Disruption of Var2csa Gene Impairs Placental Malaria Associated Adhesion Phenotype

Nicola K. Viebig1, Emily Levin2, Sébastien Dechavanne3, Stephen J. Rogerson4, Jürg Gysin3, Joseph D. Smith2, Artur Scherf4*, Benoit Gamain1*

1 Unité de Biologie des Interactions Hôte-Parasite, Institut Pasteur and CNRS, Paris, France, 2 Seattle Biomedical Research Institute, Seattle, Washington, United States of America, 3 Unité de Parasitologie Expérimentale, Université de la Méditerranée, Marseille, France, 4 Department of Medicine, University of Melbourne, Royal Melbourne Hospital, Parkville, Victoria, Australia

Infection with Plasmodium falciparum during pregnancy is one of the major causes of malaria related morbidity and mortality in newborn and mothers. The complications of pregnancy-associated malaria result mainly from massive adhesion of Plasmodium falciparum-infected erythrocytes (IE) to chondroitin sulfate A (CSA) present in the placental intervillous blood spaces. Var2CSA, a member of the P. falciparum erythrocyte membrane protein 1 (PIEMP1) family is the predominant parasite ligand mediating CSA binding. However, experimental evidence suggests that other host receptors, such as hyaluronic acid (HA) and the neonatal Fc receptor, may also support placental binding. Here we used parasites in which var2csa was genetically disrupted to evaluate the contribution of these receptors to placental sequestration and to identify additional adhesion receptors that may be involved in pregnancy-associated malaria. By comparison to the wild-type parasites, the FCR3 Δvar2csa mutants could not be selected for HA adhesion, indicating that var2csa is not only essential for IE cytoadhesion to the placental receptor CSA, but also to HA. However, further studies using different pure sources of HA revealed that the previously observed binding results from CSA contamination in the bovine vitreous humor HA preparation. To identify CSA-independent placental interactions, FCR3 Δvar2csa mutant parasites were selected for adhesion to the human placental trophoblastic BeWo cell line. BeWo selected parasites revealed a multi-phenotypic adhesion population expressing multiple var genes. However, these parasites did not cytoadhere specifically to the syncytiotrophoblast lining of placental cryosections and were not recognized by sera from malaria-exposed women in a parity dependent manner, indicating that the surface molecules present on the surface of the BeWo selected population are not specifically expressed during the course of pregnancy-associated malaria. Taken together, these results demonstrate that the placental malaria associated phenotype can not be restored in FCR3 Δvar2csa mutant parasites and highlight the key role of var2CSA in pregnancy malaria pathogenesis and for vaccine development.

INTRODUCTION

Plasmodium falciparum causes the most severe form of human malaria, with over two million deaths per year. At particular risk of developing severe, life-threatening malaria-associated complications are children and women during their first pregnancy [1]. Whereas adults in high transmission regions usually develop protective clinical immunity to malaria, primigravid women are highly susceptible to a placental form of infection [2]. Complications of pregnancy-associated malaria (PAM) result mainly from massive placentation of Plasmodium falciparum-infected erythrocytes (IE) in the placental intervillous blood spaces [3]. Placental sequestration impacts both mother and fetus, contributing to premature delivery, intrauterine growth retardation, stillbirth, maternal anaemia, and increased neonatal and maternal mortality [4]. Whereas sequestration in the peripheral microvasculature is associated with IE that bind CD36 and variably to other host receptors, chondroitin sulfate A (CSA) expressed by placental syncytiotrophoblasts has been described as a common receptor involved in IE placental sequestration [3]. With successive pregnancies, women develop protective antibody responses that block CSA binding and recognize geographically diverse placental isolates [5,6], suggesting that a vaccine against PAM is feasible. To design a vaccine to protect pregnant women and their fetuses, it is therefore crucial to define the range of host receptors and parasite ligands involved in placental sequestration.

