ROLE OF CD44 PROTEIN EXPRESSED BY SYNCYTIOTROPHOBLAST CELLS IN THE PATHOGENESIS OF MALARIA DURING PREGNANACY

by

SIMON ODERA OWINO

(Under the Direction of Julie M Moore)

ABSTRACT

Pregnant women continue to suffer from serious complication due to malaria, particularly worse in first time pregnancies. Susceptibility to infection is due to *Plasmodium falciaprum* phenotype that expressing adhesive protein VAR2CSA able to interact with placental chondroitin sulfate A (CSA) on fetal cells, syncytiotrophoblast (ST). The resulting sequestration of infected red blood cells (iRBC) in the intervillous space is associated with placental malaria pathology, marked by accumulation of inflammatory cells and damage of the ST. While it is know that ST-adherent iRBC stimulate immune activation of ST, it is yet unclear and remains necessary to determine the signaling molecule on ST as well as the synergistic role other cells, such as monocytes play in mediating immune response. In this study, we explored these question using primary trophoblast cells, a placental choricarcinoma cell line and a monocytic cell line. Here we determined the role of proteoglycan molecule, CD44 in promoting binding of ST-adherent iRBC and the downstream stimulatory of ST via Src kinase family of proteins. Also, we determined role of Toll-like receptor 2 (TLR-2) in monocyte cells and ST cell stimulation,

marked by secretion of interleukine-8 (IL-8) cytokine and determined that iRBC profoundly affect the immune activation of these cells, addatively on ST and negatively on monocytes

Taken together, these results suggest that during placental malaria, CD44 plays an important receptor and stimulation function on ST by providing CSA ligand for ST-adheren iRBCs and that the ST stimulation is to some degree orchestrated by Src kinases. Also, monocytes and ST cells TLR-2 response is differentially influenced by iRBCs, positively and negatively for ST and monocytes respectively.

INDEX WORDS: Placenta, Malaria, Syncytiotrophoblast, Src kinase, CD44, Proteoglycan, Chondroitin Sulfate A, Pregnancy, Monocytes, Toll-like receptors

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DEDICATION

I dedicate this work to Elizabeth, Sarah and Sean. This has come to fruition because of you and I did not have to think of this. It would be a gross understatement if I were to say that this was my sole effort, but rather, I would like to resound the enormous sacrifice and effort that my dear wife and best friend, Elizabeth, put in the years to ensure that I get this far. To our beloved children Sarah and Sean, thank you so much for being patient with dad and also for being great company to mum. This was more a family business than my own.

SHUKRAN NA ASANTE SANA!

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CHAPTER 1

INTRODUCTION

Epidemiological studies estimate that malaria accounts for nearly 250 million clinical cases annually, claiming approximately 700 thousand lives, and imposing an enormous impact on human health. Partial immunity is developed only over years of exposure, leaving children mainly under the age of 5 years susceptible. While this partial immunity does not provide sterilizing protection, it is adequate to reduce disease severity. Pregnant women, however, are unique, since irrespective of prior exposure and immunity, they become extremely susceptible to infection. This is mostly severe during their first pregnancy, with better outcome in subsequent pregnancies. The renewed virulence emanates from the mature late stages of *Plasmodium falciparum*, known to sequester in host microvasculature where infected red blood cells (iRBCs) adhere to host endothelium and escape clearance in the spleen. This cytoadhesion phenomenon is key to development of pathology associated with malaria. In particular, placental malaria (PM) is due to emergence of a parasite phenotype capable of binding to chondroitin sulfate A (CSA), a sulfated glycosaminoglycan, secreted from and found at the surface of specialized fetal epithelial syncytiotrophoblast (ST) cells in contact with maternal placental circulation. A parasite protein, Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP-1), exported to the surface of iRBCs, is the principle CSA ligand. It is currently estimated that up to 200,000 infants die annually due of consequences of PM. Premature delivery, maternal anemia and low birth weight are commonly outcomes associated with PM. The underlying biological interactions promoting this susceptibility to malaria during pregnancy and the associated poor birth outcomes remain

largely unknown, but hypothesis pointing toward involvement of cellular proteoglycan (PG) molecules have been advanced.

Proteoglycans are macromolecules made up of protein cores decorated with sulphated sugar polymers, glycosaminoglycans. These molecules have a number of biochemical functions, including coagulation, cell-cell adhesion and maintenance of cell matrix integrity. To date, other than describing the sugar component promoting binding to iRBCs, the role of proteoglycans bearing CSA in the pathology of PM has not been assessed. Considering the nature of CSA on ST, it is, therefore, important to investigate protein cores with which it is associated and assess the role they play in the placental response to malaria infection. Studies have demonstrated receptor as well as signaling properties of proteoglycans (PG) molecules on cells. The role of thrombomodulin, a CSA bearing PG, in coagulation has been clarified and aggrecan has been hypothesized as promoting iRBC binding; and engagement of CD44 PG has been shown to result in CD4+ T cell activation. Moreover, engagement of CD44 by hyaluronan, its principal ligand, has been shown to result in changes of the cytokine secretion profile of cells, marked by production of tumor necrosis factor (TNF), a cytokine involved in systemic inflammation. The dual role of CD44 PGs as receptor and signaling molecule on other cells, provide precedence for us to study its role on ST during PM (since this still is largely underappreciated). Furthermore, given reports of the gravidity dependent increase of CSA in placenta during PM, and the fact that the effects of PG abundance on binding of infected erythrocytes on PG has not been exhaustively explored, prioritizes stidying these PG molecule. PG such as CD44 is an integral component of ST cells plus these could not only be important as iRBCs receptors but also have significant influence in the ST cell response to iRBCs and malarial toxins, as well. Therefore it remains critical to provide an in depth understanding on whether changes in CSA abundance are infection

driven or due to host genetic differences. We address this problem using our established cell culture model to provide the vital information on molecular interactions between iRBCs and the ST. This study mainly addressed CD44-PG receptor functions and immunological consequences of engagement on ST cells, both pivotal for understanding regulation and function during PM. By demonstrating the role of CD44-PG in promoting iRBC binding to and stimulation of ST cells, we provide critical information promoting further understanding of parasite/host interaction during PM. Additionally, we provide knowledge on the course of pathology associated with malaria infection during pregnancy.

Also, we assess the role of malaria toxin, glycosylphosphatydlyinositol (GPI) in driving innate immune responses on ST cells as well as monocytes and the overall impact on the microimmune environment.

To decipher the role of CD44 PG during PM and further expand our understanding of the immunologic response of ST to malarial infection, we followed these four objectives.

(1) Establish CD44 as a CSA bearing receptor for iRBCs on ST cells

(2) Establish CD44 expression variability as a function of exposure to malaria, and

(3) Determine the functional significance of CD44 identified in Aim 1 in syncytial cell responses to iRBCs and malarial toxins.

(4) Examine the immunologic impact of interaction of ST with malarial components known to be released from iRBCs.

Significance

Malaria pathophysiology is associated with host immunologic responses. To date, little is known about the CSA-bearing PGs that mediate ST-iRBC interactions or how binding of infected erythrocytes to placental ST CSA may influence net PG core protein abundance and function. Without detailed understanding of how host (i.e., ST) and parasite interact and respond to each other, especially in terms of development, maintenance and modulation of cellular responses in the pregnant uterus, therapeutic methodologies for management of malaria during pregnancy cannot be fully realized. A detailed understanding of interaction between infected erythrocytes, CSA and CSA-bearing PGs on ST is fundamental. The systems developed through this study provide a stage upon which concise study of PG core proteins involved in placental malaria is feasible. Only through characterization of these protein cores will it be possible to fully understand proteins involved in CSA expression on ST cells, and functional changes induced by iRBCs engagement of CSA. Subsequently, this information will provide critical information relevant in understanding of the biology of the maternal/fetal interface in context of PM. This study assessed the extent to which the ST response is influenced by CD44 in response to engagement of CSA moieties on this cell surface PG by CSA adherent iRBCs, as well as effect of malaria components such as hemozoin (malaria pigment) through a series of biochemical and immunological investigations. The findings from this study demonstrate that ST CD44 proteoglycan is an integral part of ST cell response to iRBCs and malarial toxins and that abundance of protein cores of CSA bearing glycan are influenced to some degree by PM, and that these protein cores may dictate cellular phosphorylation patterns that account for immunologic activation of ST cells.

This study also enhances our understanding of general biology of maternal/fetal interface important for reproductive biology in general and other infectious diseases.

Hypothesis:

Our overarching hypothesis for the proposed research is that CD44-PG is a functionally relevant receptor for iRBCs on ST cells during PM. This study is designed to advance knowledge in the field of malaria pathogenesis and glycobiology, providing better understanding of parasite-host interaction, key for promoting development of tools important for improving overall quality of health of pregnant women and their unborn children. Our experimental model included an array of molecular and cellular biology techniques applied on both trophoblast cell line (BeWo) and primary placental cells from both malaria naïve and exposed populations. To test our hypothesis and achieve overall objectives, the following three specific aims were pursued:

SPECIFIC AIM 1: Establish CD44-PG as a CSA expressing receptor on ST cells, capable of interacting with iRBCs.

Working hypothesis 1: Binding of iRBCs to CSA moieties on ST cells is directly influenced by availability of CD44-PG.

These hypotheses were addressed by experimentally asking the following questions:

1. Will CSA moieties on CD44-PG promote iRBC binding on ST membrane?

2. What is the consequence of knocking out CD44 on STs in terms of iRBC engagement to CD44-PG on ST cells?

SPECIFIC AIM 2: Determine to what extent malaria infection influences abundance of CD44 protein and its effect on placental ST cells function.

Working hypothesis 2: Disease status will influence CD44 protein abundance.

Working hypothesis 3: Engagement of CD44-PG on ST cells results in cellular activation.

These hypotheses were addressed by asking the following questions:

3. Will ST cells with CD44 knocked down express marked difference in tyrosine phosphorylation profile post exposure to iRBCs?

4. Will women in endemic areas have differential CD44 protein expression?

SPECIFIC AIM 3: Determine to what extent a parasite toxin like malarial GPI might influence ST and its immediate immune environment

Working hypothesis 4: Engagement of the GPI receptor, TLR2, will immunologically activate both ST and monocytic cells

Working hypothesis 5: Activation of monocytic cells via TLR2 will influence cell surface phenotype.

These hypotheses were addressed by asking the following question:

5. Will ST cells and monocytes immunologically respond to a GPI analog, Pam3C SK4, in terms of chemokine secretory response and expression of CD44?

In summary, defining the role CD44 PG plays in anchoring iRBC and influencing ST cellular response to malaria infection offers critical information in understanding malaria pathogenesis.

