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Binding ICAM-1 and Is a Target of 
Cross-Reactive, Adhesion-Inhibitory Antibodies

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A Novel Domain Cassette Identifies *Plasmodium falciparum* PfEMP1 Proteins Binding ICAM-1 and Is a Target of Cross-Reactive, Adhesion-Inhibitory Antibodies


Cerebral *Plasmodium falciparum* malaria is characterized by adhesion of infected erythrocytes (IEs) to the cerebral microvasculature. This has been linked to parasites expressing the structurally related group A subset of the P. falciparum erythrocyte membrane protein 1 (PfEMP1) family of IE adhesion ligands and to IEs with affinity for ICAM-1. However, recent evidence has cast doubt on both these associations, tempering hopes of the feasibility of developing a vaccine based on ICAM-1-binding PfEMP1. In this study, we report the identification of a domain cassette (DC) present in group A var genes from six genetically distinct *P. falciparum* parasites. The three domains in the cassette, which we call DC4, had a high level of sequence identity and cluster together phylogenetically. Erythrocytes infected by these parasites and selected in vitro for expression of DC4 adhered specifically to ICAM-1. The ICAM-1-binding capacity of DC4 was mapped to the C-terminal third of its Duffy-binding–like β3 domain. DC4 was the target of broadly cross-reactive and adhesion-inhibitory IgG Abs, and levels of DC4-specific and adhesion-inhibitory IgG increased with age among *P. falciparum*–exposed children. Our study challenges earlier conclusions that group A PfEMP1 proteins are not central to ICAM-1–specific IE adhesion and support the feasibility of developing a vaccine preventing cerebral malaria by inhibiting cerebral IE sequestration. *The Journal of Immunology*, 2013, 190: 240–249.

*Plasmodium falciparum* causes the most severe type of malaria in humans. The disease cost ∼700,000 lives in 2010 alone, mainly among children <5 y of age in tropical Africa (1). The pathogenesis of *P. falciparum* malaria is closely associated with the ability of infected erythrocytes (IEs) to adhere to vascular host receptors, a process that leads to tissue-specific inflammation, circulatory obstruction, and organ dysfunction (2). IE adhesion is mediated by members of the highly polymorphic *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, encoded by ∼60 var genes per parasite genome, and the receptor specificity of these 150–400 kDa proteins is determined by the constituent two to seven Duffy-binding–like (DBL) and cysteine-rich interdomain region (CIDR) domains (3).

Despite the extensive variation of the *var* genes, they can be divided into three major groups (A, B, and C) based on sequence, chromosomal location, and promoter sequence (4, 5). Transcription of group A genes, which are less diverse than the other groups, has been related to severe malaria in several studies (6–11), which fits the restricted serological diversity of *P. falciparum* from patients with severe malaria and the relatively rapid acquisition of immunity to complicated disease (12–14). Particular subtypes of DBL and CIDR domains can be found in combination in short tandem domain cassettes (DCs) shared by many different parasite strains (15), and two such cassettes (DC8 and DC13) found in group A PfEMP1 proteins were recently implicated in the pathogenesis of cerebral *P. falciparum* malaria (16–18). The key event of this grave complication is accumulation of IEs in the cerebral microvasculature, and ICAM-1 has been suggested as an important receptor for IEs adhering in the brain (19–21). However, IEs selected for expression of DC8 or DC13 by panning on cerebral microvascular endothelial cells were found not to bind to ICAM-1 (16, 17). Furthermore, all but one of the ICAM-1–binding DBL domains identified so far belong to a fairly diverse set of DBLβ domains from group B or C PfEMP1 proteins (22–24), and it has been proposed that group A PfEMP1 proteins are not under selection for ICAM-1 binding (24). These and other findings have cast doubt on the importance of ICAM-1 binding and the involvement of group A PfEMP1 proteins in the pathogenesis of cerebral malaria. Even if ICAM-1 is in fact important for cerebral IE adhesion, the diversity of the above-mentioned
ICAM-1–binding DBLβ domains in group B and C PfEMP1 proteins make them unlikely candidates for development of a vaccine preventing cerebral malaria by inhibiting IE sequestration in the brain. On this basis, the current study was designed to investigate if the ICAM-1–binding domain of the previously identified group A PfEMP1 protein PFD1235w (7, 25) is part of an interclonally conserved DC, which would make such an ambitious vaccine goal more realistic.

