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Multiple *Plasmodium falciparum* Erythrocyte Membrane Protein 1 Variants per Genome Can Bind IgM via Its Fc Fragment Fcμ

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The *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) adhesive proteins expressed on the surfaces of infected erythrocytes (IEs) are of key importance in the pathogenesis of *P. falciparum* malaria. Several structurally and functionally defined PfEMP1 types have been associated with severe clinical manifestations, such as cerebral malaria in children and placental malaria in pregnant women. PfEMP1 that can bind the Fc part of IgM (Fcμ) characterizes one such type, although the functional significance of this IgM binding to PfEMP1 remains unclear. In this study, we report the identification and functional analysis of five IgM-binding PfEMP1 proteins encoded by *P. falciparum* NF54. In addition to the VAR2CSA-type PFL0030c protein, already known to bind Fcμ and to mediate chondroitin sulfate A (CSA)-specific adhesion of IEs in the placenta, we found four PfEMP1 proteins not previously known to bind IgM this way. Although they all contained Duffy binding-like ε (DBLε) domains similar to those in VAR2CSA-type PfEMP1, they did not mediate IE adhesion to CSA, and IgM binding did not shield IEs from phagocytosis of IgG-opsonized IEs. In this way, these new IgM-binding PfEMP1 proteins resemble the rosette-mediating and IgM-binding PfEMP1 HB3VAR06, but none of them mediated formation of rosettes. We could map the capacity for Fc-specific IgM binding to DBLε domains near the C terminus for three of the four PfEMP1 proteins tested. Our study provides new evidence regarding Fc-dependent binding of IgM to PfEMP1, which appears to be a common and multifunctional phenotype.

Many microorganisms express molecules that can bind immunoglobulins independently of the antigen specificity of the antibodies. A prominent example is the antibody-binding proteins found in the cell wall of the bacterium *Staphylococcus aureus* (1, 2). These proteins have high affinities for the conserved elements in the Fc and Fab parts of various antibody classes, and they appear to serve an immunoevasive function, as binding of antibodies to these proteins interferes with phagocytosis of antibody-opsonized bacteria (3).

Some erythrocytes infected by the malaria parasite *Plasmodium falciparum* bind IgM, but not IgG, independently of the specificity of the antibodies (4, 5). This Fc-mediated binding of IgM has been described for infected erythrocytes (IEs) that bind to the sulfated glycosaminoglycan chondroitin sulfate A (CSA) (5) and for IEs capable of forming rosettes (several uninfected erythrocytes adhering to a central IE) (4). Both IE phenotypes are related to expression of particular types of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). Thus, adhesion of IEs to CSA requires expression of the atypical PfEMP1 type VAR2CSA, which has nanomolar affinity for CSA and is responsible for placental IE sequestration (6–9). Rosetting can be mediated by several different PfEMP1 proteins that have a semiconserved N-terminal head structure made up of certain subtypes of Duffy binding-like α (DBLα)–cysteine-rich interdomain region γ/δ (CIDRγ/δ) domains (10–13), and it appears to depend mainly on relatively low-affinity interactions with a range of host carbohydrates (14–17).

The function of Fc-dependent binding of IgM to *P. falciparum* IEs is not fully understood (reviewed in reference 18). In the case of VAR2CSA-type PfEMP1, it appears to be mainly immunoevasive, as it can protect IEs from specific IgG recognition and immune destruction without compromising the CSA-adhesive function of the antigen (19). However, such masking is ineffective in the case of rosette-mediating PfEMP1 antigens (15), where bind-

ing of IgM to PfEMP1—in combination with other serum factors—seems to function to increase the low-affinity adhesive interactions involved in rosetting (15, 20). Given the apparent clinical importance of IgM binding (4, 21), it is of interest to know how many IgM-binding PfEMP1 variants exist within the PfEMP1 repertoire of a single *P. falciparum* clone and how IgM binding is related to the structural and functional characteristics of the involved PfEMP1 proteins. We therefore set out to identify IgM-binding PfEMP1 proteins in *P. falciparum* NF54. We show that the genes for at least five IgM-binding PfEMP1 variants exist in the genome of this parasite. In addition to PFL0030c, which is the VAR2CSA-type antigen in *P. falciparum* NF54, we found four others (PFL0020w, PF07_0139, MAL6P1.4, and MAL6P1.316). Surprisingly, these did not mediate rosetting in functional assays and do not possess structural features indicative of being rosette mediating. Our study shows that Fc-mediated binding of IgM to

PfEMP1 proteins is not limited to those that can adhere to CSA or mediate formation of rosettes.

MATERIALS AND METHODS

Recombinant PfEMP1 proteins and specific antisera and monoclonal antibodies. Recombinant proteins representing full-length PFL0030c and single- and triple-domain constructs of MAL6P1.4, MAL6P1.316, PFL0020w, and PFL0030c were produced in a baculovirus expression system, essentially as described previously (15, 22). The domain nomenclature proposed by Rask et al. in 2010 is used throughout (23). Antisera against MAL6P1.4, MAL6P1.316, and PFL0020w were raised in rats (24), and the human monoclonal antibody PAM1.4, specific for several VAR2CSA-type PfEMP1 proteins, including PFL0030c, was generated as described elsewhere (25, 26). Nonimmune IgM binding to recombinant PfEMP1 constructs was quantified by enzyme-linked immunosorbent assay (ELISA) as described previously (15).

Malaria parasite cultivation and *in vitro* selection procedures. *P. falciparum* NF54 parasites (27) were grown *in vitro* in O Rh$^+$ erythrocytes at 37°C in a controlled atmosphere, using complete culture medium (RPMI 1640 supplemented with 0.5% AlbuMax II [Life Technologies BV, Nærum, Denmark]), essentially as described previously (28). The *P. falciparum* NF54-derived and pVBH-transfected clone G6 was generated as described elsewhere (29–31). It was maintained in the same way as the parental strain *P. falciparum* NF54, except that blasticidin (10 mg/ml; Life Technologies) was added to shut down transcription of endogenous *var* genes and to erase the epigenetic memory. IEs were selected for surface expression of defined PfEMP1 proteins by immunomagnetic selection using (i) PfEMP1-specific rat antisera followed by biotinylated anti-rat antibody (Dako) and streptavidin-coupled Dynabeads (Fisher Scientific) or (ii) PAM1.4 followed by protein A-coupled Dynabeads (Fisher Scientific), essentially as described previously (32). For selection of IgM-binding IEs, we used human IgM (Sigma) coupled to M-450 epoxy beads (Life Technologies) according to the manufacturers' instructions. The genotypic identity of the parasites and the absence of *Mycoplasma* contamination were verified regularly as described previously (33).

