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\textit{J Immunol} published online 28 August 2015
http://www.jimmunol.org/content/early/2015/08/28/jimmunol.1501404

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/08/28/jimmunol.1501404.DCSupplemental

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Mapping the Binding Site of a Cross-Reactive *Plasmodium falciparum* PfEMP1 Monoclonal Antibody Inhibitory of ICAM-1 Binding

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The virulence of *Plasmodium falciparum* is linked to the ability of infected erythrocytes (IE) to adhere to the vascular endothelium, mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). In this article, we report the functional characterization of an mAb that recognizes a panel of PfEMP1s and inhibits ICAM-1 binding. The 24E9 mouse mAb was raised against PFDB1235w DBLβ3 D4, a domain from the group A PfEMP1s associated with severe malaria. 24E9 recognizes native PfEMP1 expressed on the IE surface and shows cross-reactivity with and cross-inhibition of the ICAM-1 binding capacity of domain cassette 4 PfEMP1s. 24E9 Fab fragments bind DBLβ3 D4 with nanomolar affinity and inhibit ICAM-1 binding of domain cassette 4—expressing IE. The antigenic regions targeted by 24E9 Fab were identified by hydrogen/deuterium exchange mass spectrometry and revealed three discrete peptides that are solvent protected in the complex. When mapped onto a homology model of DBLβ3 D4, these cluster to a defined, surface-exposed region on the convex surface of DBLβ3 D4. Mutagenesis confirmed that the site most strongly protected is necessary for 24E9 binding, which is consistent with a low-resolution structure of the DBLβ3 D4:24E9 Fab complex derived from small-angle x-ray scattering. The convex surface of DBLβ3 D4 has previously been shown to contain the ICAM-1 binding site of DBLβ domains, suggesting that the mAb acts by occluding the ICAM-1 binding surface. Conserved epitopes, such as those targeted by 24E9, are promising candidates for the inclusion in a vaccine interfering with ICAM-1—specific adhesion of group A PfEMP1 expressed by *P. falciparum* IE during severe malaria. *The Journal of Immunology*, 2015, 195: 000–000.

Human malaria caused by *Plasmodium falciparum* parasitemias remains a serious health problem. In 2013, an estimated 198 million cases of malaria resulted in 584,000 deaths, mostly in sub-Saharan Africa (1). The majority of deaths occurred in children <5 y of age.

Parasite virulence is linked to the ability of infected erythrocytes (IE) to adhere to the inside of host blood vessels, leading to inflammation, tissue obstruction, and organ dysfunction (2). IE adhesion is mediated by the surface expression of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins, which are able to bind to various host receptors present on the endothelium.

The multidomain PfEMP1 proteins are encoded by ~60 divergent var genes and consist of Duffy-binding–like (DBL) and cysteine-rich interdomain region protein domains (3), which can be divided into several major types (α, β, γ, etc.) and subtypes based on sequence similarities (4, 5). DBL domains generally contain three subdomains, which fold together to form a conserved α-helical core with loop insertions of variable sequence and length. Specific DBL and cysteine-rich interdomain region domains group together to form domain cassette (DC) families that are found across parasite isolates (5).

A frequently described PfEMP1 receptor is ICAM-1, and binding of IE to ICAM-1 during infection is linked to the development of symptoms of severe malaria, such as cerebral malaria (6–8). ICAM-1 is a membrane-bound protein with five extracellular domains (D1–D5) and is expressed by endothelial cells and leukocytes. ICAM-1 mediates leukocyte adhesion and migration to inflamed sites by binding to LFA-1 and Mac-1 (9, 10).

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Received for publication June 23, 2015. Accepted for publication July 23, 2015.

M.K.H. was supported by a Wellcome Trust investigator Award, F.L. and M.K.H. were supported by a project grant from the Medical Research Council (Grant G0901062). A.B., R.W.O., L.J., L.K.B., and A.T.R.J. were supported by grants from the Faculty of Health and Medical Sciences, the University of Copenhagen, the Aase and Ejnar Danielsens Fond, the Dagmar Marshalls Fond, the Oda and Einar Barfsensens Fond, and the Novo Nordisk Fond. Y.A. was supported by the Danish Council for Independent Research (Grant 4004-00032). P.M. was supported by European Union Grants CZ.2.16/3.1.00/24023 and CZ.1.05/1.1.00/02.0109.

The nucleotide sequences presented in this article have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank) under accession numbers KJ418726 and KJ418727.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DBL, Duffy-binding–like; DC4, domain cassette 4; Dmax, maximum particle diameter; HDX MS, hydrogen/deuterium exchange mass spectrometry; IE, infected erythrocyte; PDB, Protein Data Bank; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; Rg, radius of gyration; RU, response unit; SAXS, small-angle x-ray scattering; SPR, surface plasmon resonance.

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Surface expression of the recently identified DC4 containing PfEMP1s leads to ICAM-1–specific adhesion of IE, which is mediated by the DBLβ3_D4 PfEMP1 domain (11, 12) and appears to be involved in the pathogenesis of severe disease (13). Naturally acquired Abs against DC4 DBLβ3_D4 are cross-reactive and cross-inhibitory of ICAM-1 binding across members of DC4 and other DC types (12), suggesting that the DC4 DBLβ3 domains are attractive vaccine candidates.