Materials and Methods
Cloning and sequencing of var genes
Genomic DNA from 60 different Ghanaian P. falciparum isolates from an earlier study (14) was examined. Using primer 875M 5'-ATGGGGAATGCATCATCA-3' in combination with primer 382M 5'-ATGGGGAATGCATCATCA-3' (BM021, BM057), 384M 5'–AACGC-AGAAGATA GAAATC-3' (BM028), we could PCR amplify a pfd1235w-like three-DC (DC4: DBL3, CIDR3, DBLβ3) (see Fig. 1) from the five isolates indicated. Amplicons were cloned into the pCR2.1-TOPO vector. Sequences were then classified and named by finding the most significant HMM matches. Correct predictions were achieved for 1892 out of 1898 database and selecting the most significant (lowest E-value) nonoverlapping hits. The ability of the HMM to predict domain classes was tested by searching all sequences with known annotation (15) against the HMM database and at PlasmoDB (http://plasmodb.org/plasmo/) and the National Center for Biotechnology Information (NCBI) database.

PfEMP1 domain classification, sequence identity calculation, and phylogenetic trees
The PfEMP1 protein groups and subgroups were classified using Hidden Markov Models (HMM) using HMMer 3.0 software (http://hmm.janelia.org/). The ability of the HMM to predict domain classes was tested by searching all sequences with known annotation (15) against the HMM database and selecting the most significant (lowest E-value) nonoverlapping HMM matches. Correct predictions were achieved for 1892 out of 1898 DBL and CIDR domain groups and subgroups. The new PfEMP1 protein sequences were then classified and named by finding the most significant nonoverlapping hits in the HMM database, as described previously (15). Where this nomenclature leads to ambiguity (when more than one domain of the same subgroup is present in a given PfEMP1 protein), the location of the domain in question was specified (as indicated along the top of Fig. 1).

TABLE I. Comparison of DBLα, CIDRα, and DBLβ domains in ICAM-1–binding and –nonbinding regions of PfEMP1 proteins

<table>
<thead>
<tr>
<th>Region</th>
<th>No. of Sequences Compared</th>
<th>DBLα</th>
<th>CIDRα</th>
<th>DBLβ</th>
<th>Percenta</th>
<th>Percenta</th>
<th>Percenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC4</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>64</td>
<td>351</td>
<td>77</td>
<td>250</td>
</tr>
<tr>
<td>ICAM-1–binding regions in IT4</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>52</td>
<td>415</td>
<td>44</td>
<td>264</td>
</tr>
<tr>
<td>Other</td>
<td>226</td>
<td>94</td>
<td>205</td>
<td>47</td>
<td>442</td>
<td>35</td>
<td>280</td>
</tr>
</tbody>
</table>

aThe percentage identity among amino acid sequences.
bThe average number of amino acids in sequences compared.

Thus, DBLβ3 denotes a DBL domain of subgroup β3, whereas DBLβ3_D4 specifies that the domain is the fourth domain in the given PfEMP1 protein. The average amino acid identity of domain classes was calculated using the Praline multiple sequence alignment tool (http://www.ibi.vu.nl/programs/pralinewww/) using default settings. Multiple alignments of DBLα, CIDRα, and DBLβ domains were made using ClustalW2 (ftp://ftp.ebi.ac.uk/pub/software/clustalw2/2.0.12/) and analyzed by neighbor-joining methods (26) using Mega 4.0 software (27) to create unrooted cladograms. The number of bootstrap trials was 500. Amino acid sequences of DBLβ domains tested for ICAM-1 binding were aligned with MUSCLE v. 3.7 software (28). One hundred maximum-likelihood bootstrap trees were constructed using the phdres program of RAxML v. 7.2.5 software (29, 30), and, from these, an extended 50% majority rule consensus tree was built. The PFD1235w-DBLβ3_D4 and PFD1235w-DBLβ3_D5 domains were modeled using the HHpred server (31) with default settings and the DBLβ domain structural information provided by Juillerat et al. (32) as template.

Recombinant proteins
Most of the recombinant PfEMP1 domain constructs used were produced in a baculovirus system, using DNA amplified with specific primers as described previously (7) (Supplemental Tables I and II). In a similar way, we produced permutation hybrids of three regions of PFD1235w-DBLβ3_D4 (4A: aa 739–930; 4B: aa 931–1029; and 4C: aa 1230–1221) and three regions of PFD1235w-DBLβ3_D5 (5A: aa 1239–1425; 5B: aa 1426–1522; and 5C: aa 1523–1689). The DNA sequences of the six hybrids (5A-4B-4C, 4A-5B-4C, 4A-4B-5C, 5A-5B-4C, and 5A-4B-5C) were amplified by overlapping PCR using the primers listed in Supplemental Table I. Exceptions were BM028-DBLβ3 and IT4 var16-DBLβ5, which were produced in Eschericia coli as described (33 and A. Brown, unpublished observations).

