

Short communication

Adhesion of *Plasmodium falciparum*-infected red blood cells to CD36 under flow is enhanced by the cerebral malaria-protective trait South–East Asian ovalocytosis

Alfred Cortés^{a,*}, Mata Mellombo^a, Charles S. Mgone^{b,1}, Hans-Peter Beck^c,
John C. Reeder^b, Brian M. Cooke^d

^a Molecular Parasitology Laboratory, Papua New Guinea Institute of Medical Research, Madang, P.O. Box 378, MP511, Papua New Guinea

^b Papua New Guinea Institute of Medical Research, Goroka, P.O. Box 60, EHP441, Papua New Guinea

^c Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Basel 4051, Switzerland

^d Department of Microbiology, Monash University, Vic. 3800, Australia

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Sequestration of *Plasmodium falciparum* infected red blood cells (IRBCs) in the microvasculature is widely accepted to be a major factor contributing to the more severe pathogenesis of *P. falciparum* malaria compared to disease from other human malaria parasites, but little is known about the precise pathogenic consequences of sequestration via particular receptors. In order to better understand the relationship between receptor binding and disease, we studied the cytoadherence properties of IRBCs from individuals carrying the South–East Asian ovalocytosis (SAO) trait, which confers strong protection against cerebral malaria.

Several genetic traits have persisted in human populations because they confer a selective advantage against malaria. Among them, the SAO trait is peculiar because it confers protection against cerebral malaria but not against malaria parasitemia, mild malaria disease or some other complications of the disease, such as severe anemia [1,2]. Although the SAO trait is presumed to be lethal in utero in homozy-

gotes and is seen only in the heterozygous state [3], it reaches a high prevalence in some parts of the Western Pacific, and the presence of this trait correlates with malaria endemicity in Papua New Guinea [3].

The SAO trait is determined by a 27 bp deletion in the gene encoding the most abundant integral RBC membrane protein, band 3 [4]. This protein functions as an anion transporter in the RBC, but also contributes to the maintenance of RBC integrity by anchoring the RBC membrane to the underlying cytoskeleton. The presence of the mutated band 3 results in defective anion transport [5], reduced expression of several RBC antigens [6] and major abnormalities in the morphological and mechanical properties of the RBC, such as an oval shape and markedly increased membrane rigidity [7–9].

While early studies suggested that SAO RBCs were almost completely resistant to in vitro invasion by *P. falciparum* [8,10], others have shown that they can be invaded to at least 50% of the level for normal RBCs [9]. Working with freshly collected SAO RBCs, we found that they are highly resistant to invasion in vitro by some, but not all lines of *P. falciparum* [11]. However, the specific protection against cerebral malaria observed in SAO individuals suggests that the selective advantage conferred by this trait acts via post-invasion processes. Rosetting (the adhesion of IRBCs with one or more uninfected RBCs) has been implicated as a risk factor for cerebral malaria, but SAO RBCs bound to IRBCs as well as normal cells [12], indicating that it is unlikely that

Abbreviations: SAO, South–East Asian ovalocytosis; RBCs, red blood cells; IRBCs, infected red blood cells; TSP, thrombospondin; ICAM-1, intercellular adhesion molecule-1; CSA, chondroitin sulfate A

* Corresponding author at: National Institute for Medical Research, Mill Hill, London NW7 1AA, UK. Tel.: +44 20 8816 2402; fax: +44 20 8816 2730.

E-mail address: acortes@nimr.mrc.ac.uk (A. Cortés).

¹ Present address: The African Malaria Network Trust (AMANET), P.O. Box 33207, Dar es Salaam, Tanzania.

protection against cerebral malaria in SAO operates through the rosetting mechanism. On the other hand, sequestration of IRBCs in the microvasculature of the brain is a major factor associated with the development of cerebral malaria [13], and altered adhesiveness of SAO IRBCs could provide a plausible explanation for the protection observed [1–3].

