

**Evidence for Globally Shared, Cross-reacting Polymorphic Epitopes in the Pregnancy
Malaria Vaccine Candidate VAR2CSA**

Running Title: Shared Polymorphic Epitopes in VAR2CSA

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Abstract

Pregnancy-associated malaria (PAM) is characterized by the placental sequestration of *Plasmodium falciparum*-infected erythrocytes (IEs) with the ability to bind to chondroitin sulfate A (CSA). VAR2CSA is a leading candidate for a pregnancy malaria vaccine, but its large size (~350 kDa) and extensive polymorphism may pose a challenge to vaccine development. In this study, rabbits were immunized with individual VAR2CSA Duffy binding-like (DBL) domains expressed in *Pichia pastoris* or *var2csa* plasmid DNA and sera were screened on different CSA-binding parasite lines. Rabbit antibodies to three recombinant proteins (DBL1, DBL3, DBL6) and four plasmid DNAs (DBL1, DBL3, DBL5 and DBL6) reacted with homologous FCR3-CSA IEs. By comparison, antibodies to the DBL4 domain were unable to react with native VAR2CSA protein unless it was first partially proteolyzed with trypsin or chymotrypsin. To investigate the antigenic relationship of geographically diverse CSA-binding isolates, rabbit immune sera were screened on four heterologous CSA-binding lines from different continental origins. Antibodies did not target conserved epitopes exposed in all VAR2CSA alleles, however, anti-sera to several DBL domains cross-reacted on parasite isolates that had polymorphic loops in common with the homologous immunogen. This study demonstrates that VAR2CSA contains common polymorphic epitopes that are shared between geographically diverse CSA-binding lines.

INTRODUCTION

Pregnancy associated malaria (PAM) is a major cause of poor mother/child health and leads to maternal anemia, prematurity, low birth weight and increased infant morbidity and mortality (9). PAM is caused by *Plasmodium falciparum* infected erythrocytes (IEs) that selectively bind to chondroitin sulfate A (CSA) in the placenta (16). In areas of stable malaria transmission, women acquire antibodies against CSA-binding parasites over successive pregnancies (4,18,24,30), and a lack of these antibodies appears to contribute to the high susceptibility to malaria for primigravid and HIV-infected women (15,21,25,37). Naturally acquired antibodies from multigravid women recognize placental IEs and CSA-binding parasites collected around the world (4,18,24,30), suggesting that the target epitopes may be globally conserved or restricted, which means a vaccine may be feasible.

Several lines of evidence implicate a parasite protein, termed VAR2CSA, in placental binding and immunity to malaria during pregnancy. VAR2CSA is a member of the *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) family encoded by *var* genes. PfEMP1 proteins are clonally variant adhesive proteins expressed at the surface of IEs (22). The *var2CSA* transcript is specifically upregulated in IEs selected to bind to CSA (33) and the transcript is highly abundant in parasites isolated from infected placentas (13,42). VAR2CSA contains multiple CSA-binding domains (19), and laboratory-adapted parasites engineered with *var2csa* gene disruptions lose the ability to bind CSA and to react specifically with serum from pregnant women (14,44). Furthermore, recombinant VAR2CSA proteins are recognized in a gender and parity-dependent manner (2,43) and VAR2CSA-specific IgG is associated with protection from one of the major adverse consequences of pregnancy malaria, delivery of a low birth weight infant (32). These data establish VAR2CSA as a leading candidate for a pregnancy malaria vaccine, but a crucial question remains to be addressed,

namely how to generate a broad protective antibody response to a large and polymorphic protein.

Whereas numerous studies have shown that maternal antibodies are capable of recognizing geographically diverse CSA-binding isolates (4,18,24,30), more detailed serological characterizations have suggested that the human IgG response against placental isolates appears to be significantly focused on polymorphic regions in VAR2CSA (1,6,12,41). After exposure, pregnant women appear to acquire a repertoire of variant-specific antibodies, some of which cross-react with different placental isolates (5,6,30,41). Although there is still limited understanding of the antigenic relationship of different VAR2CSA alleles, sequence comparisons have revealed extensive segmental gene relationship between different *var2csa* genes (8,39).

We recently showed that it was possible to generate anti-sera reactive with native VAR2CSA by immunizing with individual Duffy binding-like (DBL) recombinant proteins produced in *Baculovirus* but not by expressing the same domains in *Escherichia coli*, possibly due to protein folding issues (2). Furthermore, these antibodies were able to partially cross-react on different CSA-binding parasite lines (27). However, there has been limited investigation whether vaccine sera target diverse, conserved, or common epitopes of the native VAR2CSA molecule. In this study, we used recombinant proteins produced in the methylotropic yeast *Pichia pastoris* or plasmid DNA to immunize rabbits with the six VAR2CSA DBL domains. Our results show that both approaches elicit highly variant-specific antibodies to CSA-binding IEs, but also demonstrate the presence of shared polymorphic epitopes with world-wide geographic distribution. The significance of these findings for variant antigen diversification and PAM vaccine development are discussed.

MATERIALS AND METHODS

Parasite and mammalian cell lines. *P. falciparum* parasites were grown in O+ erythrocytes and human serum. Clinical isolate PI0711 was isolated from peripheral blood of a pregnant woman with placental malaria (17), and selected on CSA prior to analysis. Laboratory isolates, FCR3, 7G8, HB3, and 3D7, were initially selected on the human placental BeWo cell line (45) or CSA and subsequently maintained by panning on 200 µg/ml bovine CSA (Fluka Biochemika) coated on plastic Petri dishes. FCR3 is isogenic with the IT4 parasite due to historical contamination (31) and considered to have the same *var2CSA* gene. Chinese Hamster Ovary K1 cells (CHO-K1) and CSA-deficient CHO-745 derivative lines were cultured in F-12 Kaigh's medium (Gibco). CHO-745 cell lines transfected with CD36 (CHO-745-CD36) (10) or VAR2CSA DBL domains (19) were maintained under selection by G418 at 500 µg/ml (Invitrogen). CHO-745 (CSA negative), CHO-745-CD36, and CHO-K1 (CSA positive) cells were used to confirm the binding phenotype of parasites in this study.

Design of DBL synthetic genes. *var2csa* synthetic genes were designed with optimized codons for human expression so they could be shuttled between different plasmid vectors for human, rabbit, and yeast cell expression. Potential N-glycosylation sites were removed from synthetic genes by converting asparagine to glutamine or by replacing asparagine with an amino acid from another VAR2CSA allele.