CHAPTER 2

LITERATURE REVIEW

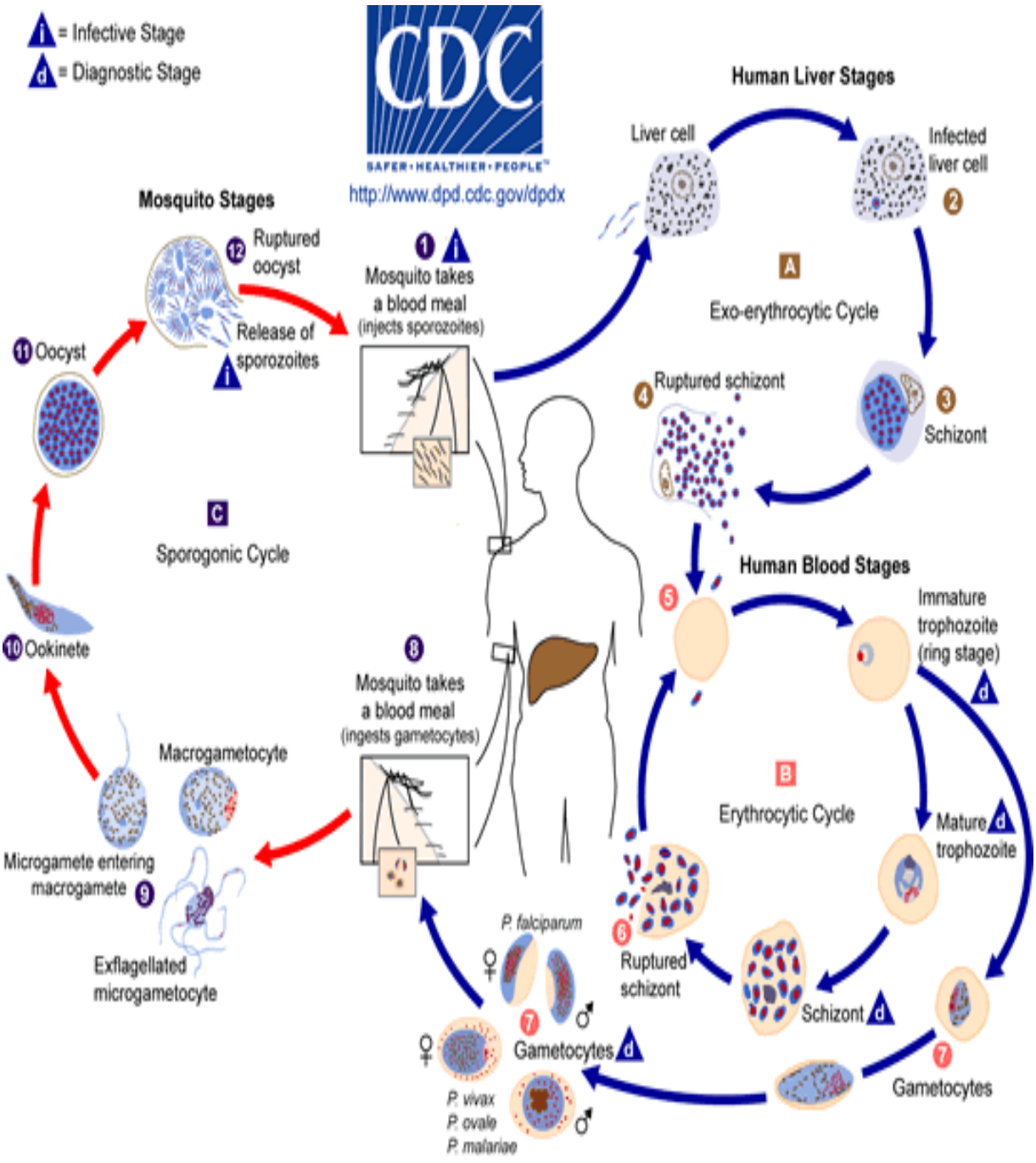
MALARIA

Life cycle of the malaria parasite

Malaria is a mosquito-borne disease mostly confined to the tropics and caused by a protozoan parasite of the genus *Plasmodium*. Five species are known to cause disease in humans: *Plasmodium falciparum* is by far most virulent, causing lethal infection. Other parasites, *P. ovale, P. vivax, P. malariae* and lately *P. knowlesi* also cause disease in human beings [1, 2].

The malaria parasite has a complex multistage life cycle divided between an insect vector and the human host [1]. Infection with malaria occurs when a person receives an infective bite from a female Anopheles mosquito. During a blood meal, the infected female mosquito injects microscopic sporozoites into the human blood stream. These sporozoites target quiescent liver cells where they rapidly divide, undetected, and transform into schizonts,that undergoes replication and mature into merozoites. After this stage, the merozoites burst out of the hepatocytes. The merozoites invade red blood cells where they transform into asexually reproducing trophozoites, then schizonts, which mature to new merozoites infective to erythrocytes or gametes that will be taken up by another mosquito during a blood meal, thus ensuring continuation of the life - cycle and survival of the parasite [3]. This sequential invasion by the parasite during the erythrocyte stage coincides with symptoms associated with infection which include: nausea, fatigue, joint pain, fever and vomiting, followed by anemia due to excessive destruction of red blood cells [4] (see figure 1 below).

Figure 2.1. Life cycle of malaria parasite



Parasite/host interaction during malaria infection

The global malaria burden is huge, approximately 2.2 billion people are exposed to *P. falciparum* annually and roughly 149 to 274 million estimated clinical cases reported are attributed to this parasite. The majority of reported deaths occur in sub-Saharan Africa, mostly among children under five years of age [4, 5]. In addition, pregnant women, especially those in their earlier pregnancies, are at elevated risk of infection despite previously acquired anti-malarial immunity [6-8].

The classical response to malaria infection involves filtration of damaged, malaria-infected cells by the spleen and to some extent, macrophage phagocytosis of iRBCs [9, 10]. Ultimately, effective parasite clearance is dependent on early detection of iRBCs by antigen presenting cells, triggering both cellular and humoral immune responses. An inflammatory-type immune response, championed by monocytes, promotes production of high levels of interferon gamma (IFN-γ) and tumor necrosis factor (TNF), both cytokines required for amplifying the inflammatory signal, leading to recruitment of a large number of immune cells to sites of iRBC cytoadhesion and enhanced phagocytosis [11]. Furthermore, sequential bursting of iRBCs and ensuing cellular debris impose a filtration problem in the spleen, hence increases in spleen size, i.e splenomegaly [12-15]. During synchronized iRBC rupture, released merozoites re-infect other erythrocytes and malarial toxins, such as malaria parasite pigment, (hemozoin; Hz) and parasite glycosylphosphatidylinositols (GPIs) released from bursting iRBCs, together act to stimulate increased cytokine and chemokine secretion by immune cells, often associated with spikes in fever and joint pains experienced during infection [16-22]. These inflammatory responses, though important for parasite clearance, left uncontrolled, often lead to tissue damage, especially in immune privileged organs such as brain and placenta [23-25].

Epidemiology of Malaria

Malaria infection is governed by many factors; patient's age and immunity are most important [26, 27]. Malaria endemicity, described as transmission rate per year has been characterized based on disease prevalence. Regions experiencing malaria transmissions all through the year with seasonal surges are termed hyper-endemic, while mesoendemic areas experience seasonal malaria outbreaks. In hyper endemic regions, adults acquire partial immunity to the parasites characterized by their T cells having high potency in secreting interferon gamma (IFN-γ) in response to parasite antigens, and production of anti-parasitic antibodies, both phenomena important in fighting malarial infection [28]. Also, pregnant women in these areas have been shown to be able to make pregnancy specific anti- iRBC adhesion antibodies during PM, with multigravid women producing more antibodies [29-32].

Clinical Manifestations of Malaria

Malaria is characterized by a combination of different signs and symptoms, dependent on infective species, the geographical setting, and an individual's level of immunity [33]. When a person is first infected, parasites establish in hepatocytes, an asymptomatic stage which lasts between seven to fourteen days. As merozoites burst out of the liver cells to infect erythrocytes, antigens released by the parasites trigger host immune responses marked by a surge of type I cytokine production, known to cause fever. As mature late stage parasites rupture from iRBCs, immunogenic malaria antigens are released that evoke secretion of TNF and IFN cytokines by macrophages and T cells, both cytokines responsible for causing cycles of fever, a common presentation during malaria infection, accompanied by headache, aches and general malaise. Other severe complications may present singly or in combination: cerebral malaria due to

accumulation of iRBC in the brain, anemia, renal failure, hypoglycemia, fluid electrolyte and acid based disturbance, pulmonary oedema, circulatory collapse and shock [33]. Prolonged malaria infection may lead to anemia (hemoglobin <50g/L or hematocrit <15%) due to continual destruction of erythrocytes, and cytoadhesion of iRBCs results in cerebral malaria, and PM in pregnant women (discussed later).

Control and Treatment of Malaria

Over the years, the fight against malaria has taken a number of approaches that include mass drug administration, environmental sanitation, application of insecticides and most recently, emphasis on use of insecticide-treated bed nets. Although promising results have been realized, global eradication of malaria poses a huge challenge, mainly due to widespread development of drug resistance by the parasite and insecticide resistance by the host vector, the mosquito [34]. The problems have led to a shift of focus, gravitating towards development of vaccines that will hopefully confer lasting immunity, especially in risk groups such as children, non-immune individuals and pregnant women. Vaccines have been argued to be the most reliable and cost effective strategy for reducing the burden of malaria disease [35-37], as is the case with other diseases. For all these noble approaches to be realized, an in-depth understanding of basic parasite/ host interaction and immunology is paramount.

Host ligands utilized by the parasite

The Plasmodial parasite, it seems, has successfully coevolved with its host and acquired ability to exploit not only surface receptors on erythrocytes, but also other host receptors on endothelial and epithelial cells. For instance, glycophorin A, a major carrier of red cell sialic acid

residue, is exploited by merozoites during entry into red blood cells [38]. Once established in the red cell, the parasite synthesizes proteins that modify the iRBC membrane [39-42]. As such, parasite gene products are exported to the iRBC membrane, a mechanism associated with parasite virulence that promotes binding of iRBCs to host ligands on micro vesicles and epithelium, impeding clearance of iRBCs. The *P. falciparum* genome contains a family of highly polymorphic genes, *var*, that encode *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), important for cytoadhesion of iRBCs to host receptors on microvasculature, an evasive mechanism for escaping splenic clearance [42, 43]. This interaction leads to sequestration of iRBC containing late stage parasites in distal organs such as brain and placenta [44, 45]. Cells in these organs are known to express adhesion receptors suitable for parasite binding. For example, multiple receptors such as CD36 and Intercellular adhesion molecule-1 (ICAM-1) promote iRBC adhesion [46] to the endothelium in microvasculature. In fact, CD36 has been associated with severe malaria in children [47] and polymorphisms in CD36 are deemed protective against cerebral malaria [48]. In the placenta, CSA, a glycosaminoglycan on placental epithelial ST cells, is the principle iRBC ligand [44, 45, 49], an interaction that ensures parasite survival, promoting chronic infection characterized by a host of undesirable immune responses and culminating in pathological consequences often with increased disease burden in pregnant women.

As a defense mechanism, certain mutations related to erythrocytes, only common in malaria endemic region, have been noted. These mutations have been shown to impede parasite entry and or survival in the red cells, as well as preventing cytoadhesion of iRBCs, conferring protection against malaria in a poorly understood manner [50-55]. For instance, sickle cell anemia, a disorder in red cells, limits ability of erythrocytes to carry oxygen and will at the same

time limit parasite infectivity [52, 53, 55, 56]. Another example involves deficiency of the enzyme glucose -6- phosphate dehydrogenase; heterozygous carriers seem to be more protected against malaria caused by *P. falciparum* [57, 58]. And recently, these abnormalities were found to impair cytoadherence of iRBCs, an important event in both parasite survival and malaria pathogenesis in humans [59]. Other abnormalities have also been reported, namely alpha and beta- thelassamieas, where the presence of hemoglobin S has been shown to impair the ability of *P. falciparum* to thrive in the erythrocyte by reducing oxygen tension [60, 61]. In addition to these mutations, T cells from individual in malaria endemic regions have been shown to secret high levels of inflammatory cytokines, mainly IFN-γ and TNF, both important for parasite clearance [62]. These individuals also, as a protective measure, produce high amounts of antibodies that opsonize iRBCs, promoting phagocytosis and preventing cytoadhesion, as well [63-65]. Put together, these defenses, natural or acquired, improve disease resistance in malaria endemic regions.

MALARIA AND THE IMMUNE SYSTEM

P. falciparum infection, as described above, follows a complex but predictable pattern of movement from the liver, the site of initial infection, to RBCs, with intervening brief windows of extracellular circulation before a new cell is invaded. At each stage of growth, the parasites are subject to a variety of unique, antigen-specific and innate immune responses mounted by the host.

The liver stage is marked by sporozoite invasion of hepatocytes and is largely an asymptomatic stage, while the erythrocytic stage, characterized by cyclic rupture of iRBCs, produces the clinical symptoms of malaria, discussed earlier [66]. Malarial antigens and toxins,

GPIs and Hz, released during these stages of invasion drive both host innate and adaptive immune responses [66-69]. Notably, due to repeated exposures to these antigens and components, individuals in holoendemic areas, irrespective of gender, develop a robust antibody repertoire, deemed protective against subsequent infections [70]. Additionally, this immunity though not sterilizing, yields low-grade parasitemia and episodes of clinical disease in the face of repeated exposures throughout life [71-73].

Strong immune responses post malaria infection are marked by production of malaria-specific antibodies mediating a number of anti-parasitic effector functions, including inhibition of cytoadhesion and invasion of erythrocyte, as well as promoting antibody-dependent phagocytosis of infected cells. Different isotypes of immunoglobulins (IgGs) have been identified to play a role in responses to malaria [74, 75].

Immunogenic components of malarial parasites that interact with human host

Many immunogenic antigens of malarial parasite are considered important for host defense and include merozoite surface proteins (MSPs) on the merozoite surface and proteins in the apical organelles [65, 76]. These and other antigens on the plasma membrane of iRBCs are highly polymorphic [66], thus complicating immunity against malaria [77-80]. Therefore, a diverse antibody repertoire capable of blocking antigens involved in invasion and tissue adhesion is favored for anti-parasite immunity [81, 82]. This variability, in essence, accords the parasite the ability to evade the immune response and is therefore considered an important virulence factor [83].

Predominantly, variant parasite antigens on iRBCs are encoded by the multi-gene family *var* [84]. The gene products are highly variant polypeptides of 200-350 kD presenting several

binding sites that promote adhesion of iRBCs to host receptors on vascular endothelium of capillaries and post-capillary venules resulting in parasite sequestration and pathogenesis [85-88]. Notably, one variant form, *var2csa*, mediates binding to CSA in the placenta leading to PM (discussed below).