We first studied the surface of SAO IRBCs to determine if the host RBC membrane showed the well-recognized alterations known to lead to adherence to the vascular endothelium [14]. Freshly collected SAO or normal RBCs were invaded in vitro with *P. falciparum* (3D7-A line) and grown to the pigmented trophozoite stage. Immunofluorescence analysis with antibodies against the conserved cytoplasmic region of the parasite-derived cytoadherence ligand, PfEMP1 [15], revealed that this molecule was present in SAO IRBCs and showed a similar punctuate pattern of distribution to normal IRBCs, which corresponds to PfEMP1 exported to the surface of the IRBC (Fig. 1A, top panel). Pooled human serum from malaria exposed individuals, which recognizes PfEMP-1 in addition to other parasite-derived or parasite-modified antigens on the IRBC surface, also gave a similar pattern of staining between normal and SAO IRBCs, with the majority of the IRBCs showing a strong rim of fluorescence that is characteristic of membrane-associated proteins (Fig. 1A, middle panel). Staining with the monoclonal antibody 1C4, which recognizes a parasite-induced modified form of band 3 [16] that has been postulated to mediate cytoadherence, revealed an identical pattern in SAO or normal IRBCs (Fig. 1A, bottom panel). Furthermore, electron microscopy revealed that SAO IRBCs develop the electron-dense surface protrusions termed knobs that are essential for IRBC sequestration and adhesion under flow (Fig. 1B).

As expected from the presence of PfEMP1, modified band 3 and knob structures at the surface of SAO IRBCs, static (non-flow) binding assays revealed that SAO IRBCs interacted with the major host-derived receptors for sequestration, CD36, ICAM-1, TSP and CSA. Binding assays were first performed with naturally infected RBCs from four SAO and four normal individuals attending a health centre in the Madang North Coast area. IRBCs from SAO or normal individuals bound to TSP, CD36 (either purified or expressed on the surface of CHO cells) and ICAM-1 (expressed on CHO cells) at comparable levels for each receptor in static binding assays (Fig. 1C). The level of binding to the different receptors varied between different isolates, as expected [17]. Binding to ICAM-1, an adhesion trait implicated in the development of cerebral malaria [17,18], was evident in the four SAO isolates, and one of them bound at high level. It is also noteworthy that binding to TSP, which may be mediated by modified band 3 [15,19], occurred in SAO IRBCs at similar level as in control IRBCs.

For a more quantitative comparison of the level of adhesion of SAO and normal IRBCs, we invaded in vitro SAO and normal RBCs with well characterized laboratory-adapted lines of *P. falciparum* and determined their level of binding in static assays. 3D7-A parasites were preferentially used

for these experiments because they invaded SAO RBCs efficiently, whereas invasion of SAO RBCs by several other laboratory-adapted parasite lines and field isolates was highly inefficient [11]. 3D7-A parasites in SAO or normal IRBCs bound at similar levels to TSP (Fig. 1D). Binding to soluble immobilized CD36 was significantly lower in SAO IRBCs ($P < 0.05$), but occurred at similar levels between SAO and normal IRBCs when CD36 was expressed on the surface of CHO cells (Fig. 1D).

Similar binding to TSP and CD36 in SAO or normal RBCs is not a peculiarity of 3D7-A parasites. Although assays performed with other parasite lines had to be carried out at low parasitemias, it was clear that SAO or normal RBCs infected with 3D7-B parasites also bound to TSP and CD36 at similar levels (Fig. 1D), as did the unrelated parasite line E8B (not shown). Additionally, CS2 parasites in normal or SAO cells bound to CSA at similar levels (Fig. 1D). CS2 show high level binding to CSA, an adherence trait implicated in the development of placental malaria [20]. The parasite lines tested did not bind to ICAM-1 at significant levels at the low parasitemias obtained after in vitro invasion of SAO cells.

Static binding assays revealed that SAO IRBCs interact with the major host derived receptors for sequestration, and the level of binding was similar for SAO or control IRBCs. However, the altered cellular mechanical properties induced by SAO band 3 make it highly likely that these IRBCs show a different circulatory behaviour in vivo. To investigate this possibility, we studied the level of adhesion of normal and SAO 3D7-A infected RBCs to CD36 under well-defined conditions of flow that mimic those in the post-capillary venules where sequestration preferentially occurs in vivo [21]. Under these conditions, the level of binding of SAO IRBCs was consistently about twice the level for control IRBCs in seven matched pairs of SAO-control RBCs donors (Table 1). Considering the controlled way in which the experiments were done, the increase can only be attributed to the SAO trait. This unexpected result clearly indicates a shear-dependent increase in the efficiency of IRBC immobilization via CD36 in SAO. We could not study binding of SAO IRBCs to other receptors under flow conditions, because 3D7-A parasites do not bind to CSA or ICAM-1, and despite our efforts to identify a parasite line with significant levels of binding to ICAM-1 at the parasitemias obtained after in vitro invasion of SAO RBCs, which included testing numerous clinical isolates, no such line was identified. All field isolates identified that bound to ICAM-1 at high levels, as well as the ICAM-1 binding parasite line E8B, did not invade SAO RBCs in vitro efficiently enough to perform flow binding assays. Furthermore, some preliminary experiments revealed no major difference between SAO and normal IRBCs in their binding to TSP under flow (data not shown), but this was not studied in detail because under flow conditions the interaction of IRBCs with TSP is highly unstable and occurs at very low levels [22].

