Insights into the Cytoadherence Phenomenon of *Plasmodium vivax*: The Putative Role of Phosphatidylserine

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Plasmodium vivax is the most geographically widespread and the dominant human malaria parasite in most countries outside of sub-Saharan Africa and, although it was classically recognized to cause benign infection, severe cases and deaths caused by *P. vivax* have remarkably been reported. In contrast to *Plasmodium falciparum*, which well-known ability to bind to endothelium and placental tissue and form rosettes is related to severity of the disease, it has been a dogma that *P. vivax* is unable to undergo cytoadherent phenomena. However, some studies have demonstrated that red blood cells (RBCs) infected by *P. vivax* can cytoadhere to host cells, while the molecules participating in this host–parasite interaction are still a matter of speculation. In the present overview, we address the evidences currently supporting the adhesive profile of *P. vivax* and, additionally, discuss the putative role of phosphatidylserine—a cell membrane phospholipid with cytoadhesive properties that has been detected on the surface of *Plasmodium*-parasitized RBCs.

Keywords: *Plasmodium vivax*, cytoadhesion, rosetting, eryptosis, phosphatidylserine

INTRODUCTION

Plasmodium vivax is the most geographically widespread and the second most prevalent parasite causing malaria in the world, with about 35% of global population living at risk of infection (1) and an estimated 8.5 million symptomatic cases in 2015 (2). *P. vivax* contributes significantly to malaria cases outside of sub-Saharan Africa, where it accounts for 41% of the cases, of which 65% occur in South-East Asia, 19% in Eastern Mediterranean, and the remaining in Western Pacific (9%) and American (7%) regions (2).

Despite its considerable impact in global public health, *P. vivax* was for long time a neglected parasite. The reasons for this scenario include: the low global prevalence [4% (2)], when compared with the most prevalent and lethal malaria parasite, *Plasmodium falciparum*; the failure to adapt to *in vitro* culture conditions; as well as the classically recognized benign profile of infection (3). However, in the last decade, severe cases and deaths due to *P. vivax* infection have remarkably been reported in all endemic regions, driving the attention of the academic community to the real importance of *P. vivax* (4). Moreover, the occurrence of severe forms of malaria in *P. vivax* infections, such as cerebral malaria and placental malaria, which were previously reported to be exclusively associated with *P. falciparum*, suggests that *P. vivax* can, to some extent, present pathogenic profiles similar to *P. falciparum* (5–8).

It is known that the main pathological phenomenon related to high virulence of *P. falciparum* is the sequestration of parasitized red blood cells (pRBC) to vascular endothelium and placenta, which allows late-stage forms of parasite to evade splenic phagocytosis, while provoking host damage by obstructing blood flow and inducing local pro-inflammatory response (9). Additional factors contributing to the pathogenesis of *falciparum* malaria comprise rosetting of pRBC with non-parasitized red blood cells (nRBC) as well as clumping of pRBC mediated by platelets (10). All these cytoadhesive events of *P. falciparum*-pRBC are recognized to be mediated by a large and diverse family of parasite antigens, named *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), that is expressed on the surface of pRBC and shows affinity to several host receptors, including intercellular adhesion molecule 1 (ICAM-1), platelet-endothelial cell adhesion molecule 1, chondroitin sulfate A (CSA), hyaluronic acid (HA), thrombospondin (TSP), and CD36 (10). Since homologous of *var* genes, which encode PfEMP1, have not been identified in other *Plasmodium* species and the cytoadherence of pRBC was not consistently documented in non-*falciparum* malaria, it was believed that the cytoadherence phenomenon of pRBC was restricted to *P. falciparum* infection (11–14). However, some evidences currently support that red blood cells (RBCs) infected by *P. vivax* (Pv-pRBC) also undergo cytoadherence events, as addressed below in the present paper.

EVIDENCES OF *P. vivax* CYTOADHESION

Since Pv-pRBC lack adhesive knob structure and, especially, because all parasite stages can be observed in the peripheral blood of patients, it has been a dogma that *P. vivax* lacks the ability to cytoadhere and, therefore, to sequester. However, in 2010 it was demonstrated, for the first time, that Pv-pRBC are able to cytoadhere *in vitro* to host cells (15). In this study, Pv-pRBC obtained from Brazilian non-severe patient samples were tested by static and flow cytoadhesion assays using human lung endothelial cells (HLEC), monkey brain endothelial cells, and human placental cryosections. Although the number of Pv-pRBC adhered under static conditions was lower than that observed for pRBC infected by *P. falciparum*, the strength of interaction with endothelium was similar. Moreover, it was shown using transfected Chinese Hamster Ovarian (CHO) cells that the binding of Pv-pRBC to ICAM-1-expressing cells was twice as high as to non-transfected cells or CD36-expressing cells and that the adherence to placental cryosections and HLEC was inhibited by soluble CSA, suggesting involvement of both ICAM-1 and CSA in adhesive processes of *P. vivax*. In fact, the adhesive capacity of Pv-pRBC to HLEC and the involvement of ICAM-1 were later recorded in studies using *P. vivax* isolates from Colombia (16). Moreover, a treatment with chondroitinase reversed the adherence of *P. vivax* isolates from the Asia-Pacific region to immobilized CSA, while it was additionally observed that hyaluronidase disturbed Pv-pRBC adhesion mediated by HA (17). Nevertheless, the degree of commitment of each host adhesive receptor studied until now is still a matter of speculation. For instance, in the study with Thai patients described above, all *P. vivax* isolates were adherent to immobilized CSA and HA, but none adhered to ICAM-1 (17),