Although no crystal structure exists currently for a DBLβ–ICAM-1 complex, this interaction has been studied in a number of different ways. Studies with truncated or mutated ICAM-1 constructs show that the binding site for DBLβ domains locates to the D1 domain of ICAM-1, and experiments with truncated and chimmeric proteins have mapped the ICAM-1 binding site to the C-terminal end of DBLβ (14–17). In addition, ICAM-1 binding is gained when replacing the C-terminal subdomain of an ICAM-1 nonbinding DBLβ3 with that of the ICAM-1 binding PFD1235w DBLβ3_D4 (12). Homology modeling (18) and small-angle x-ray scattering (SAXS) (19), together with mutagenesis studies (20), further suggest that the interaction surface is on the convex surface of the DBLβ domain. However, the exact amino acids involved in DBLβ binding to ICAM-1 are yet to be determined.

The identification of DBLβ region(s) targeted by protective Abs and a detailed mapping of ICAM-1 binding epitopes will be an essential step toward designing a PfEMP1-based vaccine potentially protective against malaria. Using modern methods for the characterization of Ab–Ag complexes, such as hydrogen/deuterium exchange mass spectrometry (HDX MS), surface plasmon resonance (SPR), and SAXS, we characterized an mAb (24E9) that binds to the convex surface of DC4 DBLβ3 domains and interferes with the DBLβ3:ICAM-1 interaction. We show that 24E9 mAb targets epitopes conserved between DC4 DBLβ3 domains from genetically distant parasite isolates and inhibits ICAM-1 binding of IE by blocking the predicted ICAM-1 binding site on DBLβ. This provides important knowledge for choosing components for a vaccine aimed at preventing PfEMP1-mediated adhesion of IE during severe malaria.

Materials and Methods

Recombinant protein expression and purification

Full-length, wild-type PFD1235w DBLβ3_D4 was subcloned into a modified pET15b vector and expressed as an N-terminal, hexahistidine-tagged protein in Escherica coli Shuffle 3030 cells (New England Biolabs) for 16 h at 25°C. The cells were pelleted, washed, and lysed, and DBLβ3_D4 was purified using Ni-NTA-Sepharose (Qiagen). The hexahistidine-tag was removed by overnight cleavage at 4°C using Tobacco etch virus protease. Tobacco etch virus protease and uncleaved protein were removed by reverse immobilized-metal affinity chromatography, and DBLβ3_D4 was further purified by size exclusion chromatography using a Superdex 75 16/60 column (GE Healthcare).

PFD1235w DBLβ3_D4 protein used for mouse immunization to generate hybridomas was subjected to an additional purification step. DBLβ3_D4 was allowed to bind to ICAM-1:D1-D5:Fc coupled to a HiTrap NHS-activated HP column (GE Healthcare). Bound DBLβ3_D4 was eluted from ICAM-1 on the column and buffer exchanged into PBS. For generation of DBLβ3_D4 mutants, a set of 5’ phosphorylated primers that included the coding sequence for the F2b or F3a regions of DBLβ3_D5 was used to amplify the DBLβ3_D4-encoding pEi15b vector by PCR. The PCR products were circularized by blunt-end ligation using T4 ligase (Life Technologies), and the mutants were expressed and purified as described previously (19). ICAM-1_D1 was expressed and purified from E. coli BL21(DE3) as described previously (22).

CD spectroscopy

Far-UV CD spectroscopy experiments were carried out with a J-815 Spectropolarimeter (Jasco) equipped with a computer-controlled Peltier temperature control unit. All samples were dialyzed into 10 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2, and measurements were taken at a protein concentration of 0.1 mg/ml using a 1 nm path cell. Spectra were acquired at 20°C at wavelengths between 195 and 260 nm. For thermal unfolding, the temperature was raised from 20° to 95°C in 0.5°C increments, and spectra were recorded between 200 and 250 nm wavelength.

Hybridoma production

24E9 hybridomas were produced according to standard protocols (23). One CB6FI mouse (Harlan) was immunized s.c. with 30 μg PFD1235w DBLβ3_D4 in CFA (Sigma-Aldrich) followed by two additional boosters of 15 μg protein in IFA (Sigma-Aldrich). A final i.v. boost of 15 μg protein in PBS was given 3 d before the mouse was sacrificed and the spleen was taken out. Single spleen B lymphocytes were made from the whole spleen and fused to SP2/0-Agi4 Myeloma cells (ATCC) in a 1:2 ratio using polyethylene glycol 4000. Spleen and myeloma cell mixture was diluted in 80 ml cell media (RPMI, 20% FBS, glutamine, penicillin/streptomycin) containing HAT media supplement (Sigma-Aldrich) to select for fused cells. A total of 100 μl/well was added to eight deep, flat-bottom, 96-well plates (Fisher Scientific) containing peritoneal macrophages from two BALB/C mice (Taconic) serving as feeder cells. Cells were grown for 1 wk at 37°C, 5% CO₂ before changing the cell supernatant to cell media supplemented with HT media supplement (hypoxanthine and thymidine; Sigma-Aldrich). Two weeks after fusion, wells with growing cells were identified under a microscope and the cells were moved into fresh 96-well plates. After 1 wk, confluent cell supernatant from each well was tested for the presence of DBLβ3_D4-reactive Abs using ELISA. To obtain true monoclonal hybridomas, we cloned cells from positive wells by limiting dilution. All animal procedures were approved by the Danish National Committee (Dyreforsøgstilsynet) in agreement with permit no. 2008/561-1498.

mAb purification

24E9 monoclonal hybridomas were expanded and seeded at ~10% confluence in 175-cm² cell flasks containing 70 ml cell media [RPMI, 10% low IgG FBS (Lonza), HT media supplement (Sigma-Aldrich), glutamine, penicillin/streptomycin]. After incubation for 1 wk at 37°C, 5% CO₂ cell supernatant was centrifuged, sterile-filtered, and buffer-exchanged into PBS before purifying mAb using a HiTrap protein G column (GE Healthcare) according to the manufacturer’s instructions.