P. falciparum, is known to express variant proteins on the surface of iRBCs. During PM, VAR2CSA protein, a member of PfEMP-1 (*var*) protein family [90, 91], predominates and interacts with placental epithelial cells, STs, via chondroitin sulfate A (CSA) [89], an adhesion ligand. VAR2CSA is a product of the *var2csa* gene and each parasite only encodes for one or two *var2csa* genes among the 50 to 60 total vars [92]. PfEMP-1 is a highly polymorphic multidomain protein [94] with only one var gene transcribed at any one time [95] in a yet not well-understood mono-allelic exclusion manner [96, 97]. This phenomenon accounts for parasite selective advantage over host defense by switching phenotypes in an event of immune pressure. In addition, binding of iRBCs to host cell receptor is domain dependent; cystine-rich interdomain region (CIDR) and duffy binding like domains (DBLs), both consisting of numerous sub-domains are critical [98]; three sub-domains (alpha beta and gamma) for CIDR and five sub-domains (alpha, beta, gamma, delta and epsilon) for DBLs are of importance. Of note, the number of DBLs and CIDR vary between PfEMP-1s, hence difference in size and adhesive phenotype [98, 99]. Importantly, analysis of *var* genes revealed high diversity amongst the genes of worldwide parasite isolates, although they can be summarized to fit into two broad categories: type 3 var gene and *var2csa* [84]. *var2csa* is relatively conserved in comparison to other *var* genes [84] and the protein, VAR2CSA, appears to be solely responsible for binding to CSA in the placenta [100], an interaction that induces pregnancy specific antibodies [101]. Appreciably, most PfEMP-1/*var* genes have < 50% amino acid sequence identity between

individual DBL domains [99]. The N-terminal region with domain 1-2 is important in *var2csa* binding to CSA [102-108]. The challenging nature of *P. falciparum* infection can be traced to its ability to regulate and switch genes, providing an enormous problem in identifying conserved T cell epitopes. During pregnancy as already mentioned, only one gene, *var2csa*, has been associated with the expression of PfEMP-1 [90] and that anti-adhesive antibodies from multigravidae women block binding to CSA in culture systems, irrespective of geographical source of the placental parasite [109-111]. Therefore, development of anti-adhesion antibodies marks immune maturation in multigravidae women [29, 31, 32]. Moreover, it has been suggested that, recirculation of memory T lymphocytes within intervillous blood, may account for maintenance of local memory immunity to placenta malaria in multigravidae women [93].

Additional malaria antigens that interact with host ligands

Other multi-gene antigen families have been identified the iRBC. Rifins, a product of a highly polymorphic *rifin* gene, occurs in at least 200 copies, mostly located subtelomerically on several parasite chromosomes [30, 112-114]. Furthermore, other antigenic components, namely (MSP)-1, have been identified as importance immune targets capable of inducing antibody production, and have found applications in vaccine development [115-118]. Despite having numerous antigenic variable sequences, the C-terminal amino acid sequence (19KD) of MSP-1 is highly conserved, favoring it as a vaccine candidate [116].

During erythrocyte stage of infection, sequential release of parasites from iRBC inadvertently leads to release of cellular components containing parasite antigens such as GPIs and Hz [65]. In circulation, both of these components have been shown to be potent immune stimulators [119].

The malaria pigment, Hz, a toxin by-product generated by parasites during hemoglobin metabolism, is crystalline and found sequestered in a parasitophorous vacuole within the iRBCs [120]. Acquisition of Hz by macrophages has been shown to impair eyrthropoiesis, and in addition to influencing macrophage function by increased secretion of TNF, in excess will reduced phagocytic capacity as well as production of macrophage inhibitory factor (MIF); an important regulator of innate inflammatory responses. In fact, impaired MIF production has been associated with severe malarial anemia in children [11, 121]. Also, during PM presence of Hz on macrophages and ST cells has been used as a marker for chronic malaria infection and shown to alter cellular function, in terms of cytokine production. [119]

GPIs are important glycolipids attached to the C-terminal and of proteins during post-translational modification and used to anchor proteins onto plasma membranes of cells. A large number of *P. falciparum* parasite proteins are GPI anchored, and the GPIs are essential for parasite survival [122]. Parasite GPIs are structurally distinct from host GPIs in their lipid content thus making them recognizable as foreign by host innate mechanisms [122, 123]. Studies have shown that parasite GPIs released into circulation during iRBCs rupture are sensed by host cells TLR-2, triggering an immune response marked by increased inflammatory cytokine production. Polymorphisims in the TLR2 gene may affect GPIs induced disease pathogenesis [123]. Furthermore, IgG from *P. falciparum*-exposed individuals significantly inhibit the GPI-induced activation of macrophages *in vitro* [124].

Innate immunity: Role of TLRs in malaria immunity

Early recognition innate and adaptive anti-parasitic responses are highly beneficial in the control of malarial infection. For this to be feasible, immune cells need to recognize pathogen-

associated molecular patterns on parasite antigen. In fact, during rupture of infected cells, malarial toxins such as GPIs and Hz are released, being potent activators of immune cells via TLRs as discussed above [125-127]. Indeed, increased IFN-γ induced TLR-2/4 and TLR-9 sensitivity to malarial antigens has been linked to elevated inflammatory responses [128]. Monocytes, neutrophils and a large proportion of resident macrophages from malaria-infected individuals engulf Hz, which is a potent modulator of cytokine profile during malaria [19, 129-131]. In addition to mediating cytokine release, Hz has been identified as a key immunosuppressant; affecting both the antigen processing and immunomodulator function of macrophages [21]. This effect has substantial benefits regarding macrophage insensitivity to other infections [132, 133].

Importantly, innate immune responses are necessary in shaping factors that dictate the outcome of adaptive immunity [134]. Indeed, modulation of cytokine profile, biased towards secretion of inflammatory type, improves macrophage phagocytosis of iRBCs and has also been associated with, to a great degree, the presence of highly opsonic antibodies in serum of individual living in endemic areas [64, 65].

Antibody mediated immunity

After many exposures, malaria infection induces production of antibodies against polymorphic antigens expressed during the parasite erythrocytic stage; a response deemed protective against *P. falciparum* malaria [32, 74, 135]. In fact, high levels of IgG1 and IgG3, both cytophilic, are considered protective against malaria [136]. And, in some cases, significantly elevated levels of IgG2, able to bind FcgammaRIIA in individuals possessing the

H131 allele on monocytic cells, as well as increased IgG2/IgG4 and IgG3/IgG4 ratios, have been associated with protection against *P. falciparum* [136].

In both human and animal studies, antibody responses have been shown to be both beneficial as discussed above or may, in some instances lead to undesirable outcome. Elevated IgE and IgE anti-malarial antibodies, for instance, have been associated with development of cerebral malaria in children [137], and a case of IgA related nephropathy in adults has been reported as well [138,139].

Mechanisms of antibody protection

Antibody protection against malaria infection works via various mechanisms. This protection is primarily through opsonization of iRBC by antibodies; in essence promoting phagocytosis, inhibition of cytoadhesion and erythrocyte invasion, and antibody dependent cytotoxicity or direct lysis of infected cells [63, 64]. The opsonizaton of iRBCs with immunoglobulin or complement enhance recognition and eventually target the iRBCs for phagocytic clearance by specialized macrophages. Generally, the level of iRBC uptake by these phagocytes is stage dependent, with trophozoites and schizonts (mature stage) being more rapidly internalized than the immature rings [140]. This interaction, inadvertently, induces release of factors such as TNF that although cytolytic to the parasite upregulates endothelial markers such as CD36 but may cause tissue lesions as well [11, 141, 142]. Collectively, antibodies act to preferentially target the iRBCs for internalization by the macrophages [143].

Cell mediated immunity: Role of T cells in anti-malarial immunity

Malaria infection induces adaptive cell-mediated immune responses against both pre-erythrocytic and erythrocytic parasite stages [144, 145]. Functionally, differentiated CD4+ T cells have been identified in naturally exposed individuals; *in vitro* experiments showed these cells to be hyper-proliferative in response malarial antigens, suggesting a memory type response beneficial in parasite clearance and protection against malaria [28, 142, 146].

Cytokines in anti-malarial immunity

The course of malaria infection is highly dependent on a tight balance between pro and anti-inflammatory cytokines secreted by the various cells in response to malaria antigens. Production of pro-inflammatory cytokines such as IFN-γ, TNF, IL-1, IL-6, IL-12 and others, crucial in regulating course of infection by inducing parasite-killing action, may in excess, induce undesirable outcomes leading to irreversible tissue damage, hence pathology associated with malaria [11, 142, 147-149]. In particular, T cell derived IFN-γ, is a potent anti-erythrocytic-stage parasite cytokine associated with protection against malaria re-infection in Africa [142]. Importantly, IFN-γ stimulates macrophage microbicidal function, increasing their production of IL-12; a response associated with protection against both murine and human pre-erythrocytic and blood infection [69, 144, 145, 148]. In fact, IL-12 in combination with IL-4 enhances *in vitro* murine erythropoiesis [150]. On the other hand, IFN-γ induced TNF production, increases monocytes phagocytic capacity by inducing increased Fc receptor expression, upon engagement of TNF receptor. Also, IFN-γ promotes production of inducible nitric oxide synthase (iNOS) by macrophages, a response beneficial in controlling malaria pathogenesis [151]. Conversely, both cytokines discussed above, despite being crucial in malaria control are also responsible for spikes

in fever, a classical malaria presentation and furthermore, TNF has also been linked to severe malarial outcomes; for example anemia and cerebral malaria [152, 153]. Most importantly, an anti-inflammatory cytokine, IL-10, counteracts the production and possible cytopathic effect of pro-inflammatory cytokines and is associated with milder form of infection [153, 154]. Additionally, *In vitro* experiments using a two-chambered system demonstrated a CXCL8, CCL3 and CCL4 chemokine driven response on STs upon stimulation with malaria antigens and toxins in an ERK1/2 dependent fashion [119]. Notably, Hz-stimulated ST cells were found to elicit specific migration of peripheral blood mononuclear cells (PBMCs) towards the ST chamber [119].

PREGNANCY

Pregnancy is a complex process marked by fertilization of an ovum and successful implantation of the resulting zygote into endometrial wall of the uterus, punctuated by cell differentiation, leading to formation of an inner cell mass and trophectodermal cells. The latter adhere to the uterus forming the placenta while the former cells develop into the embryonic cells of the fetus [155, 156]. The human placenta is defined as hemochorial, since during invasion of uterus by trophectodermal cells, a lacunae is formed, allowing maternal blood to flow over the fetal syncytialized cells. As such, any compromise of these cells will lead to dire consequences to pregnancy. The placenta, therefore, is a complex fetal organ with the following specific roles; 1) To conduct selective exchange of a wide range of substances between mother and the fetus, for example, transport of nutrients and gasses and protecting the fetus from toxic chemicals or bacteria and other pathogens. For this to happen successfully, a wide range of transport mechanisms are involved, including passive diffusion, pinocytosis, active transport and

phagocytosis. 2) To maintain pregnancy by secreting an array of molecules important for fetal survival. 3) To provide an immunologic barrier between the embryo and the mother and as such protect the foreign implanted embryo from maternal antigenic attack and also preventing any foreign antigens from attacking embryo. Therefore, placental integrity is pivotal to pregnancy success and any faults will pose a great risk in the developmental process of the embryo [157, 158].

HUMAN PLACENTA

Placental development starts immediately after conception, and for 9 months, remains the principle excretory, endocrine, metabolic, and respiratory organ for the growing fetus. During its development, the blastocyst rapidly increases in size, eventually bursting out of the zona pellucida immediately followed by implantation into the mid-line of the upper part of the posterior wall of the uterus. A change in the structure of the trophoblast ensues, leading to interdigitation of the uterine microvilli and trophoblast cell membrane. Trophoblastic cells penetrate uterine epithelium into the underlying stroma triggering invasion processes that result in trophoblastic cells differentiating into two layers. These two layers are extravillous cytotrophoblast and STs, forming a continuous fused single layer of cells with regenerative cytotrophoblasts underneath [159-162], to replenish damaged ST cells. Continual growth of ST leads to delineation of extracytoplasmic cavity forming a spongy structure, bathed with maternal blood via the spiral arteries [163-165]. At term, on average, a healthy placenta would weigh approximately 500 grams with a diameter and thickness of 20cm and 3 cm respectively. Typically, large volumes of blood flow through the placenta at an approximate rate of 500 to 700ml per minute. A large network of capillaries is a common feature of placentas; in fact, it is

estimated that if stretched, these capillaries would be about 50 km long. The ST is thus a fused single layer epithelium performing barrier functions, regulating nutrients and waste products exchange between the fetus and mother. These cells were long thought to be immunological inert, but now evidenced to be immunological active [119, 166, 167]. In essence, damage to the ST is tantamount to consequences that may compromise pregnancy.