Recombinant protein expression in *Pichia pastoris*. Synthetic genes encoding FCR3 VAR2CSA DBL domains (accession AY372123); DBL1 (residues 58-383), DBL2 (residues 536-858), DBL3 (residues 1221-1541), DBL4 (residues 1594-1888), DBL5 (residues 2003-2270) and

DBL6 (residues 2322-2590) were cloned into the pPIC9K vector containing an N-terminal α -factor secretion signal (Invitrogen). Synthetic genes contained a His₆-tag on the C-terminus. Constructs were digested with *SacI* and transformed into electrocompetent *P. pastoris* strain GS115. The transformation results in DNA insertion at the AOX1 locus generating a His⁺ Mut⁺ phenotype. Yeast clones were grown in Buffered complex Medium (BM) (1% yeast extract, 2% peptone, 1% yeast nitrogen base, 1M potassium phosphate buffer pH 6.0) plus 2% Glycerol (BMG) as a carbon source. Starting from an overnight culture, yeast cultures were grown for 3 days at 20°C with shaking at 250 rpm. After each 24h interval, recombinant protein expression was induced by adding methanol to 0.5% final concentration. Recombinant proteins were harvested 72h post-induction. Proteins were purified using nickel-nitrilotriacetic acid-agarose (Ni-NTA, Qiagen) on an Econo-Pac chromatography column (Biorad) and eluted by a step-gradient from 30mM to 450mM imidazole. Since FCR3-DBL3 was produced at lower levels, this domain was also cotransformed with a plasmid (pPICZ α A-PpPDI), which overexpresses *P. pastoris* protein disulfide isomerase (PDI) to increase recombinant protein production, and fermented in 5L bioreactors as described previously (40). Recombinant proteins identity was confirmed by mass spectrometry.

Plasma Samples. The human plasma samples used in these studies were collected from East African donors, under protocols approved by the relevant ethics review committees. Study participants provided written, informed consent before donating samples, and included adult men and multigravid women from Kenya (28) as well as from Tanzania (2).

Recombinant protein characterization. *P. pastoris* recombinant proteins were analyzed in 4-20% SDS-PAGE gels under reduced or non-reduced conditions (Biorad). Gels were stained with Coomassie blue or transferred to a nitrocellulose membrane and detected by Western blot using anti-His tag antibodies (Invitrogen). The DBL1, 3, 4 and 6 recombinant proteins were tested by ELISA against immune and non-immune sera according to published methods (32).

Construction of VR1051/DBL plasmids for DNA immunization. The six synthetic genes used for *P. pastoris* protein production (minus the His₆-tag) were cloned into the VR1051 vector (34). VR1051 contains a Tissue Plasminogen Activator (TPA) leader sequence to direct recombinant proteins into the secretory system and expresses proteins fused to the P2P30 universal helper T-cell epitope. In addition, we cloned 3D7-DBL2x (residues 542-853, accession XM_001350379) and A4-CIDR1 (residues 402-846, accession L42244). Plasmid DNA was prepared using Endofree plasmid DNA purification kit (Qiagen). Inserts were confirmed by DNA sequencing. All constructs tested less than 10 Endotoxin Unit/ml (Pyrosate Kit PSD10, Associates of Cape Cod Incorporated).

Immunizations. Immunizations were performed at R&R Rabbitry according to animal immunization guidelines. In brief, rabbits received 100 or 500 µg of recombinant protein in complete Freund's adjuvant for first immunizations and half as much protein in five subsequent immunizations (incomplete Freund's adjuvant). For DNA immunization, rabbits were immunized 6-7 times intramuscularly by injection of 500 µg of DNA with Vaxfectin adjuvant (molar ratio DNA:vaxfectin of 2:1) according to published methodologies (20).

Real-Time PCR. Quantitative real-time PCR was performed on RNA harvested ~10-12h post-invasion as previously described (44). Published cross-reactive primers to the VAR2CSA DBL4 domain (33,42) or gene-specific primers to the two HB3 *var2CSA* genes were used. Primer sequences for HB3 allele A: forward 5' TGACGAAAATTTATTAAAGC-3' and reverse 5' TTCACACTTGTTTTTCATCTC-3' and for HB3 allele B: forward 5' TCAGCAAAATTCATCTGATG-3' and reverse 5' CAATTGATCTGTACCTGTA-3'. The DBL4 and HB3 primer sets were optimized to the same conditions as the 56 IT4 *var* primers and housekeeping controls (44). Results are shown as the fold difference in expression relative to the house-keeping *Pf* adenylosuccinate lyase (*Asl*) (PFB0295w) (44).

Flow cytometry analysis. For flow analysis, a confluent monolayer of CHO-745 cells was lifted by PBS containing 4-8mM EDTA and 1 million CHO-745 cells were incubated with 1/25 dilutions of rabbit anti-VAR2CSA sera. The mAb 179 (6µg/ml) (Affymax Research Institute) and 12CA5 mouse ascites fluid (1/250) (mAb HA.11 from BAbCo) were used to monitor the expression of DBL recombinant proteins on CHO-745 transfectants (19). Primary antibodies were detected using Alexafluor 488 conjugated goat anti-rabbit IgG (1/500, Molecular Probes) and Alexafluor 488 conjugated goat anti-mouse IgG (1/1000, Molecular Probes).

Mature-stage IEs were washed and resuspended in a 100 µl volume of PBS/1% BSA containing 1/25 dilutions of rabbit sera pre-adsorbed on uninfected erythrocytes. As controls, mouse monoclonal BC6 was used to determine A4var PfEMP1 expression in the A4ultra culture (36), and pools of immune plasma from males or multigravid women 1/10 dilutions were used. Bound antibodies were detected with FITC conjugated goat F(ab')₂ anti-human IgG (Beckman, PN

IM1627) or Alexafluor 488 conjugated goat anti-rabbit IgG. Infected erythrocytes were detected using Ethidium Bromide (Sigma) at 0.02mg/ml. Samples were analyzed in a Beckman Coulter Epics XL-MCL (Tree Star Inc). Preimmune sera were used to set a gate to determine the percentage of CHO-745 cells transfectants or infected erythrocytes reactive with immune sera and to calculate the difference in Median Fluorescence Intensity (MFI) between preimmune and immune sera. Data were converted to normalized MFI by subtracting the MFI value of preimmune serum from the MFI value of immune serum.

Antibody Inhibition of IE binding to CHO cells. FCR3-CSA IEs were enriched by 0.7% porcine gelatin flotation and resuspended at 1×10^7 IEs/ml in RPMI pH7.2. Infected erythrocytes were pre-incubated for 1h at 37°C in a 1/25 final dilution of pre-immune or immune serum. After washing, IE-antibody complexes were added to CHO-K1 cells (CSA+) and incubated for 1 hr at 37°C in RPMI 1640 binding medium containing a 1/25 dilution of rabbit antibodies. The percentage of IE binding inhibition in the presence of immune serum was calculated relative to preimmune serum.

Protease treatment of IEs surface antigen. For protease treatments, IEs were washed 3 times in PBS (pH7.2) and incubated with Trypsin-TPCK-treated (Sigma) or Chymotrypsin-TLCK-treated (Sigma) at 1mg/ml or 0.1mg/ml in PBS for 15 or 30min at 37°C. Control samples were incubated with PBS alone. Digestion was stopped by addition of 1mg/ml soybean Trypsin/Chymotrypsin inhibitor (Sigma) for 5 min or culture medium containing 10% human serum. Protease treated IEs were tested by flow cytometry for reactivity with rabbit antibodies as described above.