IgG subtyping

The IgG subtype and L chain class of 24E9 mAb were determined using an IsoQuick Kit for Mouse Monoclonal Isotyping (Sigma) according to the manufacturer’s instructions.

Fab fragmentation

Purified 24E9 mAb was buffer-exchanged into cleavage buffer (0.1 M sodium phosphate pH 6.4, 0.3 M NaCl, 2 mM EDTA, 5 mM L-cysteine, 1.5 mM 2-ME) and concentrated to 1 mg/ml. Papain-agarose (Sigma-Aldrich) was added in a 20:1 ratio and incubated overnight at 37°C. Papain-agarose was removed by centrifugation, and the Fab portion and uncleaved mAb were removed from the supernatant by purification on a protein A column (GE Healthcare). Fab fragments in the flow-through were further purified by size exclusion chromatography.

Western blot

Purified 24E9 mAb was tested for reactivity against reduced (+DTT) and nonreduced (–DTT) PFD1235w DBLβ3_D4. Purified PFD1235w DBLβ3_D4 and PFD1235w DBLβ3_D5 (control; 0.5 μg) were separated by SDS-PAGE under both conditions on a NuPAGE Novex 4–12% Bis-Tris gel in MOPS SDS Running buffer (Invitrogen) and subsequently blotted onto a Hybond-C Extra NC membrane (GE Healthcare). The membrane was blocked using 2.5% skimmed milk in dilution buffer (PBS, 1% BSA). The 24E9 mAb was diluted to 10 μg/ml in dilution buffer and added to the membrane. Bound 24E9 mAb was detected by anti-mouse IgG (P260; Dako) 1:1000 in dilution buffer using a chemiluminescent detection kit (Thermo Scientific).

ELISA

Hybridoma screening. Hybridoma cell supernatants were screened for PFD1235w DBLβ3_D4-reactive Abs using ELISA. Duplicate wells of
MaxiSorp microtiter plates (Nunc) were coated with DBLβ3_D4 (50 µl; 1 µg/ml; 0.1 M glycine/HCl buffer pH 2.75; overnight; 4°C) and blocked with blocking buffer (PBS, 0.5 M NaCl, 1% Triton X-100, 1% BSA, pH 7.2). A total of 100 µl undiluted cell supernatant was added (1 h; room temperature). The plates were washed in PBS + 1% Triton X-100, and bound Ab was detected with an anti-mouse Ig-HRP (Dako; 1:3000 in blocking buffer). After 1 h of incubation, plates were developed using OPD tablets (Dako) according to the manufacturer’s instructions. The OD value was read at 490 nm using a VERSAmax microplate reader (Molecular Devices) and Softmax Pro v4.7.1.

mAb reactivity. Microtiter plates were coated with 50 µl, 2 µg/ml recombinant proteins in glycine/HCl buffer and blocked with blocking buffer. 24E9 mAb (50 µl; 3-fold dilutions starting at 10 µg/ml; 1 h; room temperature) was added, and washing was performed as described above. Bound Ab was detected with anti-mouse Ig-HRP (Dako; 1:3000 in blocking buffer; 1 h; room temperature).

Reducing ELISA. Microtiter plates were coated (50 µl; 2-fold dilutions starting at 64 µg/ml; glycine/HCl buffer; overnight; 4°C) with 24E9 mAb or the PFD1235w DBLγ-specific AB01 mAb (24) and blocked with PBS + 1% BSA. Plates were washed in PBS and PFD1235w DBLβ3_D4 or PFD1235w DBLγ (50 µl; 2 µg/ml; PBS ± 50 mM DTT; 1 h; room temperature) were added to the plates coated with 24E9 or AB01, respectively. Bound DBLβ3_D4 or DBLγ was detected by use of an anti-penta-His HRP Ab (1:3000 in blocking buffer; 1 h; room temperature; QIAGEN). Washing and detection were performed as described above.

ICAM-1 inhibition ELISA. Microtiter plates were coated with recombinant ICAM-1-Fc (50 µl; 2 µg/ml; glycine/HCl buffer; overnight; 4°C) and blocked with blocking buffer. DBLβ3_D4 domains (1–16 µg/ml) were added simultaneously with mAb 24E9 added in 2-fold dilutions ranging from 0.25 to 32 µg/ml. Mouse IgG (Life Technologies) was added as control. ICAM-1-bound DBLβ3_D4 was detected using anti–penta-His HRP Ab (1:3000 in blocking buffer; 1 h; room temperature; QIAGEN). Washing and detection were performed as described above.

Sequencing

The mouse Ig L and H chain variable genes of the 24E9 mAb were sequenced to determine the amino acid sequences of the CDRs. cDNA was made from single 24E9 hybridoma cells using a QIAGEN OneStep RT-PCR Kit with degenerate primers designed to target mouse Ig variable regions (25). cDNA was amplified using Phusion HF polymerase (New England Biolabs), and PCR products were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s instructions. Sequence data were collected on a 3100-Avant Genetic Analyzer (Applied Biosystems). The nucleotide sequences of 24E9 CDRs can be retrieved from GenBank using accession numbers KJ418726 (H chain) and KJ418727 (L chain) (http://www.ncbi.nlm.nih.gov/genbank).