IMMUNOLOGY OF THE PLACENTA

Immunologically, placenta, being a semiallogeneic organ, eludes maternal immune recognition via various mechanisms, some not yet well established. Mostly, down regulation of class I major histocompatibility complex (MHC) antigen expression on trophoblast cells is more pronounced and important for preventing tissue rejection [168]. During normal pregnancy, large amounts of trophoblast debris is shed from placenta into maternal circulation; phagocytosis of these debris influence immune microenvironment marked by switching macrophage phenotype such that they are likely to deviate maternal immune responses towards tolerance and away from inflammation [169]. Also, phagocytosis of trophoblast debris increased macrophage expression of indoleamine 2,3 dioxygenase (IDO), a key immunosuppressive enzyme important in activated catabolism of tryptophan [169]. Moreover, induction of IDO, in dendritic cells is up regulated during experimental malaria infection and its inhibition slightly suppresses parasite density in association with enhanced proliferation and IFN-γ production by CD4+ T cells in response to malaria parasite [172]. Furthermore, other deficiencies, such as deficiencies in vitamin D have been shown to influence placental immune state [170, 171]. Also, ST cells have the capacity to secret cytokines and chemokines in response to antigenic challenge, acting to modulate maternal immune system, as is the case of PM [119, 167].

The mammalian placental epithelium is heavily glycosylated, as a mechanism for attachment to uterine wall as well as shock absorption [173-175]. Glycosylation, however, has been shown to also benefit pathogens in mediating virulence. During placental malaria, for example, chondroitin sulfate A glycosaminoglycan (GAG), has been implicated in binding of iRBCs and sequestration in the placenta [176, 177]. This phenomenon highlights the importance of GAGs as a central component in malaria pathogenesis as discussed later in this chapter.

During placental development, endovascular invasion juxtaposes fetal cells with uterine wall promoting ST cell direct contact with maternal blood. Furthermore, studies have shown ST cells as a primary source of monocyte inflammatory protein (MIP)-1 alpha, a chemokine responsible for the migration of monocytes, natural killer cells and T cells, all comprising decidual granulated leukocytes (DGL). Such responses contribute to recruitment of decidual leukocytes towards the intervillous space (IVS), in close proximity to the ST and act to manipulate microimmune environment at the maternal-fetal interface as discussed above [166, 167, 178]. In fact, to create a favorable immune environment for the fetus, anti-inflammatory responses are more favored than pro-inflammatory [179-181].

Placental malaria

Selective sequestration of malaria infected erythrocytes in the placental IVS and on ST cells facing maternal blood circulation is the classical presentation of PM [182]. In response to sequestered iRBCs, ST cells secrete chemical signals, chemokines, attracting a host of immune cell into the IVS [166, 167, 178, 183, 184] that proliferate causing occlusion of IVS; resulting in placental insufficiency, a danger to both mother and fetus (See illustration below figure 2).

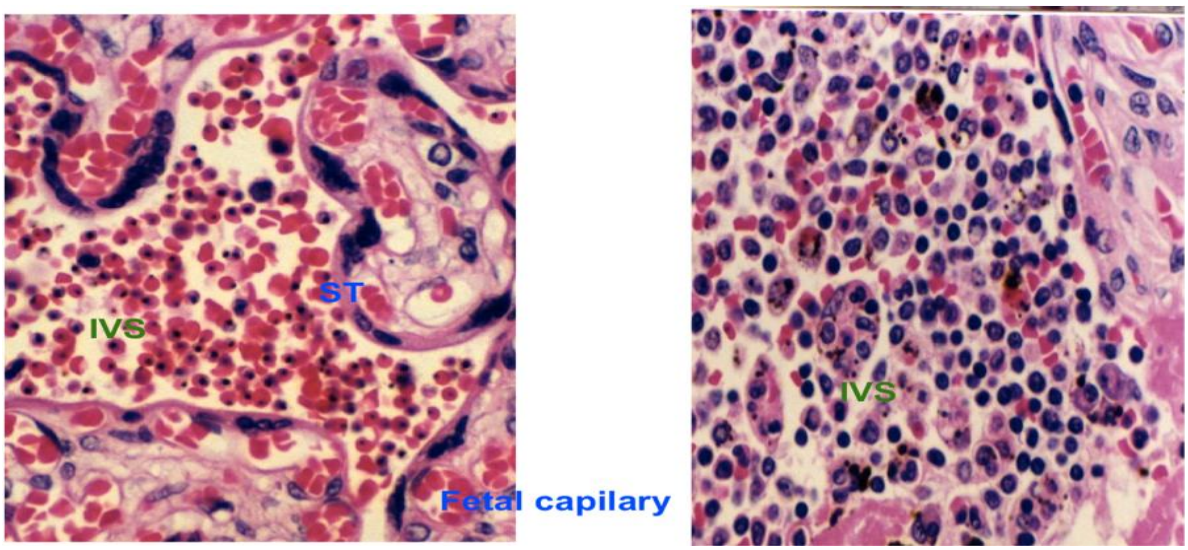


Figure 2.2. Microscopic evidence of pathology associated with PM

(Courtesy of Dr. Julie Moore)

Figure legend. Hematoxylin and eosin stain (H&E stain or HE stain) showing Pathology associated with PM. Left panel shows a healthy, normal PM negative placenta, and PM positive placenta, right panel; increased cellular infiltration and disruption of IVS is evidenced.

Consequences of PM include; low birth weight, anemia, and preterm delivery and in some instances abortion result from placental malaria [182, 185]. It is noteworthy to remember that pathology of PM emanates from interactions between PfEMP-1 and chondroitin 4-sulfate proteoglycan (CSPG) [166, 167, 178, 183, 184, 186].

CSPG is the major ligand responsible for placental parasite sequestration. In general, proteoglycans are large macromolecules constituting a protein core decorated with gylcan moieties; glycosaminoglycans are linear, sulfated and negatively charged [187-190]. These molecules contain disaccharide repeat regions, composed of uronic acid (D-glucouronic or L-iduronic) and amino sugars (D-galactosamine or D-glucosamine) and (4 s or 6s) sulfation pattern

and (beta 1-4, beta 1-3, beta 2-6) glucosodic linkage [191]. With exception of hyaluronic acid, all glycosaminoglycans are found covalently linked to a core protein [192]. For Chondroitin sulfate and heparan sulfate, the linkage involves a specific tetrasaccharide composed of two galactose residues, a xylose and a glucuronic acid residue and coupling to the protein is through an O-glycosidic bond to a serine residue [193-195]. Chondroitin sulfate-bearing proteoglycans include: Decorin, versican, aggrecan, thrombomodulin and CD44 [196-199]. Importantly, these proteins have been linked to critical biochemical functions such as regulating cell adhesion, migration, proliferation, and differentiation [196-198, 200, 201]. Of note, the glycosylation processes are marked by protein post-translational modification rather than straightforward protein synthesis from DNA and a number of important transcripts encoding specific glycosylation enzymes have been identified. In particular, from mouse studies, enrichment of GlcNAc-4,6-O-sulfotransferase, C5-epimerases, and 3-O-sulfotransferases were shown to be involved in late GAG biosynthesis during embryogenesis [201]. Different types of chondroitin sulfates are found in proteoglycans (table 1 below); however, of importance in PM is chondroitin 4-sulfate (C4S) proteoglycan, which predominantly interacts with iRBCs. Considering that CSA is an iRBC receptor in the placenta, and that CD44 is a CSA bearing PG and has been shown to inhibit cytoadhesion of iRBC binding to CD36 [203], highlight the importance CD44 proteoglycan. As such, the impetus for this study is to determine role of CD44 CSA bearing core protein in PM, establish the influence of malaria on CD44 protein abundance, and also establish ST cellular response with regard to CD44 expression.

Chondroitin sulfate type	Disaccharide repeat	Source	Common name
A	GlcAβ1-3GalNAc4S	Cartilage and other tissues	Chondroitin-4-sufate
B	IdoAα1-3GalNAc4S	Skin; tendon	Dermatan sulfate
C	GlcAβ1-3GalNAc6S	Cartilage and other tissues	Chondroitin-6-sulfate
D	GlcA2Sβ1-3GalNAc6S	Shark cartilage; brain	Chondroitin-2, 6-sulfate
E	GlcAβ1-3GalNAc4, 6diS	Squid; secretory granules	Chondroitin-4, 6-sulfate

Table1: A summary of different types of chondroitin sulfate found on mammalian cells

Table legend: GlcA = glucuronic acid, GalNAc = N-acetylgalactosamine, S = sulfate, the letters α and β denote the carbon where glycocydic linkage occurs. These descriptions were partially adapted from National Library of Medicine – Medical Subject Heading (MeSH) web page

SUMMARY AND GAPS IN KNOWLEDGE

Our understanding of iRBC placental cytoadhesion and its consequences has increased tremendously over the past decade. This, however, created more questions than answers with regard to CSA and chondroitin sulfate proteoglycan (CSPG) interaction and how this interaction influences ST cell function.

Notably, CSPG, a known ligand for iRBCs is heavily expressed on ST, thus, promoting sequestration of malaria iRBCs within the placental IVS as discussed earlier. In addition, ST cells have been demonstrated to be competent secretors of potent inflammatory molecules, chemokines and cytokines that can change the dynamics of cell trafficking within the IVS in an event of antigenic stimulation [166, 167]. A good example, shows that stimulation of ST cells in culture with bacterial lipopolysaccharide, (LPS) a known toll like receptor 4 (TLR4) agonist [166], and iRBCs [167] leads to tyrosine phosphorylation of mitogen activation protein kinases (MAPKs) resulting in downstream transcription of genes necessary for production of pro-inflammatory cytokines [166, 167]. These cytokines are the primary causes of gross pathology in tissues if in excess, a response deleterious to placenta and hence fetus [205]. In this scenario, sequestration of iRBCs within the IVS followed by morphologic changes characterized by excessive macrophage infiltration, malarial pigment deposition, fibrinoid deposition, trophoblastic basal lamina thickening, have far reaching consequences: ST necrosis or ultrastructural damage such as partial microvilli loss [186], result in placental insufficiency. Preterm delivery, low birth weight of infant and sometimes abortion may occur [207, 208] (see illustration in figure 2).

Put together, these observations strengthen the need for disease intervention strategies to combat the huge problem malaria posses to the mother and her unborn fetus. What we have proposed to do is in line with understanding parasite/ host interaction and is critical for pushing forward knowledge base of strategies geared towards alleviating the health burden of mothers in malaria endemic.

To achieve the full potential of developing novel chemotherapeutics and immune prophylaxis for pregnant women, more studies remain paramount. The roles of cytoadhesion and pro-inflammatory responses during placental malaria pathogenesis are well documented, but how ST cells orchestrate this role is not yet well understood. It is not clear whether binding of iRBCs to CSPG influence the net abundance of available CSPG, and if so, by what means. Some ideas put forward so far suggest that given the nature of CSPG, they posse a protein backbone that mediates attachment to cellular membrane and in some cases these proteins are transmembrane with cytoplasmic domains bearing residues that can be phosphorylated and act to recruit adaptor molecules important for cellular signaling. These ideas have not yet been exhaustively explored in the context of PM, therefore warranting an in depth assessment. So far, our laboratory has shown malarial components such as hemozoin, parasite GPI and iRBCs as causing overt stimulation of ST cells [119, 166, 167, 178]. These observations, together with differential CSPG levels in malaria infected women [209], lay precedence for studies toward discerning involvement of CSPG core proteins in eliciting such responses. Also, the role these core proteins plays at influencing net immune response in the placenta are of key interest. Therefore, studies defining proteoglycans in PM are important in providing the much needed explanations. It is plausible to expect some level of placental malaria disease driven modulation of CSA core proteins. To answer some of these questions, careful manipulation of an in vitro system developed for studying iRBCs binding to ST is needed to investigate the responses elicited by ST cells during interaction with iRBCs and malarial components such as GPI and Hz. In this study, we developed such a system, which we have used to attempt to discern the role of CD44 CSPG core protein in malaria pathogenesis.

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CHAPTER 3

CD44 IS A FUNCTIONALLY RELEVANT RECEPTOR FOR ADHERENT *PLASMODIUM FALCIPARUM* IN THE PLACENTA

[1]

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ABSTRACT

Plasmodium falciparum infected red blood cells (iRBCs) accumulate in the maternal blood space of the placenta during malaria infection, culminating in pathological consequences deleterious to pregnancy success. The fetal cell in contact with maternal placental blood is a syncytialized epithelium called syncytiotrophoblast (ST). The placenta has a rich supply of low sulfated chondroitin sulfate A (CSA), a principle ligand for VAR2CSA parasite protein, present on the surface of placenta-adherent iRBCs, but the critical proteoglycans bearing CSA that participate in placental adherence and influence the course of infection have not been studied. Given that ST is immunologically active in the presence of iRBCs, here we examined the role of CD44 proteoglycan, a known CSA bearing molecule with a trans-membrane cytoplasmic domains adept at signaling functions, in iRBC/ST functional interactions.