Human ICAM-1 was produced as a chimera with human IgG1-Fc (34, 35) in transfected HEK293-F cells (Invitrogen), using FreeStyle MAX reagent (Invitrogen) according to the manufacturer’s instructions. Recombinant protein was purified from the supernatant after 6 d using ProPur Protein G Ab purification kits (Nunc). The protein was eluted using 0.2 M glycine/HCl buffer (pH 2.5) and neutralized in suspension using Tris/HCl buffer (1 M, pH 9).

Accession numbers for nucleotide sequences and protein data available at PlasmoDB (http://plasmodb.org/plasmo/) and the National Center for Biotechnology Information.
Biotechnology Information (http://www.ncbi.nlm.nih.gov/genbank/) are as follows: PFD1235w (XP_001351561), PF11_0008 (XP_001347692), PF13_0003 (XP_001349740), PFD0020c (XM_001351285), PF08_0140 (XM_001349476), PF08_0141 (XM_001349477), IT4var02 (AY372122), IT4var16 (AAAS9259), IT4var64 (EF158102), BM021 (JF712900), BM028 (JF712901), BM048 (JF712902), BM057 (JP037695), and BM66 (JF712903).

PfEMP1-specific antisera

Rat antisera to recombinant PfEMP1 constructs (Supplemental Table II) were generated by subcutaneous immunization with 10–20 μg protein in Freund’s complete adjuvant, followed by several booster vaccinations in IFA. Preimmunization sera were collected and used as negative controls in the flow cytometry experiments. All of the animal experiments described in this study were conducted according to the guidelines described in act numbers LBK 1306 (23/11/2007) and BEK 1273 (12/12/2005) and approved by The Danish Animal Procedures Committee (“Dyreforsøgstilsynet”) as described in permit number 2008/561-1498.

P. falciparum–reactive human Abs

Pooled plasma from nine P. falciparum–exposed Liberian adults (36) was used for affinity purification of Abs reactive with PFD1235w-DBLa3-D4, PFD1235w-DBLb-D5, Dd2var32-DBLa1.7-DBLb1, and IT4var16-DBLb5. In brief, the recombinant proteins (1 mg) were dialyzed against coupling buffer and subsequently coupled to HiTrap NHS-activated HP columns as described by the manufacturer (GE Healthcare). The pooled Liberian plasma was the diluted 1:1 in PBS and affinity purified on the HiTrap column. Following elution in glycine buffer (0.1 M, pH 2.8), the Abs were neutralized in Tris buffer (1 M, pH 9) and dialyzed against PBS. The same plasma pool, depleted of reactivity with PFD1235w-DBLb3-D4, PFD1235w-DBLb5-D5, and the 4A-5B-SC and 5A-4B-4C hybrids of these domains, was also used in some experiments. To that end, we coupled the proteins (200 μl; 0.2 mg/ml in PBS) to Epoxy M270 Dynabeads according to the manufacturer’s instructions (Invitrogen). The plasma pool (200 μl) was then incubated (2 h; room temperature) with the coupled Dynabeads (3 mg). Abs bound to the Dynabeads were eluted in glycine/HCl (0.1 M, pH 2.75) and neutralized in Tris buffer (1 M, pH 9).

Pooled plasma from P. falciparum–exposed Tanzanian donors (37) was used in competition assays of PfEMP1 binding to ICAM-1, whereas individual plasma samples were used to study the age dependency of acquisition of Abs interfering with binding of PFD1235w-DBLb3-D4 to ICAM-1.

Malaria parasites and in vitro selection procedure

The P. falciparum clone 3D7 and two Ghanaian patient isolates (BM021 and BM057) described previously (14) were maintained in long-term in vitro culture and selected for IE surface expression of DC4-containing PfEMP1 proteins by repeated Ab selection as described (25). In brief, we used rabbit antisera to PFD1235w-DBLb13 (25) to select 3D7 and PFD1235w-CIDRox1.6 (25) to select BM057, whereas the above-mentioned rat antisera to JF712900-DBLb11 was used to select BM021. The identity of the isolates was routinely verified by genotyping as described (38), and Mycoplasma infection was regularly excluded using the MycoAlert Mycoplasma Detection Kit (Lanza) according to the manufacturer’s instructions.

Flow cytometry analysis of IE surface-expressed PfEMP1 proteins

P. falciparum IEs were DNA-labeled with ethidium bromide and surface-labeled with antisera or immune plasma followed by FITC-conjugated secondary Abs as described previously (25). FITC fluorescence data from ethidium bromide–positive cells were collected on a Cytomics FC 500 MPL flow cytometer (Beckman Coulter) and analyzed in WinList version 6.0 (Verity Software House).