Malaria parasites and flow-cytometry analysis

The 3D7 P. falciparum clone and one Ghanaian patient isolate (BM057) were cultured in vitro (26) and were selected for DC4 PIEMPI1 IE surface expression by repeated Ab selection as described previously (12). The identity of the isolates was routinely verified by genotyping as described previously (27), and Mycoplasma infection was regularly excluded using the MycoAlert Mycoplasma Detection Kit (Lonza) according to the manufacturer’s instructions.

P. falciparum IE were DNA-labeled with ethidium bromide and surface-labeled with mouse anti-berine obtained from the immunized mouse used for hybridoma production (15 µl serum/well), 24E9 mAb (100 µg/ml), or 24E9 Fab fragments (100 µg/ml). Whole Abs were labeled using an FITC-conjugated secondary anti-mouse IgG (1:100; Vector Labs), and an anti-mouse F(ab')2 IgG (1:100; Jackson Immunoresearch) was used to detect Fab fragments. FITC fluorescence data from ethidium bromide* cells were collected on a CytoFACS FC 500 MPL flow cytometer (Beckman Coulter) and analyzed in WinList version 6.0 (Verity Software House).

ICAM-1 adhesion assays under physiological flow conditions

Biochips (Vena; Cellix) were coated at 4°C overnight with recombinant ICAM-1–Fc (50 µg/ml) produced as described previously (21). Channels [400 × 100 × 20 mm (w × d × h)] were blocked for 1 h at 37°C with PBS + 1% BSA and the chip mounted onto a Leica inverted phase-contrast microscope. To generate a wall shear stress representing that within microvessels (28), we connected the biochip to an NE-100X microfluidic pump (World Precision Instruments, U.K.). Erythrocytes at 3–5% parasitemia (1% hematocrit in RPMI 1640 plus 2% normal human serum) were flowed over the biochip for 5 min. The number of bound IE per square millimeter for five separate fields was counted at 20 times magnification, and a minimum of three independent experiments was done in triplicates.

To inhibit ICAM-1 adhesion, we combined IE with 24E9 mAb (1 × 10−3; 1, 10 µg/ml) or Fab fragments of 24E9 (1 × 10−3; 1 × 10−4; 1 µg/ml) before assaying as described earlier. Mouse IgG (10 µg/ml; Life Technologies) or mouse IgG Fab fragments (1 µg/ml; Rockland) were included as negative controls. Specificity of adhesion to recombinant ICAM-1–Fc was determined by the preincubation of channels with 40 µg/ml anti–ICAM-1 (clone 15.2; AbD Serotec).

SPR

SPR measurements were conducted using a BIAcore T-100 instrument (GE Healthcare). DBLβ3_D4 was diluted to 10 mM acetyl buffer, pH 4.0, and covalently coupled to a CM5 chip (GE Healthcare) by amine coupling to a density of 400 response units (RU). ICAM-1–Fc was immobilized to a density of 400 response units (RU). SPR measurements were performed in triplicate and measurements were averaged.
Using the program SCULPTOR (34). The DBL Data Bank [PDB ID 3GK8) were docked into the resulting envelopes (PDB ID 1ZRL), and EBA-140 (PDB ID 4GF2) as templates. Structural models were refined by DAMMIN (33), using default parameters and the original pair distance distribution functions $P_D$ were calculated using DAMMIF (31) using default parameters with P1 symmetry. For both DBL$_b$3_D4 and DBL$_b$3_D4 homology model $P_D$ was generated with TASSER (c-score = -0.9) using the structures of DBL3X (PDB ID 3Q8K), NTS-DBL1s (PDB ID 2XU0), EBA-175 (PDB ID 1ZRL), and EBA-140 (PDB ID 4GF2) as templates. Structural models were visualized using PyMol Version 1.5.0.4 (Schrödinger).

Results

The 24E9 mAb is cross-reactive against DC4-containing PfEMP1 present on the surface of IE

We have previously observed that 3D7 PFD1235w DBL$_b$3_D4 elicits adhesion-inhibitory Abs that are cross-reactive to DC4-containing PfEMP1 from genetically distant parasite isolates (12). To study the specific epitopes targeted by such protective Abs in more detail, we first raised a monoclonal mouse Ab against the DBL$_b$3_D4 homology model and homology models of DBL$_b$3_D4 and a mouse Fab fragment (Protein Data Bank [PDB] ID 3GK8) were docked into the resulting envelopes using the program SCULPTOR (34). The DBL$_b$3_D4 homology model was generated with TASSER (c-score = -0.9) using the structures of DBL3X (PDB ID 3Q8K), NTS-DBL1s (PDB ID 2XU0), EBA-175 (PDB ID 1ZRL), and EBA-140 (PDB ID 4GF2) as templates. Structural models were generated using DAMMER (32). The averaged model was further refined by DAMMIN (33), using default parameters and the original pair distance distribution functions $P_D$ were calculated using GNOM (30).

Ab initio models were generated from solution scattering data by DAMMIF (31) using default parameters with P1 symmetry. For both DBL$_b$3_D4 and DBL$_b$3_D4 Fab, 20 independent DAMMIF models were averaged using DAMAVER (32). The averaged model was further refined by DAMMIN (33), using default parameters and the original pair distance distribution functions as input. SITUS was used to calculate volumetric representation from the bead models generated by DAMMIN, and homology models of DBL$_b$3_D4 and a mouse Fab fragment (Protein Data Bank [PDB] ID 3GK8) were docked into the resulting envelopes using the program SCULPTOR (34). The DBL$_b$3_D4 homology model was generated with TASSER (c-score = -0.9) using the structures of DBL3X (PDB ID 3Q8K), NTS-DBL1s (PDB ID 2XU0), EBA-175 (PDB ID 1ZRL), and EBA-140 (PDB ID 4GF2) as templates. Structural models were visualized using PyMol Version 1.5.0.4 (Schrödinger).