Using a flow cytometric approach with extracted ST proteins, we show specific CD44 protein binding on the surface of CSA-adherent iRBCs, an interaction that is dependent on CSA, since chondroitinase treatment of syncytiotrophoblast membrane proteins (SMPs) significantly reduced binding and a parasite line devoid of VAR2CSA did not capture CD44. Lentiviral knockdown of CD44 expression in the choriocarcinoma cell line, BeWo, confirms a significant role for ST CD44 in iRBC binding. Additionally, we show by western blot CD44-dependent changes in tyrosine phosphorylation status of a series of ST proteins and confirm involvement of Src Kinases using specific inhibitor, indicating a potential functional role for CD44 in the ST immunological response. By using a specific Src kinase inhibitor, we confirm involvement of these family of kinases in response to iRBC activation. Furthermore, malarial exposure appears to enhance CD44 expression by ST cells in vitro as assessed by protein detection in cell lysates by ELISA.

In summary, we provide evidence for CD44 proteoglycan potential dual role as an *in vivo* receptor for VAR2CSA expressing iRBCs as well as an ST signaling molecule modulated by malaria infection.

Key words: Placental malaria, CD44 proteoglycan, Chondroitin sulfate A, Syncytiotrophoblast

INTRODUCTION

Malaria infection during initial pregnancies bears serious consequences for both mother and fetus [1-3]. Of the five known Plasmodial species infective to human beings, *Plasmodium falciparum* is most virulent, mainly due to expression of variable genes encoding *Plasmodium falciaprum* erythrocyte membrane protein-1 (PfEMP1), an adhesive protein exported to membranes of infected erythrocytes [4-6]. As a consequence, interaction of PfEMP1with host molecules on the endothelium of microvasculature and placental epithelium impedes clearance of infected cells [5, 7-9]. Ultimately, sequestered *P. falciparum*-infected red blood cells (iRBCs) induce a myriad of inflammatory responses [10-14], which are inadequate in clearance of parasites from the maternal blood, and instead culminate in a pathological condition referred to as placental malaria (PM). The PM-induced inflammatory responses cause significant local tissue damage and are associated with poor birth outcomes [13, 15-19].

The specialized fetal epithelial cells (syncytiotrophoblast; ST) in direct contact with maternal blood in the villous placenta boast a rich supply of a low sulfated form of chondroitin sulfate A (CSA) [20, 21], a principal ligand for sequestering iRBCs that express VAR2CSA, an adhesive parasite protein on membranes of iRBCs [6, 22]. It has been shown that disruption of this gene ablates iRBCs binding to placental epithelium [23]. Importantly, we have shown immunologic consequences of interaction between VAR2CSA-expressing parasites and CSA on STs, marked by secretion of cytokines and chemokines [10, 24-27], which may play a role in infiltration of intervillous space by immune cells [26, 27], often harmful to STs integrity [18, 26]. These underlying damages and inadequate repair of STs contribute to intrauterine growth retardation and ultimately may lead to abortion or delivery of underweight babies [16].

Cross talk between mammalian cells is critical for function and survival. Such cellular interactions are mostly governed by an array of membrane bound proteins and extracellular molecules [28-34]. Protein posttranslational modification during biosynthesis is responsible for most functional specificity of molecules [35]. For example, protein modification by addition of glycan moieties alters protein properties and function. Relevant to immunologic function, CSA on the chemokine receptor CCR5 has been shown to mediate its interaction with the chemokine, RANTES/CCL5 [36], and in coagulation, CSA bearing proteoglycan (PG), thrombomodulin, engages thrombin and therefore modulates its activity [37]. In these cases, the PG inclusive of CSA is operational. In the case of *P. falciparum* iRBCs [20, 38-40], while the CSA glycan has been well characterized as an iRBC ligand, the critical protein backbone that bears CSA on STs and anchors iRBCs on the placental epithelium remains to be identified. Since the protein backbone of a PG is important in determining function, attempts have been made to elucidate protein cores responsible for CSA expression on ST cells during PM. A protein with amino acid residues consistent with aggrecan was proposed [41] and thrombomodulin has most often been suggested as a possible candidate [42-44]. However, because STs are immunologically activated post- interaction with iRBCs [10, 25-27], we decided to target a PG that would satisfy a dual role of receptor as well as signaling molecule.

CD44 proteoglycan, a principle hyaluronic acid (HA) receptor [45-49] was appealing in this context because it bears CSA and possesses a cytoplasmic domain that interacts with phosphorylated tyrosine residues of cytoplasmic molecules such as Lck, Fyn and other Src kinases [50]. This is of relevance since activation of these kinases precede changes in cellular immunologic function, as evidenced by T cell activation post engagement of CD44 by HA [50, 51] and CCL5 [36], which induce secretion of interferon gamma (IFN-γ). Moreover, CD44 was

recently shown to act as a receptor for CSA-adherent *P. falciparum* on endothelial cells and, via Src-family kinases and MEK kinase, to influence the ability of CD36 to act as a cytoadherence receptor for iRBCs with that specificity [52]. Considering these functions and the fact that CD44 is present on human placental ST cells [31] supports our interest in determining role of CD44 as a *P. falciparum* iRBC sequestration receptor important for ST cell stimulation in human placenta.

MATERIALS AND METHODS

Placental sample collection and processing

Informed consent to obtain fresh, term placenta from elective Cesarean sections was sought from women attending pre-operative counseling at Athens Regional Hospital, Athens, Georgia USA, using forms pre-approved by both Institutional Review Boards of University of Georgia and Athens Regional Medical Center. Immediately post-Cesarean delivery, placentae were collected into a suitable container containing saline with heparin and immediately transported to the laboratory for processing. Placentae were processed following our previously published protocol [10]. Upon successful purification of cytotrophoblast cells, 3.0×10^5 cells/cm^2 were cultivated on 6 and 24 well cell culture treated plates (Corning Inc., New York. USA). Following syncytium formation, normally by day 4 of culture, cells were used for experiments.

For immunohistochemical analyses, placental tissues collected from women naturally exposed to malaria in western Kenya were utilized. The client recruitment and sample collection procedures and placental sample processing were previously summarized [53, 54]. PM-negative samples were confirmed by both microscopy and PCR [53] to be infection-free.

Cultivation of P. falciparum and isolation of trophozoite-infected iRBCs

P. falciparum CS2 (CSA-binding; WT) and FCR3Δvar2csa (KO; generously provided by Artur Scherf [22] laboratory isolates were kept in continuous culture as described [10]. Cultures were maintained at 4% hematocrit in 75 cm^2 tissue culture flasks at 37°C in 25 mM HEPES, 0.05 mg/mL hypoxanthine, 2.2 mg/mL $NaHCO_3$, 0.5% Albumax, and 0.01 mg/mL gentamicin RPMI medium. The VAR2CSA KO parasites were maintained under identical conditions as CS2 with the exception of substitution of half the volume of Albumax with human O^+ serum. Cultures were confirmed to be mycoplasma negative by routine PCR analysis. Synchronous cultures were achieved by weekly/biweekly treatment with 5% D-Sorbitol.

To purify trophozoites, sorbitol synchronized cultures were enriched over a 35% to 70% percoll step gradient and centrifuged at 2000 xg. Cells at the interface were harvested and washed 3 times in PBS supplemented with 2 % fetal bovine serum (FBS). Cell numbers were determined and concentration adjusted to 10^6 cells per ml.

Cell stimulations

Syncytialized primary trophoblast cells (ST) or the choriocarcinoma cell line, BeWo, purchased from American Type Culture Collection (Manassas, Virginia USA) were used. The latter were seeded at 1 X 10^5 cells/mL in 24 well tissue culture plates and left at 37° C, 5% CO_2 for 24 hour in Ham's F12 medium supplemented with 10% fetal bovine serum and 1% antibiotics (100 μM penicillin/100 mU streptromycin) mixture. Cultures were stimulated with 10μg/ml of bacterial lipopolysaccharide [55] purchased from Sigma Aldrich (Saint Louis, Missouri USA) as positive control [25], 10 uninfected erythrocytes or 10 iRBCs per ST nucleus. Stimulations were done for five, fifteen and thirty minutes for assessing phosphorylation changes

and longer incubations, 12 and 24 hours for assessing protein expression and secretion. BeWo ST cells were cultured and treated with 10μM/ml of a pan Src Kinase inhibitor (PP1, cat# 567809, EMD Millipore, Billerica MA, USA) or vehicle (DMSO) for 30 minutes prior to five minutes stimulation with iRBC

shRNA knock down of CD44 using lentiviral particle delivery system

For targeted CD44 knock down, a pool of human SMARTvector 2.0 Lentiviral shRNA particle (VSH-5417; Thermo Fisher Scientific, Lafayette, CO) was utilized following manufacturer's instructions. The following SMARTvector 2.0 particles were used, Non-target negative control for RNAi (S-005000-01), Human GAPD positive control (S-001000-01) and three different pools for targeted CD44 knock down (SH-009999-01-10, SH-009999-02-10 and SH-009999-03-10). In order to determine multiplicity of infection (MOI), growth medium was replaced with 225 μl of serum free medium and Lentiviral control particles listed above prepared into eight five-fold dilutions; 25μl of diluted particles were added into respective wells containing 50,000 BeWo cells. After 4 hours, 1 ml of complete medium was added to cells. Successful integration of control shRNA was monitored by expression of green fluorescence protein (GFP) under a fluorescent light microscope at 72 hours post-transduction. A MOI of 0.1-0.2 was chosen and hence used for transducing cells with shRNA specific for knocking down CD44 mRNA, following the same procedure. Transduced cells sorted on the basis of GFP into GFP-intermediate and GFP-high populations using a MoFlo cell sorter (Beckman Coulter, Inc.).

Extraction of ST membrane proteins

The basal membrane was trimmed off fresh placenta and villous material extracted and minced into small half-centimeter cube sections, which were further washed in 0.9% NaCl to

remove excess blood. The tissue pieces were then incubated for 60 minutes with intermittent mixing every 10 minutes in 1x cold phosphate buffered saline (PBS) supplemented with a cocktail of protease inhibitors; 2μg/ml aproptinin, 5μg/ml leupeptin, 1μg/ml pepstatin A, 1mM sodium orthovanadate and 1mM phenylmethylsulfonyl fluoride (abcam, Cambridge, MA, USA). The resulting suspension was passed through a 20μm pore size sieve to filter out debris and large tissue pieces. The filtrate was clarified sequentially by centrifugation at 1000 xg for 10 minutes then again at 14000 xg before membrane proteins were collected using a final spin at 100000 xg. The pellet was then re-suspended in 2ml of cold 1X PBS supplemented with protease inhibitor cocktail and concentrations determined by Bradford protein assay (Thermo Scientific). Separately, cultured ST proteins were prepared post stimulation with iRBCs. Briefly, ST cells were lysed in RIPA buffer (150mM NaCl, 50mM Tris, 1% V/V NP-40, 0.5% sodium deoxycholate, 1% SDS (sodium dodesyl sulfate) and supplemented with protease inhibitor cocktail as above, with protein concentration likewise determined by Bradford protein assay.

Western blots

For analysis of protein phophorylation state, ST protein concentrations were equalized prior to separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). For resolving proteins, 12% gels were run at 150 volts for 90 minute, then proteins transferred to nitrocellulose membrane for 60 minute, blocked for 60 minute in 5% bovine serum in 1x tris buffer saline supplemented with 1% Tween-20 (TBS-T), then incubated overnight at 4°C with mouse mAb anti-phosphotyrosine (clone 4G10) from Upstate Biotechnology (Lake Placid, NY). Immunoblotted proteins were visualized with horseradish peroxidase–linked secondary antibodies (Dako, Glostrup, Denmark) using enhanced chemiluminescent (ECL) substrate

according to the manufacturer's specifications (Amersham Pharmacia Biotech). To re-probe blots, membranes were incubated in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris (tris (hydroxymethyl) aminomethane)–HCl, pH 6.7) at 55°C for 1 hour and rinsed with PBS several times prior to a second Western blot analysis for assessing expression of housekeeping proteins as loading controls; rabbit anti-human GAPDH (Cat # 5174S, Cell Signaling Technology).

