1 **A single full-length VAR2CSA ectodomain variant purifies broadly**
2 **neutralizing antibodies against placental malaria isolates**

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Abstract

Placental malaria (PM) is a deadly syndrome most frequent and severe in first pregnancies. PM results from accumulation of *Plasmodium falciparum* (Pf)-infected erythrocytes (IE) that express the surface antigen VAR2CSA and bind to chondroitin sulfate A (CSA) in the placenta. Women become PM-resistant over successive pregnancies as they develop anti-adhesion and anti-VAR2CSA antibodies, supporting VAR2CSA as the leading PM-vaccine candidate. However, the first VAR2CSA subunit vaccines failed to induce broadly neutralizing antibody and it is still unclear whether naturally acquired protective antibodies target variant or conserved epitopes. This is crucial to determine whether effective vaccines will require incorporation of many or only a single VAR2CSA allele.

Here, IgG from multigravidae was sequentially purified on five full-length VAR2CSA ectodomain variants, thereby depleting IgG reactivity to each. The five VAR2CSA variants purified ~0.7% of total IgG and yielded both strain-transcending and strain-specific reactivity to VAR2CSA and IE-surface antigen. IgG purified on the first VAR2CSA antigen displayed broad cross-reactivity to both recombinant and native VAR2CSA variants, and inhibited binding of all isolates to CSA. IgG remaining after depletion on all variants showed significantly reduced binding-inhibition activity compared to initial total IgG.

These findings demonstrate that a single VAR2CSA ectodomain variant displays neutralizing epitopes shared by multiple parasites, including maternal isolates, and suggest that a broadly effective PM-vaccine can be achieved with a limited number of VAR2CSA variants.

Key words: Pregnancy, malaria, vaccine, VAR2CSA, variant, blocking antibody

Introduction

Plasmodium falciparum (Pf) infection in pregnant women causes placental malaria (PM) when Pf-infected erythrocytes (IE) accumulate in the intervillous spaces of the placenta. PM has been linked to several adverse pregnancy outcomes (Steketee et al., 2001; Guyatt and Snow, 2001; Desai et al., 2007; Moore et al., 2017), and first-time mothers are most vulnerable (Brabin, 1983). Over successive pregnancies, PM and the related sequelae become less prevalent (Guyatt and Snow, 2001). Susceptibility to PM has been attributed to Pf parasites that bind chondroitin sulphate-A (CSA) expressed by the placental syncytiotrophoblast (Fried and Duffy, 1996), and express the variant surface antigen VAR2CSA (Salanti et al., 2003; Tuikue Ndam et al., 2005). Conversely, the decrease in PM-related poor pregnancy outcomes with increasing parity is associated with the acquisition of functional antibodies to CSA-binding IE and antibodies to VAR2CSA (Fried and Duffy, 1998; Ricke et al., 2000; Salanti et al., 2004; Ndam et al., 2015). Such functional antibodies have been characterized for two major functions: 1) blocking CSA-binding of VAR2CSA-expressing parasites; and 2) opsonizing IE to promote phagocytosis (Fried and Duffy, 1998; Ricke et al., 2000; Duffy and Fried, 2003; Keen et al., 2007; Ataíde et al., 2011).