Binding to ICAM-1 is mediated through the DBL$_b$3_D4 domains of DC4 PfEMP1 (12). We therefore tested whether the 24E9 mAb blocks this interaction. We first compared the affinity of DBL$_b$3_D4 for both 24E9 and ICAM-1 by SPR. We used 24E9 Fab fragments, leading to monovalent binding, which allowed for global fitting of the data with a one-site binding model. 24E9 Fab bound to DBL$_b$3_D4 with low nanomolar affinity, comparable with the affinity of DBL$_b$3_D4 for ICAM-1_D1-D2 and ICAM-1_D1 (Fig. 3A, Table I). Furthermore, the interaction between 24E9 Fab and DBL$_b$3_D4 showed fast association and slow dissociation rates similar to those observed for the interaction between ICAM-1_D1 and DBL$_b$3_D4 (Fig. 3A–C, Table I).

We next analyzed whether 24E9 mAb directly inhibits the DBL$_b$3_D4:ICAM-1 interaction. A chip coupled with DBL$_b$3_D4 was preincubated with different concentrations of 24E9 Fab, followed by addition of ICAM-1_D1. As a control, ICAM-1_D1 was flowed over the chip surface without prior incubation with 24E9 Fab (Fig. 3D, red curve). Preabsorption of DBL$_b$3_D4 with increasing concentrations of 24E9 Fab reduced the binding of ICAM-1_D1 in a concentration-dependent manner (Fig. 3D, 3E), demonstrating that 24E9 Fab effectively blocks the interaction between DBL$_b$3_D4 and ICAM-1_D1.

The observation that 24E9 is cross-reactive against several DC4 DBL$_b$3_D4 domains from different parasite isolates (Fig. 2) raised the possibility that 24E9 also cross-inhibits the interaction between ICAM-1 and these domains. We tested this by ELISA and found that 24E9 mAb inhibited ICAM-1 binding of PFD1235w DBL$_b$3_D4 and of the five DC4 DBL$_b$3_D4 domains in a concentration-dependent manner (Fig. 3F). Taken together, these data show that 24E9 mAb is both cross-reactive and cross-inhibitory of ICAM-1 binding to all tested DC4 DBL$_b$3_D4 domains and binds with a sufficiently strong affinity to effectively compete with ICAM-1 binding.
24E9 mAb and 24E9 Fab inhibits IE binding to ICAM-1 under flow conditions

IE expressing DC4 PfeEMP1 proteins adhere to ICAM-1 (12), a phenotype linked to sequestration of IE in the microvasculature of the brain (6, 35). We therefore tested whether the 24E9 Ab blocks this interaction. Biochips were coated with recombinant ICAM-1, and 3D7 DC4+ parasites were flowed over at 1 dyn/cm². The 24E9 mAb successfully inhibited adhesion at 1 μg/ml (67%; 133 nM) and at 10 μg/ml (79%; 1.3 mM), whereas the control mouse IgG (10 μg/ml) failed to significantly alter adhesion to ICAM-1 (Fig. 4A). Fab fragments generated from 24E9 mAb were also assessed for inhibition at 1 μg/ml (83%; 400 nM) and were...
titrated to determine the extent of activity. The Fab fragment continued to demonstrate adhesion inhibition at \( \geq 50\% \) even at 0.001 mg/ml (67\%; 400 pM; Fig. 4A), whereas the control mouse IgG Fab again failed to alter adhesion at the highest concentration tested (1 mg/ml). A second strain, BM57 DC4\(^+\), was assessed, and like 3D7 DC4\(^+\), adhesion was significantly inhibited by 24E9 mAb (0.1 mg/ml; 80\% inhibition) and 24E9 Fab (0.001 mg/ml; 83\% inhibition) at the lowest concentrations tested (Fig. 4B). The specificity of adhesion to rICAM-1 was verified by preincubating control channels with anti–ICAM-1, which significantly reduced adhesion (81\% inhibition; Fig. 4).

24E9 mAb recognizes a conformational epitope

To determine whether 24E9 mAb interacts with a conformational epitope, we used Western blotting to test the reactivity of 24E9 mAb to reduced and nonreduced DBL\(_{b3}\_D4\) as a control, we performed the same experiment with the non-DC4 PFD1235w DBL\(_{b3}\_D5\) domain. 24E9 recognized only nonreduced DBL\(_{b3}\_D4\) (Fig. 5A). We observed the same result by ELISA (Fig. 5B), where 24E9 recognized only nonreduced DBL\(_{b3}\_D4\). To test whether the loss of reactivity of the mAb toward DTT-treated PFD1235W DBL\(_{b3}\_D4\) was a result of the mAb being reduced in the ELISA, we performed the same assay using the PFD1235w DBL\(_{\gamma}\)-specific human mAb (AB01), which is only partially dependent on the correct folding of DBL\(_{\gamma}\) (24). AB01 mAb was still able to recognize DTT-treated DBL\(_{\gamma}\) (Fig. 5C) showing that a similar mAb remained intact in the ELISA. This suggests that 24E9 mAb targets a conformational epitope.