and when *P. vivax* isolates from Brazilian Amazon region was evaluated, a low frequency of pRBC adhesion to ICAM-1 and CSA was observed (18).

Corroborating the adhesive profile of *P. vivax*, it was recently reported that the schizont stage was absent in the peripheral circulation in more than half of Brazilian patients diagnosed with *P. vivax* malaria by blood smears and, even when *P. vivax* schizonts were detected, they were mostly present at low frequency (19). Moreover, *in vitro* maturation of *P. vivax* isolates provided a greater ability of Pv-pRBC to cytoadhere to HLEC than the same isolates before maturation, revealing a higher adhesive capacity of mature forms. These data indicate that *P. vivax* might be sequestered in the deep vasculature and that maturation of late stages of *P. vivax* occur outside peripheral circulation. Actually, more than 50 years ago sequestration of Pv-pRBC was proposed by Field et al. (20), who showed a disappearance of schizonts from the peripheral blood of a *P. vivax* patient. In addition, recent discoveries showing accumulation of *P. vivax* schizonts and gametocytes in the bone marrow (21); detection of a large number of intact Pv-pRBC in the spleen (22); and presence of Pv-pRBC within pulmonary microvasculature from a patient with negative blood smear at the time of death (23) support the hypothesis that *P. vivax* has the ability to sequester.

Although *P. vivax* does not present any protein homologous to PfEMP1, a group of variable proteins (VIR proteins) is expressed by this parasite species (24). In contrast to PfEMP1, VIR proteins are not clonally expressed and can additionally be found within pRBC, indicating initially that these *P. vivax* antigens have different functions from PfEMP1 ones (25). However, based on their variant nature and presence on pRBC surface, the role of VIR antigens in Pv-pRBC adhesion has been evaluated. Thus, computational analysis using a *P. falciparum*-based algorithm revealed putative adhesive protein motifs in VIR proteins (26), which could explain the capacity of *vir* gene (VIR-14) to mediate adhesion of pRBC to ICAM-1 when transfected into a non-adhesive *P. falciparum* line (27). Also, consistent with the participation of VIR proteins in the sequester phenomenon of Pv-pRBC, it was previously demonstrated that antibodies against variants of VIR proteins (VIR-A4 and VIR-E5) partially inhibit adhesion of Pv-pRBC to HLEC (15).

Therefore, there is now a growing body of evidence supporting that *P. vivax* parasites possess adhesive phenotypes. Indeed, besides adhesion to endothelium and placental tissue, it is known that *P. vivax* has the ability to form rosettes, which are defined by the binding of a pRBC with two or more nRBC. Rosetting formation in *P. vivax* infection was described more than 20 years ago (28) and has been shown to be more frequent than in *P. falciparum* infection (29, 30), but few studies have been conducted to investigate this *P. vivax* phenomenon; largely due to the absence of a *P. vivax* continuous culture method. Notwithstanding, it was already demonstrated that rosettes in *P. vivax* infection are formed by interaction of pRBC containing trophozoites, schizonts, or gametocytes with mature RBCs (normocytes), a process that involves glycophorin C receptor present on nRBC surface (30). Furthermore, *P. vivax* rosettes were shown to be stable even under high physiological shear stress and rosette formation was closely associated with induction of an increased rigidity of Pv-pRBC,

possibly contributing to sequestration of *P. vivax* in the microvasculature (31). However, differently from *P. falciparum*, both host and parasite antigens intricate on *P. vivax* rosetting as well as the relation of this adhesive phenomenon to the pathogenesis of *vivax* malaria remain unknown.

THE ROLE OF PHOSPHATIDYLSERINE (PS) IN CYTOADHESIVE PHENOMENA

While some research efforts have been dedicated to identifying *P. vivax* antigens participating in cytoadhesion of pRBC, little attention has been given to host RBC factors that have adhesive potentiality, such as PS. PS is a cell membrane phospholipid usually restricted to the inner leaflet of the lipid bilayer (32), but during apoptotic cell death processes PS is exposed on cell surface, promoting recognition and clearance of dying cells by phagocytes (33). Externalization of PS also occurs in activated platelet and transiently in activated lymphocytes and mast cells, where it is associated with procoagulant activity, homing to sites of inflammation and cell degranulation, respectively (34–36). Furthermore, it has been shown that the presence of PS on external leaflet of cell membrane is a hallmark of suicidal erythrocyte death, named eryptosis (37).