Hence, VAR2CSA represents the leading candidate for PM vaccine development. VAR2CSA is a large (~318-478 kDa) multidomain transmembrane protein, a member of the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) family encoded by *var* genes. The cysteine-rich ectodomain is formed by N-terminal sequence (NTS), six and sometimes more Duffy-binding-like (DBL) domains as well as interdomain (ID) regions (Kraemer and Smith, 2006; Doritchamou et al., 2019). Recent studies showed that VAR2CSA ectodomain structure includes a stable core (NTS-DBL1X-ID1-DBL2X-ID2-DBL3X-DBL4e-ID3) flanked by a flexible arm (DBL5e-DBL6e), and the receptor interaction involves CSA threading through two channels that formed within the stable core (Ma et al., 2021). Multiple individual DBL domains of VAR2CSA interact with CSA *in vitro* (Dahlbäck et al., 2011; Clausen et al., 2012; Ma et al., 2021) and induce functional antibodies in animals (Magistrado et al., 2011; Bigey et al., 2011; Obiakor et al., 2013; Fried et al., 2013; Doritchamou et al., 2013; Clausen et al., 2012; Srivastava et al., 2011; Nielsen and Salanti, 2015; Chêne et al., 2018). Two subunit vaccine candidates (called PAMVAC and PRIMVAC) from the N-terminal region of VAR2CSA were recently tested in phase 1 trials (Mordmüller et al., 2019; Sirima et al., 2020). Both trial teams reported that VAR2CSA subunit vaccines were safe, immunogenic and induced functional anti-adhesion antibodies in malaria-naïve and malaria-exposed women (Mordmüller et al., 2019; Sirima et al., 2020). However, functional activity was primarily against homologous parasites (same VAR2CSA sequence as vaccine) and low or absent against heterologous parasites (Sirima et al., 2020), suggesting the vaccine antigen failed to display functional epitopes shared across multiple *P. falciparum* variants.

Similarly, VAR2CSA fragments (single or double domains) fail to purify the broadly neutralizing activity of sera from PM-resistant multigravidae (Doritchamou et al., 2016). Although VAR2CSA fragments (including constructs similar to PAMVAC vaccine) purified antibodies with CSA-binding inhibitory activity against homologous parasites, broadly neutralizing activity was neither purified nor depleted after passing sera over several VAR2CSA domains and variants (Doritchamou et al., 2016). These data supports the hypothesis that functional antibodies to VAR2CSA may target

conformational epitopes not displayed by any individual domain: for example, the strain-transcending human monoclonal antibody PAM1.4 is specific for an unknown conformational epitope on full-length VAR2CSA (Barfod et al., 2007).

Naturally acquired antibodies of multiparous women exhibit broadly neutralizing anti-adhesion activity (Fried and Duffy, 1998; Ricke et al., 2000; Ndam et al., 2015). However, it is unknown whether broadly neutralizing antibodies target conserved epitopes on VAR2CSA or rather result from the cumulative repertoire of antibodies against variant specific epitopes. In this study, we investigate the hypothesis that full-length VAR2CSA ectodomain may capture naturally acquired antibodies from multigravidae plasma with greater breadth of neutralizing activity than previously achieved by multiple VAR2CSA domains. We show that a single ectodomain can purify strain-transcending IgG as well as the bulk of functional activity naturally acquired by multigravid women. In parallel, the depletion of IgG reactivities to five variants of full-length VAR2CSA resulted in significantly diminished strain-transcending anti-adhesion activity. These data suggest that full-length VAR2CSA ectodomain displays functional epitopes that are absent in VAR2CSA fragments and provide a basis to design improved vaccines.

Results

Depletion of IgG on five VAR2CSA ectodomains significantly reduces broad neutralizing activity

IgG specific to full-length VAR2CSA ectodomains was purified from a plasma pool prepared using samples from multigravid women participating in the previously described Immuno-epidemiology (IMEP) study (Fried et al., 2018) (see **Methods** and **Figure 1** for experimental design).

Figure 1: Flow chart of the experimental design

The affinity purification of IgG specific to five VAR2CSA ectodomain variants yielded a combined ~386.1 μ g of VAR2CSA-specific IgG, representing about 0.7% of the original total IgG (**Table 1**). The highest yields of purified IgG were obtained on the first two variants (NF54 and 7G8) totaling 76% of the IgG purified on all five variants.