Adhesion of P. falciparum–IE adhesion to ICAM-1

Static adhesion assays were done as described previously (25). In brief, late-stage IEs labeled with [3H]hypoxanthin were purified by gelatin flotation and allowed to adhere to monolayers of Chinese hamster ovary (CHO) cells (wild-type, ICAM-1–transfected, or CD36-transfected; all from American Type Culture Collection) in triplicate wells of flat-bottom microtiter plates (Nunc). After incubation (2 h, 37°C) on a rocking table, unbound cells were washed off using a BioMek 2000 robot (Beckman Coulter). Adhering cells were then harvested on Unifilter-96 mats (PerkinElmer) and [3H]-scintillation counted on a TopCount NXT (PerkinElmer).

Binding of recombinant PfEMP1 proteins to ICAM-1 and inhibition of this binding

Maxisorp plates (Thermo Scientific) were coated (overnight; 4°C) with recombinant PfEMP1 proteins (0.1–0.2 μg/well) in glycine/HCl buffer.
Table II. Sequence identity of PfEMP1 domains downstream of DC4

<table>
<thead>
<tr>
<th>Domain Group</th>
<th>DBL(\alpha)</th>
<th>DBL(\gamma)</th>
<th>DBL(\beta)</th>
<th>CIDR(\beta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of sequences compared</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Average sequence identity (%)</td>
<td>47</td>
<td>43</td>
<td>37</td>
<td>40</td>
</tr>
</tbody>
</table>

*Domain subgroups were not considered in this analysis.

http://www.ibi.vu.nl/programs/pralinewww/

(0.1 M, pH 2.75) and blocked with blocking buffer (PBS, 0.5 M NaCl, 1% Triton X-100, 1% BSA [pH 7.2]). Recombinant ICAM-1–Fc chimera (0.2 μg/well) was added (1 h; room temperature), followed by washing and detection of bound chimera by anti-human HRP-IgG (1:3000 in blocking buffer; 1 h) or by mouse anti-human ICAM-1 (clone RR1/1; AH Diagnostics; 1:400) followed by anti-mouse HRP-IgG (DakoCytomation; 1:1000) (39). In assays of PfEMP1 domain interference of this binding, the ICAM-1–Fc chimera (2 μg/ml) was preincubated with recombinant PfEMP1 constructs (0–50 μg/ml; 1 h; room temperature). In assays of human plasma Ab interference of the binding of ICAM-1 to PfEMP1 proteins, pooled plasma (1:10) or Dynabead-depleted pooled plasma (1:5) was added together with the ICAM-1–Fc chimera.

Cross-reactivity of human PfEMP1-specific Abs
Maxisorp plates (Nunc) were coated with recombinant PfEMP1 proteins as above. Pooled plasma (1:100) preincubated or not with recombinant PfEMP1 proteins (10 μg/ml; 1 h; room temperature), followed by washing and detection of bound IgG using HRP-conjugated rabbit anti-human IgG as described (7).

Results
DBL\(\alpha\)1.4/DBL\(\alpha\)1.1–CIDR\(\alpha\)1.6-DBL\(\beta\)3 form an interclonally conserved three-DC (DC4)
We have previously identified a group A var gene (pf\(d1235w\)) that is transcribed at high levels in P. falciparum 3D7 selected for re- cognition of IEs by IgG from semi-immune children (7). In the current study, we first used a set of pf\(d1235w\)-specific primers and a degenerate group A exon 2–specific primer to identify an additional five related genes by screening 60 genetically distinct P. falciparum isolates from Ghana. The sequence of the five new genes all contained an N-terminal series of three specific domain subgroups (DBL\(\alpha\)1.4/DBL\(\alpha\)1.1–CIDR\(\alpha\)1.6-DBL\(\beta\)3) that is also present in pf\(d1235w\) (Fig. 1). We propose the name DC4 for this series, in accordance with the nomenclature of Rask et al. (15). The sequence identity of the individual DC4 domains was substantially higher than that of previously identified ICAM-1–binding (and –nonbinding) domains (Table I), and they clustered together phylogenetically (Fig. 2). Domains downstream of DC4 were of different groups and subgroups (Fig. 1) and with low sequence identity (Table II). These data show that DC4 is a new strain-transcending DC that is likely to be of functional significance in the pathogenesis of severe P. falciparum malaria.