RESULTS

Expression of *P. pastoris* recombinant proteins. To determine conditions for producing individual DBL immunogens the six DBL domains of the IT4/FCR3 form of VAR2CSA were expressed as secreted His-tagged recombinant proteins in the methylotropic yeast *P. pastoris* (Fig. 1A). Using this system, rDBL1, rDBL4 and rDBL6 were produced at approximately 1-4mg/L of yeast culture, while rDBL3 protein was produced at lower levels. However, rDBL3 protein production could be increased to 0.5 mg/L broth by co-transforming with a plasmid that overexpresses *P. pastoris* protein disulfide isomerase (PDI) (40) and controlling fermentation conditions. In contrast, we were unable to produce appreciable amounts of rDBL2 and rDBL5 proteins, although these same construct boundaries were previously used for expressing proteins at the surface of CHO-745 cells (19).

Under SDS-PAGE reducing conditions, all four proteins migrated at the expected molecular weight (Fig. 1B and C). However, rDBL3 and rDBL1 contained minor, smaller molecular weight products. These minor products are likely nicked protein, which can occur due to limited lysis of *P. pastoris* and subsequent release of proteases (11,40). Furthermore, in contrast to the other proteins, the majority of rDBL6 migrated as a dimer or larger forms under non-reducing conditions (Fig. 1 B and C). DBL domains contain several conserved intra-domain disulfide bonds (35,38), however, it is not known whether a disulfide interaction normally occurs between DBL6 domains in the native VAR2CSA protein. All four *P. pastoris* recombinant proteins were recognized in a gender-specific manner by plasma from Tanzanian women with a history of malaria exposure during pregnancy compared to plasma samples from exposed men from the same endemic area or to non-immune samples (Fig. 1D).

Antibodies to VAR2CSA react with homologous FCR3-CSA infected erythrocytes. Rabbits were immunized with the four recombinant proteins or the six individual IT4/FCR3 VAR2CSA DBL domains cloned into a plasmid DNA vector (VR1051) (3). In addition, rabbits were immunized with plasmid DNA encoding the 3D7-DBL2 VAR2CSA and the A4var-CIDR1 domain from a non-placental type PfEMP1 protein. A4var was selected as a control because there is a specific monoclonal antibody to the A4var PfEMP1 protein (mAb BC6) that can be used to select IEs expressing this specific PfEMP1 variant from the IT4/FCR3 parasite genotype (36). The immunization protocols are summarized in Table 1.

Antibody titers as determined by end-point dilution ranged between 10^4 to 10^6 for both protein and DNA immunizations and peaked after four immunizations (data not shown). To assess antibody cross-reactivity between different VAR2CSA domains and between different allelic forms of a given domain, each serum was screened against the DBL1, DBL2 and DBL3 domains in IT4-VAR2CSA (homologous domains) and all six DBL domains from 3D7-VAR2CSA (heterologous domains) expressed at the surface of CHO-745 cells (19) (Table 2). The six DBL domains of IT4/FCR3 and 3D7 have 85%, 76%, 81%, 80%, 85%, and 64% identity, respectively. In general, anti-sera had strong reactivity to both homologous and heterologous forms of the immunizing domain but limited cross-reactivity to other VAR2CSA domains (Table 2). There were only a few examples of partial cross-reactivity between different VAR2CSA domains; IT-rDBL3 antiserum (rabbit 1) reacted with both DBL1 and DBL3 domains, IT-rDBL4 (rabbit 1) reacted with DBL4, DBL6, and DBL2 β , and IT-rDBL6 reacted with DBL6 and DBL1. Cross-reactivities between VAR2CSA domains were confined to rabbits in the protein immunization group. Therefore, anti-sera were generally highly specific to the type of domain used for immunization and VAR2CSA recombinant proteins contain cross-reactive epitopes.

To investigate whether VAR2CSA antisera could recognize native protein, rabbit sera were tested in flow cytometry on FCR3-CSA-infected erythrocytes (homologous parasites) and A4ultra-infected erythrocytes. Using gene-specific primers to the 56 known *var* genes in the IT4/FCR3 parasite genome (44), *var2CSA* was the predominant *var* gene transcribed in FCR3-CSA (greater than 19:1 ratio compared to house-keeping gene *Pf Asl* and *A4var* was the predominant *var* gene in the A4ultra culture (~6:1 ratio) (Fig. 2). Conversely, *var2CSA* and *A4var* were present at much less than 0.1:1 ratio when the gene was not “on” (Fig. 2). As expected, FCR3-CSA infected erythrocytes bound CSA and were preferentially recognized by pooled sera from multi-gravid women, while A4ultra infected erythrocytes did not bind CSA and were recognized equally well by pooled sera from women or males (Fig. 3, and data not shown).

Rabbit antibodies to the three VAR2CSA recombinant proteins (rDBL1, rDBL3, rDBL6) and four plasmid DNAs (DBL1, DBL3, DBL5 and DBL6) reacted with homologous FCR3-CSA infected erythrocytes (Fig. 3). Rabbit sera were considered negative if they produced a signal lower than plasmid DNA vector alone. In general, positive sera recognized more than 50% of IEs and the reactivity of anti-DBL1 and anti-DBL6 sera were similar or higher than pooled multigravid sera. Conversely, VAR2CSA anti-sera did not recognize A4ultra infected erythrocytes, although this parasite line was recognized equally well by anti-A4var CIDR1 sera and A4var monoclonal antibody, BC6 (Fig. 3).

In contrast to other domains, antibodies to DBL2 and DBL4 reacted poorly or not at all with infected erythrocytes. Previous studies have shown that DBL4 reactivity can be revealed by treating infected erythrocytes with protease conditions that have been shown to partially cleave VAR2CSA (2,6). To determine the effect of protease-treatment, FCR3-CSA infected erythrocytes were treated with trypsin or chymotrypsin. Both treatments led to a dose- and time-dependent increase in DBL4 reactivity and partial loss of DBL1 and DBL3 reactivity (Table 3).

Of interest, DBL4 is the most conserved VAR2CSA domain (8). Taken together, these observations suggest that the DBL4 immunogen may not be folded properly or that this domain may be less accessible to antibodies in the native protein.

None of the sera were able to efficiently block IEs binding to CSA, although we obtained a partial binding inhibition, approximately 24% of IEs-FCR3-CSA by one of the rDBL1x protein immune serum and also ~20% with the pDNA-DBL1x immune serum (data not shown). However, there was no CSA binding inhibition when sera were pooled from DBL1-DBL3 or DBL4-DBL6 (DNA immunization) or DBL1 and DBL3 (protein immunization) (data not shown). Taken together, antibodies generated to individual VAR2CSA DBL domains were able to recognize native PfEMP1 protein, but unable to block infected erythrocyte binding.