Eryptosis occurs in senescent RBC and can precociously be triggered by a variety of endogenous and xenobiotics stimuli (38, 39). Similarly to apoptosis of nucleated cells, eryptotic processes are characterized by many morphological and biochemical changes, i.e., Ca^{2+} influx, cysteine protease activity, PS exposure, cell shrinkage, and plasma membrane microvesiculation, with externalized PS rendering RBC susceptible to clearance by splenic phagocytes (40). Accordingly, overinduction of PS-exposing eryptotic RBC is believed to contribute to the development of anemia related to several clinical disorders, as reported in both experimental and human malaria (41, 42). But, additionally, PS on RBC surface is also considered one of the factors responsible for thrombo-occlusive events in pathologies such as sickle cell disease, chronic renal failure, retinal vein occlusion, and diabetes; in part, by mediating RBC adherence to endothelium as well as cell aggregation (43–46).

Indeed, adherence of PS-exposing RBC to endothelium is observed *in vitro* under dynamic flow conditions mimicking venular wall shear stress (47, 48) and takes place through interaction of PS with the scavenger receptors CD36 or CXC chemokine ligand 16 (CXCL16) expressed on endothelial cell membrane, as well as with TSP, which is found in the basement membrane and extracellular matrix of endothelium and that can be exposed by vascular injury (45, 48, 49). Moreover, soluble plasma TSP can interact with CD36 and, in this manner, could operate as a bridge to adherence of PS-exposing RBC (50, 51). Such interactions involving PS, CD36, TSP, and CXCL16 have also been involved in RBC-platelet aggregation, agreeing with the presence of CD36 and CXCL16 in platelet membrane (52–54). Thus, if parasite antigens able to provide pRBC adhesiveness are absent, *P. vivax* could explore host adhesive molecules to mediate cytoadhesive events of pRBC.

Interestingly, it has previously been demonstrated that intraerythrocytic plasmodia development progressively induces PS exteriorization on pRBC, with larger exposure at the late

stages of parasite maturation (55, 56), which possibly result from eryptosis stimulation. Schizogonic process is described to activate non-selective cation channels in host pRBC membrane, allowing the entry of Ca^{2+} necessary for parasite intracellular growth, which, in turn, leads to the activation of phospholipid scramblases responsible for PS exposure (57). Although PS externalization has not been evaluated in *P. vivax* infection, it was already detected by flow cytometry in RBC infected by *P. falciparum*, *P. berghei*, and *P. yoelii* (41, 58, 59) and in *P. falciparum*, the binding of late-stage pRBC exposing PS to CD36-expressing cells as well as immobilized CD36 and TSP was inhibited by annexin V, PS-containing liposomes or glycerophosphorylserine—a soluble form of PS (60), indicating that PS could, at least in part, support cytoadhesive phenomena of pRBC in malaria. Consistent with this possibility, a relationship between cytoadhesive activity and PS exposure was also reported when knobby and knobless *P. falciparum* strains, which differentially induce PS externalization on late-stage pRBC, were studied (60) and, more recently, it was also shown that PS-expressing RBC can operate as nuclei for RBC aggregation induced by *P. falciparum*-conditioned medium (61).

Importantly, studies conducted on *P. berghei* ANKA experimental infection with CD36-deficient rodents have demonstrated that CD36 is an essential receptor for sequestration of schizont-stage pRBC, which occurred mostly in the capillaries of lungs and adipose tissue, but not in the brain, where endothelial expression of CD36 is low or absent (62). Indeed, besides being incriminated in acute tissue injury induced by *P. berghei* ANKA-pRBC accumulation in lung (63), CD36 is known as an important receptor mediating pRBC sequestration, non-related to brain and placental tissue in *P. falciparum* malaria (64), and its expression on surface of platelets and RBC has been implicated in clumping and rosetting processes of *P. falciparum*-pRBC, respectively (65, 66). However, it is noteworthy that, in contrast to *P. falciparum*, which expresses the adhesin PfEMP1, but similarly to *Plasmodium chabaudi*, whose late-stage forms undergo CD36-dependent cytoadhesion *in vitro* (67), no putative parasite ligand for CD36 has been identified in genome of *P. berghei*, or even other species of *Plasmodium* displaying cytoadherence phenotypes, such as *P. vivax* (14, 68), reinforcing the premise that alternative pathways, not based on the expression of parasite adhesins, could mediate CD36-dependent cytoadhesion of late-stage pRBC.