***var* gene transcription analysis.** For analysis of *var* gene transcription by quantitative real-time PCR, we used cDNA generated from ring-stage parasite RNA and *P. falciparum* 3D7 *var* gene-specific primers as described in detail elsewhere (6, 34, 35). Transcription levels relative to those of the seryl-tRNA synthetase housekeeping gene were calculated by the $2^{-\Delta CT}$ method.

Antibody labeling of IEs. The binding of nonimmune IgM and PfEMP1-specific antibody to IEs was quantified by flow cytometry, essentially as described previously (19). In brief, we used magnetically activated cell sorting (MACS) to purify late-stage IEs, labeled with ethidium bromide (10 mg/ml), serial dilutions of human IgM, and secondary antibody. Human IgA was used as a negative control for IgM. In some experiments, IgM (100 nM) was preincubated (20 min, room temperature) with equimolar concentrations of mouse monoclonal antibodies specific for human Fcμ2 (HB57), Fcμ3 (5D7), or Fcμ4 (1G6) (all described in reference 36) or mouse control IgG (Sigma). Three replicates were measured in each experiment. The purification of late-stage IEs by MACS was assessed by ethidium bromide staining as described above, and IE surface expression of the expected PfEMP1 protein was verified by labeling with PfEMP1-specific antisera or human monoclonal antibody. All list-mode data files were analyzed using Winlist 6.0 (Verity, Topsham, ME).

Single-cell cloning of IgM-binding IEs. *P. falciparum* G6 grown under blasticidin pressure to erase the epigenetic memory of *var* gene transcription was released from the selection pressure for 2 weeks to obtain a population with highly heterogeneous *var* gene transcription, as described previously (31). MACS-purified late-stage IEs were labeled with human IgM (10 nM, 30 min, 4°C), washed thrice in sterile phosphate-buffered saline (PBS) supplemented with fetal bovine serum (2%), stained with phycoerythrin-conjugated donkey anti-human IgM (Jackson Immuno-Research, Newmarket, United Kingdom) (1:200, 30 min, 4°C), and

washed thrice as described above. An Aria-II fluorescence-activated cell sorter (BD Biosciences, San Jose, CA) was used to single-cell sort IgM-positive IEs into round-bottomed 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark) containing complete culture medium (200 μl/well) and uninfected erythrocytes (2 μl/well). Some wells received 50 IEs rather than a single IE. The 96-well plates were incubated for 2 weeks (37°C, 5% CO$_2$), with a change of medium thrice weekly and addition of uninfected erythrocytes (2 μl/well) twice weekly. Small aliquots of IEs were stained with ethidium bromide (2 μg/ml), IgM (10 nM), and fluorescein isothiocyanate-conjugated anti-human IgM (Sigma; 1:150) and used to assess parasite growth and IgM binding by flow cytometry, using a Beckman Coulter FC500 instrument (Beckman Coulter, Fullerton, CA). Clones with IgM-positive IEs were transferred and expanded further in tissue culture flasks.

Adhesion of IEs to CSA. Adhesion of IEs to CSA-expressing BeWo cells was determined essentially as described previously (37). In brief, ring-stage IEs were labeled with [^{3}H]hypoxanthine for 24 h. BeWo cells (LGC Promochem, Borås, Sweden) were grown to confluence in flat-bottomed 96-well plates (Thermo Fisher Scientific, Denmark) and blocked with bovine serum albumin (5% in PBS, 1 h). MACS-purified [^{3}H]hypoxanthine-labeled late-stage IEs (2 × 10^5 IEs/well) were added and incubated (37°C, 1 h). Unbound IEs were washed off by use of a Biomek 2000 automated plate washer (Beckman Coulter). After washing, MACS-purified [^{3}H]hypoxanthine-labeled late-stage IEs (2 × 10^5 IEs/well) were added to three empty wells as a measure of maximal binding. The IEs were harvested onto Optiplate-96 filters (PerkinElmer, Waltham, MA), Microscint40 scintillation liquid (PerkinElmer; 50 μl/well) was added, and [^{3}H]hypoxanthine incorporation was determined on a TopCount NXT scintillation counter (PerkinElmer).

Rosetting assay. Ring-stage IEs were incubated overnight (37°C, 5% CO$_2$) in RPMI 1640 supplemented with either nonimmune human serum (10%) or AlbuMax II (0.5%). After labeling of parasites with ethidium bromide (25 μg/ml), rosetting frequencies were assessed by counting 200 ethidium bromide-stained IEs, using wet-slide preparations and fluorescence microscopy. Rosettes were defined as IEs having two or more adhering uninfected erythrocytes. All assays were done in triplicate.

Phagocytosis assay. Phagocytosis of antibody-opsonized IEs was measured as described previously (38). In brief, MACS-purified late-stage IEs were stained with ethidium bromide (100 μg/ml, 10 min, room temperature), washed twice in RPMI supplemented with 2% fetal bovine serum, labeled with either human IgM or human IgA (50 nM), and opsonized with pooled plasma pools from nonexposed or *P. falciparum*-exposed adults (1:10, 30 min, room temperature). After washing as described above, the IEs were incubated in 4-ml tubes (30 min, 37°C, 5% CO$_2$) with cells of the human monocytic leukemia line THP-1 (TIB-202; LGC Standards, Borås, Sweden) at a 20:1 ratio. Following lysis of remaining free IEs (15 mM NH$_4$Cl, 10 mM NaHCO$_3$, 1 mM EDTA), the proportion of ethidium bromide-positive THP-1 cells was determined by flow cytometry using an FC500 instrument (Beckman Coulter).