Cytoadhesion is mediated through the P. falciparum erythrocyte membrane protein-1 (PIEMP1), encoded by members of the vari gene family [7–9]. Gene disruption has been used to show that var2CSA is the primary PIEMP1 protein mediating CSA-binding and the only CSA-binding protein that displays a placental antigenic phenotype [10,11]. However, it is still controversial if CSA is the only placental receptor involved during PAM. Therefore if additional host receptors are involved, the corresponding parasite ligands need to be characterized in order to develop efficient vaccines. Experimental evidence suggests that IE in the placenta interact with neonatal Fc receptors via surface bound non-immune IgG [12] and cytoadhere to hyaluronic acid (HA) [13,14]. Therefore, FCR3 Δvar2csa mutant parasites are not only a useful tool to evaluate if additional PIEMP1 besides var2CSA have a role in placental IE cytoadhesion, but could also

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* To whom correspondence should be addressed. E-mail: bgamain@pasteur.fr (BG), ascherf@pasteur.fr (AS)
identify additional host receptors on the syncytiotrophoblasts or in the placental intervillosus space.

In this study, we used FCR3-var2csa mutant parasites to investigate if var2csa is essential for HA cytoadhesion and if the parasite genome encodes for other parasite ligands that mediate binding to this receptor. In addition, we used the human placental-derived trophoblastic BeWo cell line [15–17] to identify other putative unknown receptors present on the surface of syncytiotrophoblasts that could play a role in placental sequestration. Using these approaches, we were unable to define new parasite adhesion ligands beyond var2CSA that were recognized by sera of malaria-exposed women in a parity dependent manner. Our results strongly support the concept that the massive accumulation of IE in the placenta is predominantly mediated through CSA specific cytoadhesion and that var2CSA is the key virulence factor involved in the pathogenesis of PAM.

RESULTS

Var2csa is essential for IE cytoadhesion to purified hyaluronic acid preparations

Var2csa was previously reported to be transcriptionally upregulated in both CSA [18,19] and HA binding parasites [20]. To evaluate if other PfEMP1 besides var2CSA could mediate IE cytoadhesion to HA, we tested the capability of the FCR3-var2csa mutant clone 1F1 to gain a HA binding phenotype upon selection on bovine HA (bHA) immobilized to plastic Petri dishes. Whereas FCR3-CD36 wild type parasites, displaying a CD36 binding phenotype before selection, gained binding to bHA after only four rounds of selection, no specific enrichment was observed for the 1F1 FCR3-var2csa mutants (Fig. 1A).

The HA-selected IE adhesion phenotype was further examined on bHA, CSA and CD36 coated to plastic Petri dishes (Fig. 1B). While wild type FCR3 IE selected on bHA (FCR3-HA) or with the CSA binding phenotype (FCR3-CSA) bound to bHA and in higher numbers to CSA, 1F1 FCR3-var2csa mutant clone IE selected on bHA (1F1-HA) maintained their CD36 binding phenotype and did not acquire any binding to CSA and bHA (Fig. 1B). In comparison to the FCR3-CSA IE, 3D7-CSA IE revealed a very low binding to CSA and no binding to bHA. No cytoadhesion to BSA or CSC was observed for any of the IE (data not shown). As expected from previous studies [18–20], FCR3-CSA and FCR3-HA parasites transcribed a full-length var2csa transcript, while FCR3-var2csa 1F1-HA IE transcribed a truncated non-functional var2csa transcript [10] (Fig. 1C). Furthermore, using a probe to the semiconserved var exon II, transcripts of around 9 kb were identified in ring-stage RNA of FCR3-CD36 and in the mutant clone 1F1-HA (data not shown). Taken together, these data indicate that var2csa is essential for cytoadhesion of the late stage FCR3-IE to both the placental receptor CSA and to bHA and that no other var genes in FCR3-var2csa mutants were able to compensate for HA binding in the absence of var2csa.

Evaluation of the HA binding specificity

Although these results seem to implicate var2CSA as being the parasite ligand for HA binding, several studies have raised doubts on the specificity of HA cytoadhesion as it has been reported that bHA preparations used to select IE contain low to moderate levels of CSA contamination [21–23]. To test the HA binding specificity of FCR3-HA IE, binding inhibition assays were performed as described previously [24] using Streptococcus HA (sHA) preparations known to be free of CSA from three different commercial sources. Sonication has been shown to increase HA binding inhibition activity [24], so HA preparations were tested plus or minus sonication. Whereas bHA with or without sonication treatment totally abrogated IE cytoadhesion to bHA coated on plastic Petri dishes, the three commercially available sources of sHA used in this study completely failed to inhibit binding to bHA, whether sonicated or not (Fig. 2A). Furthermore, soluble CSA or bHA were able to completely cross-inhibit FCR3-HA cytoadhesion to bHA as well as to CSA (Fig. 2B). In addition, chondroitinase ABC treatment, but not Streptomyces hyaluronidaticus hyaluronidase treatment inhibited IE adhesion to the two receptors, CSA and bHA (data not shown). Therefore, binding of FCR3 IE to bHA is caused by CSA contamination in the HA preparation.