DC4-positive IEs adhere to ICAM-1
P. falciparum parasites causing severe malaria in children tend to express structurally related PfEMP1 proteins (group A) (5, 7, 13, 14). PFDF1235w is among these and mediates IE binding to ICAM-1 (25). To test whether DC4 generally confers IE adhesiveness to ICAM-1, we used specific antisera to select three genotypically distinct parasite isolates (3D7, BM021, and BM057) for IE surface expression of DC4-containing PfEMP1 proteins (Fig. 3A). Although none of the isolates bound to ICAM-1–transfected CHO cells before selection, they all bound to these cells at high levels following selection for IE surface expression of a DC4-containing PfEMP1 protein (Fig. 3B). IE adhesion to wild-type or CD36-transfected CHO cells was unaffected by this selection. Ab selection of P. falciparum 3D7 for reactivity with the DC4-negative PfEMP1 protein PF11_0008 (3D7 only) was achieved as described (7).

FIGURE 3. Erythrocytes infected by P. falciparum parasites expressing a DC4-containing PfEMP1 protein adhere to ICAM-1. Surface expression of DC4-containing PfEMP1 proteins on erythrocytes infected by P. falciparum 3D7, BM021, or BM057 before (DC4−) and after (DC4+) repeated selection for reactivity with DC4-specific antisera (A). The ability of these IEs to bind to wild-type (CHO-wt), CD36-transfected (CHO-CD36), and ICAM-1–transfected (CHO-ICAM1) CHO cells before (−) and after (+) selection for surface expression of DC4 (3D7, BM021, and BM057) or the DC4-negative PfEMP1 PF11_0008 (3D7 only) (B). Mean adhesion (three independent experiments) relative to the adhesion of PDF1235w+ 3D7-IEs to ICAM-1–transfected CHO cells in (B) is shown. Error bars indicate SD.
(25) did not induce IE adhesion to ICAM-1 (Fig. 3B). These data show that DC4 identifies PfEMP1 proteins that can adhere to ICAM-1, an adhesion phenotype that has been associated with sequestration of IEs in the cerebral microvasculature (reviewed in Ref. 40).

The ICAM-1–binding site of DC4-containing PfEMP1 proteins is located in the DBLβ3 domain

To identify the DC4 domain(s) mediating binding to ICAM-1, we assessed the ability of recombinant constructs representing each of the extracellular domains of PFD1235W (Fig. 1) to bind to ICAM-1. Despite their high structural similarity, only the N-proximal (DBLβ3_D4) of the two DBLβ3 domains in PFD1235w, which lies within DC4, bound ICAM-1 (Fig. 4A). All of the six DC4-DBLβ3 domains from the DC4-containing PfEMP1 proteins identified (Fig. 1), as well as the non-DC4 IT4var16-DBLβ5, bound ICAM-1 in a concentration-dependent manner, whereas two DBLβ3 domains (PFD1235w-DBLβ3_D5 and PF13_0003-DBLβ3) that do not form part of a DC4 cassette (Fig. 4B) did not bind to ICAM-1. All of the six DC4-DBLβ3 domains and IT4var16-DBLβ5 competed with PFD1235w-DBLβ3_D4 for ICAM-1 binding, whereas the non-DC4 PFD1235w-DBLβ3_D5 and PF13_0003-DBLβ3 domains did not (Fig. 4C). These data show that the ICAM-1–binding site in DC4 is within DBLβ3_D4.

DBLβ3 domains in DC4 are structurally related

Until now, the majority of described ICAM-1–binding sites in PfEMP1 are located in DBLβ5 domains of group B PfEMP1 proteins (22–24). The exceptions are PF11_0521-DBLβ3 (group A; 23), Dd2var32-DBLβ1 (group A), and IT4var01-DBLβ5 (group C; 22). A phylogenetic analysis of the 16 ICAM-1–binding DBLβ3 domains described earlier and in this paper, as well as 19 DBLβ3 domains not binding ICAM-1 (22, 23, 41, 42), showed that the ICAM-1–binding DBLβ3 of DC4 form a cluster separate from other ICAM-1–binding and –nonbinding domains (Fig. 5). It remains to be seen whether DC4 domains use similar or different binding surfaces to mediate their interactions with ICAM-1 when compared with previously characterized group B ICAM-1–binding DBL domains. However, the distinct clustering of these domains makes it possible that they have evolved separate and distinct ICAM-1–binding surfaces.