The epitope targeted by 24E9 partially overlaps with the potential ICAM-1 binding site of PFD1235w DBL\(_{b3}\_D4\)

To identify the specific peptides and surface features recognized by 24E9, we used HDX MS, a powerful, modern immunological method to examine epitopes bound by Abs under native conditions (36, 37). We analyzed the DBL\(_{b3}\_D4\):24E9 Fab complex by measuring deuterium uptake over 200 min deuteration time for 83 partly overlapping peptides from DBL\(_{b3}\_D4\), alone or in complex with 24E9 Fab. These correspond to 79\% of the DBL\(_{b3}\_D4\)

<table>
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<th>Interaction</th>
<th>( k_a (\times 10^5 \text{M}^{-1} \text{s}^{-1}) )</th>
<th>( k_d \left( \times 10^{-3} \text{s}^{-1} \right) )</th>
<th>( K_D (\text{nM}) )</th>
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FIGURE 4. 24E9 mAb and 24E9 Fab inhibit IE binding to ICAM-1 under flow conditions. Inhibition of adhesion by 24E9 mAb and 24E9 Fab of 3D7 DC4\(^+\) and BM57 DC4\(^+\) to rICAM-1 coated onto Biochips. Abs were titrated at 0.1–10 \( \mu \text{g/ml} \) (24E9 mAb) and 0.001–1 \( \mu \text{g/ml} \) (24E9 Fab). Each condition was run in triplicate for a minimum of three independent experiments and expressed as average number bound per square millimeter compared with untreated controls. Mouse IgG and mouse IgG Fab fragments were added as controls. Statistical significance was determined via one-way ANOVA with Tukey’s multiple comparison test. **\( p < 0.05 \), ***\( p = 0.0001 \).
primary sequence (Fig. 6A). Comparison of the level of deuteration highlighted three distinct regions, P1, P2, and P3, which show a reduction in deuterium uptake in the complex with 24E9 when compared with that of free DBLβ3_D4 (Fig. 6A), indicating that these regions are masked by 24E9 Fab. Similar results were also observed for 20 min deuteration time (data not shown). P1 is located in the N-terminal third of DBLβ3_D4 (subdomain 1), P2 in the center region (subdomain 2), whereas P3 is near the C-ter-

FIGURE 5. 24E9 mAb recognizes a conformation epitope on PFD1235w DBLβ3_D4. (A) Western blotting of PFD1235w DBLβ3_D4 (D4) and DBLβ3_D5 (D5). +DTT (reduced), −DTT (nonreduced). Lane 1, Prosieve protein marker (M) visualized by phosphorescent paint as dots. Lanes 2 and 3, DBLβ3_D4 (+DTT). Lanes 4 and 5, DBLβ3_D5 (+DTT). Arrow shows nonreduced DBLβ3_D4 (lane 3) recognized by 24E9 mAb. (B) 24E9 mAb ELISA reactivity against reduced (+DTT) and nonreduced (−DTT) PFD1235w DBLβ3_D4. (C) AB01 mAb ELISA reactivity against reduced (+DTT) and nonreduced (−DTT) DBLγ of PFD1235w. Mean OD values are shown for three independent experiments. Error bars indicate SD.

FIGURE 6. The 24E9 Fab binds to the convex surface of PFD1235w DBLβ3_D4. (A) Peptides from DBLβ3_D4 that were identified in mass spectra are represented by bars overlaying the primary sequence. The secondary structure, derived from a homology model of DBLβ3_D4, is shown below the sequence. The level of protection of individual peptides, as determined by comparing the %D incorporation over 200 min for free DBLβ3_D4 with that for DBLβ3_D4 bound to 24E9 Fab, is color coded according to the scale bar. Highly protected areas are in red, whereas unprotected areas are in gray. Three highly protected regions were P1 (residues 110–121), P2 (193–220), and P3 (357–388), as indicated. (B) HDX MX results were mapped onto a model of the PFD1235w DBLβ3_D4 domain. Protected areas are color coded as shown in (A). (C) Surface of PFD1235w DBLβ3_D4 model as shown in (B). (D) Potential ICAM-1 binding sites on the DBLβ3_D4 model as predicted by Bertonati and Tramontano (18) (green) and determined by Bengtsson et al. (12) (light green).
minus of the protein and part of subdomain 3. When mapped on a homology model of DBL\(b_3\)\(_D4\), all three regions cluster to a well-defined, surface-exposed area (Fig. 6B, 6C), in accordance with the observation that 24E9 targets a conformational epitope (Fig. 5A–C). The size of this protected area is 2887 \(\text{Å}^2\), which is comparable with the total \(\sim 2800 \text{Å}^2\) surface-exposed area of the variable loops of a Fab fragment.

The area protected by 24E9 Fab lies on the convex surface of DBL\(b_3\)\(_D4\). Mutational and modeling studies of non-DC4 DBL\(b\) \(b\) domains previously showed that this surface contains the ICAM-1 binding site (18–20, 38). Amino acids equivalent to residues important for the interaction between group B DBL\(b\) \(b\) domains and ICAM-1 (Fig. 6D, dark green) (18) partly overlap with P1, P2, and P3. Furthermore, the ICAM-1 binding site of DBL\(b_3\)\(_D4\) has been mapped to the C-terminal third of the domain (Fig. 6D, light green) (12). This includes region P3, which shows the strongest protection from deuteration in the DBL\(b_3\)\(_D4::24E9\) Fab complex. These observations indicate that the epitopes targeted by 24E9 are mostly conserved only among 24E9 binding DBL\(b_3\) \(b\) domains (Fig. 7A) and are strongly protected in the DBL\(b_3\)\(_D4::24E9\) Fab complex, suggesting that these motifs might directly contribute to 24E9 binding. To test this, we generated mutants by swapping the P2b and P3a peptides from DBL\(b_3\)\(_D4\) for the equivalent regions of the DBL\(b_3\)\(_D5\) domain (Fig. 7B), which is not recognized by 24E9. These mutants were expressed and purified as native DBL\(b_3\)\(_D4\), and their folding was confirmed by CD spectroscopy (Supplemental Fig. 1). The binding of the mutants to 24E9 mAb was analyzed by SPR, which showed that the exchange of the P2b peptide had little effect on 24E9 affinity, whereas exchange of the P3a led to a complete loss of Ab binding (Fig. 7C, Table II). This demonstrates that P2 makes a minor contribution to 24E9 binding, whereas the P3 region contains an essential determinant of Ab binding.