Selection of var2csa disrupted IE on the placental BeWo cell line and phenotypic analysis

Currently, there are no P. falciparum animal models for placental sequestration or PAM disease. However, recently, it has been shown that placental isolates adhere strongly to the human placental-derived trophoblastic BeWo cell line and that this is a quick and easy alternative to select IE for the CSA-binding phenotype [15–17]. In order to identify other putative unknown receptors present on the surface of syncytiotrophoblasts that could play a role in placental sequestration, we selected the FCR3-var2csa variant clone 1F1 on the BeWo cell line. After six pannings on the BeWo cell line, the 1F1-BeWo selected parasite population bound to the BeWo cells, however, in approximately five-fold lower numbers than the FCR3-CSA wild type parasites (Fig. 3A). In contrast, 1F1 parasites selected to bind CD36 did not bind BeWo cells (Fig. 3A).

BeWo cells are heterogeneous cells [25] expressing numerous potential parasite cytoadhesion receptors including CSA, intercellular adhesion molecule-1 (ICAM-1) [15] and also the neonatal Fc receptor [12,26]. However, these cells do not express CD36, CD31, E-selectin and vascular cell adhesion molecule-1 (VCAM-1) [15]. To examine the 1F1-BeWo parasites binding phenotype, cytoadhesion experiments to different host receptors coated on plastic Petri dishes were performed. Whereas 1F1-CD36 parasites bound only to CD36, 1F1-BeWo IE adhered in low numbers to two different sources of recombinant human ICAM-1 and to CD36, but did not bind to CSA (Fig. 3B).

To characterize the respective receptors that FCR3-CSA and 1F1-BeWo IE were using to adhere to BeWo cells, binding inhibition assays were performed. While antibodies to ICAM-1 had no effect on FCR3-CSA IE adhesion, they inhibited 1F1-BeWo IE cytoadhesion by 40%, indicating that parts of the population display an ICAM-1 binding phenotype (Fig. 4A). By comparison, chondroitinase ABC treatment of the BeWo cells partially inhibited FCR3-CSA IE cytoadhesion, but 1F1-BeWo IE binding was not altered (Fig. 4B). No cytoadhesion inhibition of either parasite line was observed after either hyaluronidase treatment of the cells or antibodies to CD36 (Fig. 4). These results demonstrate that the 1F1-BeWo IE binding interaction to the BeWo cells is CSA and HA independent.

Binding of non-immune IgG on the IE surface of CSA binding parasites expressing var2CSA such as FCR3-CSA was previously reported to be involved in IE adhesion to the neonatal Fc receptors expressed by the syncytiotrophoblasts [12,14]. As BeWo cells have been described to express the neonatal Fc receptor [26], we assessed the capacity of FCR3-CSA and 1F1-BeWo IE grown in the presence of human sera to bind to the BeWo cells. Although under our experimental conditions non-immune immunoglobulins can be detected on the surface of BeWo cells and FCR3-CSA IE but not on the surface of the 1F1-BeWo IE (data not shown), pre-incubation of late stage FCR3-CSA and 1F1-BeWo IE with 200 μg/ml protein A, but not with BSA, resulted for both parasite
lines in partial and non-specific inhibition of adhesion to the BeWo cells (data not shown). The fact that this partial inhibition was independent of the parasite phenotype and of the presence of immunoglobulins on the IE surface suggests a non-specific inhibition and a minor role for PfEMP1 in this interaction. Taken together, our data shows that selection of FCR3D var2csa parasites for cytoadhesion to the placental derived BeWo cell line, results in a CSA-independent, multi-phenotypic parasite population.

