβ9_D8	No[10]	A	
	N26	A4393	KJ866959	DBLβ3	No[10]	A	
	N27	IT4	IT4VAR13[7]	DBLβ3_D4	Yes[10+12]	B	
	N28	1983	JQ691647	DBLβ3_D4	No[10]	A	

N29	MN35	KM364034	DBLβ6	No[10]	A	
N30	Dd2	VAR52	DBLβ7_D4	No[10]	A	
N31	HB3	VAR01	DBLβ7_D4	No[10]	A	
N32	1983	JQ691649	DBLβ6_D4	No[10]	A	
N33	IT4	IT4VAR16[8]	DBLβ5_D4	Yes[10+12]	B	Genomic DNA using forward/reverse primers: 5'-ATCCCGGGTGTGCTGAACCTAATGGTAG-3'/ 5'-ATGCGGCCGCTACAAGCACACGCATCATC-3'

[1] Nomenclature as described in (15). [2] Yes indicates DBLβ_motif domains that bind ICAM-1 (M1-M15, N27 and N33), No indicates domains that do not bind ICAM-1 (remainder). [3] All domains were group A except for two group B DBLβ domains as indicated (N27 and N33). [4] a.k.a. PF3D7_1150400. [5] a.k.a. PF3D7_0425800. [6] a.k.a. KOB85388. [7] a.k.a. ABM88750. [8] a.k.a. AAS89259. [9] From genomic DNA, using previously described primers (15, 33), except where indicated. [10] (Data in ref. 33). [11] Unpublished data. [12] (Data in ref. 62). [13] (Data in refs. 15, 33, 62).