Binding kinetic analysis. We used a Biacore 2000 instrument (GE Healthcare, Hillerød, Denmark) to measure the affinity of IgM for recombinant PfEMP1 constructs by surface plasmon resonance analysis as described previously (15). Following gel filtration, the recombinant PfEMP1 domain constructs (50 ml/min for 60 s), followed by buffer (120 s), were flowed over IgM (750 relative units) immobilized on a CM4 biosensor chip (GE Healthcare) by amine coupling. After each run, the chip was regenerated with 20 mM NaOH. All experiments were performed in HEPES buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% [vol/vol] surfactant P20, pH 7.4, 20°C). The specific binding response to IgM was calculated by subtracting the response to an uncoupled chip and to buffer injection. The kinetic sensorgrams were fitted to a global 1:1 interaction model to allow calculation of the k_a, K_d (dissociation constant), and K_D (equilibrium dissociation constant) values, using BIAevaluation software v. 4.1 (GE Healthcare).
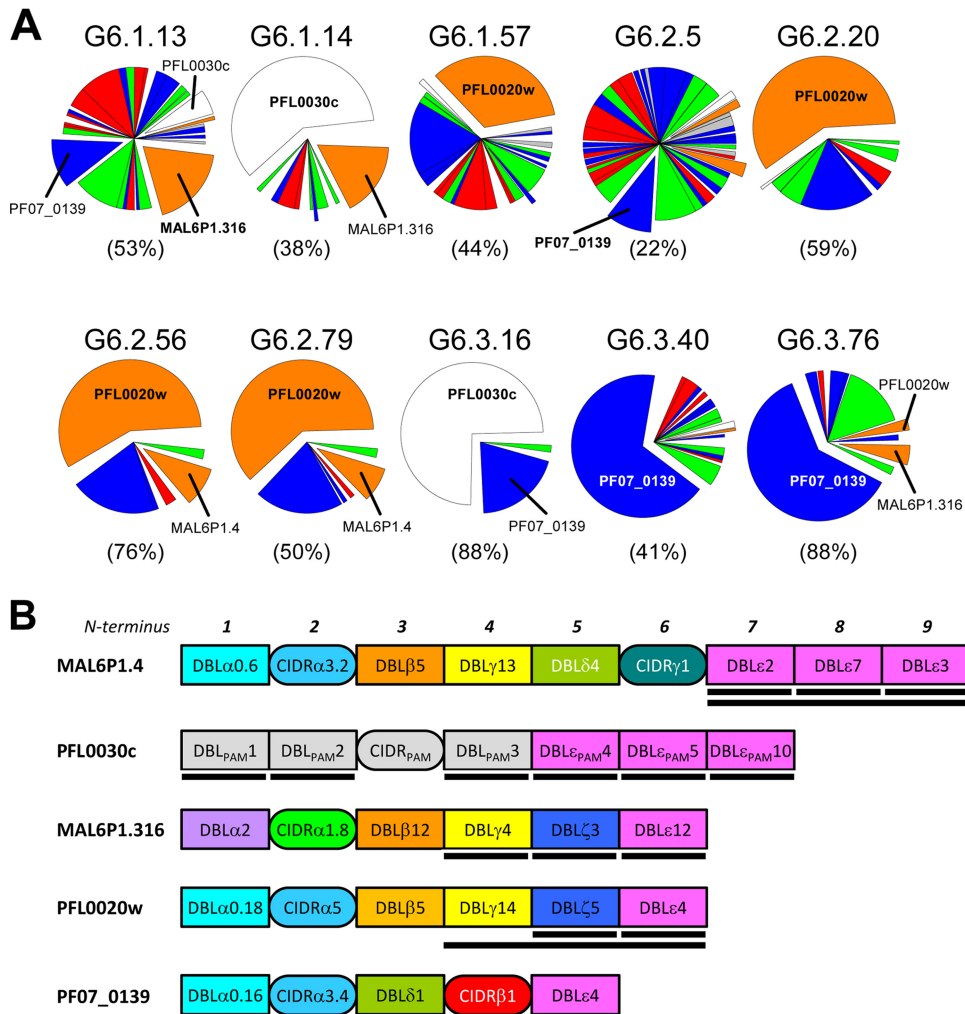
FIG 1 *var* gene transcription in *P. falciparum* G6 following subcloning. (A) Distributions of *var* transcripts in progenies of single-cell-sorted subclones and 50-cell-sorted sublines (G6.1.13 and G6.1.14) of IgM-positive *P. falciparum* G6 following 3 weeks of expansion *in vitro*. Transcripts are color coded as described by Fastman et al. (31), according to the grouping scheme described by Lavstsen et al. (61). The groups identified are group A (gray), group B/A (orange), group B (blue), group B/C (green), group C (red), and VAR2CSA (white). The major transcripts are identified by name, and all candidate genes selected are shown as "exploded pie slices." The percentage of corresponding IEs binding IgM is indicated in parentheses below the pie for each subclone. (B) Domain structures of the PfEMP1 proteins encoded by major *var* gene transcripts in these subclones and sublines. The orientation and domain numbers are indicated in italics along the top of the panel. Recombinant PfEMP1 constructs used in the present study are indicated by underlining.

Statistical analysis. One-way analysis of variance followed by Holm-Sidak multiple pairwise comparisons was used to evaluate the capacity of Fcμ-specific antibodies to inhibit binding of IgM to IEs, and Student's *t* test was used to evaluate the ability of IgM and IgA to inhibit phagocytosis of IgG-opsonized IEs. *P* values of <0.05 were considered statistically significant.