Several var genes are transcribed in 1F1-BeWo parasites
Cytoadhesion of late stage IE is mainly mediated by PfEMP1 that is encoded by members of the var multi gene family [7–9]. Recently, the var gene repertoire was obtained from the FCR3/CS2/IT4 parasite genotype [27]. To identify the var gene(s) predominantly transcribed in the 1F1-CD36 and 1F1-BeWo parasite populations, gene-specific primers were designed to the 5' known var genes (materials and methods) and quantitative real-time PCR was performed on RNA extracted from ring stage parasites at 10 h post-invasion (Fig. 5). Prior to var transcriptional analysis, 1F1-CD36 IE were resected on recombinant human CD36 and 1F1-BeWo parasites were analyzed after six rounds of selection on the BeWo cells. As expected, both 1F1-derived parasite lines express a partial, non-functional var2csa transcript as a result of the gene disruption event [10]. This transcript was detected by primers to the 5' end of the gene, but not to the 3' end of the gene. The 1F1-CD36 parasite line also expresses one dominant var gene, var34, and a second gene at lower level (var47). The var34 and var47 transcripts are also present in the 1F1-BeWo parasite population, plus four additional var genes (var5, var6, var7, and var51) (Fig. 5). These results were confirmed by Northern blot analysis (Fig. S1). The results of this var gene transcriptional analysis indicate that the 1F1-BeWo population expressed multiple var genes displaying a multi-adhesive phenotype.

1F1-BeWo selected parasites do not show characteristics of placental parasites
The BeWo cell line is considered to be a model that can be used to study interactions between IE and placental syncytiotrophoblasts. To test if the 1F1-BeWo parasite line has the characteristics of
a placental parasite population it was investigated for adhesion to normal human placenta cryosections under flow conditions at 0.05 Pa. In this experiment, FCR3-CSA parasites, used as a control, adhered mainly to the syncytiotrophoblast lining. In contrast, 1F1-BeWo and 1F1-CD36 IE showed weaker binding and adhered mostly to the villous tissue and not specifically to the syncytiotrophoblast lining (Fig. 6A, and data not shown). To examine whether 1F1-BeWo selected parasites were recognized in a gender-specific manner they were tested by flow cytometry and live immunofluorescence assay using sera from Malawian male and pregnant women. Using these sera, only FCR3-CSA IE were recognized by sera pools of malaria-exposed women in a parity dependent manner (Fig. 6B and C). In contrast, FCR3-CD36, 1F1-BeWo and 1F1-CD36 were recognized equally well by sera pools of malaria-exposed males, primigravid and multigravid women (Fig. 6B and C). Taken together, the low binding to the syncytiotrophoblast lining and the parity independent surface reactivity suggest that erythrocyte surface molecules expressed by 1F1-BeWo selected population are not specifically expressed during the course of pregnancy-associated malaria.

Figure 2. Binding of the FCR3-HA selected parasites to HA and CSA is inhibited by soluble bovine HA and soluble CSA, but not by *Streptococcus* HA. A. Evaluation of the HA binding specificity of the FCR3-CD36 IE selected on HA was determined without (control) or with 200 µg/ml of different HA preparations sonicated or not in the binding medium. HA from bovine vitreous humor (bHA) and different HA from *Streptococcus* (sHA) were tested for their ability to inhibit IE binding to bHA. Data are the mean number (±SEM) of IE per mm² adhering to receptor-coated plastic Petri dishes, as determined in three independent assays in duplicate spots. B. Soluble bHA and CSA cross-inhibit cytoadhesion of FCR3-HA IE to bHA as well as CSA. FCR3-HA IE were pre-incubated with 200 µg/ml soluble CSA as well as untreated or treated bHA prior to the binding assay. The ability to inhibit IE cytoadhesion to bHA (left panel) and CSA (right panel) was examined. Data are the mean number (±SEM) of IE per mm² adhering to receptor-coated plastic Petri dishes, as determined in three (A) or two (B) independent assays in duplicate spots.