VAR2CSA antibodies partially cross-react on geographically diverse CSA-binding infected erythrocytes. To study the antigenic relationship of different CSA-binding variants, rabbit sera were tested against four different CSA-binding parasite lines; 3D7-CSA (Amsterdam, origin ambiguous), HB3-CSA (Central America), 7G8-CSA (South America), and a clinical isolate from a pregnant woman, PI0711 (Africa). These four parasite isolates all appear to have distinct origins from the FCR3 parasite, whose exact provenance is unknown due to historical contamination but groups with South East Asian isolates by DNA genotyping (26). Whereas FCR3, 3D7, HB3, and 7G8 parasites have been cloned *in vitro* and represent single parasite genotypes (7,29), the PI0711 has only recently been adapted to *in vitro* cultivation and is likely to contain more than one parasite genotype (17).

Based upon quantitative RT-PCR, the three long-term adapted parasite isolates all transcribed *var2CSA* at greater than 3:1 ratios while *var2csa* was transcribed at approximately 1:1 ratio in PI0711 compared to *Pf Asl* house-keeping control gene (Fig. 2). Of interest, HB3 have

two different *var2CSA* alleles (alleles A and B), and the HB3-CSA culture appeared to be a mixture of both variants. Consistent with transcriptional data, all four CSA-binding lines were recognized in a gender-specific manner by endemic sera, although the proportion of IEs reacting with pooled maternal antibodies differed between isolates and the median fluorescent intensity (MFI) for all four CSA-binding lines was less than FCR3-CSA (Fig. 4), which may indicate weaker variant antigen surface expression.

VAR2CSA immune sera cross-reacted with these parasites in different patterns, and none of the anti-sera recognized all four isolates (Fig. 4), indicating that vaccine sera did not target conserved epitopes exposed in the native protein. Instead, antibody cross-reactivity appeared to be due to shared polymorphic epitopes. For instance, DBL3 antibodies cross-reacted with 7G8-CSA and HB3-CSA lines and were very weakly cross-reactive with P10711, but did not label 3D7-CSA above the negative control. This pattern of reactivity corresponded with molecular modeling and sequence analysis showing that FCR3 is identical to 7G8 at polymorphic segments 1, 3, and 5, and shares identity to HB3 alleles at segments 3 and 5, but has no polymorphic segments in common with 3D7 (Fig. 5). The presence or absence of common polymorphic epitopes also correlated with the antibody cross-reactivity to the DBL1 and DBL5 domains (data not shown). Therefore, common and pan-geographically shared polymorphic epitopes were found in multiple VAR2CSA DBL domains.

DISCUSSION

Considering the broad protective antibody response that women develop to placental isolates (18), the development of a vaccine to prevent malaria during pregnancy appears feasible. Indeed, the variant surface molecules expressed by placental variants have been found to be highly immunogenic and higher levels of surface reactive IgG has been correlated with improved maternal haemoglobin levels and infant birthweight (15,37).

In this study, we used plasmid DNA and *P. pastoris* to immunize rabbits with VAR2CSA recombinant antigens. *P. pastoris* has previously been used to express high levels of functionally active DBL recombinant proteins from PfEBA-175 for x-ray crystallography (38) and in a form that was highly immunogenic in all animals tested and produced parasite growth inhibitory antibodies (46). We were able to produce 4 of the 6 VAR2CSA domains, although DBL3 production required small-scale fermentation in combination with a modified *P. pastoris* host (40), due to its poor expression. By this way, sufficient materials were produced to test by immunization.

Our analysis showed that DBL recombinant proteins contain cross-reactive epitopes, but only a subset of these are exposed in the native protein. This has implications for VAR2CSA vaccine development because “non-native” epitopes are likely to be boosted when several domain variants are combined as components in a vaccine but will not lead to productive immune responses. Therefore, it may be necessary to design vaccination approaches that dampen antibody responses to non-native epitopes. In contrast, we were unable to target conserved epitopes in the native protein using the vaccination schemes tested here. However, our results demonstrate the existence of common polymorphic epitopes in geographically diverse CSA-binding parasite lines, and suggest that this “patch-work” epitope relationship contributes to antigenic cross-reactivity between different CSA-binding lines. Thus, one mechanism used by

the parasite to evade immunity may be to create combinatorial diversity at flexible loops in the DBL domains. Remarkably, most VAR2CSA polymorphic loops appear to consist of two or three basic sequence types and minor point mutation variations on these (8). The basic loop types are globally conserved across isolates (8,39), which may contribute to the broad antibody reactivity of maternal antibodies (4,18,24,30) that could theoretically develop after exposure to diverse VAR2CSA alleles during pregnancy. Whether functional binding sites are under greater restriction for antigenic diversification is unknown, although natural adhesion blocking antibodies appear to target variable epitopes (5,6,41). Although the specificity of natural antibodies may differ from vaccine-elicited antibodies, this concept of shared polymorphism may have broader implications for understanding how pregnant women and children develop anti-disease immunity after relatively few clinical episodes (23).

In terms of vaccine development, these findings reinforce the candidature of VAR2CSA as a potential PAM vaccine target and demonstrate that recombinant proteins produced in the commercially scalable *P. pastoris* system are able to elicit antibodies to the native protein. Using knowledge about VAR2CSA sequence diversity and overlap (8), it may be possible to design VAR2CSA vaccine mixtures that promote broader antibody reactivity or target polymorphism surrounding the CSA interaction site.

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Figure legends

FIG. 1. Production of FCR3-VAR2CSA recombinant proteins using *P. pastoris*. A, Schematic diagram of FCR3-VAR2CSA domain organization. Potential CSA-binding domains are colored black (19). DBL amino acid boundaries are labeled and position of the mutated N-glycosylation sites are indicated by dots (black for glutamine replacement and grey for conservative amino acid replacements). At the bottom, five μg of rDBL1, rDBL4 and rDBL6 and 3 μg of rDBL3 were loaded on a 4-20% gradient Tris-HCl PAGE gel under non-reduced and reduced conditions and stained with Coomassie blue stain (B) or detected using anti-His Tag antibodies (C). Molecular weight standards are shown alongside. D, ELISA assay showing gender-specific recognition of *P. pastoris* rDBL proteins by human IgG. Three groups of human plasma were tested; 30 *P. falciparum* exposed women (W), 12 *P. falciparum* exposed men (M), 5 non-immune adults (Non Im). Median (central bar), central 50% (boxes), central 90% (whiskers), and outlier (circles) levels of human plasma IgG reactivity with recombinant proteins. Statistics were done by Kruskal-Wallis test. Each of the four groups had an overall significant p value, pairwise comparisons within each group achieving significance are shown (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

FIG. 2. Transcriptional analysis of *var* genes in CSA-binding parasite lines. *var* gene transcription was compared from FCR3-CSA and A4ultra parasite lines using the full set of 56 IT4 *var* primers. The FCR3-CSA parasite line predominantly transcribes *var2csa*. The A4ultra parasite line predominantly transcribes *A4var* and expresses *AFBR6 var* at lower levels. Gene expression is compared to the house-keeping gene *Pf* adenylysuccinate lyase. In the insets, *var2csa* transcription was separately analyzed using two primers specific to the

VAR2CSA DBL4 domain (DBL4e var2csa and 79a PFL0030c) or with allele-specific primers to HB3 *var2CSA* alleles A and B.