RESULTS

Identification of candidate IgM-binding PfEMP1 proteins in *P. falciparum* NF54. We used the *P. falciparum* NF54-derived clone G6 to identify IgM-binding PfEMP1 proteins. This clone, which is transfected with a pVBH plasmid containing a blasticidin resistance gene controlled by a *var* promoter, initially transcribed the *var* gene *pfd1015c* (31). Culturing this parasite under high blasticidin selection pressure results in a complete shutdown of endogenous *var* transcription and erasure of epigenetic memory, and removal of drug pressure allows *in vitro var* switching to begin (31). We labeled IEs with IgM and single-cell sorted IgM-positive IEs

into 96-well plates. Four cell-sorting control lines with 50 IEs per well were also established. After *in vitro* expansion for 3 weeks, we had 19 growing cultures originating from individual single-cell-sorted IEs. *var* gene transcription was assessed in all lines containing IgM-positive IEs (eight from subclones and two from the 50-cell control sublines). Each of the subclones transcribed multiple *var* genes as a result of shifts in *var* transcription during the period of *in vitro* expansion (Fig. 1A). Nevertheless, all but one subclone (G6.2.5) and one 50-cell subline (G6.1.13) showed dominant expression of a single *var* gene. One subclone (G6.3.16) and one 50-cell subline (G6.1.14) mainly transcribed *pfl0030c*. Four clones (G6.1.57, G6.2.20, G6.2.56, and G6.2.79) mainly transcribed *pfl0020w*, two (G6.3.40 and G6.3.76) predominantly transcribed *pf07_0139*, and both the 50-cell sublines showed prominent transcription of *mal6p1.316*. The relative transcription levels of the identified candidate *var* genes were reflected in the proportions of erythrocytes infected by the subclones and sublines that bound IgM (Fig. 1A).

The PfEMP1 protein encoded by the *pfl0030c var* gene in *P. falciparum* NF54 is the CSA-adhering VAR2CSA-type protein PFL0030c (6, 7). This protein is known to bind IgM via Fcμ (19, 39), and several DBL domains have been implicated in this binding (39, 40). Only the three C-terminal DBL domains in PFL0030c are of a type (DBLε) that is also found in non-VAR2CSA-type PfEMP1 proteins. All the non-VAR2CSA-type candidate IgM-binding PfEMP1 proteins we identified above contained C-terminal DBLε domains (Fig. 1B). However, we recently mapped the Fcμ-binding domain in the rosetting PfEMP1 protein HB3VAR06 to its C-terminal DBLζ_8 domain, and two of our candidate *var* transcripts (*pfl0020w* and *mal6p1.316*) additionally contain a DBLζ domain each (Fig. 1B). Together, these data point to C-terminal DBLε and DBLζ domains as being relevant for Fc-mediated IgM binding.

Verification of the IgM-binding properties of candidate PfEMP1 proteins. To verify the predicted IgM-binding capacity of our candidate PfEMP1 proteins, we next subjected previously unselected *P. falciparum* NF54 parasites to repeated selection with either the VAR2CSA-specific monoclonal antibody PAM1.4 or rat antisera to PFL0020w and MAL6P1.316. In addition, we selected IEs by using a rat antiserum to the PfEMP1 protein MAL6P1.4, because two of our subclones (G.6.256 and G6.2.79) showed prominent transcription of *mal6p1.4* (Fig. 1A). MAL6P1.4 contains three C-terminal DBLε domains (Fig. 1B), making it a strong IgM-binding candidate. We did not have an antiserum specific for PF07_0139 and instead subjected subclone G6.3.40 to repeated rounds of selection for IgM binding. As a negative control, we used *P. falciparum* NF54 selected by an antiserum specific for the intercellular adhesion molecule 1 (ICAM-1)-binding PfEMP1 protein PFD1235w (33, 41). These selection protocols all resulted in parasites with dominant transcription of the expected *var* gene (Fig. 2A), and all the selected IEs bound IgM to various degrees, except for the IEs selected for expression of PFD1235w (Fig. 2B and C).

Determination of the IgM domain involved in binding to PfEMP1. We previously showed that the Cμ3-Cμ4 domains of IgM bind to VAR2CSA-type PfEMP1 (19) and to the rosetting PfEMP1 HB3VAR06 (15). We therefore measured the ability of monoclonal antibodies specifically recognizing the Cμ2, Cμ3, and Cμ4 domains of human IgM to interfere with IgM binding to IEs expressing each of the IgM-binding PfEMP1 proteins studied here. In each case, all three Fcμ-specific antibodies significantly ($P < 0.001$ in all cases) reduced the binding of IgM to the IEs (Fig. 3), although the Cμ2-specific antibody was less effective than the other two antibodies at inhibiting IgM binding to the VAR2CSA-type PFL0030c protein, in accordance with previous findings (19).

Determination of the PfEMP1 domains involved in Fc-specific binding of IgM. Multiple domains have been implicated in Fc-dependent binding of IgM to PfEMP1 (15, 39, 40, 42, 43). We recently used multiple recombinant single- and multi-DBL-domain constructs to map this type of IgM binding to the penultimate C-terminal DBL (DBLζ_8) domain in the rosette-mediating PfEMP1 HB3VAR06 (15). Taking a similar approach here, we used ELISA to identify the domains involved in IgM binding, employing an array of recombinant proteins representing single and triple domains of PFL0030c, PFL0020w, MAL6P1.4, and MAL6P1.316, as well as a construct representing the full ectodomain of PFL0030c (see Fig. 1B for an overview of the recombinant antigen constructs used). With this approach, we unequiv-
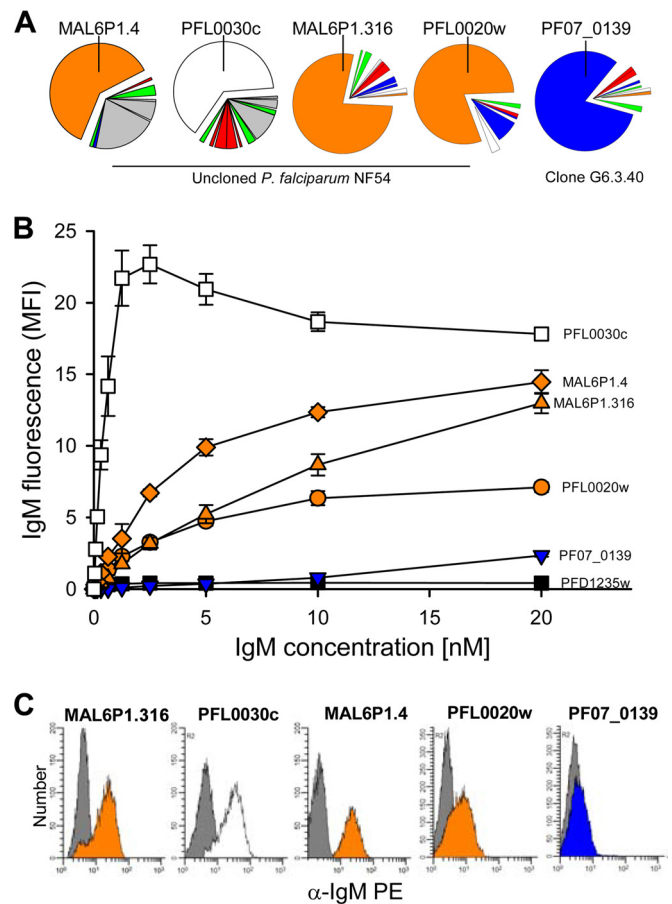