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No PAM Phenotype in *Var2csa* KO
Figure 3. Phenotypic analysis of FCR3:var2csa 1F1 IE selected on the human placental derived trophoblastic BeWo cell line. A. Cytoadhesion of IE to BeWo cells was compared for the FCR3:var2csa clone 1F1 selected on BeWo cells (1F1-BeWo), the 1F1 control parasites selected on recombinant human CD36 (1F1-CD36) and the wild-type FCR3-CSA parasites. Data are the mean number (±SEM) of IE per mm² adhering to BeWo cells as determined in at least two independent assays in duplicate. B. Erythrocytes infected with *P. falciparum* 1F1-CD36 (white bars) and 1F1-BeWo (gray bars) were analyzed for cytoadhesion to CSA, CD36 (left panel), recombinant human ICAM-1 (rhICAM-1) and ICAM-1 Fc (right panel). Data are the mean number (±SEM) of IE per mm² adhering to receptor-coated plastic Petri dishes as determined in at least two independent assays in duplicate.

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Figure 4. Anti-ICAM-1 antibody inhibits 1F1-BeWo IE binding to BeWo cells. A. and B. Binding specificity of FCR3-CSA (white bars) and 1F1-BeWo IE (gray bars) to BeWo cells was determined using various inhibitors. BeWo cells were either pre-incubated with adhesion blocking anti-ICAM-1 or anti-CD36 antibodies at 5 μg/ml (A) or pre-treated with *Streptomyces hyalurolyticus* hyaluronidase (2S units/ml) or chondroitinase ABC (0.5 units/ml) for 1 h at 37°C (B). Data are the mean percentage (±SEM) of IE binding compared to the appropriate control as determined in three independent experiments.

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DISCUSSION

Cytoadhesion of late stage IE in the placenta is a crucial event in the development of severe malaria complications during pregnancy especially in primigravid women [28]. Although it is generally accepted that a vaccine that would protect against PAM should target the var2CSA molecule, a crucial question remains to be addressed, namely if non-CSA mediated adhesion events could replace CSA adhesion in the placenta. Using our recently described var2csa deficient parasite line FCR3Δvar2csa 1F1 [10], we were able to evaluate the role of non-CSA placental adhesion receptors.

In this study, we showed that var2csa is linked to the interaction with certain HA preparations bound to plastic and that this phenotype is lost in var2csa deficient mutant parasites. However, we failed to prove the specificity of HA in the binding assays, leading us to the conclusion that binding of IE to bHA is mediated by CSA contamination in the HA preparation. These results are in disagreement with several studies reporting HA specific IE cytoadhesion [13,14,24,29,30], but supported by other studies that raised doubts on the specificity of HA mediated IE cytoadhesion [22,23,31]. More recently, Muthusamy et al. demonstrated that HA is not present in the intervillous space of the human placenta [31], indicating that HA is unlikely to be involved in placental sequestration.

In addition to CSA and HA, the binding of non-immune immunoglobulins to the IE surface was reported to be an important ligand for IE adhesion to the placental syncytiotrophoblasts via neonatal Fc receptors [12]. This study implicated a non-var2csa-type var gene (TM284S2 var1) in placental binding. To address whether other parasite ligands can supplant placental binding in the absence of functional var2CSA, we selected 1F1 FCR3Δvar2csa mutant parasites on BeWo cells. We obtained a parasite line that displayed a mixed, but CSA-independent binding phenotype. 1F1-Bewo cells adhered much weaker than
FCR3-CSA IE to BeWo cells. This observation supports previous results indicating that var2CSA is the primary ligand for CSA in the FCR3 genotype. The CSA-independent IE adhesion with BeWo cells was investigated for its possible interaction with the neonatal Fc receptor. IE adhesion to the BeWo cells was partially inhibited with protein A. This inhibition was, however, independent of the parasite phenotype and of the presence of immunoglobulins on the IE surface, suggesting a non-specific inhibition and a minor role for PfEMP1 in this interaction. In addition, several studies have concluded that neonatal Fc receptors are not expressed on the syncytiotrophoblasts surface and may therefore not be accessible to IE cytoadhesion [32–34]. Taken together, our data show that the neonatal Fc receptor is not a major placental cytoadhesion receptor for FCR3 parasites under the experimental conditions.