Table 1: Purification yields of purified IgG

Order of depletion	IgG	Yield
-	Total IgG	58000 μ g
1	Fv2-NF54 IgG	145.6 μ g
2	Fv2-7G8 IgG	148.9 μ g
3	Fv2-FCR3 IgG	53.4 μ g
4	Fv2-HB3 IgG	14.5 μ g
5	Fv2-Camp IgG	23.7 μ g

Variable levels of IgG reactivity to seven recombinant VAR2CSA ectodomains was measured in the pre-depletion total IgG. As expected, IgG reactivity to NF54, 7G8, FCR3, HB3 and MC variants were successfully depleted from the total IgG pool, as demonstrated by the absence of reactivity in each post-depletion sample (**Figure 2A and Supplemental Figure S1A**). Of note, substantially reduced reactivity to M920 and M200101 (not used in the affinity purification assay) was seen in the post-depletion total IgG, indicating that cross-reactive IgG to these two antigens was also depleted.

Figure 2: Depletion of VAR2CSA-specific IgG and activity of Total IgG purified from Malian multigravidae

Similarly, reactivity to native VAR2CSA measured on the IE surface by flow cytometry was absent in the post-depletion IgG when tested on NF54, FCR3 and MC isolates (**Figure 2B and Supplemental Figure S1B**). Further, reactivity of post-depletion total IgG to native VAR2CSA of heterologous maternal isolates (M2022170, M2001190 and M0736) was substantially reduced versus the pre-depletion total IgG.

Importantly, depletion of IgG on the five VAR2CSA variants also reduced CSA-binding inhibition activity in the total IgG, being abolished against some isolates and significantly reduced against others (**Figure 2C**). The inhibition activity of the original total IgG sample was >75% against all parasite lines including recently adapted maternal isolates, except for FCR3 whose binding was inhibited 33% (**Figure 2C**). After the depletion of VAR2CSA-specific IgG, the inhibition activity was substantially reduced, with the % reduction (measured as ((pre-depletion % inhibition-post-depletion % inhibition)/(pre-depletion % inhibition) x 100) ranging from 45% against M2022170 to 87% against NF54 (**Figure 2D**). CSA-binding inhibition activity was substantially reduced against the three maternal isolates (including M2022170), implying that a significant fraction of cross-inhibitory IgG to the maternal isolates were also depleted on the heterologous VAR2CSA antigens.

Highly cross-reactive IgG is purified on the first full-length VAR2CSA antigen

VAR2CSA-purified IgG exhibited cross-reactivity in ELISA to different variants including M920 and M200101 (**Figure 3A**), although the degree of this dropped substantially after the first purification. As expected, reactivity was largely absent when IgG was tested against VAR2CSA variants previously used in the series of purifications/depletions. Thus, NF54-purified IgG (NF54-IgG) reacted to homologous and heterologous VAR2CSA antigens at the highest level of any IgG, while IgG purified on MC (the last antigen used in the purification/depletion series) showed no cross-reactivity to NF54, 7G8, FCR3 or HB3 variants. Notably, each variant purified specific reactivity to itself, albeit at lower OD values than that seen with the initial NF54 purification, which may indicate the greater abundance and/or greater affinity of cross-reactive versus variant-specific IgG acquired by multigravidae.

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Figure 3: Reactivity of the purified VAR2CSA-specific IgG to different full-length VAR2CSA antigens and *P. falciparum* isolates

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Similar to ELISA, flow-cytometry revealed strain-transcending reactivity to native antigen, though this decreased after the first (NF54) and again after the second (7G8) affinity columns. NF54-IgG showed the greatest breadth of reactivity (defined as the number of isolates recognized) with detectable reactivity to IE surface of all parasites including the maternal isolates (known to be polyclonal parasite samples). NF54-IgG also displayed the highest level of reactivity to all the isolates (**Figure 3B**). Compared to NF54-IgG, IgG subsequently purified on FCR3 and MC ectodomains demonstrated lower surface reactivity to the homologous parasite and lower cross-reactivity to the maternal isolates. Although the 7G8-IgG and HB3-IgG were not tested against their homologous parasite line, these antigen-purified IgG displayed some cross-reactivity to different heterologous parasites that was lower compared to NF54-IgG. As with ELISA, reactivity by flow cytometry was lost against parasite isolates (ie, NF54 and 7G8) after their corresponding recombinant ectodomain had been used in the series of depletion/purifications, attesting to the complete depletion of homologous IE-surface reactivity by each homologous antigen.

