Placental Chondroitin Sulfate A–Binding Malarial Isolates Evade Innate Phagocytic Clearance

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Pregnancy-associated malaria is characterized by the accumulation of parasitized erythrocytes (PEs) and monocytes in the placenta, and they are believed to directly contribute to adverse birth outcomes. Although most parasite isolates adhere to CD36, placental isolates express novel variant surface antigens (VSAs) and bind to chondroitin sulfate A (CSA). CSA-binding PEs are rarely observed outside of pregnancy, and most primigravid women lack immunity and must rely on innate immune mechanisms to clear these placental parasite variants. We hypothesized that differences in VSA expression and adhesive phenotype between pregnancy-associated (CSA-binding) and non–pregnancy-associated (CD36-binding) isolates may have direct implications for the failure of primigravid women to control the placental parasite burden through innate phagocytic pathways. We demonstrate here, both in vitro and in vivo, that there is a nonopsonic phagocytic defect for CSA-binding PEs. The ability of CSA-binding PEs to evade innate clearance pathways may contribute to the parasite accumulation and recruitment of monocytes that characterize placental malaria.

Individuals living in areas of stable malaria transmission gradually acquire immunity during childhood and, as adults, are generally protected from high parasitemia and clinical disease [1]. However, an important exception to this occurs with pregnancy-associated malaria. Plasmodium falciparum infections in pregnant women, especially in primigravid women, are more frequent and more severe than those in nonpregnant women and in men [6, 7]. Placental parasite isolates express novel variant surface antigens (VSAs) on the erythrocyte, and these VSAs confer a distinctive adhesive phenotype. Although most isolates of P. falciparum bind to the scavenger receptor CD36, placental isolates preferentially bind to glycosaminoglycans, such as chondroitin sulfate A (CSA), that are expressed on placental syncytiotrophoblasts and uniquely do not adhere to CD36 [7]. Adhesion to both receptors is mediated through the expression of VSAs, in particular the polymorphic P. falciparum erythrocyte membrane protein 1 (PfEMP-1), which is encoded by the var multigene family. The cysteine-rich interdomain region of PfEMP-1 has been implicated in adherence to CSA [8–10]. It has been reported that structural constraints in different
PfEMP-1 domains preclude simultaneous adherence to both receptors [11].

CSA-binding PEs are rarely observed outside of pregnancy, and nonpregnant and primigravid women generally lack acquired immunity to these parasites [12]. Because primigravid women lack antibodies to CSA-binding PEs, they must rely on innate immune mechanisms to control and clear pregnancy-associated parasite variants. For reasons that are not fully understood, innate responses are inadequate to prevent parasite accumulation in the placenta. Previous studies have demonstrated that innate recognition and phagocytosis of PEs occurs primarily through the engagement of the pattern-recognition receptor CD36 on monocytes and macrophages [13–15]. We hypothesized that differences in VSA expression and adhesive phenotype between pregnancy-associated (CSA-binding) and non–pregnancy-associated (CD36-binding) isolates may have direct implications for the failure of primigravid women to control the placental parasite burden through innate phagocytic pathways.

To investigate this hypothesis, we studied parasite isolates that bind to CD36 and isolates selected to bind to the placental receptors CSA and hyaluronic acid (HA) [16, 17] and examined the cytoadherence characteristics of these isolates and the capacity of macrophages to clear them through innate pattern-recognition receptor–mediated (nonopsonic) phagocytic pathways. The isolates selected to bind to CSA express var2csa as their dominant var transcript and display antigenic and adhesive phenotypes that are similar to those of clinical isolates that cause placental malaria [9, 10, 17–20]. We demonstrate, both in vitro and in vivo, that malarial isolates that express pregnancy-associated VSAs and that bind to CSA evade innate clearance mechanisms.