To assess the potential role of non-var2CSA PfEMP1 variants in PAM pathogenesis, we characterized the binding and antigenic phenotype of BeWo selected parasites. By comparison to FCR3-CSA IE expressing var2csa, 1F1-BeWo IE do not sequester specifically to the syncytiotrophoblast lining of normal human placental cryosections nor are they recognized in a gender-specific manner by endemic sera suggesting they are not expressed during the course of pregnancy-associated malaria.

Similar to our work, Duffy et al. [11] have generated var2csa-disruption mutants in a parasite line, termed CS2, which is isogenic to the FCR3 parasite used here. While CS2:var2csa were able to weakly recover a CSA-binding phenotype, these minor variants were not recognized by pregnant women sera in a parity dependent manner and therefore are unlikely to have an important role in pregnancy associated malaria. Recently, the
var gene repertoire was obtained from the FCR3/CS2/1F4 parasite genotype [27]. Interestingly, the varP gene tag described to be upregulated in these CS2 var2csa var gene deficient mutants is identical to the var6 gene upregulated in our 1F1-BeWo parasite line. Therefore it is possible that the var6 protein possesses a very low affinity CSA binding domain. However, none of the var genes upregulated in 1F1-BeWo parasites has significant relationship to the TM284S2 var1 gene [12], which, unlike var2csa, is not conserved across P. falciparum isolates.

While the new var genes upregulated on BeWo-selected IE are unlikely to have a role in PAM pathogenesis, they do provide new information about var-associated binding phenotypes. Whereas 1F1-CD36 parasites bound only to CD36, 1F1-BeWo IE adhered in low numbers to human ICAM-1 and to CD36. As the 1F1-CD36 parasites do not bind to ICAM-1 we can exclude that var34 and var47 are involved in the observed 1F1-BeWo ICAM-1 binding phenotype. As BeWo cells do not express CD36, the CD36 binding phenotype of 1F1-BeWo likely arose due to a PIEM1 variant that happened to encode CD36 binding activity function in addition to the host receptor adhesion trait that led to its selection on BeWo cells. This is not surprising because CD36 binding is a common adhesion trait in many different PIEM1 variants [35]. Because var34 is found in FCR3 parasites with different phenotypes we suggest that high “on” rates for the var34 var gene in FCR3 parasites may explain the frequently observed subpopulation expressing var34.

With regard to the development of a vaccine aiming to protect pregnant women and their fetuses from severe disease, our results are encouraging. Using FCR3 parasites and a defined placental binding model, we were unable to confirm a role for HA as well as for non-immune immunoglobulins in IE placental sequestration nor to select for additional parasites with a placental antigenic phenotype, besides var2CSA. Although we cannot exclude that by using another laboratory strain or another selection system we would have observed the same data, our experimental findings strongly point to var2CSA as the main parasite ligand mediating high affinity and high density cytoadhesion to the placenta. Compared to typical PIEM1 proteins, var2CSA is exceptionally conserved between parasite isolates from different regions of the world [36,37]. This could help explain how multigland women in malaria endemic areas develop antibodies that recognize erythrocytes infected with placent al and CSA binding parasites in a strain transcendent manner. In conclusion, the analysis of a mutant parasite (var2csa KO) has been a valuable tool to reevaluate previously described interactions of IE with the placenta. Our results indicate that vaccine development should focus on the var2CSA molecule.

**MATERIALS AND METHODS**

**Parasite and cell culture**

The *P. falciparum* FCR3 and 3D7 strains were cultivated according to standard conditions [38] in O+ human erythrocytes in RPMI 1640 containing L-Glutamine (Invitrogen) supplemented with 5% human serum (PAA Laboratories GmbH), 0.25% Albumax I (Invitrogen), 1× hypoxanthine (c.c.pro) and 20 μg/ml gentamicin (Sigma). FCR3/Var2csa clone 1F1 [10] was grown in the presence of 2.5 mM WR99210 (Jacobus Pharmaceutical Company). For selection on BeWo cells and for flow cytometry, parasites were grown in RPMII 1640 containing L-Glutamine supplemented with 0.5% Albumax I, 1× hypoxanthine and 20 μg/ml gentamicin. Cultures were synchronized as described previously [39]. To maintain knob-positive parasites, cultures were routinely selected by gelatin flotation using Plasmion (Fresenius Kab) [40]. The human choriocarcinoma placenta BeWo cell line was cultured as described [15]. Parasites and cells were tested *Mycoplasma* negative by PCR.