The ICAM-1–binding site of PFD1235w is located in the C-terminal third of DBLβ3_D4

To pinpoint the ICAM-1–binding site in DBLβ3_D4, we produced six hybrid DBLβ3 domains (H1–H6). The hybrids represented all possible permutations of the N-terminal, central, and C-terminal parts of the two highly similar but functionally distinct DBLβ3 domains in PFD1235w: the DBLβ3_D4 domain that binds ICAM-1 and the DBLβ3_D5 domain that does not (Fig. 6A, 6B). Only the three hybrids containing the C-terminal part of DBLβ3_D4 (H1, H2, and H4) retained the ICAM-1–binding capacity of native PFD1235w-DBLβ3_D4 (Fig. 6C) and competed efficiently with PFD1235w-DBLβ3_D4 for binding to ICAM-1 (Fig. 6D). The very end of DBLβ3_D4 was not required for binding, as a DBLβ3_D4 construct truncated of the 12 C-terminal amino acids (T1) bound ICAM-1 comparable to DBLβ3_D4 (Fig. 6C). The three ICAM-1–binding hybrids also competed for binding to ICAM-1 with two non-DC4 DBLβ3 domains, namely Dd2var32-DBLβ1 (Fig. 6E) and the phylogenetically more distant (group B) IT4var16-DBLβ5 (Fig. 6F). To exclude the possibility that improper folding was the cause of the absent ICAM-1–binding
property of the three remaining hybrids, we used them to immunize rats and tested the reactivity of the antisera with IEs expressing PFD1235w. All of the antisera efficiently labeled PFD1235w IEs, strongly supporting that the recombinant constructs used for immunization were correctly folded (Fig. 6G). To further substantiate our mapping of the ICAM-1–binding site of PFD1235w to the C-terminal third of DBL$\text{b}_3$D4, we depleted an ICAM-1 adhesion-inhibitory plasma pool of Abs that reacted with a hybrid (H4) containing this subdomain. This reduced the ability of the plasma pool to inhibit adhesion of PFD1235w-DBL$\text{b}_3$D4 to ICAM-1 to the same extent as depletion with native DBL$\text{b}_3$D4 (Fig. 7). Depletion of the plasma pool with native DBL$\text{b}_3$D5 or with a DBL$\text{b}_3$ hybrid not containing the C-terminal part of DBL$\text{b}_3$D4 (H3) had no effect (Fig. 7). We conclude that residues 1029–1209 form an essential part of the ICAM-1–binding site.

FIGURE 5. Phylogeny of ICAM-1–binding and –nonbinding DBL$\text{b}$ domains. Maximum-likelihood phylogram of 16 ICAM-1–binding DBL$\text{b}$ domains (red) and 19 DBL$\text{b}$ domains not binding to ICAM-1 (blue). The shaded area indicates DBL$\text{b}$ domains in DC4. The edge numbers indicate the bipartition bootstrap support (%), whereas the UpsA, UpsB, or UpsC group that each gene belongs to is indicated by an uppercase letter within a box next to the gene names. Scale bar indicates amino acid substitutions per site.

DC4 is target of broadly cross-reactive and adhesion-inhibitory IgG Abs

The sequence identity of DC4 domains (Table I) and their phylogenetic clustering (Fig. 2) suggest that they might be serologically cross-reactive. We therefore immunized rats with recombinant proteins representing various DC4 domains from the six DC-containing PfEMP1 proteins identified (Fig. 1). The resulting antisera reacted with the surface of erythrocytes infected by heterologous parasites expressing a DC4-containing PfEMP1 protein, whereas none of them reacted with erythrocytes infected by 3D7 parasites expressing the PfEMP1 protein PF11_0008, which binds to PECAM-1 (25) and does not contain a DC4 cassette (Fig. 8). On this basis, we proceeded to examine whether natural $P$. falciparum infections also induce cross-reactive DC4-specific IgG Abs and the functional significance of such cross-reactivity. We found that DBL$\text{b}_3$D4 from all of our six DC4-containing PfEMP1 proteins showed extensive cross-reactivity in competition ELISA using pooled immune plasma, whereas the other DBL$\text{b}$ domains tested did not (Fig. 9A). To assess the functional significance of this cross-reactivity, we next used pooled immune plasma that could inhibit ICAM-1–specific adhesion to DBL$\text{b}$ domains. Plasma IgG from this pool that had been affinity-purified on PFD1235w-DBL$\text{b}_3$D4 inhibited the ability of ICAM-1 to bind to each of the DBL$\text{b}_3$D4 of our six DC4-containing PfEMP1 proteins (Fig. 9B). The PFD1235w-DBL$\text{b}_3$D4–specific IgG also inhibited ICAM-1 binding to DBL$\text{a}$.7-DBL$\text{b}$1 from one DC4-negative group A PfEMP1 protein (Dd2var32), whereas ICAM-1 binding to DBL$\text{b}$5 from a group B PfEMP1 protein (IT4var16) was unaffected. Conversely, affinity-purified Dd2var32-DBL$\text{a}$.7-DBL$\text{b}$1–specific IgG inhibited the ability of ICAM-1 to bind to DBL$\text{b}_3$D4 from each
of the DC4-containing PfEMP1 proteins, whereas IT4var16-DBL\textsubscript{b}5–specific IgG did not. As expected, affinity-purified PFD1235w-DBL\textsubscript{b}3\_D5–specific IgG or nonimmune IgG did not inhibit binding of ICAM-1 to any of the tested DBL\textsubscript{b} domains. In a final set of experiments, we showed that levels of IgG inhibiting ICAM-1 binding to PFD1235w-DBL\textsubscript{b}3\_D4 were higher among Tanzanian children aged 5–9 y than among 2–4-y-old children from the same community (Fig. 9C). We conclude from these experiments that DBL\textsubscript{b}3\_D4 of DC4 induces cross-reactive IgG Abs that interfere with PfEMP1-specific adhesion of IEs to ICAM-1 and are acquired in an age-dependent manner among people exposed to natural \textit{P. falciparum} infection.