FIG 2 IgM binding and relative *var* gene transcription in *P. falciparum* NF54 following antibody selection. (A) Distributions of *var* transcripts in *P. falciparum* NF54 following antibody selection for IE surface expression of MAL6P1.4, PFL0030c, MAL6P1.316, PFL0020w, and PF07_0139. The color coding and layout are as described in the legend to Fig. 1. (B) Titration of IgM binding to erythrocytes infected by the corresponding subclones and sublines. Means (symbols) and standard deviations (error bars) for triplicate measurements are shown. MFI, mean fluorescence intensity. (C) Histograms showing representative flow cytometry data on all selected parasite cultures, with (orange/white/blue) or without (gray) IgM. PE, phycoerythrin.

ocally identified DBLε_PAM5 as the IgM-binding domain in the VAR2CSA-type PFL0030c protein (Fig. 4A). This is the penultimate C-terminal DBL domain in that protein and was the most prominent IgM-binding domain in an earlier study of VAR2CSA-type PfEMP1 (39). Similarly, we mapped the IgM-binding capacity of PFL0020w and MAL6P1.4 to their C-terminal domains (DBLε4_6 and DBLε3_9, respectively) (Fig. 4B). MAL6P1.4 contained an additional IgM-binding domain (DBLε2_7). We were unable to map the IgM-binding domain of MAL6P1.316 with our recombinant constructs.

We used surface plasmon resonance analysis to show that the binding affinity of IgM for each of the domains identified to bind IgM by ELISA was high, with K_D values in the nanomolar range (Table 1), which is the same range as that reported previously (15). Triple-domain constructs had higher affinities (lower K_D values) than single-domain constructs, which may be related to the construct conformation but likely also reflects the fact that two IgM-binding domains (DBLε2_7 and DBLε4_9) were present in our MAL6P1.4 triple-domain construct (Fig. 4 and Table 1).
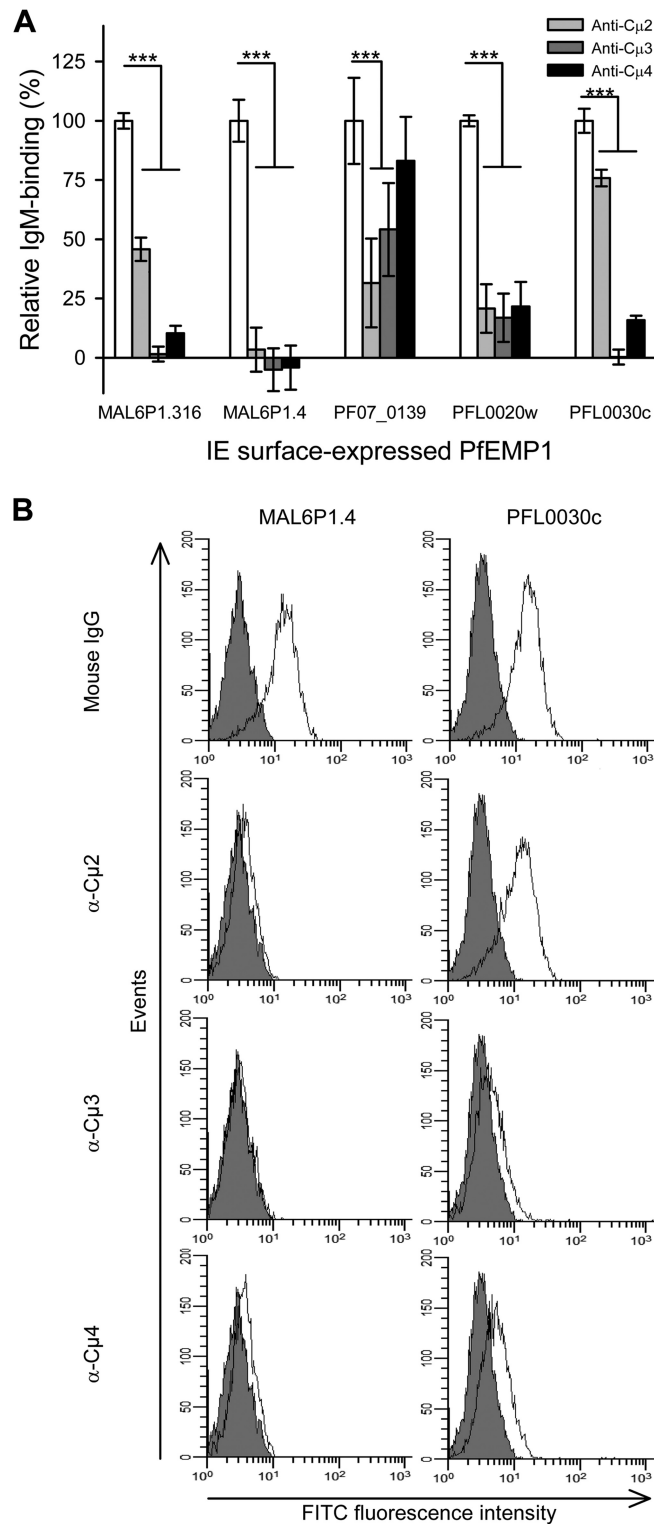
Infection and Immunity