MATERIALS AND METHODS

Reagents. Endotoxin-free RPMI 1640 and heat-inactivated fetal calf serum (FCS) were obtained from Wisent. Monoclonal anti–mouse CD36 IgA (clone 63) was obtained from Sigma-Aldrich. Monoclonal anti–human CD36 IgG (clone FA6-152) was obtained from Beckman Coulter. CSA (chondroitin 4-sulfate), chondroitinase ABC, hyaluronidase, trypsin, and all other reagents were obtained from Sigma-Aldrich.

Parasite culture. P. falciparum isolates were obtained from Wisent. Cultures were treated with mycoplasma removal agent (ICN) and were determined to be mycoplasma free by polymerase chain reaction before use. Isolates E8B and CS2 were gifts from S. Rogerson (University of Melbourne, Melbourne, Australia). E8B—which adheres to CD36, intercellular adhesion molecule 1, and, to a minor extent, CSA and HA—was derived from the laboratory isolate ITG by panning on endothelial cells [21], CS2, which binds to CSA and HA but not to CD36, was derived from E8B by panning on CHO cells and on immobilized CSA [22]. The isolate 3D7-CSA was generated by panning on CHO cells followed by selection on plastic-bound bovine trachea CSA, as described elsewhere [22].

Cytoadherence assay. CHO cells that had been stably transfected with human CD36 (CHO-CD36 cells) or that had been mock transfected (CHO-mock cells) were plated in 12-well polystyrene culture plates (VWR) at a density of 2 × 10^5 cells/well in RPMI 1640 with 10% FCS (RPMI 10). Synchronized trophozoites were added at a ratio of 20 PEs to 1 CHO cell in 0.3 mL of RPMI 10 and incubated with rotation for 90 min at room temperature. Unbound PEs were removed by washing. Cytoadherence was quantified microscopically by counting the number of bound PEs per CHO cell in 100 CHO cells/well. Where indicated, CD36 on CHO cells was blocked by pretreatment with an anti-CD36 monoclonal antibody (FA6-152; 10 µg/mL) for 20 min. To block CSA binding, either CSA was cleaved by pretreatment of CHO cells with chondroitinase ABC (0.5 U/mL) for 1 h at 37°C (pH 8.0) or PEs were preincubated with soluble CSA (1 mg/mL) for 30 min at room temperature. In some experiments, PEs were pretreated with 0.05% trypsin-EDTA for 30 min at 37°C, to cleave PfEMP-1 from the erythrocyte surface [23].

In vitro phagocytosis. In vitro phagocytosis assays were performed as described elsewhere [13, 15]. Thioglycollate-elicited macrophages from CD36+/+ wild-type (WT) mice (CD36-WT macrophages) and from CD36−/− knockout (KO) mice (CD36-KO macrophages) were collected by peritoneal lavage with ice-cold PBS. Human peripheral-blood monocytes were purified using ficoll and Percoll gradients [13]. Murine macrophages and human monocytes were plated at densities of 1.25 × 10^5 and 2.5 × 10^5 cells/well, respectively, on glass coverslips (Fisher Scientific) in 24-well plates and cultured for 3 days before use. Fc receptors were blocked by pretreatment of macrophages with Fc fragments (20 µg/mL) for 20 min at 4°C. Synchronized trophozoites were added at a ratio of 20 PEs to 1 macrophage and incubated with rotation for 4 h at 37°C. In some experiments, PEs were preincubated in nonimmune human serum (1:1 dilution) before being used in phagocytosis assays. For these assays, macrophage Fc receptors were not blocked. Coverslips were washed in ice-cold water for 30–40 s, to lyse nonphagocytosed PEs, and were then fixed and stained with Diff-Quick (Fisher Scientific). Phagocytosis was quantified microscopically, with 500 cells counted per coverslip, and is presented as percentage phagocytosis (the percentage of macrophages with at least 1 internalized PE, determined on the basis of defined morphologic criteria, as described elsewhere [13, 15]). Where indicated, CD36 was blocked by preincubation of macrophages with an anti-CD36 monoclonal antibody for 20 min at 4°C (clone FA6-152 for human macrophages and clone 63 for murine macrophages; 10 µg/mL). To examine the
of CSA, either chondroitinase ABC (0.5 U/mL) was used to cleave CSA on macrophages or PEs were pretreated with soluble CSA (1 mg/mL), as described above. In some experiments, macrophages were pretreated with hyaluronidase (0.5 U/mL) for 1 h at room temperature, to cleave HA.