Binding to ICAM-1 and most other endothelial receptors varies between different parasite isolates, but most if not all

field isolates (with the exception of parasites that sequester in the placenta) bind to CD36, including those that bind strongly to ICAM-1 [14,17]. Although CD36 is widely believed to be the principal receptor for sequestration of IRBCs [14,17,22], it is virtually absent from endothelial cells in the brain [13,18] and is unlikely to play a major role in sequestration of IRBCs in this organ. Thus, the higher efficiency of sequestration via CD36 in SAO individuals could determine a different organ distribution of sequestered IRBCs, such that fewer parasites would be sequestered in non-CD36 expressing organs like the brain, and preferentially targeted to other vascular beds where CD36 is abundantly expressed, including the microcirculation of non-vital organs and tissues like muscle and skin [18]. CD36-expressing vascular beds might act like a trap for IRBCs outside the brain in SAO individuals. However, the interaction of CD36 with IRBCs is undoubtedly complex. In addition to mediating sequestration, it also plays a role in non-opsonic phagocytosis of IRBCs and in parasite induced

modulation of the immune system [23]. Thus, it is possible that the increased efficiency of the interaction of CD36 with IRBCs in SAO also has an effect on these processes.

Increased sequestration via CD36 in SAO would not alter the distribution of sequestered IRBCs if it was paralleled by an identical increase in sequestration via other endothelial receptors present in the brain. However, it is unlikely that the interactions that mediate sequestration in the brain are affected by the presence of SAO band 3 in the same way as the interaction with CD36. In the brain, sequestration might be mediated by ICAM-1, and/or other receptors such as TSP, VCAM-1, PECAM-1 (CD31), fraktalkine, E-selectin and P-selectin, in addition to CD36 on platelets bridging IRBCs with brain endothelial receptors [24], although the physiological relevance of all these interactions with IRBCs is questionable and not well established [14]. Some of these receptors cannot mediate adherence of IRBCs under flow conditions, whereas others, notably ICAM-1, predominantly mediate rolling in-