FIG 3 IgM domains involved in binding to PfEMP1 proteins. (A) Involvement of Fcμ domains in the binding of IgM to PfEMP1 as determined by the interference of monoclonal antibodies to various Fcμ domains (Cμ1, Cμ2, and Cμ3) with IgM binding to selected PfEMP1 proteins. Overall means (histograms) and standard deviations (error bars) for three independent experiments are shown. Statistically significant differences ($P < 0.001$) relative to the negative controls (mouse IgG) are indicated by asterisks. (B) Representative flow cytometry data for MAL6P1.4 and PFL0030c. Each histogram shows an overlay of no IgM (gray) and IgM with anti-IgM or control mouse antibodies (white). FITC, fluorescein isothiocyanate.
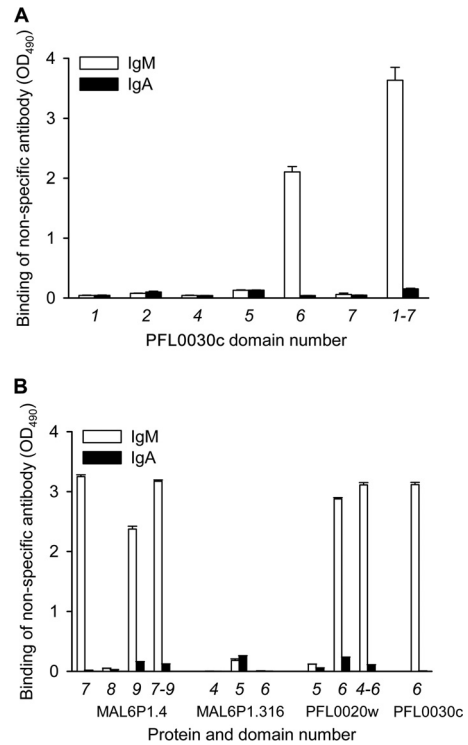

FIG 4 PfEMP1 domains involved in IgM binding. The graphs show the ability of domains in VAR2CSA-type (A) and non-VAR2CSA-type (B) PfEMP1 proteins to bind IgM (white) and IgA (black). Means (histograms) and standard deviations (error bars) for triplicate measurements from a representative experiment (of three) are shown. Domains are numbered as described in the legend to Fig. 1B.

All constructs displayed rapid association kinetics, with k_a values of 1×10^6 to $10 \times 10^6 \, \text{M}^{-1} \, \text{s}^{-1}$. The dissociation kinetics were more variable but were slower for the triple-domain constructs than the corresponding single-domain constructs, suggesting that the higher-order conformation of the PfEMP1 proteins contributes to retention of IgM once it is bound.

CSA-binding and rosetting properties of the new IgM-binding PfEMP1 proteins. Fc-dependent IgM binding to PfEMP1 has previously been shown for parasites expressing CSA-adherent VAR2CSA-type or rosette-mediating PfEMP1 variants (reviewed in reference 18). We therefore tested the abilities of erythrocytes infected by *P. falciparum* expressing either PFL0030c or each of the four new non-VAR2CSA-type PfEMP1 proteins to bind to CSA and to form rosettes. We included parasites expressing the PfEMP1 protein HB3VAR06 as a positive control in the rosetting assays, as this PfEMP1 is known to mediate IgM-dependent rosette formation (15). IEs expressing the VAR2CSA-type PFL0030c protein adhered strongly to CSA-expressing BeWo cells, in accordance with previous reports (6, 37), while none of the other PfEMP1 proteins mediated significant adhesion to this receptor (Fig. 5A). None of the five IgM-binding PfEMP1 proteins studied here was able to mediate formation of rosettes (Fig. 5B). We previously reported that HB3VAR06-mediated rosetting requires soluble serum factors (15, 20), but serum did not lead to rosetting in IEs expressing any of our new IgM-binding PfEMP1 proteins (Fig. 5B).

IgM-mediated interference with phagocytosis of IgG-opsonized infected erythrocytes. Fc-dependent IgM binding to

TABLE 1 Surface plasmon resonance analysis of IgM binding to recombinant PfEMP1 domains

PfEMP1	Domain(s)	Mean ± SD[a]		
		k_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (nM)[b]
MAL6P1.4	DBLε2_7-DBLε7_8-DBLε3_9	$1.21 \times 10^6 \pm 1.02 \times 10^6$	$7.19 \times 10^{-4} \pm 2.73 \times 10^{-4}$	2.07 ± 2.78
	DBLε2_7	$7.14 \times 10^5 \pm 5.32 \times 10^5$	$5.03 \times 10^{-3} \pm 1.24 \times 10^{-3}$	14.80 ± 7.43
	DBLε7_8	NA	NA	NA
	DBLε3_9	$8.58 \times 10^5 \pm 6.18 \times 10^5$	$9.63 \times 10^{-3} \pm 0.58 \times 10^{-3}$	66.06 ± 56.22
PFL0020w	DBLζ14_4-DBLζ5_5-DBLε4_6	$3.25 \times 10^6 \pm 0.63 \times 10^6$	$3.71 \times 10^{-3} \pm 1.15 \times 10^{-3}$	2.36 ± 2.31
	DBLε4_6	$5.23 \times 10^5 \pm 5.25 \times 10^5$	$9.92 \times 10^{-3} \pm 2.97 \times 10^{-3}$	66.52 ± 45.38
PFL0030c	DBLε$_{PAM}$5_6	$5.96 \times 10^5 \pm 3.1 \times 10^5$	$3.98 \times 10^{-2} \pm 1.97 \times 10^{-3}$	140.02 ± 77.91

[a] NA, not available because the domain did not bind IgM.
[b] The K_D values were calculated based on a 1:1 binding model from 3 to 6 independent analyses, with 4 to 8 different concentrations tested in duplicates per analysis, and the values are shown with composite standard deviations.