In vivo phagocytosis. To evaluate in vivo phagocytosis of CD36- and CSA-binding PEs, $5 \times 10^7$ PEs were injected into the peritoneal cavity of Wistar rats or spontaneous hypertensive rats (SHRs). SHRs are derived from Wistar rats but possess a spontaneous mutation that makes them CD36 deficient [15]. Three hours after injection, peritoneal cells were collected, washed, and allowed to adhere to glass coverslips. Coverslips were washed, fixed, and stained with Diff-Quick. A small volume of the peritoneal-lavage fluid was also immediately cytospun onto microscope slides, fixed, and stained. Phagocytosis was quantified microscopically by counting the number of monocytes with at least 1 internalized PE.

Statistical analysis. All experiments were performed in duplicate or triplicate and were repeated at least 3 times. Data are presented as means ± SEs, unless stated otherwise. Statistical significance ($P < .05$) was determined by Student’s $t$ test.

RESULTS

We wished to examine whether differences in VSA expression and adhesive phenotype between pregnancy-associated (CSA-binding) and non–pregnancy-associated (CD36-binding) isolates might explain, at least in part, the failure of primigravid women to control the placental parasite burden through innate phagocytic pathways. To investigate this hypothesis, we studied 2 parasite isolates, CS2 and its parental clone, E8B, and examined their cytoadherence characteristics and the capacity of macrophages to clear these isolates through innate pattern-recognition receptor–mediated (nonopsonic) phagocytic pathways. E8B and CS2 express different VSAs, including forms of PfEMP-1 that confer differing receptor specificities [16]. E8B has been shown to predominately bind to CD36. CS2 was derived from E8B by repeated selection on CSA and preferentially binds to the placental receptors CSA and HA [17]. CS2 (and other isolates selected on CSA) express var2csa as their dominant var transcript and display antigenic and adhesive phenotypes similar to those of clinical isolates that cause placental malaria [9, 10, 17–20]. Adhesion of freshly collected placental parasite isolates to CSA has been strongly correlated with adhesion to low-sulfated placental chondroitin proteoglycans, and binding to either receptor is strongly and negatively correlated with low birth weight [19]. VSAs on CS2 PEs and other isolates selected on CSA also display the same sex- and parity-dependent IgG recognition as do fresh clinical pregnancy-associated malarial isolates [6, 20]. CS2 PEs are recognized by antibodies from multigravid women but not by antibodies from uninfected primigravid women or men who are from areas where malaria is endemic, and anti-CS2 antibodies recognize CSA-binding P. falciparum lines but not CD36-binding isolates and are able to block the adherence of CSA-binding PEs to CSA [17, 18, 20].

Cytoadherence of pregnancy-associated and non–pregnancy-associated PEs. To determine whether the avidity of the binding of E8B (CD36-adhering) PEs and CS2 (CSA-adhering) PEs to target cells was similar and, thus, unlikely to explain any differences observed in phagocytic uptake, we performed cytoadherence assays using CHO-CD36 and CHO-mock cells. CHO cells constitutively express CSA on their surface. Both E8B PEs and CS2 PEs adhered avidly to CHO-CD36 cells (figure 1), but only CS2 PEs adhered to CHO-mock cells. Pretreatment of CHO cells with an anti-CD36 monoclonal antibody significantly inhibited the adherence of E8B PEs to CHO-CD36 cells but did not affect the adherence of CS2 PEs to either CHO-CD36 or CHO-mock cells. Both pretreatment of CHO cells with chondroitinase ABC (to cleave CSA) and pretreatment of PEs with soluble ABC inhibited the adherence