A single full-length VAR2CSA variant purifies the bulk of naturally acquired binding-inhibitory IgG

Strong cross-inhibitory activity of IgG purified on VAR2CSA was observed in assays that measure inhibition of parasite binding to CSA (**Figure 4 and Supplemental Figure S2**). IgG binding-inhibition activity was normalized (nBIA) as a percentage of the pre-depletion total IgG, with the assumption that maximum inhibition of each parasite line is measured in IgG before depletion. NF54-IgG contained the largest fraction of the inhibitory activity, with mean nBIA values >50% against all isolates and hovering around 100% for most (**Figure 4A**). This included three recently adapted heterologous maternal isolates, which showed both the lowest (M2022170) and highest (M2001190) nBIA levels respectively at 56% and 111% inhibition. Interestingly, titration of the inhibitory capacity of NF54-IgG against the homologous parasite indicated that blocking is achieved at IgG concentration as low as 10µg/mL (**Supplemental Figure S3**). 7G8-IgG showed lower levels of nBIA than NF54-IgG against all heterologous isolates, except against the maternal isolate M2022170 where 7G8-IgG showed similar levels of nBIA to NF54-IgG; as expected 7G8-IgG (and subsequent variant-purified IgG) showed essentially no activity against NF54 parasites (**Figure 4B-E**). Subsequent purifications of IgG on ectodomain variants continued to yield some level of homologous activity, as seen with FCR3 and MC (**Figure 4C, E**). Taken together, the IgG purified on VAR2CSA variants clearly demonstrated homologous inhibition activity while displaying high levels of cross-inhibition activity with initial purifications that generally waned with successive purifications. Despite significant heterologous IE surface reactivities captured on the NF54 antigen, subsequent IgG purifications on other variants captured blocking activity against homologous parasites.

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Figure 4: CSA-binding inhibitory activity of VAR2CSA-specific purified IgG from multigravidae

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Discussion

VAR2CSA is the leading vaccine candidate to protect malaria-exposed pregnant women against PM and related adverse outcomes. Two vaccines based on VAR2CSA fragments have been recently tested in clinical trials (Mordmuller et al., 2019, Sirima et al., 2020), showing good safety and immunogenicity but a general inability to induce broadly neutralizing antibodies, such as those described in sera of PM-resistant multigravid women (Fried and Duffy, 1998; Ricke et al., 2000; Duffy and Fried, 2003; Ndam et al., 2015). The limitations of VAR2CSA domain-based subunit vaccines have also been established from the studies of naturally acquired antibodies in PM-resistant multigravidae, where single domains or domain combinations from different variants failed to deplete heterologous binding-inhibition activity (Doritchamou et al., 2016). Among the probable explanations, the restricted array of protective epitopes displayed by VAR2CSA subunit vaccines, as well as the contribution of antibodies targeting other variants or antigens (including non-VAR2CSA proteins), are of major interest. It is therefore likely that larger fragments of VAR2CSA would offer a broader spectrum of functional epitopes including conformational epitopes and those present in other fragments missing from the subunits tested. Supporting this hypothesis, the present data demonstrate that strain-transcending naturally acquired anti-adhesion antibodies can be purified on a single recombinant VAR2CSA ectodomain, providing additional evidence that define VAR2CSA as the major target of functional antibodies.

This conclusion is well-supported by previous studies showing that naturally acquired anti-adhesion antibodies in PM target VAR2CSA (Bigey et al., 2011; Doritchamou et al., 2016), even though other invariant proteins are highly expressed by placental parasites (Francis et al., 2007; Fried et al., 2007; Tuikue Ndam et al., 2008; Bertin et al., 2013). Anti-adhesion activity of antibodies targeting these invariant proteins has not been reported thus far, and whether these proteins play a role in parasite binding to CSA is still unclear. Our data demonstrate that the removal of IgG specific to five variants of VAR2CSA ectodomain significantly reduced/abolished IE-surface reactivity and the CSA-binding inhibition activity of total IgG from multigravidae. This observation suggests that antibodies to surface-expressed non-VAR2CSA proteins might have limited or no blocking activity against CSA-binding parasites, although a synergistic effect of these antibodies with anti-VAR2CSA cannot be excluded.