**Low-resolution structure of the DBL\(b_3\)\(_D4::24E9\) Fab complex**

To understand better the architecture of the DBL\(b_3\)\(_D4::ICAM-1\) complex, we performed small-angle x-ray scattering analysis of the DBL\(b_3\)\(_D4\) domain alone or in complex with 24E9 Fab (Fig. 8). The Rg determined from the composite scattering curve (Fig. 8A) was higher for the DBL\(b_3\)\(_D4::24E9\) Fab complex than for DBL\(b_3\)\(_D4\) alone (Table III). The increased Porod volume and

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**Table II.** Kinetic parameters derived from SPR experiments on 24E9 mAb interacting with mutant version of PFD1235w DBL\(b_3\)\(_D4\)

<table>
<thead>
<tr>
<th>Interaction</th>
<th>(k_a (\times 10^7 \text{M}^{-1} \text{s}^{-1}))</th>
<th>(k_d (\times 10^{-5} \text{s}^{-1}))</th>
<th>(K_D (\text{pM}))</th>
<th>(R_{max} (\text{RU}))</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBL(b_3)(_D4) wild type::24E9 mAb</td>
<td>6.4</td>
<td>7.7</td>
<td>1.2</td>
<td>229.2</td>
<td>One-site</td>
</tr>
<tr>
<td>DBL(b_3)(_D4::P2b)(_D5::24E9) mAb</td>
<td>1.87</td>
<td>4.1</td>
<td>2.19</td>
<td>239.2</td>
<td>One-site</td>
</tr>
<tr>
<td>DBL(b_3)(_D4::P3a)(_D5::24E9) mAb</td>
<td>2.2</td>
<td>24</td>
<td>110</td>
<td>8.64</td>
<td>One-site</td>
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the apparent molecular mass were also consistent with formation of a 1:1 complex between 24E9 Fab and DBL\textsubscript{b}3\textsubscript{D4} (Table III).

The distance distribution function shows a more skewed profile for DBL\textsubscript{b}3\textsubscript{D4}::24E9 Fab than that for DBL\textsubscript{b}3\textsubscript{D4} alone (Fig. 8B), indicating that binding of 24E9 Fab results in a more elongated particle (39). Accordingly, the $D_{\text{max}}$ increases from 9.4 nm for DBL\textsubscript{b}3\textsubscript{D4} to 12.2 nm for the complex (Table III).

Envelopes for DBL\textsubscript{b}3\textsubscript{D4} and DBL\textsubscript{b}3\textsubscript{D4}::24E9 Fab were generated by ab initio modeling based on the scattering data, and the homology model of DBL\textsubscript{b}3\textsubscript{D4} was manually docked into these envelopes together with a model Fab fragment. Comparison of the two envelopes reveals that the DBL\textsubscript{b}3\textsubscript{D4}::24E9 Fab complex is more elongated, with an additional mass protruding from DBL\textsubscript{b}3\textsubscript{D4}, corresponding to 24E9 Fab (Fig. 8C, 8D). Simultaneous docking into this envelope positions the Ag binding loops of the Fab toward the regions identified as being protected in the DBL\textsubscript{b}3\textsubscript{D4}::24E9 Fab complex are color coded as in Fig. 5. The structure of a mouse Fab fragment (green, PDB ID 3GK8) was used as a model for 24E9 Fab.

**Discussion**

The presence of inhibitory Abs that bind to the variable surface Ag P\textsuperscript{fEMP1} correlates with naturally acquired, protective immunity against P\textsuperscript{fEMP1}-mediated IE adhesion during severe malaria (38, 40, 41). However, the epitopes targeted by such functional Abs and the mechanism by which they prevent IE adhesion are still unknown. In this study, we used immunological and biophysical methods to demonstrate that an mAb raised against a single DC4 DBL\textbeta domain recognizes epitopes conserved between DC4 DBL\textbeta domains and prevents ICAM-1 binding by both purified domains and IE, occluding the ICAM-1 binding site on the surface of a DBL\textbeta domain.

Of biological relevance, the 24E9 mAb and Fab fragments inhibit 3D7 DC4\textsuperscript{+} IE at picomolar to subnanomolar concentrations (Fig. 4). Titration of the Abs not only confirmed the specificity of 24E9, but also illustrated how effective the Ab remained at low concentrations. Despite having only one Ag binding site, the 24E9 Fab is a more potent inhibitor of ICAM-1 binding than 24E9 mAb (Fig. 4A). This lower efficacy of the full-length and thus bulkier IgG molecule might be explained by steric hindrance and partially restricted access to the binding site of the native P\textsuperscript{fEMP1} as compared with the smaller Fab fragment. 24E9 also successfully inhibited the ICAM-1 adhesion of erythrocytes infected by a genetically distinct parasite, BM57 DC4\textsuperscript{+}. The mAb 24E9 is therefore able to inhibit ICAM-1 binding of DC4 DBL\textsubscript{b}3\textsubscript{D4} domains from a number of different parasite isolates, indicating that these domains share a common antigenic epitope.