![Figure 1](https://example.com/figure1.png)

Figure 1. Cytoadherence of E8B (CD36-binding) and CS2 (chondroitin sulfate A [CSA]–binding) parasitized erythrocytes (PEs). E8B PEs and CS2 PEs cytoadhered equally well but to different receptors. Cytoadherence assays were performed using CHO cells that had been stably transfected with human CD36 (CHO-CD36 cells) or that had been mock transfected (CHO-mock cells), as described in Materials and Methods. Both CHO-CD36 and CHO-mock cells express CSA. Cytoadherence is shown as the no. of PEs bound per CHO cell. The black bars represent control-treated CHO cells; the dark gray bars represent CHO cells pretreated with anti-CD36 monoclonal antibody; the light gray bars represent CHO cells exposed to PEs pretreated with trypsin; the white bars represent CHO cells pretreated with chondroitinase ABC (to cleave CSA); and the hatched bars represent PEs pretreated with soluble CSA. Data are the mean ± SD results of a representative experiment repeated 3 times. *Statistically significant difference (Student’s $t$ test), for the following comparisons: CHO-CD36 E8B control vs. anti-CD36 ($P = .042$) and vs. trypsin ($P = .0036$); CHO-CD36 CS2 control vs. chondroitinase ABC ($P = .0053$) and vs. soluble CSA ($P = .0089$); CHO-mock CS2 control vs. chondroitinase ABC ($P = .0069$) and vs. soluble CSA ($P = .0072$). •Statistically significant difference (Student’s $t$ test), for the comparison CHO-mock E8B control vs. CHO-CD36 E8B control ($P = .0059$).
of CS2 PEs to CHO-CD36 and CHO-mock cells but did not affect E8B PE cytoadherence. Adherence of PEs to CD36 has been previously shown to be trypsin sensitive, a result of the cleavage of PfEMP-1 from the surface of the PE [23]. Pretreatment of E8B PEs with trypsin resulted in a significant reduction in adherence to CHO-CD36 cells, whereas CS2 PE cytoadherence was not significantly inhibited by pretreatment with trypsin. Collectively, these data demonstrate that the avidities of the binding of E8B and CS2 are largely equivalent, although there are clear differences in receptor specificities (E8B binding to CD36 and CS2 binding to CSA).

**In vitro analysis of nonopsonic phagocytosis of CSA-binding and CD36-binding PEs.** Primigravid women lack or have limited acquired immune responses to CSA-binding PEs and must generally clear these parasite variants in the absence of opsonizing antibodies. To determine the molecular basis of innate recognition and phagocytic clearance, we examined the nonopsonic uptake of CD36-binding and CSA-binding PEs by human monocyte–derived macrophages and by murine CD36-WT and CD36-KO macrophages. The scavenger receptor CD36 has been identified as a major sequestration receptor for PEs but is also a pattern-recognition receptor that contributes to innate recognition and uptake of apoptotic cells and PEs by monocytes and macrophages [13, 15, 24]. Because CSA-binding isolates are unique in their inability to bind to CD36, we hypothesized that this may enable them to evade innate clearance pathways and contribute to parasite accumulation in the placenta. To test this hypothesis, we examined the phagocytosis of E8B (CD36-binding) PEs and CS2 (CSA-binding) PEs by murine CD36-WT and CD36-KO macrophages. E8B PEs were avidly phagocytosed by CD36-WT macrophages. In contrast, phagocytosis of CS2 PEs by CD36-WT macrophages was significantly reduced (figure 2A). In the absence of CD36, phagocytosis of E8B PEs was reduced by ~90%. Monoclonal-antibody blockade of CD36 inhibited phagocytosis of E8B PEs by CD36-WT macrophages only, whereas trypsinization of PEs resulted in a 70% decrease in the phagocytosis of E8B PEs and a small but significant decrease in the phagocytosis of CS2 PEs by CD36-WT macrophages. Both trypsin-sensitive and trypsin-resistant cytoadherence to CSA have been reported [17] and may partially explain the inhibition of phagocytosis of CS2 PEs by CD36-WT macrophages seen with PE trypsinization. However, these observations may also be attributable to the presence of low residual levels of CD36 binding, which has been observed for CS2 [16] and could also account for why trypsin sensitivity was observed only when CD36 was present. Treatment of macrophages with soluble CSA, chondroitinase ABC, or hyaluronidase did not affect phagocytosis of either E8B PEs or CS2 PEs.