Enzyme Linked Immunosorbant Assays (ELISA)

To assess CD44 expression, 100 μg of proteins isolated from stimulated ST cells were used for overnight coating of Immulon II plates (Thermo Scientific, Rochester, NY USA) in duplicate wells. Wells were washed four times in 1x phosphate buffered saline (PBS) supplemented with 0.1% bovine serum (BSA) and 0.05% Tween-20. Blocking for non-specific binding was done using 1% BSA in PBS for 30 minutes at room temperature. Plates were then incubated with 1 μg/ml of anti-CD44 (IgG2a k; MCA89, AbD Serotec, Raleigh, NC) for 2 hour at room temperature. After primary antibody incubation, washing was done as detailed above then 0.25ug/ml of polyclonal biotin anti mouse IgG (cat # 13-4013-85 eBioscience San Diego California, USA) was added to each well. Plate was then incubated for 30minutes with a 1/200 dilution of streptavidin-horseradish peroxidase (HRP) conjugate enzyme system (R&D Systems). Thermo Scientific Pierce TMB chromogenic substrate used to detect HRP activity.

Matched antibody pairs and recombinant standards were used for quantification by ELISA in assessment of macrophage migration inhibitory factor (MIF), interleukin (IL)-8, and CCL3 (R&D systems) and IL-6 (BD Biosciences) analytes in culture supernatants following procedures as described by the manufactures.

Immunohistochemistry

Five μm thick paraffin embedded placental tissues mounted on glass slides were subjected to deparaffinization, hydration and antigen retrieval protocol [56]. Endogenous peroxides in tissues were quenched by incubating with 30% hydrogen peroxide for 30 minutes at room temperature and washed once in PBS. Blocking for non-specific binding was done using 4% BSA in PBS for ten minute prior to addition of mouse anti-CD44 (IgG2a k; MCA89, AbD Serotec, Raleigh, NC) at 10μg/ml concentration and incubated at room temperature for one hour. Following primary incubation, tissues were washed in PBS four times. Polyclonal rabbit anti-mouse (Millipore, Bedford, MA) antibody was used at 2μg/ml for one hour then slides washed as above. Color development done using a rabbit specific DAB substrate kit (cat # 760-4311, Ventana Medical systems). Tissues were then dehydrated and cover slips mounted using flow texx (cat # 14-390-3, Thermo Scientific).

Flow cytometry

Sorbitol-purifed trophozoites were labeled with hydroethidine (HE) at 2 μg/ml per 10 million iRBCs for 45 minutes and washed three times. To assess CD44 binding, 1 X 10^6 cells were incubated with 1000 μg of ST lysate proteins and incubated for 60 minutes at 4^o C with gentle shaking. Detection of CD44 bound on to erythrocytes was done using monoclonal mouse anti-CD44 (IgG2a k; MCA89, AbD Serotec, Raleigh, NC) isotype followed by signal amplification using a polyclonal biotin anti mouse IgG (cat # 13-4013-85) and streptavidin FITC (cat # 11-4317-87) (eBioscience San Diego California, USA). Data were acquired on a FACSCalibur Becton Dickinson (BD Biosciences) cytometer and analyzed using FloJo software (Version 9.5.2, Tree Star, Inc., OR).

Parasite binding assay

BeWo cells were grown for 48 hours in 12 well tissue culture plates. Percoll-enriched, HE-stained iRBCs were washed three times in binding medium (RPMI-1640 medium supplemented with 25mM Hepes and 10% human O^+ serum), enumerated and added to BeWo cells in binding medium at a ratio of 10:1. Following a one hour incubation, the wells were washed three times with binding medium, using gentle rocking and vacuum aspiration to remove medium. Enumeration of bound cells was done by capturing 10 low power photomicrographs per well using a fluorescent microscope (Leica DM IRBE, Ludi Electronic Production Ltd.) with a digital camera (model C4742-95, Hamamatsu) and analyzing fluorescence intensity with Image-J (version 2; rsbweb.nih.gov/ij/).

Statistical analysis

Graphing and statistical analysis were done using GraphPad software (version 5; GraphPad Sofware Inc., CA)

RESULTS

CD44 interacts with VAR2CSA on infected red blood cells via CSA

To determine that CSA-bearing CD44 can mediate iRBC adherence to ST, placenta-derived membrane proteins were incubated with CS2 parasites, a *P. falciparum* line shown to have a stable CSA binding phenotype [57] and probed with antibodies specific to CD44. To evaluate the extent to which this reaction is attributable to VAR2CSA, a VAR2CSA null (KO), non-CSA adherent parasite line, was employed. As shown in Figures 3.1A and 3.1B, interaction of placenta-derived CD44 with iRBCs was significantly influenced by the presence of

VAR2CSA, with significantly reduced binding to VAR2CSA null iRBCs (Figure 3.1C; $p < 0.05$, n=3; unpaired t-test). Pre-treatment of the placental membrane proteins with increasing concentrations of chondroitinase ABC, a CSA digesting enzyme, resulted in significantly diminished CD44 binding at 2 units per milliliter (Figure 3.1D; $p < 0.05$, n =3, unpaired t- test), confirming the dependence of the CD44/VAR2CSA interaction on the presence of CSA.

The importance of cell surface CD44 in CSA-dependent iRBC adherence to trophoblast was assessed using a lentiviral short hairpin RNA knockdown approach, evaluated by protein expression (Figure 3.2A) and GFP expression on BeWo STs. Cells were sorted into intermediate and bright GFP-expressors and then assessed for iRBC binding; iRBCs were stained with a red DNA intercalating dye, HE (Figure 3.2B). While non-transduced BeWo cells and intermediate GFP cells readily captured CS2 iRBCs *in vitro,* significantly fewer cells bound to GFP-bright, CD44 knock down cells (Figure 3.2B and 3.2C).

CD44 expression on ST is influenced by interaction with CSA-adherent iRBCs

There is evidence in the literature that CSA expression on ST is increased in the context of PM [58]. Our preliminary data assessing glycan-associated transcripts support this observation (Appendix 1). Therefore, it was of interest to examine the extent to which CD44 expression is influenced by iRBC exposure and VAR2CSA-iRBC engagement of cell surface CSA. Toward this end, primary ST was exposed to VAR2CSA intact (CS2; WT) and null (KO) iRBCs, as well as uninfected RBCs (uRBC) for 24 hours. Assessment of CD44 levels on proteins isolated from these cells by ELISA method showed CSA-adherent iRBC (VAR2CSA intact)-dependent CD44 up-regulation on ST cells compared to non-adherent (VAR2CSA null) iRBCs, (Figure 3.3A; $p < 0.05$, unpaired t test). This observation notwithstanding, immunohistochemical staining of fixed

paraffin embedded placental tissues from malaria-exposed Kenyan women, while confirming CD44 expression (Figure 3.3B), did not reveal differences in antigen staining intensity on ST in infected relative to uninfected placentas (Figure3.4C), with intervillous leukocytes showing a similar level of staining in the context of infection (Figure 3.4B). Interestingly, this analysis revealed a slight tendency for decreased leukocyte CD44 expression in infected samples relative to the malaria-free counterparts (Figure 3.4D). Despite the absence of a clear relationship between infection and CD44 expression on ST, linear regression analysis of CD44 staining intensity with parasite density on thick smears revealed parasite density as a positive predictor of CD44 levels on ST (Figure 4E; $R^2 = 0.35$, $p = 0.016$).

ST activation by iRBC exposure is influenced by CD44

Since ST is known to respond immunologically to iRBCs and other malarial components [10, 25-27], and given that CD44 binds to CSA-adherent iRBCs and is known to have signaling function [52], it was important to discern the role it may play in ST cell activation. To assess this, intracellular phosphotyrosine species were examined by western blot following exposure of intact and CD44 knockdown BeWo cells to uRBCs and both CS2 and KO iRBCs. This analysis revealed that several proteins migrating between 30-40 kDa, at ~50 kDa, at ~58 kDa, and at 100-110 kDa were differentially activated in cells exposed to CSA-adherent versus non-adherent iRBCs (Figure 3.5A). Moreover, most of these proteins were less abundant in CD44 KO cells; proteins migrating at ~50 kDa were notably absent from both CS2/WT iRBC-exposed CD44 KO cells and KO iRBC-exposed cells (regardless of lentiviral transduction). Furthermore, by using a specific inhibitor for Src kinases, PP1, a specific reduction of phosphorylated proteins in the 50

to 60 kD range was observed with iRBC exposure, suggesting that CD44 stimulation activities are mediated by part via Src family of kinases (Figure 3.5B).

DISCUSSION

While substantial progress has been made towards increasing our understanding of the role CSA plays in mediating *P. falciparum*-iRBC binding to ST and sequestration in the IVS, the nature and role of proteoglycans that bear CSA on ST cells has not been elucidated. The present study made use of two cell types, primary trophoblast and BeWo choriocarcinoma cells, to address this problem. BeWo cells are comparable to primary cells in many aspects, including the ability to secret signature placental derived hormones [59] in culture, and as we demonstrate, to express CD44-associated CSA on the cell membrane. Notably, BeWo cells in contrast to primary ST cells can be propagated indefinitely as mononuclear cells *in vitro*, greatly expanded to large amounts and are easily manipulated for functional studies like the one reported here. In this study, and backed by our prior experiences [10, 25-27] both cells have been useful tools for exploring *Plasmodium*/trophoblast interactions.

The general processes leading to accumulation of iRBC in the placenta are well understood. However, the nature of proteoglycans that express CSA on the ST membrane have not been determined, although thrombomodulin [42-44] and a protein with amino acid residues consistent with aggrecan [41] have been suggested as possible candidates. Based on evidence supporting mitogen activated protein kinase dependent ST cell immunologic activation post-interaction with iRBCs [10, 25-27], as well as a report of CD44 acting as a signaling receptor for CSA-adherent iRBCs on endothelium [52], it was of interest to target a proteoglycan that would satisfy a dual role of receptor as well as signal transducer. In this report, we show that CD44, a

known CSA-bearing proteoglycan, is present on ST cells and promotes binding of iRBCs as well as influences net tyrosine phosphorylation patterns in ST cells.

In this study, two parasite strains, CS2, which is a known CSA binder [60] and an FCR3delta mutant var2csa, a non-CSA binder (knock out (KO)) [23] together with ST cell membrane proteins were used to demonstrate involvement of CD44 in iRBC binding to ST. As expected, there was notable difference in amount of CD44 detected when ST membrane proteins were incubated with the two parasite lines, with VAR2CSA-intact iRBCs having significantly higher mean fluorescent intensity compared to KO iRBCs. Notably, CD44 binding to iRBC was diminished, when proteins were pretreated with chondrointinase ABC, an enzyme that cleaves CSA. The residual binding post CSA cleavage could suggest that CD44 may be binding to iRBC using an alternative mechanism. Given that HA, a known CD44 ligand is found as a pericellular coating on live cells [61], including red blood cells [62], could provide a plausible explanation to the residual CD44 binding phenomenon.

It is known that adhesion molecules can serve as receptor-signaling molecules capable of influencing cellular activation [63]. Several studies have shown that iRBC adhesion to ST induces intracellular signaling by triggering activation of mitogen-activated protein kinase (MAPK) pathway [25-27]. Moreover, RANTES (CCL5) chemokine, induces the formation of a signaling complex composed of CD44, src kinases, and adapter molecules, triggering the activation of the p44/42 mitogen-activated protein kinase (MAPK) pathway [36]. Together, these observations set the premise for investigating the impact of CD44/iRBC interaction in influencing ST cellular activation. Here, by knocking down CD44 on BeWo cells, we demonstrate a reduction in iRBC binding capacity. Furthermore, we also show an altered tyrosine phosphorylation pattern, with reduced activity upon CD44 knock down. It is of interest

to note that phosphorylation of 60 kDa protein is consistent with tyrosine kinase of the Src family, Fyn and Lyn, both important for cellular activation [36, 64] and in fact by using a pan Src kinase inhibitor, PP1, we confirm involvement of Src family molecules. This is a novel observation, showing the potential of CD44 acting as a signaling receptor in ST. Careful identification of the phosphorylated protein tyrosine kinases will be necessary to fully appreciate the functional significance of this activation.

A full understanding of the functional implications of binding of iRBCs to ST during PM is still lacking, however, the ST is known to be highly immunoreactive, and capable of secreting cytokines and chemokines [25-27]. We are in the process of testing the effect of CD44 loss at altering these secretion patterns. Our preliminary results suggest that knockdown of CD44 actually increases MIF secretion capacity by BeWo cells (Appendix 2), which was an unexpected observation. It deserves mention that CD44 acts as the signaling component of the MIF receptor, CD74 [65]. Clearly, further examination of the role of CD44 in ST and how it might influence immunologic responses by these cells is warranted. Also, it remains important to evaluate any chemotactic responses of immune cells towards ST molecules in relation to CD44 mediated signaling. These studies will have important implications for the understanding of the biology of PM, as they will provide critical information with regard to ST cell immunologic stimulation. Furthermore, since trophoblasts are involved in manipulation of local immune response, potentially participating in both protective and pathogenic immune mechanisms, an understanding of signaling receptors involved in these responses, has significant implications for the prevention and control of this important public health problem.