**Table III.** Experimental values derived from PFD1235w DBL\textsubscript{b}3\textsubscript{D4}::24E9 Fab SAXS experiments

<table>
<thead>
<tr>
<th></th>
<th>$R_g$ (nm)</th>
<th>$D_{\text{max}}$ (nm)</th>
<th>$V_{\text{Porod}}$ (nm$^3$)</th>
<th>$M_{\text{exp}}$ (kDa)</th>
<th>$M_{\text{app}}$ (kDa)</th>
<th>$\chi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBL\textsubscript{b}3\textsubscript{D4}</td>
<td>2.69</td>
<td>9.39</td>
<td>91.13</td>
<td>55</td>
<td>54.45</td>
<td>4.57</td>
</tr>
<tr>
<td>DBL\textsubscript{b}3\textsubscript{D4}::24E9 Fab</td>
<td>3.81</td>
<td>12.16</td>
<td>140.17</td>
<td>105</td>
<td>91.05</td>
<td>3.456</td>
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The experimental $R_g$ ($R_g_{\text{exp}}$) was determined using AutoRG (8), the $D_{\text{max}}$, was derived from GNOM (30), and the Porod volume ($V_{\text{Porod}}$) was determined by using PRIMUS (29). The expected molecular mass ($M_{\text{exp}}$) is shown for DBL\textsubscript{b}3\textsubscript{D4} and the DBL\textsubscript{b}3\textsubscript{D4}::24E9 complex. The apparent molecular mass ($M_{\text{app}}$) was calculated from the volume excluded in the final DAMMIN (33) model divided by 2. The $\chi$ value represents the best fit of 20 low-resolution shape reconstructions using ab initio modeling.
Using HDX MS, we have identified three regions that cluster on the convex surface of PFD1235W DBLβ3_D4 and that show reduced hydrogen-deuterium exchange in the presence of 24E9. One of these three peptides, the P3a motif of region P3, was protected most strongly (Fig. 6) and is absolutely required for Ab binding (Fig. 7C). Indeed, this motif is strictly conserved only among DBLβ3 domains recognized by 24E9. In addition, the P2b motif of region P2 is conserved in most 24E9-binding DBLβ3 domains, but varies significantly between nonbinders (Fig. 7A). However, this motif plays only a minor role in 24E9 binding, as demonstrated by a slight reduction in $K_d$ when it is mutated (Table II). In contrast, P1 and the remaining regions of P2 and P3 show a substantial degree of conservation between both 24E9-binding and nonbinding DBLβ3 domains (Fig. 7A), making it more likely that these amino acids are not part of the 24E9-binding site, but instead are sterically protected from hydrogen-deuterium exchange by the presence of the Ab binding to the neighboring epitopes. Our mapping data also suggest a mechanism by which 24E9 inhibits ICAM-1 binding, because epitopes recognized by mAb 24E9 cluster on the convex surface of DBLβ3_D4 that is predicted to contain the binding site for ICAM-1 (15, 19, 20, 38). In addition, region P3, which contains the main determinant of 24E9 binding, lies within subdomain 3 of DBLβ3_D4. This subdomain forms a significant part of the convex surface of DBLβ3_D4, and a previous study suggested that it is required for the interaction with ICAM-1 (12). These findings indicate that 24E9 exerts its inhibitory function by masking the ICAM-1 binding site of DBLβ3_D4. These conclusions are further supported by our low-resolution shape reconstruction, determined by SAXS, which shows that 24E9 Fab adopts an orientation relative to the DBLβ3_D4 that is similar to that of ICAM-1-D1-D2 bound to the DBLβ domain of IT4ivar13 (19).

The identification of the convex surface of DBLβ3 domains as the main target of inhibitory Abs provides important knowledge for choosing the components of a vaccine aimed at preventing P3EMP1-mediated adhesion of IE during severe malaria. A detailed mapping and structural characterization of the ICAM-1 binding sites of DBLβ3 domains from group A and B P3EMP1 and the identification of conserved surface features involved in this interaction are now needed to guide future decisions about how to design immunogens that elicit Abs inhibitory of ICAM-1 binding. Our observation that an Ab raised against a single DBLβ3_D4 domain prevents the interaction between ICAM-1 and DBLβ3_D4 domains from genetically distantly parasite isolates demonstrates the existence of conserved antigenic epitopes. These might be used to specifically induce the production of Abs that cross-inhibit ICAM-1 binding by an important set of ICAM-1 binding DBLβ domains. Because DC4 DBLβ3_D4 domains are found in group A P3EMP1, which have been associated with increased IE adhesion and severe malaria (6, 15, 42), such conserved epitopes are promising candidates for inclusion in a vaccine that interferes with the P3EMP1::ICAM-1 interaction and confers strain-independent protection against severe malaria.

Acknowledgments

The authors thank Anna L. Jensen, Vera V. Pinto, Mette U. Madsen, Marianne A. Andersen, Michael B. Dalgaard, Kirsten P. Zimling, and Maiken H. Visti for excellent technical assistance. We acknowledge Maxime Rome from the Botanical Garden in Lyon for providing pitcher fluid from Nepenthes carnivorous plants, giving the opportunity to evaluate the activity of native nepenthesin in HDX MS conditions.

Disclosures

The authors have no financial conflicts of interest.

References