**Discussion**

Severe \textit{P. falciparum} malaria, particularly cerebral malaria (CM), has repeatedly been associated with parasites transcribing a structurally related and serologically cross-reactive subset of PfEMP1 proteins called group A (4, 5, 7, 9). The pathogenesis of CM has furthermore been associated with cerebral sequestration of IEs binding to ICAM-1 (19, 40). In this study, we describe a novel tandem three-domain PfEMP1 region \textit{[a so-called DC (15)]} called DC4, which identifies serologically cross-reactive group A PfEMP1 proteins that bind to ICAM-1. The domains characterizing DC4 were originally observed in the group A PfEMP1 protein PFD1235w (7, 25), but we show in this study that other PfEMP1 proteins from genetically distinct parasite isolates also possess this cassette of phylogenetically related domains. We furthermore show that erythrocytes infected by parasites expressing DC4-containing PfEMP1 proteins can adhere to ICAM-1. DC4-containing PfEMP1 proteins show high sequence identity within DC4 but considerable diversity in sequence and domain
architecture downstream of the cassette. This suggests that the ICAM-1–binding site lies within DC4, and, indeed, we could map it to the DBLβ3 D4 domain within DC4. Phylogenetic analysis of this domain in different DC4-containing proteins revealed a tight cluster that excluded other DBL β3 domains, including PFD1235w-DBLβ3 D5, which lies outside DC4 of that PFEPl protein and has no affinity for ICAM-1.

Although at least two previously described DBL β3 domains contain an ICAM-1–binding site, many others do not (23, 24). By testing hybrids of the ICAM-1–binding PFD1235w-DBLβ3 D4 domain and immediate downstream DBLβ3 D5 domain that does not bind ICAM-1, we could show that only aa 1029–1209 of DBLβ3 D4 were required for binding to ICAM-1. This region roughly corresponds to the part of DBLβ3 D4 that was previously known as C2 (aa 1103–1222), but which is now included in DBLβ (15, 22). Furthermore, we could demonstrate that the ICAM-1 binding of this 181-aa stretch was retained when placed downstream of nonbinding stretches. Both of these results are at variance with earlier reports indicating that both the C-terminal and N-terminal parts of DBL β are required for binding to ICAM-1 and that the C-terminal part of the DBL β domain (the former C2 domain) cannot be interchanged among ICAM-1–binding domains (22, 42, 43). Our conclusion that only the C-terminal part of DBLβ is required for ICAM-1 binding is based on gain-of-function hybrid data rather than relying only on loss-of-function chimera data and is furthermore supported by Ab-depletion data showing that residues in the N-terminal part of the domain are not targeted by Abs inhibiting its binding to ICAM-1.

Only one (PF11_0521) of the many ICAM-1–binding PFEPl proteins described previously (22–24, 42, 43) belongs to the group A that has repeatedly been shown to be associated with severe P. falciparum malaria (5, 13, 14). Furthermore, it has been proposed that group A PFEPl proteins are not under selection for ICAM-1–binding DBL β domains (22), and large group B and group C PFEPl proteins appear to be under selection for concomitant adhesion to ICAM-1 and CD36 (24, 44). Because IE adhesion to CD36 is associated with uncomplicated malaria (21), these observations can be seen as arguments against an important role for ICAM-1–specific IE adhesion in the pathogenesis of severe P. falciparum malaria, not least CM. Very recent studies showing that in vitro selection of P. falciparum for expression of PFEPl proteins mediating adhesion to human brain microvascular endothelial cells leads to IEs not binding to ICAM-1, and to parasites selectively transcribing DCs other than DC4 (namely, DC8 and DC13), point in the same direction (16, 17).