FIG. 3. Reactivity of VAR2CSA anti-sera with FCR3-CSA and A4ultra infected

erythrocytes. Sera from DNA immunizations (1 rabbit) or protein immunizations (2 rabbits) were tested against homologous (FCR3-CSA) or heterologous (A4ultra) parasite isolates by flow cytometry. The results show the mean and standard deviation of the normalized Median Fluorescence Intensity (MFI) (black bar) and percentage of infected erythrocytes reactive (%IE) (grey bar) from 4 distinct experiments for FCR3-CSA and in triplicate for A4ultra. The histogram provides an example of how the normalized MFI and % IEs reactive were calculated for serum raised against pDNA-A4-CIDR1 reacting with A4ultra. % IE indicates the shift or percentage of infected erythrocytes that label with immune sera above the level of preimmune sera. Normalized MFI is the difference in surface fluorescence intensity between preimmune and immune sera. Pooled sera from multigravid Kenyan women or males were used as controls for gender-specific recognition of CSA-binding parasite lines. Immune sera reactivities below the pDNA VR1051 vector alone were considered negative.

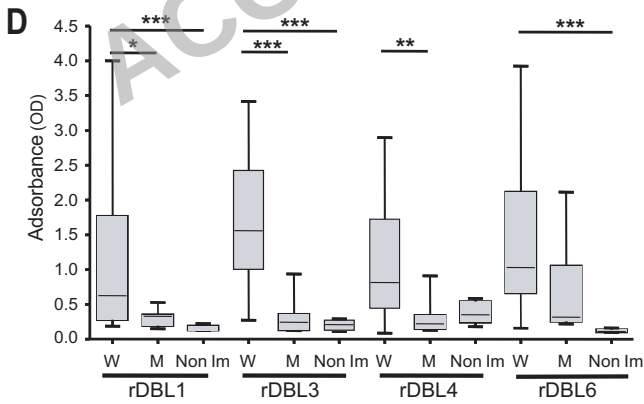
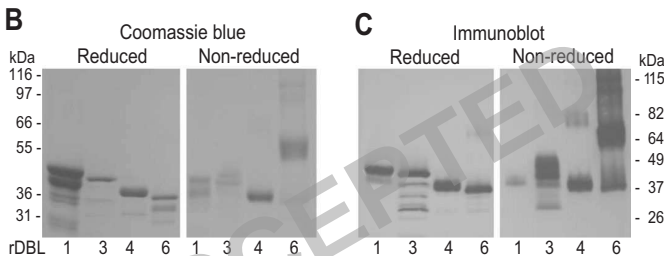
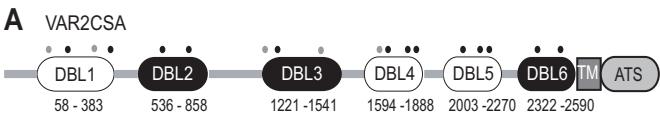
FIG. 4. Cross-reactivity of VAR2CSA anti-sera with geographically diverse CSA-

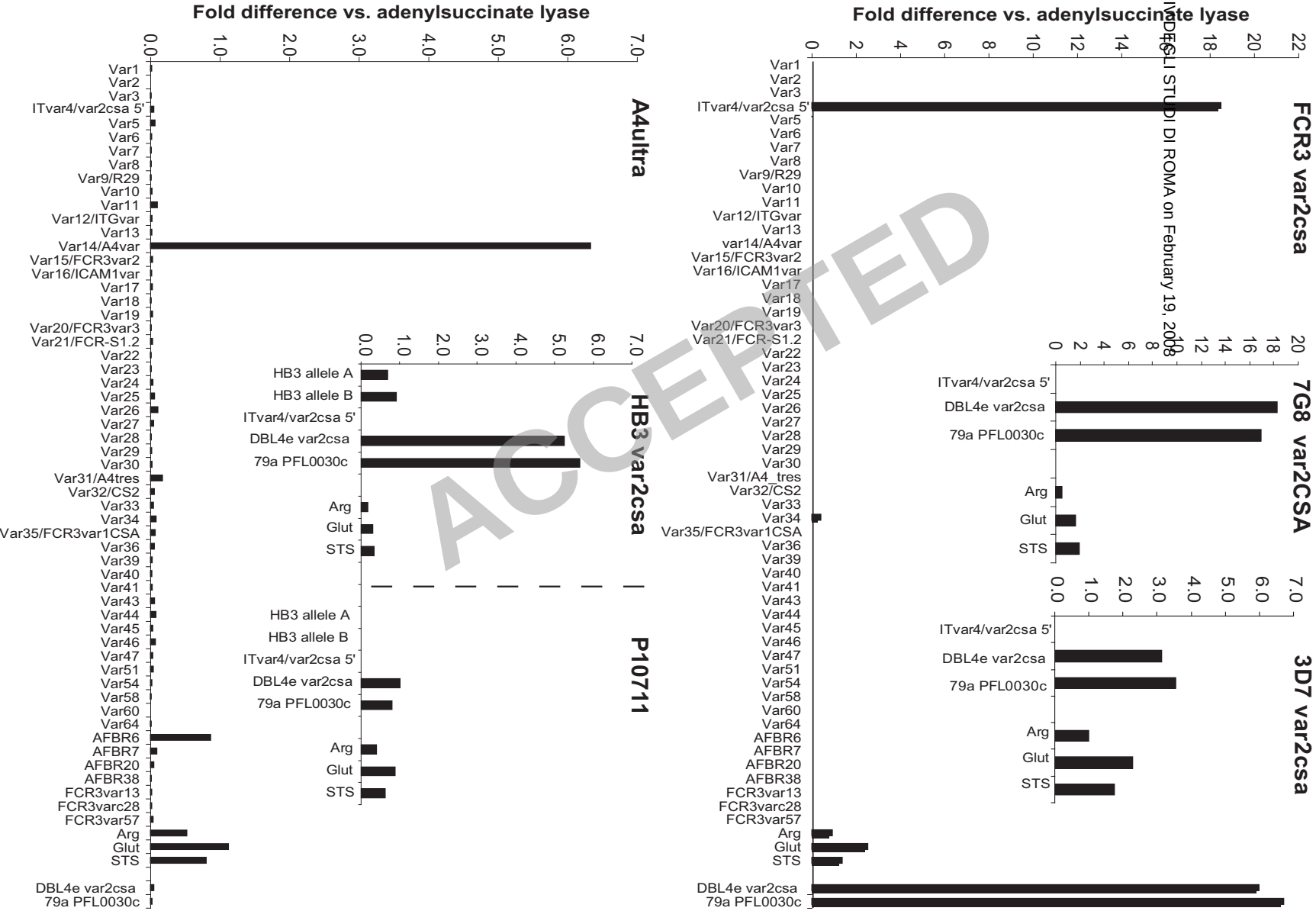
binding isolates. Antisera to the FCR3 VAR2CSA recombinant proteins were tested against four other different CSA-binding lines (3D7, 7G8, HB3 and P10711 clinical) from different continental regions. Sera were screened from all the plasmid DNA immunizations and from one of the two rabbits that were most reactive to FCR3CSA after protein immunization. Data were analyzed at least in duplicate as described in Figure 3. Pooled sera from multigravid Kenyan women or males were used as controls for gender-specific recognition of CSA-

binding parasite lines. Immune sera reactivities below the pDNA VR1051 vector alone were considered negative.