Naturally acquired antibodies of malaria-exposed multigravid women recognize placenta-binding parasites from diverse geographical origins (Fried and Duffy, 1998; Ricke et al., 2000). One leading hypothesis is that pregnant women acquire antibodies to both cross-reactive and variant-specific epitopes over successive pregnancies and exposure to diverse placenta-binding parasites. We show here that the purification of IgG to VAR2CSA recombinants resulted in loss of IE surface reactivity against the three homologous parasites, and significantly reduced heterologous cross-reactivity of total IgG to maternal isolates. Consequently, the VAR2CSA-purified IgG exhibited strong cross reactivity to all isolates tested in this study, with NF54-IgG (the first purification in the series) demonstrating the highest level of cross-reactivity, and MC-IgG (the last purification) showing modest surface cross-reactivity to the maternal isolates. Overall, it may be difficult to estimate the fraction of variant-specific or cross-reactive antibodies contributed by each variant to the measured activities, knowing that each sequence of VAR2CSA could have multiple epitopes that are variably

shared with some or all variants (Benavente et al., 2018; Otto et al., 2019). However, highly cross-reactive antibodies can be purified from a single recombinant VAR2CSA ectodomain, suggesting that immunization with a single variant may prove highly effective.

The full-length VAR2CSA ectodomain-based vaccine approach has been previously investigated in preclinical studies in which antisera raised in rodent models had strong homologous functional activity (Khunrae et al., 2010). However, these antibodies generated in rodents did not cross-inhibit different CSA-binding isolates, while exhibiting a broadly strain-transcending surface reactivity to these isolates (Avril et al., 2011). In contrast to this observation, our data demonstrate that naturally acquired IgG specific to a single variant and purified from multigravidae sera cross-inhibited multiple isolates. Notably, most of the binding-inhibition activity to CSA-binding parasites was depleted on the first variant (NF54) and the resulting IgG purified on NF54 demonstrated strain-transcending blocking activity to all isolates tested in this study. This finding suggests that one VAR2CSA variant might induce antibody with broadly neutralizing activity, acknowledging the challenges in developing a full-length vaccine (reviewed in (Doritchamou et al., 2021)), including the possibility that non-functional epitopes may interfere with the optimal functional antibody response. It is also possible that the flanking flexible arm composed of DBL5 ϵ -6 ϵ or DBL5 ϵ -7 ϵ (in some cases) may play a role in masking functional epitopes on the core (NTS-ID3) structure of VAR2CSA (Doritchamou et al., 2019; Ma et al., 2021). Future studies that investigate differences in the host immune response to VAR2CSA in human versus other species will also elucidate any discrepancies between the functional activity of human versus rodent antibody.

To conclude, we report that naturally acquired antibody purified on VAR2CSA ectodomains exhibited strong strain-transcending surface reactivity and blocking activity to CSA-binding isolates. Interestingly, IgG purified against a single variant (NF54, used first in the series of depletions/purifications) captured the bulk of strain-transcending IE reactivity and CSA-binding inhibition activity from total IgG of PM-resistant multigravidae. Therefore, the full-length VAR2CSA ectodomain appears to display both variant-specific and shared epitopes targeted by functional antibodies and as such, would be a credible alternative to the VAR2CSA subunit approach in PM vaccine development.

Materials and Methods

Plasma pool, recombinant full-length VAR2CSA and *P. falciparum* cultures

For this study, a plasma pool was prepared using samples from multigravid women participating in the previously described Immuno-epidemiology (IMEP) study (Fried et al., 2018). Briefly, pregnant women aged 15–45 years without clinical evidence of chronic or debilitating illness were enrolled from 2010-2013 into a longitudinal cohort study of mother-infant pairs conducted in Ouélessébougou, Mali. All participants provided signed informed consent after receiving a study explanation form and oral explanation from a study clinician in their native language. The study protocol and study procedures were approved by the institutional review board of the National Institute of Allergy and Infectious Diseases at the US National Institutes of Health (ClinicalTrials.gov ID NCT01168271), and the Ethics Committee of the Faculty of Medicine, Pharmacy and Dentistry at the University of Bamako, Mali.