Like all *in vitro* systems this cell culture approach has some limitations. For example, we did not completely replicate flow conditions of blood in the IVS of the placenta, therefore the

static condition used in this model may not reflect true *in vivo* situations. It should be possible in future work, however, to perform these studies in a flow cell. Also, *in vivo*, cells work in concert; ST is in direct contact not only to iRBC during PM but also with maternal immune cells and their secreted factors, including cytokines and chemokines. These factors may profoundly influence the expression of CD44 on the ST membrane and potentially affect its interaction with iRBC, which may influence the signaling properties. We are currently investigating how these and other parameters may influence the system, with the aim of developing a better a system that closely mimics the *in vivo* environment.

Without a concise understanding of host (ST) cell receptors involved in interaction with iRBC and modulation of immune responses in placenta during PM, full evaluation of therapeutic methodologies will not be achieved. The study described here, provides for the first time, to the best of our knowledge, a compelling argument for the involvement of CD44 proteoglycan in the immune modulation of ST cells during PM. Only through identification of signaling receptors involved in the iRBC-induced ST immune response will a complete understanding of the biology of maternal/fetal interface in the context of PM be achieved.

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Figure legends.

Figure 3.1. Interaction of placental CD44 proteoglycan with infected erythrocytes.

Proteins derived from term human placental villous material were incubated with both VAR2CSA intact [68] and null (KO) iRBCs, probed with anti-CD44 antibodies and assessed by flow cytometry. A) Representative histograms showing CD44 staining on VAR2CSA null (KO; top panels) and VAR2CSA competent (WT; bottom panels) iRBCs; isotype controls, left panels and CD44 specific staining, right panels. B) Overlay of CD44 histograms; WT depicted in blue and KO in red. C) Summary of median fluorescence intensity of CD44 staining on CS2 [68] and VAR2CSA null (KO) iRBCs (n = 3; $^{**}P < 0.01$ unpaired t test). D) Representative histogram overlay for iRBCs incubated with membrane proteins treated with 500mU/ml chondroitinase ABC in blue, intact membrane proteins in orange compared to isotype control in red, one sample representative of three placental protein preparations.

Figure 3.2. CD44 is a receptor for CSA-adherent *P. falciparum* on BeWo cells.

A) Bar graph showing percentage knock down on CD44 expression on BeWo ST cells based on CD44 elisa data. B) Intact syncytialized BeWo cells (BeWo ST) and lentiviral transduced BeWo cells sorted on the basis of integrated lentiviral GFP expression to dim (CD44 intermediate KO) and bright (CD44 greater KO) were incubated with CS2 parasites pre-treated with HE. Washed cells were assessed by fluorescence microscopy, one experiment representative of three. C) iRBC binding was enumerated as the sum of total fluorescence per 10 wells per condition ($^{*}P < 0.05$, Dunn's multiple comparison test).

Figure 3.3. Induction of CD44 expression by iRBCs on ST.

A) Primary ST was exposed to medium alone (Med), uninfected RBCs (uRBC), CS2 (iRBC WT) and VAR2CSA null (iRBC KO) strains. Extracted proteins were probed for CD44 protein by ELISA. The CD44 signal is reported as optical density. B) Immunohistochemical staining for CD44 shows clear staining on ST and on intervillous leukocytes in a placenta exposed to malaria.

Figure 3. 4. Determination of CD44 abundance on placental tissues from malaria infected and uninfected individuals.

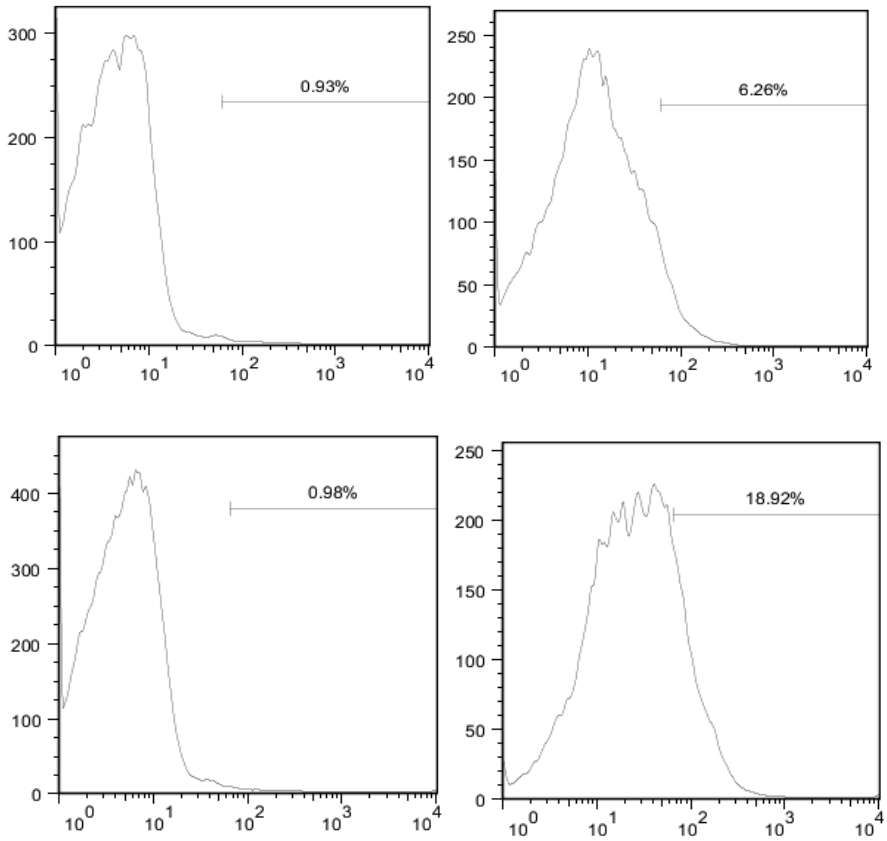
Fixed thin placental sections, 5μm thick, were probed for CD44 expression using specific antibodies as described in materials and methods section. Semi-quantitatively scored for CD44 by two independent, blinded scorers (SO and DS) on a scale of 0 to 5. Graphs show summaries of these scores with median and Standard error of means, unpaired t test statistics) A) Malaria-free and B) malaria-infected samples, showing staining scores for ST and intervillous leukocytes. C) Summary of CD44 staining score on ST and D) leukocytes (P value= 0.145, unpaired t test), by infection status. E) Linear regression analysis of CD44 with parasite density per 300 leukocytes as predictor (R square =0.35, p value = 0.016).

Figure 3.5. Tryosine phosphorylation in BeWo cells is influenced by both CSA adherence and the presence of CD44.

ST cells were cultured in 6 cell tissue culture plates for 48 hours then stimulated for five minutes with iRBC as indicated with gently rocking. For the kinase assays, the cells were pre-incubated with respective inhibitors, Src kinases (567809, PP1, EDM Millipore, MA, USA) and MEK kinases (Cell Signaling Technology Inc, MA, USA) at 10 μM, as indicated, for 30 minutes prior to the 5 minutes stimulation with VAR2CSA expressing iRBCs. Western blotting for phosphotyrosine was performed as described in Materials and methods. Arrowheads indicate molecular weights as depicted. A) Shows tyrosine phosphorylation pattern due to stimulation with adherent and non- adherent iRBCs, on intact BeWo, lentiviral vector control, lentiviral non-target control, and lentiviral and CD44 knock down. B) Show effect of Src kinase inhibitors on tyrosine phosphorylation on lentiviral vector control and CD44 knock down bewo cells; medium control, untreated, Src kinase inhibitor Src kinase vehicle only.

Figure 3.1.

A)



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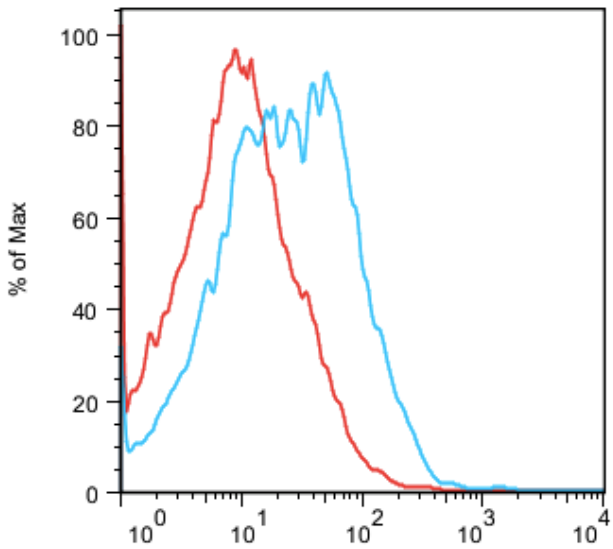
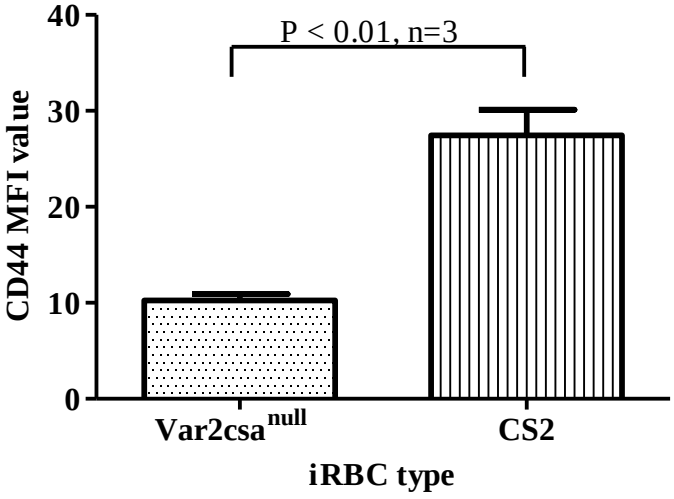


Figure 3.1. cont'd.

C)



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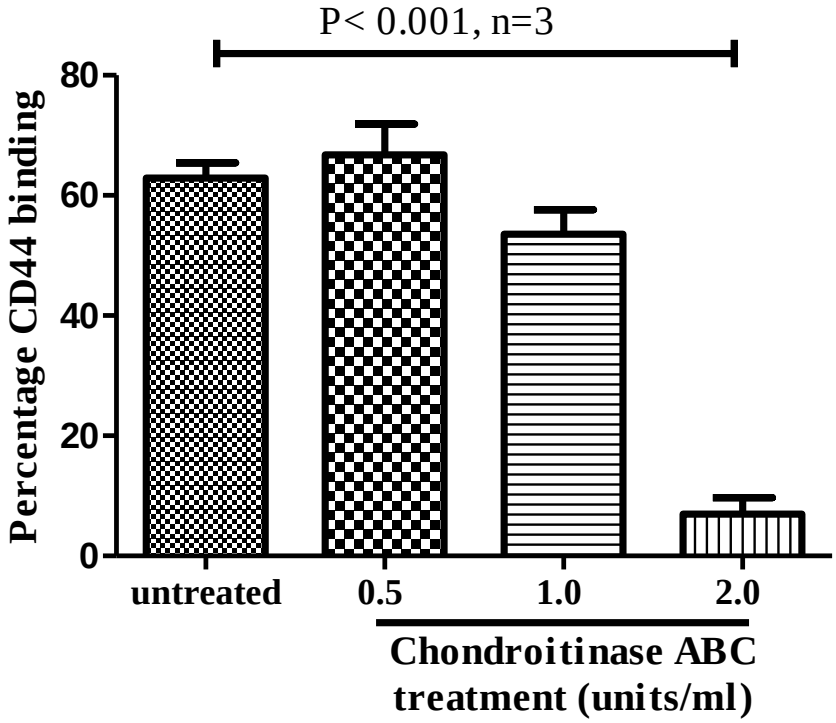
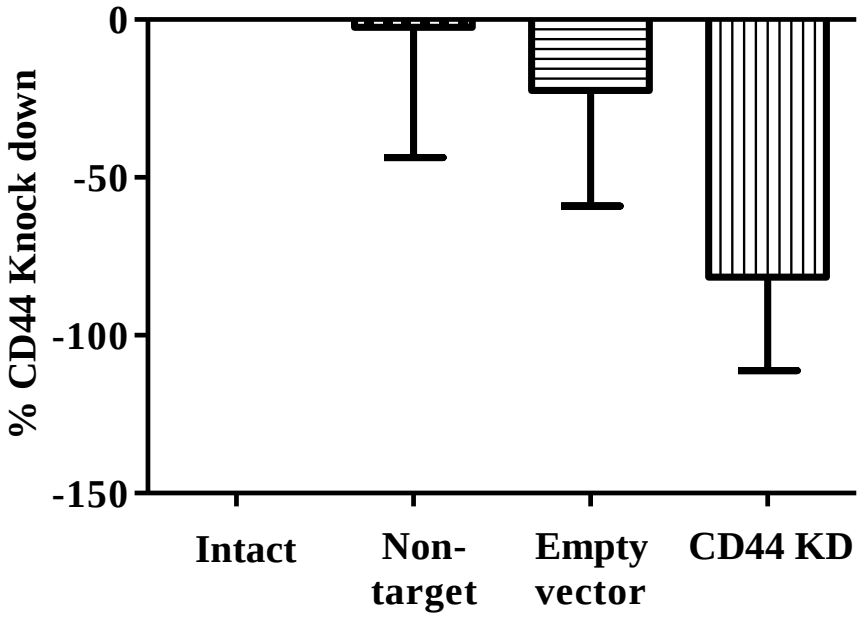


Figure 3.2.