**Binding phenotype selection**

To select the FCR3 strain and the FCR3/Var2csa clone 1F1 for specific binding phenotypes, trophozoite-stage parasitized erythrocytes (IE) were purified using Plasmion and selected either on BeWo cells or on plastic Petri dishes coated with bHA or recombinant CD36.

To obtain a CSA-binding phenotype, FCR3-CSA and 3D7-CSA IE used in this study were selected on the trophoblastic BeWo cell line as described recently [15].

Plastic Petri dishes (Falcon #1005) were coated overnight at 4°C with PBS containing 100 μg/ml HA sodium salt from bovine vitreous humor (Sigma) or 10 μg/ml recombinant human CD36-Fc (R&D Systems), washed three times with PBS and blocked with 1% BSA for 1 h at RT, before trophozoite-IE were allowed to adhere. All assays were carried out using RPMI 1640/25 mM Heps, pH 7.2 (panning buffer). BeWo cells were washed three times with panning buffer, before IE were allowed to adhere. For gelatin enrichment, 1 ml RBC pellet was mixed with 1.4 ml of parasite culture medium and 2.4 ml of Plasmion and incubated for 50 min in a 37°C water bath. The enriched IE were washed twice with panning buffer and were resuspended in 10 ml of panning buffer at a concentration of approximately 5 × 10^9 IE/ml. After incubation at 37°C in a 5% CO2 incubator for 1 h with gentle agitation every 15 min, non-adherent IE were washed away with panning buffer. Bound IE were detached with the pipette stream and returned to culture. Parasites were grown to a parasitemia of 4–10% before repeating this process three times for selection on bHA, five times for selection on BeWo cells.

**Cytoadhesion assays on immobilized receptors**

Cytoadhesion assays on receptors immobilized on plastic petri dishes were carried out as described [41,42]. Briefly, plastic Petri dishes were coated overnight at 4°C with PBS containing 1 mg/ml CSA sodium salt from bovine trachea (Sigma), 1 mg/ml chondroitin sulfate C sodium salt from shark cartilage (CSC) (Sigma), 100 μg/ml HA sodium salt from bovine vitreous humor (Sigma), 10 μg/ml recombinant human CD36-Fc Chimera (R&D Systems), 10 μg/ml recombinant human ICAM-1 (R&D Systems), 1% BSA or MAb 179 (25 μg/ml) [41]. MAb 179 coated spots were incubated with recombinant CD36 protein containing this epitope tag for 1 h at RT. All spots were blocked with 1% BSA for 1 h at RT before trophozoite-IE (5 × 10^9 IE/ml) were allowed to adhere. The average number of adherent IE (± SEM) for four different fields in duplicate spots was determined in two to three independent experiments after fixing with 2% glutaraldehyde in PBS for 2 h at RT and staining the plates with Giemsa. Pictures were taken with a Nikon camera. Lucia software was used to determine the number of bound IE.

**Inhibition assays on immobilized receptors**

HA of the different sources was dissolved overnight at 4°C on a rotating wheel at a concentration of 5 mg/ml in water. The solutions were treated by intermittent sonication, in an ice water bath, for 5 min using a Bioruptor (Diagenode) set at maximum output with 30 sec/30 sec intervals. Control HA samples were incubated in an ice water bath for an equivalent time. HA from bovine vitreous humor (Sigma), Streptococcus sp. (Calbiochem), Streptococcus equi (Fluka) and Streptococcus zoopneumoniae (Sigma) was tested. CSA was dissolved in PBS at a concentration of 5 mg/ml.
5 x 10^7 IE/ml were pre-incubated with 200 μg/ml of the different soluble HA preparations for 30 min at RT and adding the soluble inhibitors to the binding assay. 200 μg/ml CSA was used as a positive control for inhibition of cytoadhesion.