Recombinant full-length VAR2CSA ectodomains of seven *Pf* parasite lines (NF54, 7G8, FCR3, HB3, Malayan Camp (MC), M920 and M200101) were expressed in a mammalian expression system as in a previous study (Renn et al., JEM 20210848P). Three CSA-binding maternal isolates (M2022170, M2001190 and M0736) expressing VAR2CSA as well as three lab-strains (NF54, FCR3, MC) selected to bind CSA were maintained in culture for the functional assays.

Purification of IgG specific to full-length VAR2CSA ectodomains

Total IgG was initially purified from 4 mL of the multigravidae plasma pool using Protein G Sepharose 4 Fast Flow resin (GE Healthcare Life Sciences) according to the manufacturer's instructions. For VAR2CSA-specific IgG purification and depletion, an antigen-specific affinity column was prepared as previously described (Doritchamou et al., 2016) for each VAR2CSA variant. Briefly, each ectodomain variant was chemically cross-linked to N-hydroxysuccinimide (NHS)-activated Sepharose beads (GE Healthcare Life Sciences) according to the manufacturer's instructions. IgG specific to five VAR2CSA variants (NF54, 7G8, FCR3, HB3 and MC) were sequentially removed from the total IgG by passage through the affinity column and by applying the flow-through sample from one column to the next column until all specificities were depleted (**Figure 1: Flow chart**). IgG purification was performed repeatedly on each VAR2CSA antigen to ensure complete depletion of the specific IgG. The purified IgG were eluted with a lower pH Elution buffer (Invitrogen, Carlsbad, CA), neutralized with 2M Tris buffer pH 9.0, and dialyzed into PBS pH 7.4. The different aliquots of purified IgG were quantified using a Nanodrop (ND2000, Thermo Fisher Scientific, Waltham, MA).

Reactivity of the naturally acquired IgG to VAR2CSA recombinants and VAR2CSA-expressing isolates

Total IgG (before and after each variant-specific depletion) or VAR2CSA-specific IgG purified on each the five ectodomain variants was assessed by ELISA as previously described (Doritchamou et al., 2019). Briefly, recombinant VAR2CSA proteins were coated at 1 µg/mL and incubated overnight at 4°C. Plates were blocked at room temperature (RT) for 2 hours (h) and 100 µL of total IgG at 10 µg/mL and VAR2CSA-specific IgG at 0.1 µg/mL were added in duplicate wells for 1h at RT. The plates were washed and 100 µL of HRP-conjugated anti-human IgG antibody (at 1:3000 dilution) added to each well for 1h incubation at RT followed by a final wash. One hundred microliters TMB (SeraCare) was added to each well for 10 min of incubation in dark at RT and the reaction was stopped by adding an equal volume of Stop Solution (SeraCare). Optical density (OD) values at 450 nm were acquired using the MultiskanFC (Thermo Fisher) plate reader.

IgG reactivity to native VAR2CSA expressed on IE surface was assessed by flow cytometry using 6 isolates of *P. falciparum* as previously described (Doritchamou et al., 2016). Briefly, enriched mature trophozoite/schizont stages of IE were incubated with total IgG (at 100 µg/mL) or the antigen-specific IgG (at 1 µg/mL), washed, and bound IgG labeled with PE-conjugated anti-human IgG in a buffer containing 0.1% SYBR. IgG-labeled IE were quantified using an LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed in FlowJo 10 (Tree Star, Inc.). The median fluorescence intensity (MFI) was determined and background intensity from a well containing IE without immune IgG was subtracted from the MFI value detected for each test IgG.