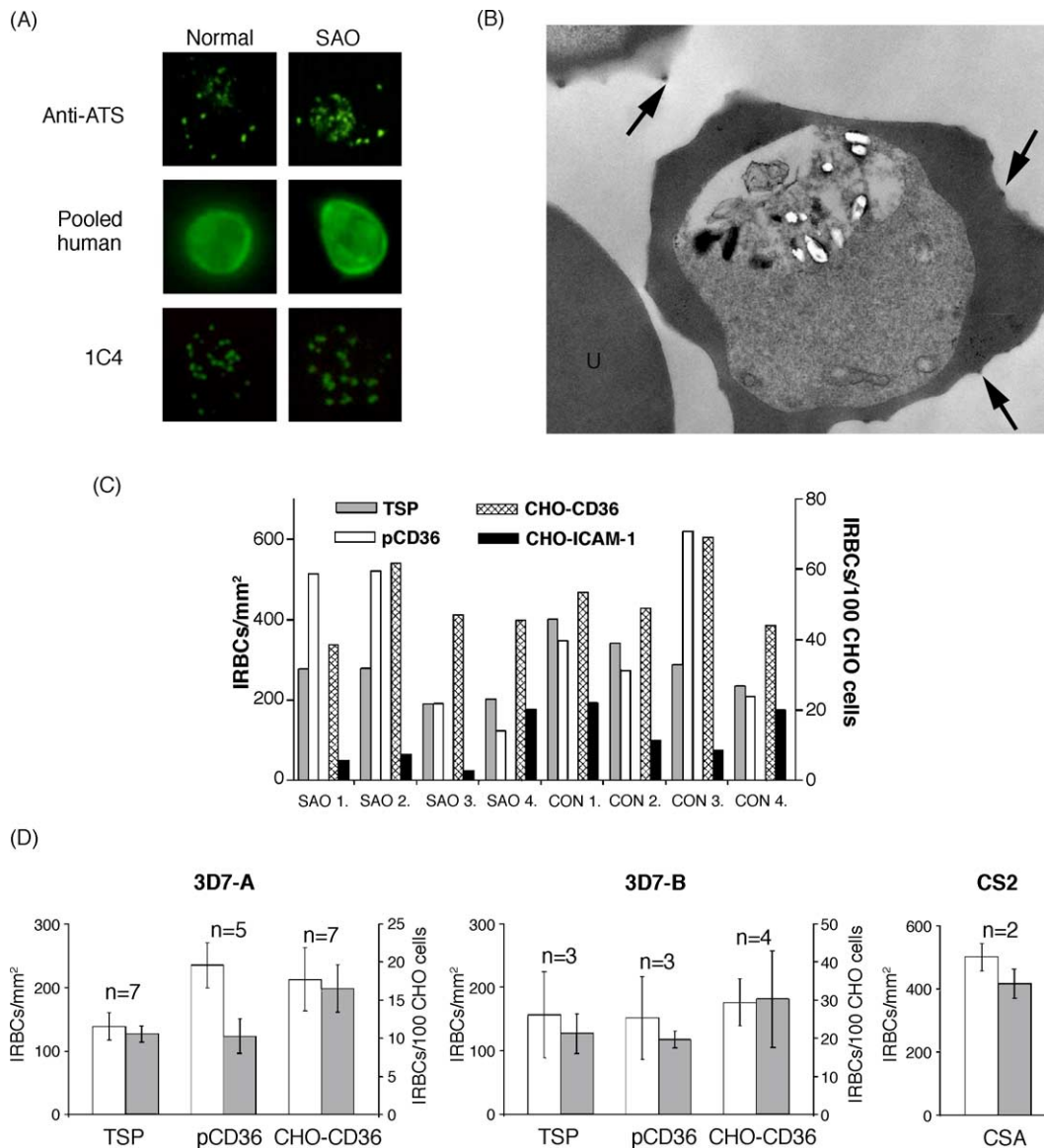


Table 1
Adhesion of flowing SAO and normal IRBCs to CD36.

RBC donor	IRBCs (mm ⁻²)	Binding SAO/binding normal
SAO 1	1403	2.6
Normal 1	541	
SAO 2	1493	2.0
Normal 2	750	
SAO 3	1554	1.9
Normal 3	826	
SAO 4	1377	2.4
Normal 4	575	
SAO 5	1333	2.0
Normal 5	675	
SAO 6	690	2.3
Normal 6	305	
SAO 7	538	2.5
Normal 7	218	

RBCs from seven SAO and seven geography-matched control adult donors were invaded in vitro with 3D7-A parasites. Binding of SAO and control IRBCs was significantly different ($P < 0.05$) by Wilcoxon signed-rank test. All samples were tested at a hematocrit of 1.5%. Samples within each SAO-normal RBC pair were tested at the same parasitemia, which ranged from 4 to 8%. IRBC binding assays were performed under controlled conditions of flow (wall-shear stress of 0.1 Pa) using CD36-coated glass microcapillary tubes as previously described in detail [21]. Under these conditions, no binding of either SAO or normal uninfected RBCs was observed.

teractions, but of all of the vascular receptors described to date to which IRBCs can bind, only CD36 mediates high-level, stable, stationary adherence, at least when acting alone [22]. Thus, sequestration in the brain probably occurs through a different rheological mechanism than sequestration via endothelial CD36, and is unlikely to be affected by the presence

of SAO band 3 to the same extent as direct stationary binding to CD36. Although this could not be proved because it was impossible to study binding of SAO IRBCs to other receptors under flow, an altered balance in the distribution of sequestered parasites between vascular beds where CD36 is present and vascular beds where it is virtually absent, like those in the brain, provides the most plausible explanation to reconcile the observations of protection against cerebral malaria and more efficient immobilization via CD36 in SAO.

The postulated protective effect of high level binding to CD36 against cerebral malaria is also supported by the results of a comprehensive study conducted in Kenya, which found that isolates from cerebral malaria patients exhibited lower adherence to CD36 than isolates from uncomplicated malaria patients [17]. Also in good agreement, a study conducted in Kenya and The Gambia found that mutations causing CD36 deficiency were associated with increased risk of cerebral malaria [25], although another study conducted in Kenya did not confirm this result [26], but found that CD36 truncations were associated with slightly lower risk of severe malarial anaemia. Also in agreement with the hypothesis that high level of binding to CD36 might have a protective effect against cerebral malaria, is the observation that expression of group A *var* genes, which encode a group of PfEMP1 molecules that are unable to bind to CD36, is associated with severe malaria [27].