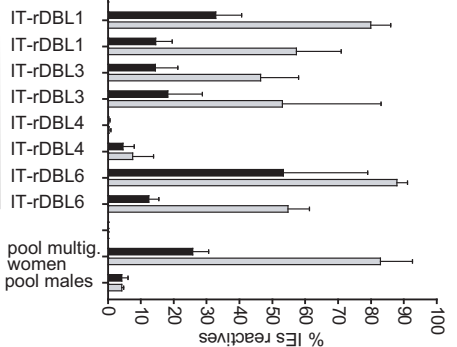
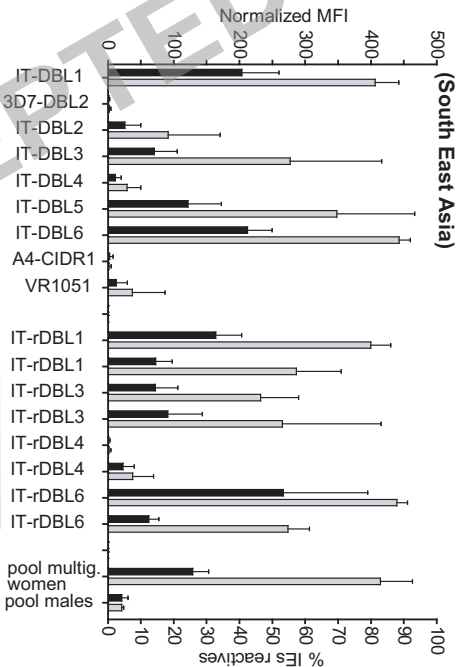
FIG. 5. Amino acid conservation and diversity between VAR2CSA DBL3 domain.

The DBL3 sequences from the four CSA-selected parasites lines were aligned. The top shows the predicted DBL3 structure modeled on EBA-175 (38), and the location of five predicted polymorphic loops in color. As determined previously by molecular modeling and Segmentation analysis (8), the sequence polymorphism at each loop is restricted and globally overlapping (colored sequence segments at the right). At the bottom, polymorphic residues that are common between IT4 and other VAR2CSA alleles are indicated with white font and black outline. The location of semi-conserved B, D, F and H blocks are indicated. The DBL structure and polymorphic segments for all but the 7G8 sequence are reprinted by permission from (8).

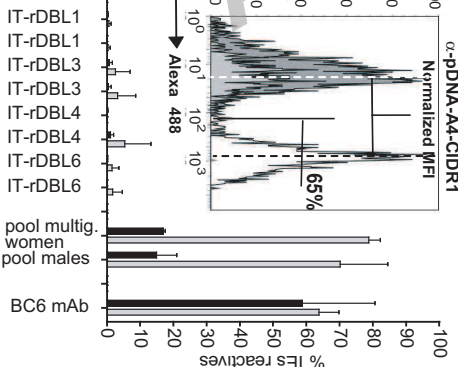
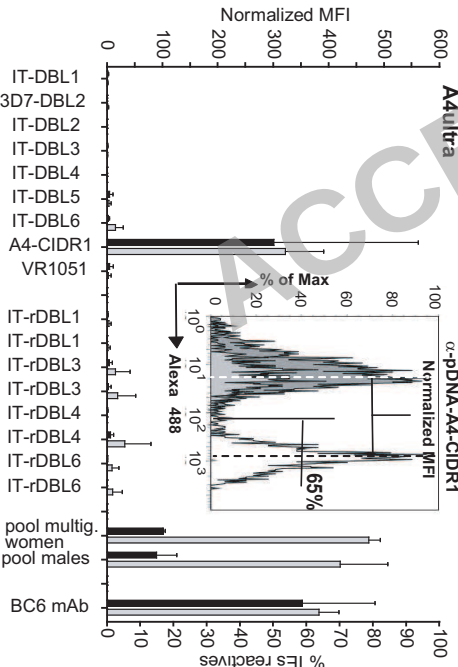




FCR3cscs (South East Asia)

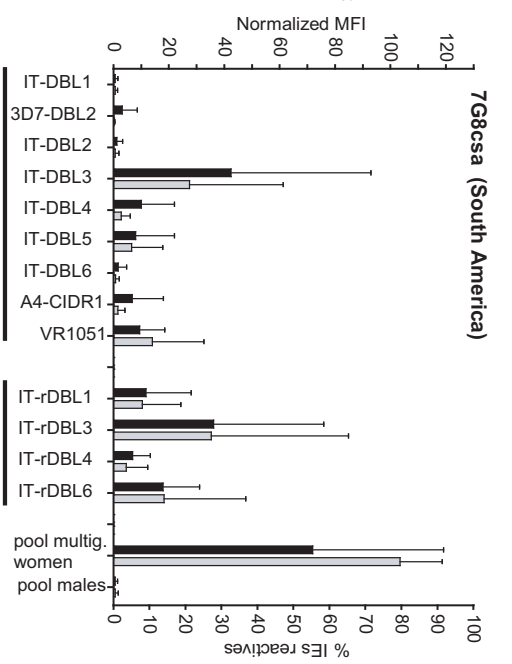
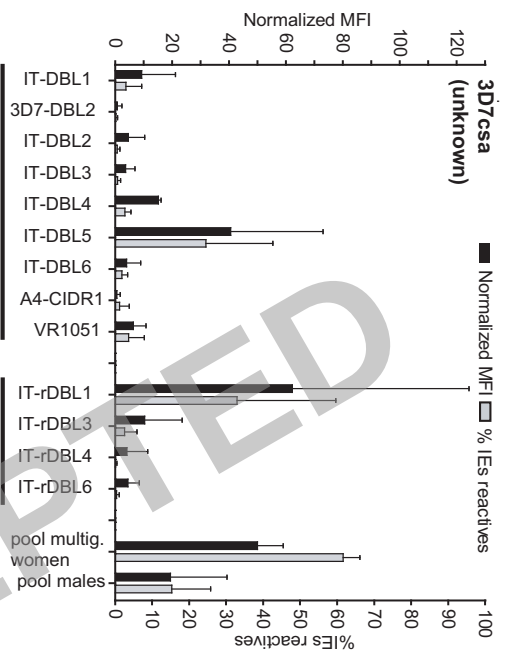