Inhibition of IE binding to CSA

The CSA-binding inhibition activity of the purified IgG was evaluated in a static binding inhibition assay using immobilized CSPG receptor as previously described (Doritchamou et al., 2016). Briefly, the proteoglycan decorin (Sigma) at 2 µg/mL in 1X PBS was coated as 15 µL spots on a 100×15 mm Petri dish (Falcon 351029) by overnight incubation at 4°C in a humid chamber. The spots were blocked with 3% BSA in 1X PBS at 37°C for 30 min. Before binding assay, enriched mature trophozoite/schizont stages of IE from different *Pf* strains were adjusted to 20% parasite density at 0.5% hematocrit and incubated with total IgG (at 1, 2 and 4 mg/mL) or VAR2CSA-specific IgG (at 0.1 mg/mL) for 30 min at 37°C. IE suspended in IgG solution were then allowed to bind duplicate receptor spots for 30 min at RT. Unbound IE were washed away and bound IE were fixed, stained and quantified by microscopy. The percentage of inhibition was calculated relative to the wells containing IE without IgG.

Author Contributions

Conceptualization: J.Y.A. Doritchamou, J.P. Renn and P.E. Duffy. Data Curation: J.Y.A. Doritchamou. Formal analysis: J.Y.A. Doritchamou. Supervision/direction: J.Y.A. Doritchamou, M. Fried and P.E. Duffy. Funding acquisition: M. Fried and P.E. Duffy. Designed experiments: J.Y.A. Doritchamou, J.P. Renn and P.E. Duffy. Investigation: J.Y.A. Doritchamou, J.P. Renn and B. Jenkins. Analyzed data: J.Y.A. Doritchamou, B. Jenkins and P.E. Duffy. Validation: J.Y.A. Doritchamou and B. Jenkins. Visualization: J.Y.A. Doritchamou. Writing (original draft): J.Y.A. Doritchamou. Writing (review and editing): J.Y.A. Doritchamou, J.P. Renn, B. Jenkins, M. Fried and P.E. Duffy.

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Competing Interests

The authors declare that no conflicts of interest are associated with this manuscript.

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Figure legends

Figure 1: Flow chart of the experimental design. VAR2CSA-specific IgG was sequentially purified from total IgG isolated in multigravidae (MG) using antigen-specific columns made with NF54, 7G8, FCR3, HB3, Malayan Camp (MC) alleles of full-length VAR2CSA ectodomain. The flow-through IgG as well as eluted IgG after each VAR2CSA columns were assessed by ELISA, in Flow cytometry for surface reactivity and binding inhibition assay using CSA-binding *Plasmodium falciparum*-infected red blood cells (RBC). Image was created with BioRender.com

Figure 2: Depletion of VAR2CSA-specific IgG and activity of Total IgG purified from Malian multigravidae. Activity of the total IgG before (Pre-depletion) and after (Post-depletion) the purification of VAR2CSA-specific IgG was assessed by ELISA **(A)** on different recombinant full-length VAR2CSA ectodomain, by Flow cytometry **(B)** and CSA-binding inhibition assay **(C)** using six isolates including three recently adapted maternal isolates (M0736, M2022170 and M2001190). Reduction in the inhibition activity after the depletion of reactivities to five variants of full-length VAR2CSA ectodomain is shown as % reduction in inhibition **(D)**. In C, histogram without IgG represent assay well with no testing IgG and similarly stained with the conjugated anti-human secondary IgG. Data in C–D represent at least two independent experiments.

Figure 3: Reactivity of the purified VAR2CSA-specific IgG to different full-length VAR2CSA antigens and *P. falciparum* isolates. Reactivity of the VAR2CSA specific purified IgG was assessed by ELISA **(A)** on seven recombinant full-length VAR2CSA ectodomain proteins including two proteins (M920 and M200101) that were not used in the purification assay. IgG were tested at 0.1 µg/mL concentration (IgG conc.) and the star (*) indicates the depleted reactivity. **(B)** Ability of the purified VAR2CSA IgG to bind native VAR2CSA on six CSA-binding isolates was evaluated by Flow cytometry. Testing IgG is indicated in red while histogram in blue represent assay well with no testing IgG and similarly stained with the conjugated anti-human secondary IgG. Median fluorescence intensity (MFI) values are indicated. Due to limited material of the purified IgG on HB3 VAR2CSA (HB3-IgG) no data (ND) is available for the maternal isolates (M0736, M2022170 and M2001190). Star (*) indicates surface reactivity to the homologous parasite.