In marked contrast to the above findings, our data indicate that group A PFEPl proteins containing the ICAM-1–binding DC4 are in fact present in a sizeable fraction of P. falciparum genomes. The CDR domain in DC4 is of a subgroup (CDRx1) that does not bind CD36 (45), and erythrocytes infected by parasites selected for expression of DC4-containing PFEPl proteins do not bind CD36. Conversely, we have previously shown that selection of 3D7 IEs for adhesion to CD36 leads to loss of PFD1235w expression (46). Dissociation between IE adhesion to CD36 and ICAM-1 has been observed previously (42), and this may be significant, because CD36 is absent or sparsely expressed on human brain endothelium (19). Furthermore, P. falciparum from CM patients (including fatal cases) were recently found to transcribe CIDRa1.6 (present in DC4) at higher levels than parasites from hospitalized children without severe disease (18). Together, these features support a role for DC4-mediated ICAM-1–specific adhesion of IEs in the pathogenesis of CM (reviewed in Ref. 40).

Acquisition of protective immunity to severe P. falciparum malaria precedes resistance to uncomplicated disease (reviewed in Ref. 47). Parasites expressing group A PFEPl proteins occur predominantly among children with limited immunity (7), and IgG to this group of Ags is acquired faster than IgG to other PFEPl groups (48, 49) and appear to be more cross-reactive (39, 50). The data presented in this study underpin these earlier findings and support the hypothesis that IgG to relatively conserved and functionally important Ab epitopes in group A PFEPl proteins are of importance in acquisition of clinical immunity to malaria. Thus, we found that DBLβ3 domains in DC4 of distinct PFEPl proteins competed efficiently with each other for binding to ICAM-1, whereas non-DC4 DBLβ domains were generally ineffective. Furthermore, rat antisera to DC4-specific domains in PFEPl proteins from distinct P. falciparum genomes could be used to select for IE adhesion to ICAM-1 and showed substantial cross-reactivity. Finally, we could show that similar cross-reactive Abs are acquired early in life by humans following natural exposure to P. falciparum parasites, including Abs to the functionally important C-terminal part of DBLβ3 D4.

Taken together, the results demonstrate the presence of an intergenomically conserved ICAM-1–binding epitope in a subset of group A PFEPl proteins and that this epitope is a target of cross-reactive Abs inhibiting IE adhesion to ICAM-1. There is conflicting evidence regarding the importance of ICAM-1–specific IE adhesion in the pathogenesis of CM, which remains poorly understood and may well involve both ICAM-1–dependent and –independent adhesion (21). Based on current evidence, DC4 appears to be an attractive candidate for inclusion in a vaccine against this severe complication of P. falciparum malaria.

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FIGURE 8. Cross-reactivity of experimental antisera to DC4-containing PFEPl proteins. Reactivity of antisera from rats immunized with DBLα1.4-CIDRa1.6, CIDRa1.6, or DBLβ3 D4 from the DC4-containing PFEPl proteins PFD1235w, JF712900 (BM021), JF712901 (BM028), JF712902 (BM048), JF037695 (BM057), and JF712903 (BM066) with erythrocytes infected by P. falciparum 3D7, BM021, BM057 and selected for expression of DC4 (DC+) or the DC4-negative PFEPl protein PF11_0008 (3D7 only). Reactivity (black boxes) or lack of reactivity (white boxes) is shown. Data are representative of a minimum of three independent experiments.
The reduction in reactivity by depletion is indicated by shading: black (>75%), dark gray (51–75%), light gray (26–50%), and white (0–25%) (A). Ab-mediated inhibition of ICAM-1 binding of DBLβ3 domains from PiEMP1 proteins containing DC4 (PFD1235w, BM021, BM028, BM048, BM057, and BM066) or not containing DC4 (Dd2var32, IT4var16) (B).

FIGURE 9. Cross-reactivity and acquisition of Abs to DC4-containing PiEMP1 proteins. ELISA reactivity of pooled immune plasma IgG with PiEMP1 proteins (coating Ag) containing (red boxes) or not containing DC4 (Dd2var32, IT4var16-DBL) (C).

The authors have no financial conflicts of interest.

References

The inhibitory capacity of an immune plasma pool, Abs from this pool purified on PFD1235w-DBLβ3 D4, Dd2var32-DBLβ1, IT4var16-DBLβ5, or PFD1235w-DBLβ3 D5, respectively, as well as a nonimmune plasma pool (Hu IgG) are shown. The inhibitory capacity is indicated by shading in (A). The ability of plasma samples from P. falciparum-exposed children aged 2–4 and 5–9 y to interfere with binding of PFD1235w-DBLβ3 D4 to ICAM-1 (C). Binding relative to binding in the absence of Abs is shown for one experiment representative of a minimum of three independent experiments. Medians (center line), central 50% (shaded boxes), central 80% (whiskers), and outliers are indicated.

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Disclosures
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