**Inhibition assays on BeWo cells**

Specificity of IE binding to the BeWo cells was determined using various inhibitors. Cytoadhesion assays were carried out as described recently [15]. Binding specificity was determined either by pre-treating the BeWo cells with 0.5 units/ml of chondroitinase ABC (Fluka) or 25 units/ml Streptomyces hyalurolyticus hyaluronidase (Calbiochem) or by pre-incubating the cells with 5 μg/ml anti-CD36 monoclonal antibody FA6/152 (Beckman Coulter) or 5 μg/ml anti-ICAM-1 monoclonal antibody 84H10 (Beckman Coulter) for 1 h at 37°C. Whereas enzyme treated cells were washed prior to IE adhesion, antibodies remained present during adhesion assays. IE were pre-incubated with the soluble inhibitor protein A from Staphylococcus aureus (200 μg/ml) or with 200 μg/ml BSA for 20 min at RT. Cytoadhesion assays were carried out in panning buffer pH 7.2. Pictures of cells were taken with a Nikon camera after fixing cells with 2% glutaraldehyde in PBS.

**Flow adhesion assays**

Adhesion assays to normal human placenta cryosections under flow conditions at a shear stress of 0.05 Pa were performed as described [43].

**Flow cytometry**

 Cultures with 3–5% parasitemia synchronous at the mid/late trophozoite stage were washed three times in PBS/0.2% BSA and resuspended in PBS/0.2% BSA to 1 x 10^7 cells/ml. Samples were stained for 30 min at RT with sera pools of malaria-exposed male, primigravidae and multigravidae from Malawi (1:20 dilution) [44]. Sera were collected from pregnant women near delivery. All women gave written consent for HIV counselling and testing, and for the use of their sera to investigate immunity to malaria. Only HIV uninfected women’s sera were used in the present study. Samples from males were collected from fathers of children admitted to the same hospital with malaria, who gave witnessed verbal consent. The use of these sera was approved by the College of Medicine Research Ethics Committee, University of Malawi. Samples from males were collected from fathers of children admitted to the same hospital with malaria, who gave witnessed verbal consent. The use of these sera was approved by the College of Medicine Research Ethics Committee, University of Malawi. Only HIV uninfected women’s sera were used in the present study. Samples from males were collected from fathers of children admitted to the same hospital with malaria, who gave witnessed verbal consent. The use of these sera was approved by the College of Medicine Research Ethics Committee, University of Malawi.

**Northern blot Analysis**

Total RNA was prepared from synchronized parasite cultures approximately 10 h and 30 h post-invasion, respectively. RNA preparation, electrophoresis, membrane transfer and hybridization were carried out as previously described [47]. The membrane was hybridized at high stringency conditions at 60°C overnight and washed twice with 0.3 x SSC, 0.1% SDS at 30 min. The probe for FCR3 varcsa DBL1-X was generated by PCR amplification from FCR3 genomic DNA using the primers 5′-tccggtggctttcctgctcctcc-3′ and 5′-gagatctaggagaggaaaggg-3′ and radiolabelling as previously described [19].

**SUPPORTING INFORMATION**

**Table S1**

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**Figure S1** Transcriptional analysis of var genes Northern blot analysis of total RNA isolated from ring (R) and trophozoite stage parasites (T) FCR3-CSA, FCR3-CD36, 1F1-CD36 and 1F1-BeWo. The membrane was hybridized with probes specific for varCSA DBL1, varCSA DBL3, var7, var34, var47 and semi-conserved varT11.1 exon II. The Northern blot data confirms the quantitative real-time PCR data that several different full-length var genes are transcribed in the multi-phenotypic parasite population 1F1-BeWo. The probes were generated by PCR.
amplification from FCR3 genomic DNA and radiolabelling as previously described [19]. For the var probes following primers were used: var26, gaagcagaaatatgcatatag and caagctggatcaacagtcatgc, var7, gcacatttggaacagcagc and cattgcgacctcattcwatc, var34, caaaccacagcagcttg and cgtgatccctgtctgtctgtc, var47, aaccacagagtttgcggc and cttaaagcaggaatagggg. 

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Author Contributions

Conceived and designed the experiments: JG AS JS BG NV. Performed the experiments: NV EL SD. Analyzed the data: JG AS JS BG EL SD. Contributed reagents/materials/analysis tools: SR. Wrote the paper: AS SR JS BG NV.

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Author/s:
Viebig, NK; Levin, E; Dechavanne, S; Rogerson, SJ; Gysin, J; Smith, JD; Scherf, A; Gamain, B

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