A4ultra



PDNA immunization

Pichia protein immunization



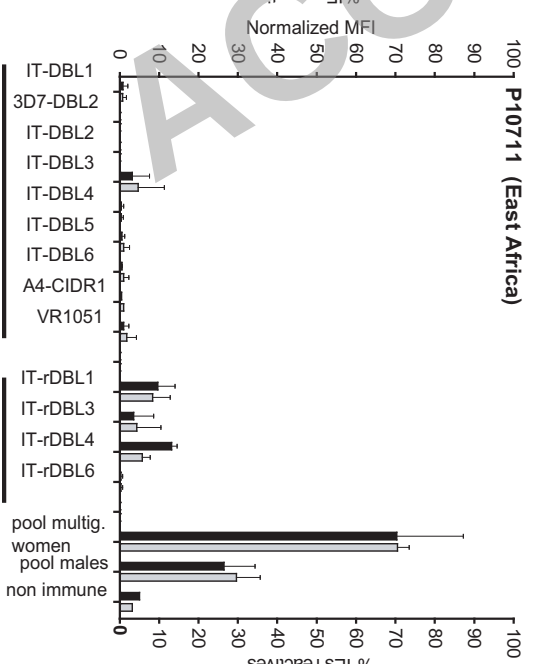
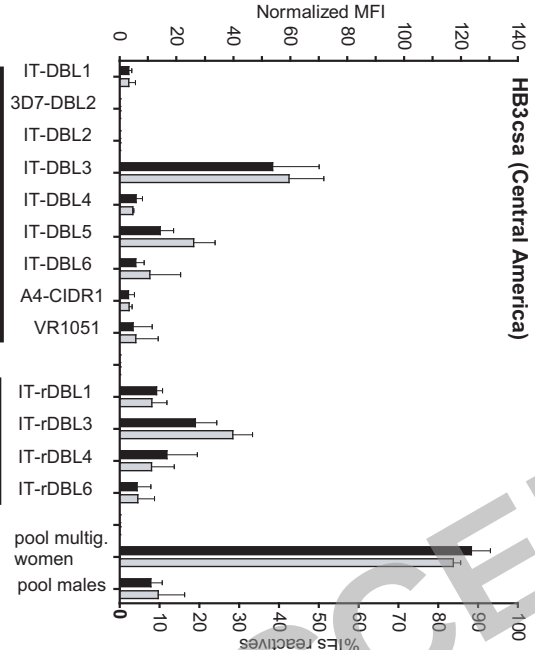
HB3cscs (Central America)

P10711 (East Africa)

P10711 (East Africa)

P10711 (East Africa)

P10711 (East Africa)

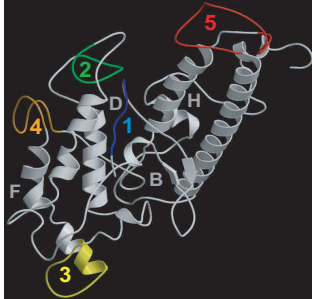


pDNA immunization

Pichia protein immunization

pDNA immunization

Pichia protein immunization



DBL3 VAR2CSA

IT	SKTYDGKIF	KN.....DKKG.QKKG....NDP	KGTPQKDKIGGVGS	CAITKINK.KNNNSIFNGD	TINGKNEKKCINSKSGQGDKIQGA
3D7	PKIYDGKIF	RNEM.....KG.QKEK.EEKNNS	KGTTKQNGKT..VGS	CAITKINKKQKKNGTFSID	TNNG.....QGDKIQGD
7G8	SKTYDGKIF	RNPMKEGGEDGKGQKEGGEAANNNS	KGTPQKDKIGGVGS	CAITKIKK.QKKNGTFNNG	TINGKNEKKCINSKSGQGDKIQGA
HB3-B	PKRYDGFIS	RNPMKEGGEDGKGQKEGGEAANNNS	QETTKTKDKIGG..S	CAITKIKK.QKKNGTYTGN	TINGKNEKKCINSKSGQEDKVEGA
HB3-A	PKRYDGFIS	RNPMKEGGEDGKGQKEGGEAANNNS	KGTPQKDKIGGVGS	CGIKTINKKQKKNGTYTGN	TINGKNEKKCINSKSGQEDKVEGA



IT	LNATNYIRGCQSKTYDGKIFPGKGGEKQWICKDTTHGDTNGACIPPRTONLCVGLWDKSYGGRSNIKNDTKELLKEKIKNAIHKETELLYEYHDTGTAIISKN.....DKKG.QKKG....NDP
3D7	CSEPIYIRGCQPKIYDGKIFPGKGGEKQWICKDTTHGDTNGACIPPRTONLCVGLWDKRYGGRSNIKNDTKESLKQKIKNAIQKETELLYEYHDKGTAIISRNPM.....KG.QKEK.EEKNNS
7G8	LNATNYIRGCQSKTYDGKIFPGKGGEKQWICKDTTHGDTNGACIPPRTONLCVGLWDKSYGGRSNIKNDTKESLKKNLKNAIQKETELLYEYHDKGTAIISRNPMKEGGEDGKGQKEGGEAANNNS
HB3B	LNATNYIRGCQPKRYDGFISPGKGGEKQWICKDTTHGDTNGACIPPRTONLCVGLWDKRYGGRSNIKNDTKESLKKNLKNAIQKETELLYEYHDKGTAIISRNPMKEGGEDGKGQKEGGEAANNNS
HB3A	CNEPIYIRGCQPKRYDGFISPGKGGEKQWICKDTTHGDTNGACIPPRTONLCVGLWDKRYGGRSNIKNDTKESLKKNLKNAIQKETELLYEYHDKGTAIISRNPMKEGGEDGKGQKEGGEAANNNS

IT	NGLPKGFCHAVQVSFIDYKNMILGTSVNIYEHIGKLQEDIKKIEKGTPOQKDKIGGVGSSTENVNAWVKGIEREMWDVRCVCAITKINK.KNNNSIFNGDECCVSPPTGNDEDQSVSWFKWGEQFCIE
3D7	NGLPKGFCHAVQVSFIDYKNMILGTSVNIYEHIGKLQEDIKKIEKGTPTKQNGKT..VSGAENVNAWVKGIEGEMWDVRCVCAITKINKKQKKNGTFSIDECGIFPPTGNDEDQSVSWFKWGEQFCIE
7G8	NGLPKGFCHAVQVSFIDYKNMILGTSVNIYEHIGKLQEDIKKIEKGTPOQKDKIGGVGSSTENVNAWVKGIEGEMWDVRCVCAITKIKK.QKKNGTENGDECCVSPPTGNDEDQSVSWFKWGEQFCIE
HB3B	NGLPKGFCHAVQVSFIDYKNMILGTSVNIYEHIGKLQEDIKKIEQERTTKDKIGG..SGADKVDNWWVKGIEGEMWDVRCVCAITKIKK.QKKNGTYTGNCECVSPPTGNDEDQSVSWFKWGEQFCIE
HB3A	NGLPKGFCHAVQVSFIDYKNMILGTSVNIYEHIGKLQEDIKKIEKGTPOQKDKIGGVGSADKVDNWWVKGIEGEMWDVRCVCAITKINKKQKKNGTYTGNCECVSPPTGNDEDQSVSWFKWGEQFCIE