These experiments were extended to examine nonopsonic phagocytosis of E8B and CS2 PEs by human macrophages. As
with murine macrophages, E8B PEs were avidly phagocytosed, whereas phagocytosis of CS2 PEs was significantly less than that of E8B PEs (figure 2B). Phagocytosis of E8B PEs was inhibited by monoclonal-antibody blockade of CD36 and by trypsination of PEs. Furthermore, monocyte-derived macrophages from male and female donors behaved similarly, with both showing a defect in the uptake of CSA-binding PEs. In summary, although nonopsonized E8B PEs were phagocytosed avidly in a CD36-dependent manner by both murine and human macrophages, an innate phagocytic defect for CS2 PEs was observed.

To confirm that our observations were not unique to CS2, the nonopsonic phagocytosis experiments were repeated using another CSA-binding isolate, 3D7-CSA [25], and the results were compared with those for the CD36-binding parental isolate 3D7. As was observed for CS2, phagocytosis of the 3D7-CSA PEs by CD36-WT macrophages was decreased by >90%, compared with phagocytosis of 3D7 PEs (mean ± SD percentage phagocytosis for 3D7 vs. 3D7-CSA PEs, 27.6% ± 7.0% vs. 1.4% ± 0.3% [P = .017]). Human macrophages displayed a phagocytic defect for 3D7-CSA PEs similar to the one they displayed for CS2 PEs. Thus, a general defect in the innate recognition and uptake of CSA-binding PEs was observed.

To examine whether nonspecific adsorption of immunoglobulin to the erythrocyte surface alters the innate phagocytic uptake of PEs, we preincubated E8B and CS2 PEs with nonimmune human serum and then examined the uptake of these PEs by murine CD36-WT macrophages and human macrophages. The phagocytosis profiles were similar to those of nonopsonized PEs. Both murine CD36-WT macrophages and human macrophages phagocytosed nonimmune human serum–opsonized E8B PEs significantly better than did nonimmune human serum–opsonized CS2 PEs (phagocytic index for E8B vs. CS2 PEs, 10.5% ± 4.4% vs. 3.2% ± 1.2% for murine macrophages [P = .040] and 10.6% ± 1.9% vs. 1.2% ± 1.3% for human macrophages [P = .018]). Therefore, even in the presence of nonimmune human serum, a defect in the uptake of CSA-binding PEs was still observed.

**In vivo analysis of nonopsonic phagocytosis of CSA-binding and CD36-binding PEs.** To extend our observations in vivo, we examined nonopsonic phagocytosis of CD36-binding and CSA-binding PEs in SHRs, which harbor a spontaneous mutation that renders them CD36 deficient [26], and in Wistar rats (the parental strain), which express normal levels of CD36 [15]. Highly synchronized E8B or CS2 trophozoite-stage PEs were injected into the peritoneal cavity of Wistar rats and SHRs. Three hours later, the peritoneum was lavaged and phagocytosis of PEs by macrophages was quantified. In agreement with our