VAR2CSA-type PfEMP1 markedly inhibits the binding of specific IgG to the IE surface and subsequent phagocytosis of opsonized IEs (19). However, this is not the case for parasites expressing the rosette-mediating HB3VAR06 protein, as IgM binding has a limited effect on the phagocytosis of antibody-opsonized IEs (15). To test whether IgM could inhibit binding of PfEMP1-specific IgG to IEs positive for each of the new IgM-binding PfEMP1 proteins identified here, we opsonized the IEs with immune human plasma

in the absence or presence of IgM and subsequently measured phagocytosis by using a robust *in vitro* assay (38). Phagocytosis of IEs expressing the VAR2CSA-type PFL0030c protein was markedly (about two-thirds) and significantly reduced in the presence of IgM, in accordance with previous data (19) (Fig. 6). A smaller or nonsignificant impact of IgM on IgG-dependent phagocytosis was seen for each of the other PfEMP1 proteins (Fig. 6). In this respect, they therefore resembled the rosette-mediating PfEMP1 HB3VAR06 (15), although none of them appear to be involved in rosetting (Fig. 5B).
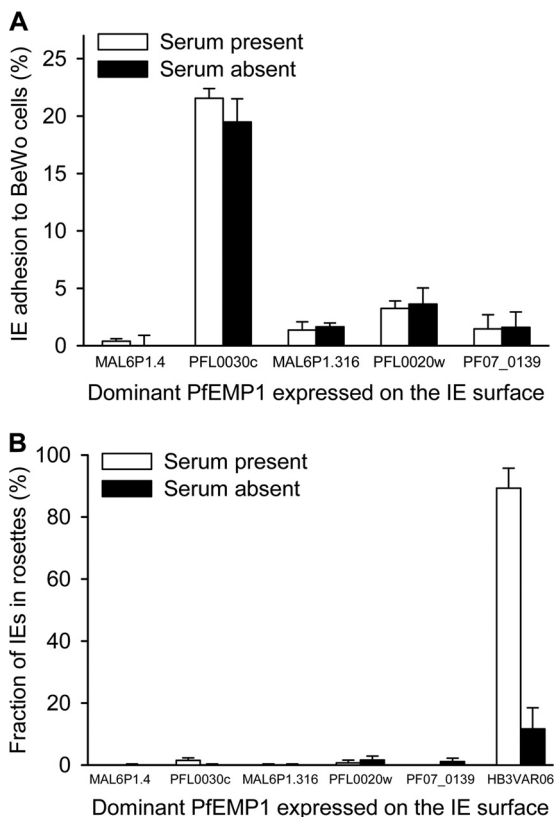
DISCUSSION

The particular virulence of *P. falciparum* parasites is related to their ability to express members of the clonally variant protein family PfEMP1 on the surfaces of the erythrocytes they infect (reviewed in reference 44). The PfEMP1 proteins mediate adhesion of IEs to different host receptors in various tissues to avoid IE destruction in the spleen (45). Each parasite genome contains about 60 PfEMP1-encoding *var* genes that are transcribed in a mutually exclusive manner (46–48). Thus, normally only one PfEMP1 is expressed on the IE surface at any given time, but the parasites can switch transcription among the different *var* genes from one asexual 48-h multiplication cycle to the next, thereby
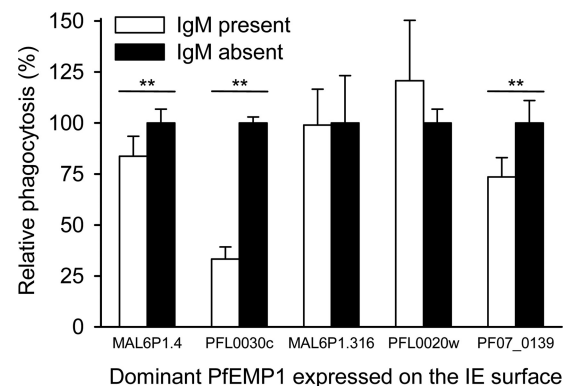

FIG 5 Functional properties of erythrocytes infected by *P. falciparum* parasites expressing defined PfEMP1. The graphs show the ability of IE surface-expressed PfEMP1 proteins to mediate adhesion to CSA on BeWo cells (A) or to mediate rosetting (B) in the presence (white) or absence (black) of serum. Means (bars) and standard deviations (error bars) for triplicate measurements from a representative experiment (of three) are shown.

FIG 6 IgM-dependent interference with phagocytosis of IgG-opsonized infected erythrocytes. The graph shows the ability of IgM (white) and IgA (black) to interfere with IgG-specific opsonization and phagocytosis of erythrocytes infected by *P. falciparum* parasites expressing defined PfEMP1. Overall means (bars) and standard deviations (error bars) for three independent experiments are shown. **, significant differences between the two bars.

changing the adhesive and antigenic properties of the IEs ([46], [47]). By now, it is clear that certain structurally defined and functionally related PfEMP1 subfamilies are involved in particular types of severe malaria (reviewed in reference [49]). The role of endothelial protein C receptor (EPCR)-adhering PfEMP1 proteins sharing domain cassette 8 (DC8) and DC13 motifs ([50], [51]) in the pathogenesis of cerebral malaria is a recent example. The similar role of DC4-containing PfEMP1 proteins adhering to ICAM-1 is another ([33]). Finally, VAR2CSA-type PfEMP1 proteins adhering to CSA have a well-established key role in the pathogenesis of placental malaria (reviewed in reference [52]). The tissue-specific adhesion mediated by these types of PfEMP1 makes it easy to appreciate their role in pathogenesis. It is much less obvious why the ability of some PfEMP1 proteins to bind IgM via Fc should be linked to malaria severity, as has repeatedly been shown (reviewed in reference [18]). Several PfEMP1 domains have been implicated in IgM binding ([15], [36], [39], [40], [42], [43]), but a unifying picture has not yet emerged. Furthermore, it is unclear whether Fc-specific binding of IgM to IEs is restricted to parasites expressing PfEMP1 proteins that can adhere to CSA or lead to the formation of rosettes. Finally, it is not known how many PfEMP1 proteins encoded by any single *P. falciparum* genome possess this phenotype. Thus, the purpose of the present study was to address these unanswered questions.