Several human traits are well known to confer a selective advantage against the development of severe clinical manifestations of malaria, but little is known about the mechanisms by which the protection is achieved. Here, we provide a possible explanation for the selective advantage conferred by SAO against cerebral malaria. Contrary to previous hypothesis that SAO IRBCs might have reduced adhesive interactions

Fig. 1. Characterization of the surface and the static binding of SAO IRBCs. (A) Immunofluorescent staining of 3D7-A-infected normal (non-SAO) and SAO RBCs with affinity purified antibody against the conserved cytoplasmic domain of PfEMP1 (top panels), pooled human sera from five malaria-exposed adults (middle panels) or monoclonal antibody 1C4 against parasite-induced modified band 3 [16] (bottom panels). Immunofluorescence was performed on acetone/methanol (9:1) fixed smears. Parasites were identified inside RBCs by counterstaining with DAPI (not shown). (B) Transmission electron micrograph of SAO IRBCs. The arrows indicate knobs. An uninfected SAO RBC is indicated by "U". Electron microscopy was performed on SAO 3D7-A infected RBCs that were purified in Percol-sorbitol gradients, then fixed in 1.25% glutaraldehyde and processed for electron microscopy following standard procedures. (C) Binding of naturally infected SAO and normal IRBCs to TSP, CD36 and ICAM-1 in a static (non-flow) adhesion assay. RBCs from SAO and normal individuals with a high density *Plasmodium falciparum* infection (>1%) were obtained from uncomplicated malaria patients attending the Alexishafen health centre (Sek, Madang Province, Papua New Guinea) and maintained in culture for 24–30 h until the majority of the parasites reached the pigmented trophozoite stage. Assays were performed at a parasitemia between 1.2 and 3% (SAO) or 1.1 and 4.2% (normal). Binding is normalized to a parasitemia of 1% mature pigmented trophozoites. Binding to purified, immobilized receptors (TSP and pCD36) is expressed as the number of IRBCs bound/mm², and binding to transfected CHO cells (CHO-CD36 and CHO-ICAM-1) expressed as the number of bound IRBCs/100 CHO cells. Duplicate or triplicate spots were used in all experiments. Static binding assays using purified receptors were performed at a 4% hematocrit essentially as described [20], but for assays with purified TSP (ICN), protein was immobilized in Petri dishes at 50 µg ml⁻¹ in Tris-buffered saline (TBS)-1.5 mM CaCl₂, then blocked with the same buffer plus 2% BSA. These TSP IRBC binding assays were performed using RPMI as the suspending and washing medium. Assays with stably transfected CHO cells were performed essentially as described [29], but using formaldehyde-fixed cells grown in hydrophobic circles in tissue-culture dishes. For fast assessment of the SAO status at the health centre, we determined the formation of echinocytes after mounting diluted blood under a coverslip, approximately as previously described [7]. The SAO status of all blood samples used in this study was confirmed by PCR as described before [4]. (D) Static binding of in vitro infected SAO and normal IRBCs. Values are the average of several independent experiments (*n*) with normal (open columns) and SAO (grey columns) RBCs, with 95% confidence interval. Binding is normalized to a parasitemia of 1% pigmented-trophozoites and expressed as IRBCs bound/mm² or IRBCs bound/100 CHO cells, as in C. In all experiments parasitemia was adjusted before the assay between the SAO and the normal culture by addition of non-infected RBCs. RBCs from eight different adult SAO donors and their corresponding geography-matched controls (non-SAO) were used for these experiments. CS2, E8B (FAF-EA8) and 3D7 laboratory-adapted parasites lines have been described previously [21]. 3D7-B [11] corresponds to 3D7 parasites that were selected for binding to ICAM-1 by panning four times in CHO-ICAM-1 cells, and had higher binding to CD36 and ICAM-1 than the parental 3D7 parasites from which they were derived. 3D7-A corresponds to 3D7 parasites obtained from a different laboratory [11]. SAO and normal RBCs were invaded with laboratory-adapted lines as previously described [11].

with vascular endothelium [1–3], we found that they have increased cytoadherence via CD36. Our results suggest that protection of SAO individuals against cerebral malaria might be mediated by an altered distribution of sequestered IRBCs. In addition, the study of the adherent behaviour of these mutant RBCs shed some light on the relationship between receptor binding and disease. Regardless of the mechanism of protection, our important and conclusive result is that in SAO, which protects against cerebral malaria, interaction of IRBCs with all receptors occurs, and binding to CD36 under flow is clearly increased, which challenges the classical view that increased cytoadhesion is always associated with more severe pathology in falciparum malaria. This is an important consideration for the development of new therapeutic strategies aimed to reduce parasite sequestration via CD36 [21,28].

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