IT	RLRYEONIREACTINGKNEKKCINSKSGQGDKIQGAACKRKCE
3D7	RLQYEKNIRDACTNNG.....QGDKIQGDCKRKCE
7G8	RLRYEONIREACTINGKNEKKCINSKSGQGDKIQGAACKRKCE
HB3B	RLRYEONIREACTINGKNEKKCINSKSGQEDKVEGAACKRKCE
HB3A	RLRYEONIREACTINGKNEKKCINSKSGQEDKVEGAACKRKCE

TABLE 1. Immunization protocol

Experimental groups	number of rabbit	number of vaccinations	vaccination doses , μ g	
			Prime	boost
Protein	IT-rDBL1	2	6	500 + CFA 250 + IFA
	IT-rDBL3	2	6	100 + CFA 50 + IFA
	IT-rDBL4	2	6	500 + CFA 250 + IFA
	IT-rDBL6	2	6	500 + CFA 250 + IFA
pDNA	IT-rDBL1	1	6	
	IT-rDBL2	1	10	
	3D7-rDBL2	1	10	
	IT-rDBL3	1	7	
	IT-rDBL4	1	6	intramuscularly DNA:Vaxfectin 2:1 with 500 μ g of DNA
	IT-rDBL5	1	6	
	IT-rDBL6	1	6	
	A4-CIDR1	1	7	
	VR1051 ^a	1	7	

CFA : complete Freund's adjuvant ; IFA : incomplete Freund's adjuvant.

^a VR1051 is a plasmid vector alone control

TABLE 2. Reactivity of transfected CHO cells with rabbit immune sera by FACS analyses.

		Median Fluorescence Intensity ^a (% CHO cells reactive ^b)										
		IT-DBL1	IT-DBL2	IT-DBL3	3D7-DBL1	3D7-DBL2	3D7-DBL3	3D7-DBL4	3D7-DBL5	3D7-DBL6	CHO-745	3D7-DBL2 β
rDBL protein immunization	IT-rDBL1	1140 (81)	45 (23)	+/-	836 (88)	+/-	-	+/-	+/-	+	+/-	-
	IT-rDBL1	1184 (86)	+	-	461 (69)	-	+/-	+/-	-	+/-	+/-	+/-
	IT-rDBL3	584 (59.5)	+/-	295 (94)	387 (61)	-	137 (29)	-	-	+/-	-	-
	IT-rDBL3	+/-	+/-	1411 (79)	+	+/-	244 (44)	+/-	-	+/-	+/-	+/-
	IT-rDBL4	-	-	-	57 (73)	-	-	104 (22)	-	177 (41)	+/-	107 (60)
	IT-rDBL4	+/-	-	+/-	+/-	149 (40)	+/-	116 (62)	+/-	-	+/-	50 (35)
	IT-rDBL6	58 (33)	-	-	42 (62)	-	-	+/-	+/-	364 (74)	-	43 (64)
	IT-rDBL6	+/-	-	61 (43)	+/-	-	+/-	+/-	+/-	314 (63)	+/-	+
pDNA immunization	IT-rDBL1	831 (71)	-	-	637 (80)	-	-	-	-	+/-	-	-
	3D7-rDBL2	+	683 (87)	+/-	+	501 (90)	+/-	+/-	+/-	+/-	-	+/-
	IT-rDBL2	-	982 (92)	-	+	478 (59)	-	+/-	-	+/-	+/-	+/-
	IT-rDBL3	+/-	-	91 (46)	+	-	85 (65)	-	-	-	-	-
	IT-rDBL4	+/-	-	-	-	-	-	185 (50)	-	-	-	-
	IT-rDBL5	-	46 (37)	+/-	-	+	-	+/-	747 (97)	-	+/-	+
	IT-rDBL6	-	-	-	-	-	-	-	+/-	1076 (86)	-	-
	A4-CIDR1	-	+/-	+/-	+/-	+/-	+/-	+/-	-	-	+/-	+
anti-Tag	VR1051	+/-	-	-	-	-	-	-	-	-	-	+/-
	mAb 179	122 (86)	261 (88)	167 (82)	301 (87)	188 (87)	157 (91)	179 (89)	232 (96)	90 (73)	N/A	313 (75)
	12CA5	414 (91)	366 (97)	399 (96)	309 (87)	355 (90)	148 (91)	355 (98)	374 (97)	119 (66)	N/A	189 (90)

^a normalized Median Fluorescence Intensity (MFI) = MFI obtained with the Immune serum - MFI obtained with the Preimmune serum

^b Preimmune sera were used to set a gate to determine the percentage of cells reactives with immune sera.

-, no reactivity ; +/-, % CHO cells reactive < 10% ; +, 10% < % CHO cells reactive < 20% ; bold, reactivity with homologous and heterologous domain

TABLE 3. IEs-FCR3csa surface reactivity with the anti-sera after protease treatment.

		after protease treatment.				
		Median Fluorescence Intensity ^a (% IEs-FCR3csa reactive ^b)				
		untreated	Trypsin 0.1mg/ml 15min	Trypsin 1mg/ml 15min	Trypsin 1mg/ml 30min	Chymotrypsin 1mg/ml 30min
rDBL protein immunization	IT-rDBL1	144 (86)	73 (71)	60 (51)	27 (23)	4 (2.5)
	IT-rDBL1	94 (72.5)	111 (77)	96 (63)	18 (14)	5 (3)
	IT-rDBL3	108 (71)	20 (17)	24 (16)	8 (13)	48 (51)
	IT-rDBL3	73 (64.5)	-	14 (9)	-	21 (39)
	IT-rDBL4	-	9 (9)	55 (31)	56 (25)	70 (45)
	IT-rDBL4	-	20 (9)	48 (20)	48 (12)	47 (34)
	IT-rDBL6	188 (88.5)	155 (88)	132 (83)	264 (86)	103 (76)
	IT-rDBL6	51 (45)	67 (56)	101 (76)	237 (87)	71 (57)
pDNA immunization	IT-rDBL1	295 (88.5)	103 (78)	100 (60)	74 (38)	11 (10)
	3D7-rDBL2	-	-	-	-	-
	IT-rDBL2	-	2 (6)	-	-	-
	IT-rDBL3	38 (41)	16 (21)	16 (8)	11 (2.5)	21 (21)
	IT-rDBL4	11 (7.5)	14 (29)	135 (82)	250 (89)	208 (89.5)
	IT-rDBL5	113 (81.5)	204 (89)	136 (77)	147 (69)	93 (65)
	IT-rDBL6	171 (87)	92 (75)	115 (76)	173 (85)	79 (73.5)
	A4-CIDR1	-	2 (2.5)	4 (9)	-	-
	VR1051	-	-	-	-	-

^a normalized Median Fluorescence Intensity (MFI) = MFI obtained with the Immune serum - MFI obtained with the Preimmune.

^b Preimmune sera were used to set a gate to determine the percentage of Infected Erythrocytes reactives with immune sera.

- none or less than 1% of IEs-FCR3csa were reactive.