Individual *P. falciparum* parasites transcribe only a single *var* gene at a time, and this mutually exclusive transcription ensures that normally only a single PfEMP1 variant is present on the surface of any given IE ([53]–[55]). Even at the population level, the diversity of PfEMP1 expression is often limited due to the presence of epigenetic memory (reviewed in references [56] and [57]). To overcome this difficulty, we selected pVBH-transfected *P. falciparum* G6 clonal parasites for resistance to blasticidin, which effectively erases the epigenetic *var* gene transcription memory ([29]–[31]). Following induction of *var* switching, we single-cell sorted IEs from this population based on their capacity to bind IgM at the IE surface, and we obtained subclones and sublines with dominant transcription of four different *var* genes ([Fig. 1]). One was *pfl0030c* ([6]), which encodes the VAR2CSA-type PfEMP1 in the *P. falciparum* 3D7 clone (from which the G6 clone is derived) and was already known to bind IgM via Fc ([19], [39], [40]). The other three (*mal6p1.316*, *pfl0020w*, and *pf070139*) encode PfEMP1 proteins not previously reported to bind IgM. However, all contain at least one DBLε-type domain, similar to the C-terminal domains previously implicated in the IgM-binding capacity of VAR2CSA-type PfEMP1 ([39], [40]). Finally, the *var* gene *mal6p1.4*, which encodes a PfEMP1 with three C-terminal DBLε domains in tandem, was also prominently transcribed in several subclones/sublines. These structural characteristics supported the IgM-binding capacity of these PfEMP1 variants. Two of the genes (*pf0020w* and *mal6p1.316*) additionally encode a C-terminal DBLζ domain. This further reinforced their candidature, as we recently mapped Fcμ-specific binding of IgM to a domain of that type ([15]). Indeed, we experimentally confirmed their ability to bind IgM ([Fig. 2]).

The Fc-specific binding of IgM to PfEMP1 proteins has consistently been shown to involve the Cμ3-Cμ4 domains in the Fc part of pentameric IgM. Our findings confirm this location of the PfEMP1-binding site in IgM ([Fig. 3]). More ambiguity exists with respect to the corresponding PfEMP1 domains involved. Thus far, at least three of the DBL domains in VAR2CSA-type PfEMP1 (DBL$_{PAM}$2_2, DBLε$_{PAM}$5_6, and DBLε$_{PAM}$10_7) have been impli-

cated ([39], [40]). We resolved this uncertainty by using recombinant constructs of each of the DBL domains in PFL0030c to map the IgM-binding site to the penultimate N-terminal domain DBLε$_{PAM}$5_6 ([Fig. 4]). Similar confusion has existed with respect to IgM binding to non-VAR2CSA-type PfEMP1 proteins, in which N-terminal cysteine-rich interdomain region α (CIDRα), central DBLβ, and C-terminal DBLζ domains have all been implicated ([15], [42], [43]). We found that DBLε domains near the C terminus were responsible for mediating binding to IgM in the PfEMP1 proteins studied here. One of them (MAL6P1.4) even contained two IgM-binding domains (DBLε2_7 and DBLε3_9), something which has not been observed previously. Overall, presently available evidence points to DBLε and DBLζ domains near or at the C terminus as the key IgM-binding elements in PfEMP1 proteins.

The functional significance of Fc-mediated binding of IgM to PfEMP1 proteins remains unclear ([18]). In the case of VAR2CSA-type PfEMP1, it appears to serve an immunoevasive role that interferes with phagocytosis of IgG-opsonized IEs without compromising the adhesive function of PfEMP1 ([19]). Our findings here support these earlier results. Thus, PF0030c-expressing IEs adhered strongly to CSA, whether IgM was present or not ([Fig. 5A]), and IgM markedly inhibited phagocytosis of IgG-opsonized IEs expressing this PfEMP1 ([Fig. 6]). In contrast to these characteristics of VAR2CSA-type PfEMP1, we recently reported that Fc-dependent binding of IgM does not protect parasites expressing the rosette-mediating HB3VAR06 protein from phagocytosis of IgG-opsonized IEs but rather may serve to augment low-affinity adhesive interactions between the PfEMP1 head structure and as yet undefined carbohydrate moieties ([15]). Fc-specific binding of IgM to the new PfEMP1 proteins identified here did not markedly protect them from phagocytosis following opsonization by antigen-specific IgG ([Fig. 6]). In this respect, they resemble the rosette-mediating PfEMP1 protein HB3VAR06, which suggests that they have an elongated conformation. Only one of them (MAL6P1.316) contains an N-terminal DBL domain of a type previously associated with rosetting (DBLα1.5, DBLα1.6, DBLα1.8, or DBLα2) ([10]–[13], [15]), and none of them mediated rosetting ([Fig. 5B]). These findings establish for the first time that the ability to bind IgM via Fcμ is not restricted to VAR2CSA-type and rosette-mediating PfEMP1 variants. They also indirectly support our hypothesis that an important function of IgM binding is to augment low-affinity adhesive interactions between the PfEMP1 head structures and host endothelial receptors ([15]). Only when such receptors are also found on erythrocytes would this lead to rosetting.

In conclusion, we have provided evidence that each *P. falciparum* genome encodes several PfEMP1 proteins with the capacity to bind Fcμ. We cannot formally rule out that other parasite-encoded IE proteins, e.g., rosette-mediating RIFINs ([58], [59]), also have affinity for Fcμ, but there is presently no evidence to support that possibility. We identified five PfEMP1 proteins in *P. falciparum* 3D7/NF54 parasites, but this is almost certainly an underestimation considering the limited number of subclones we investigated. Our findings confirm that Fc-specific binding of IgM to VAR2CSA-type PfEMP1 has an immunoevasive function. However, they also support recent data pointing to alternative roles for Fc-mediated IgM binding to other types of PfEMP1 ([15], [18], [20]). In any case, the IgM-binding phenotype is likely to be both common and important, as we showed that Fc-dependent binding of

IgM is not restricted to PfEMP1 proteins mediating either adhesion to CSA or rosetting. Although rosetting has been associated with expression of PfEMP1 proteins causing severe malaria, the relationship is not absolute (reviewed in reference 60). Indeed, several PfEMP1 proteins that mediate adhesion to EPCR and ICAM-1, phenotypes that have repeatedly been associated with severe malaria, do not mediate formation of rosettes (50). Future analysis of the interrelationship among various PfEMP1 phenotypes that have been individually associated with malaria severity may well produce important new insights. This prediction is supported by the fact that one of the PfEMP1 proteins studied here (MAL6P1.316), which bound Fcμ but did not mediate formation of rosettes, contains a CIDR of a type (CIDRα1.8) that is involved in adhesion to EPCR (51).

ACKNOWLEDGMENTS

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