Additional evidences for this proposition are also documented in *P. vivax* malaria. First, a reduction in Pv-pRBC adhesion to HLEC was achieved in the presence of anti-CD36 antibodies, although the small number of samples limited the statistical analysis concerning extension of CD36 participation in *P. vivax* cytoadhesion (15). Second, while studying cellular trafficking and the adhesive propriety of *P. vivax* VIR proteins in *P. falciparum* transgenic lines, it was shown that only one variant of VIR proteins (VIR-14) was exposed at the surface of pRBC, mediating cytoadherence to CHO cells through ICAM-1, but not CD36 (27). Thus, it is tempting to speculate that if antigens encoded by *vir* genes participate in Pv-pRBC adhesive events, it seems that it does not take place through a CD36-dependent mechanism, in which PS could play a role (**Figure 1**). In view of this possibility,
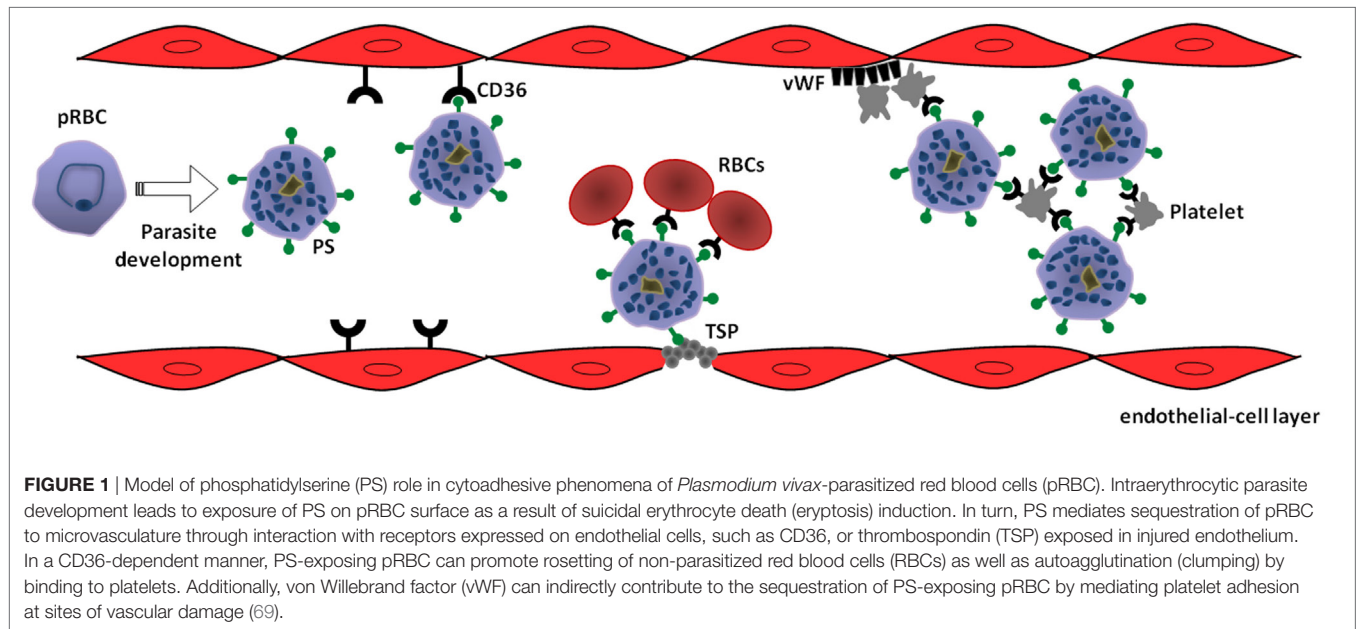
FIGURE 1 | Model of phosphatidylserine (PS) role in cytoadhesive phenomena of *Plasmodium vivax*-parasitized red blood cells (pRBC). Intraerythrocytic parasite development leads to exposure of PS on pRBC surface as a result of suicidal erythrocyte death (eryptosis) induction. In turn, PS mediates sequestration of pRBC to microvasculature through interaction with receptors expressed on endothelial cells, such as CD36, or thrombospondin (TSP) exposed in injured endothelium. In a CD36-dependent manner, PS-exposing pRBC can promote rosetting of non-parasitized red blood cells (RBCs) as well as autoagglutination (clumping) by binding to platelets. Additionally, von Willebrand factor (vWF) can indirectly contribute to the sequestration of PS-exposing pRBC by mediating platelet adhesion at sites of vascular damage (69).

studies evaluating the occurrence of PS externalization in pRBC from *P. vivax* isolates as well as the effect of blocking PS-CD36/TSP interaction on adhesive phenomena of Pv-pRBC may help to confirm the involvement of PS in *vivax* malaria.

AUTHOR CONTRIBUTIONS

PT and SL wrote the paper. Both authors read and approved the final version of the manuscript.

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