Figure 4: CSA-binding inhibitory activity of VAR2CSA-specific purified IgG from multigravidae. The inhibition capacity of the VAR2CSA-specific purified IgG was assessed on CSA-binding isolates **(A – E)**. Blocking activity (nBIA) of the purified IgG was normalized the activity obtained with the total IgG before any VAR2CSA IgG purification (Pre-depletion). Due to limited material, the purified IgG on FCR3, HB3 and MC has not been tested on the maternal isolates (M0736, M2022170 and M2001190). Black box indicates inhibition activity to the homologous parasite and empty box represent activities against heterologous parasites. Data represent at least two independent experiments.

Supplemental Figure S1: Reactivity of the post depletion samples to VAR2CSA recombinants and CSA-binding isolates. Reactivity of the initial total IgG (MG total IgG) as well as flow through (FT) IgG samples collected after each VAR2CSA-specific column was assessed by ELISA **(A)** on seven recombinant full-length VAR2CSA ectodomain proteins. IgG were tested at 10 µg/mL concentration

(IgG conc.). **(B)** Ability of the MG total IgG and FT IgG to bind native VAR2CSA on CSA-binding isolates was evaluated by Flow cytometry. Testing IgG is indicated in red while histogram in blue represent assay well with no testing IgG and similarly stained with the conjugated anti-human secondary IgG. Median fluorescence intensity (MFI) values are indicated. For maternal isolate M2022170, no data (ND) is available for FT samples collected after NF54, 7G8, FCR3 and HB3 due to limited material.

Supplemental Figure S2: CSA-binding inhibitory activity of VAR2CSA-specific IgG purified from multigravidae. The inhibition capacity of the VAR2CSA-specific purified IgG was assessed on CSA-binding isolates **(A – E)**. Percent inhibition activity of the purified IgG was determined relative to activity obtained with the control wells without any testing IgG. Due to limited material, the purified IgG on FCR3, HB3 and MC has not been tested on the maternal isolates (M0736, M2022170 and M2001190). Black box indicates inhibition activity to the homologous parasite and empty box represent activities against heterologous parasites. Data represent at least two independent experiments.

Supplemental Figure S3: Titration of homologous inhibition activity by purified VAR2CSA_{NF54} specific IgG. The homologous inhibition activity of naturally acquired IgG specific to VAR2CSA_{NF54} ectodomain was assessed by testing variable concentrations (100, 25, 10, 5 and 2.5µg/mL). Data represent at least two independent experiments.

Supplementary Information for

A single full-length VAR2CSA ectodomain variant purifies broadly neutralizing antibodies against placental malaria isolates

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This PDF file includes:

Figures S1 to S3

Supplemental Figure S1. Reactivity of the post depletion samples to VAR2CSA recombinants and CSA-binding isolates. Reactivity of the initial total IgG (MG total IgG) as well as flow through (FT) IgG samples collected after each VAR2CSA-specific column was assessed by ELISA **(A)** on seven recombinant full-length VAR2CSA ectodomain proteins. IgG were tested at 10 µg/mL concentration (IgG conc.). **(B)** Ability of the MG total IgG and FT IgG to bind native VAR2CSA on CSA-binding isolates was evaluated by Flow cytometry. Testing IgG is indicated in red while histogram in blue represent assay well with no testing IgG and similarly stained with the conjugated anti-human secondary IgG. Median fluorescence intensity (MFI) values are indicated. For maternal isolate M2022170, no data (ND) is available for FT samples collected after NF54, 7G8, FCR3 and HB3 due to limited material.

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