**Figure 3.** In vivo phagocytosis of E8B (CD36-binding) and CS2 (chondroitin sulfate A–binding) parasitized erythrocytes (PEs). CS2 PEs evade phagocytic uptake in CD36-expressing Wistar rats and CD36-deficient spontaneous hypertensive rats (SHRs). In vivo phagocytosis assays were performed as described in Materials and Methods; phagocytosis was assessed as the percentage of macrophages with at least 1 internalized PE. In panel A, the black bars represent phagocytosis by Wistar monocytes, and the white bars represent phagocytosis by SHR monocytes. Data are the mean ± SE results of 3 independent experiments. Panel B shows light-microscopy pictures of monocytes isolated from the peritoneum of Wistar rats injected with E8B (top) or CS2 (bottom) PEs. The white arrows indicate internalized PEs; the black arrows indicate noninternalized PEs. *Statistically significant difference (Student’s t test), for the comparison Wistar E8B vs. SHR E8B (P = .0079). †Statistically significant difference (Student’s t test), for the comparison Wistar E8B vs. Wistar CS2 (P = .0059).

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in vitro findings, in the Wistar rats, the phagocytic uptake of E8B PEs was ~6-fold higher than that of CS2 PEs (figure 3). Confirming a role for CD36-mediated phagocytosis in vivo, phagocytic uptake of E8B PEs in the SHR rats was decreased by ~90%. These data confirm and extend our in vitro observations; there was a defect in in vivo phagocytosis of CSA-binding PEs, compared with that of CD36-binding PEs.

**DISCUSSION**

To date, the susceptibility of pregnant women to placental malaria largely has been attributed to the lack of antibodies capable of inhibiting adhesion of PEs to placental glycosaminoglycans, such as CSA. Our observations provide new insights into pregnancy-associated malaria and suggest that, in addition to placental adhesion, the ability of CSA-binding PEs to evade innate phagocytic pathways, particularly in primigravid women, may contribute to the pathogenesis of placental malaria. Using both in vitro and in vivo models, we have demonstrated that, although CSA-binding PEs adhere avidly to target cells, they are phagocytosed at significantly lower levels than CD36-binding PEs. We have also confirmed and extended previous observations that the pattern-recognition receptor CD36 is a major receptor that mediates the uptake of non–pregnancy-associated PEs, and we have provided, via a CD36-deficient and -non-deficient rat model, the first in vivo evidence showing CD36-mediated uptake of *P. falciparum* PEs. Taken together, our data suggest that there is a defect in the innate immune clearance of placental parasites and that this defect may contribute to parasite accumulation in the placenta.

Our findings may also have implications for monocyte recruitment to the placenta in primigravid women with placental malaria. Placental monocytes contribute to altered cytokine profiles and elevated proinflammatory cytokine levels, which may be important mediators of adverse birth outcomes [4, 5, 27–29]. CD36-mediated uptake of PEs, like that of apoptotic cells, has been demonstrated to be noninflammatory and does not contribute to the induction of such inflammatory cytokines as TNF-α, high levels of which are associated with adverse birth outcomes [5, 13, 29]. The inability to engage this noninflammatory pathway to efficiently clear CSA-binding parasites in the placenta may lead to increased malarial hemozoin and glycosylphosphatidylinositol toxin exposure, local activation of monocytic and endothelial cells, cytokine and chemokine secretion, and additional monocyte recruitment [4, 5, 29].

In summary, our data indicate that evasion of innate immunity by placental PEs may play a role in the pathogenesis of pregnancy-associated malaria. A defect in the innate clearance of placental PEs would be of potentially greater consequence in primigravid women and others who either lack or have limited preexisting cytophilic antibodies to CSA-binding PEs and, thus, would be deficient in both opsonic and non-opsonic phagocytosis at the time of infection [12, 30–32]. Furthermore, our observations suggest that, in addition to CSA-adhesion blocking activity, the evaluation of potential VSA-based vaccines for pregnancy-associated malaria should assess the ability of these vaccine candidates to generate cytophilic antibodies that can overcome a defect in innate phagocytic clearance by facilitating opsonic phagocytosis and control of placental parasite burden.

**References**