A)


0
-50
-100
-150
% CD44 Knock down
Intact
Non-target
Empty vector
CD44 KD

B)

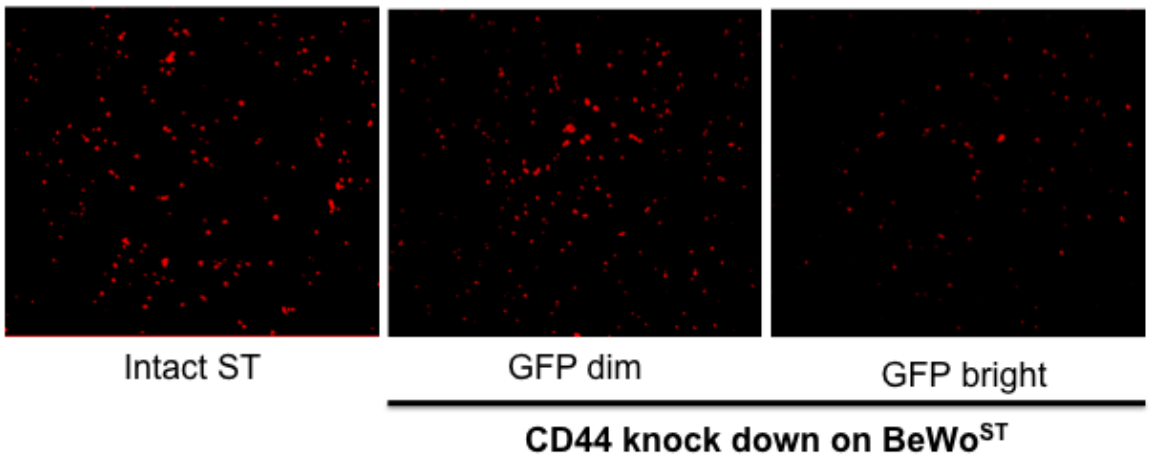

Intact ST
GFP dim
GFP bright
CD44 knock down on BeWoST

Figure 3.2 continuedC)

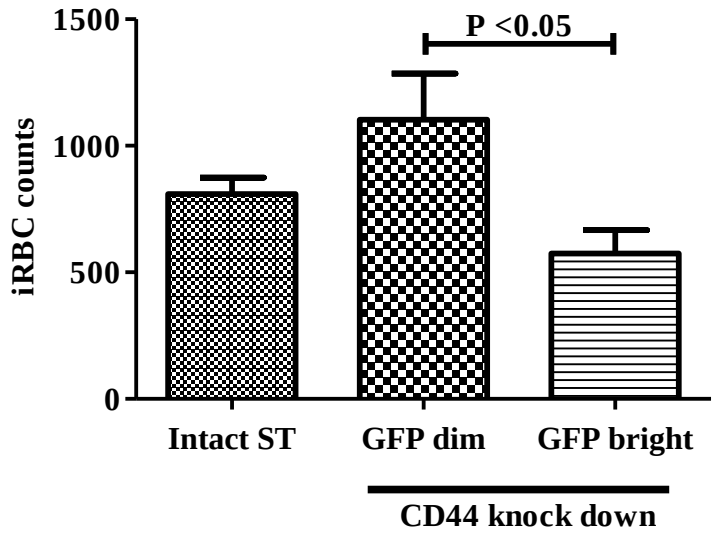
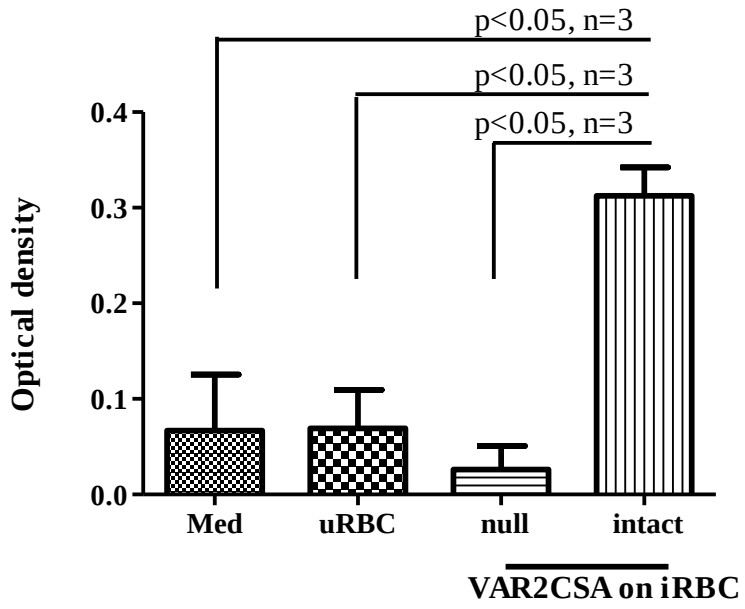


Figure 3.3.

A)


p<0.05, n=3
p<0.05, n=3
p<0.05, n=3
Optical density
0.4
0.3
0.2
0.1
0.0
Med
uRBC
null
intact
VAR2CSA on iRBC

B)

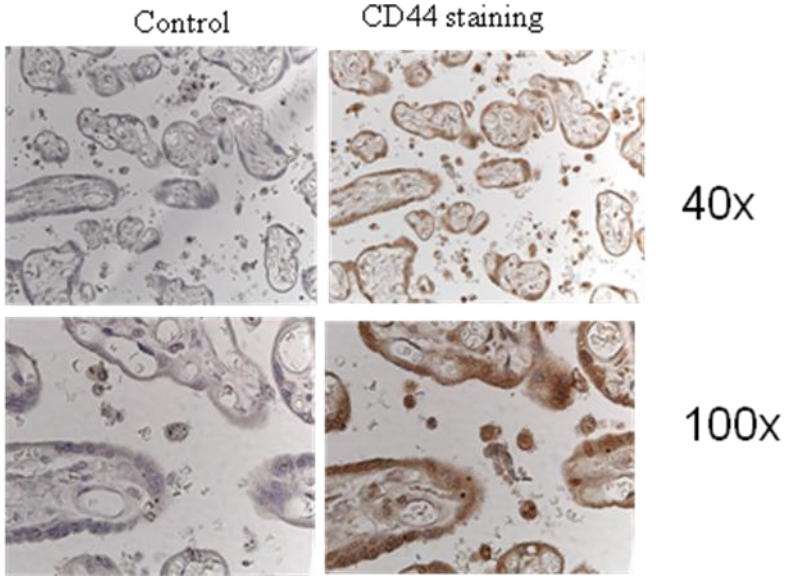
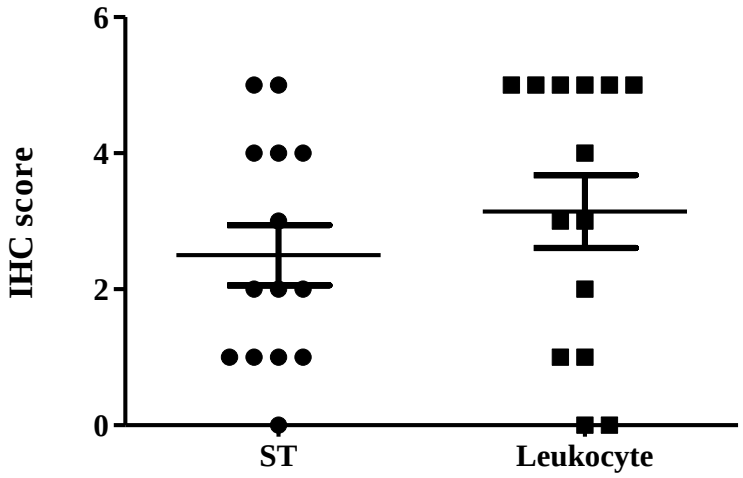

Control
CD44 staining
40x
100x

Figure 3.4

A)



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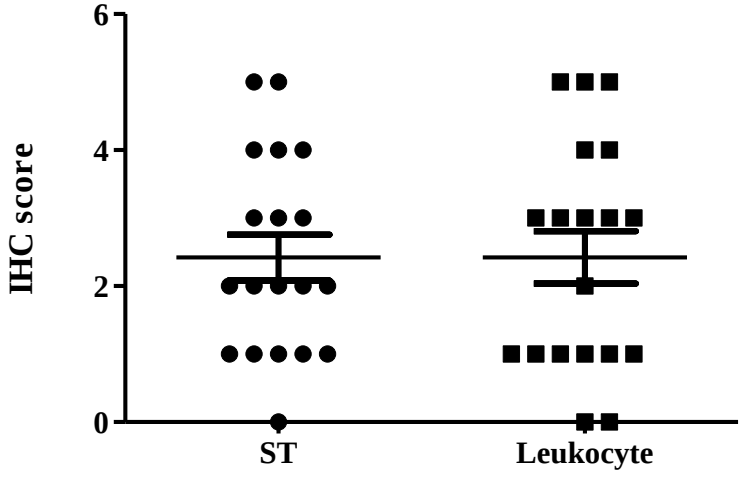
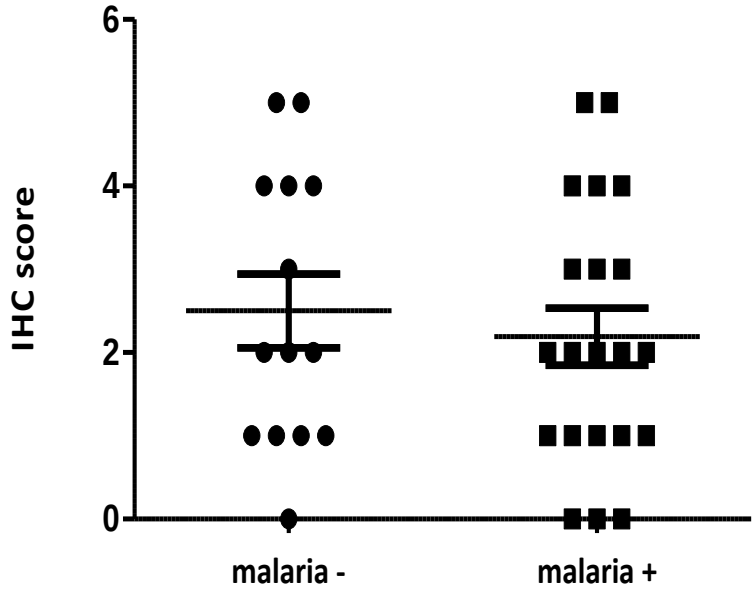


Figure 3.4 continued

C)



D)

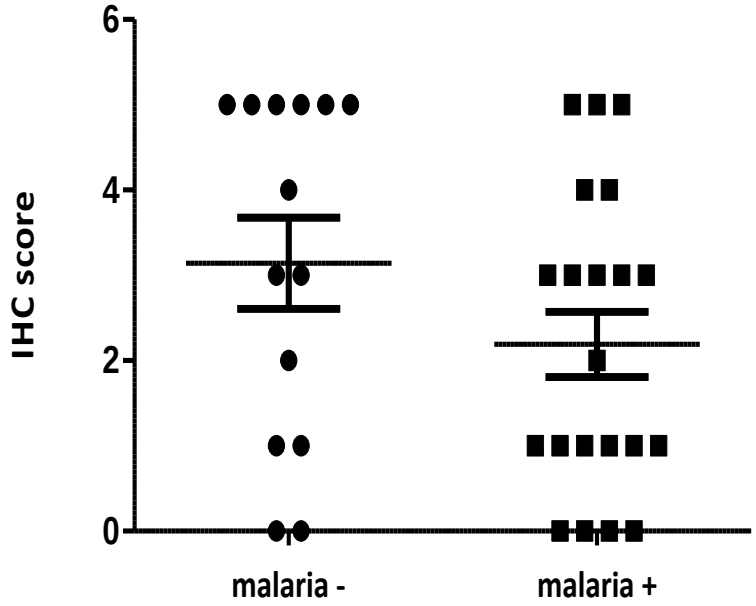


Figure 3.4 continued

E)

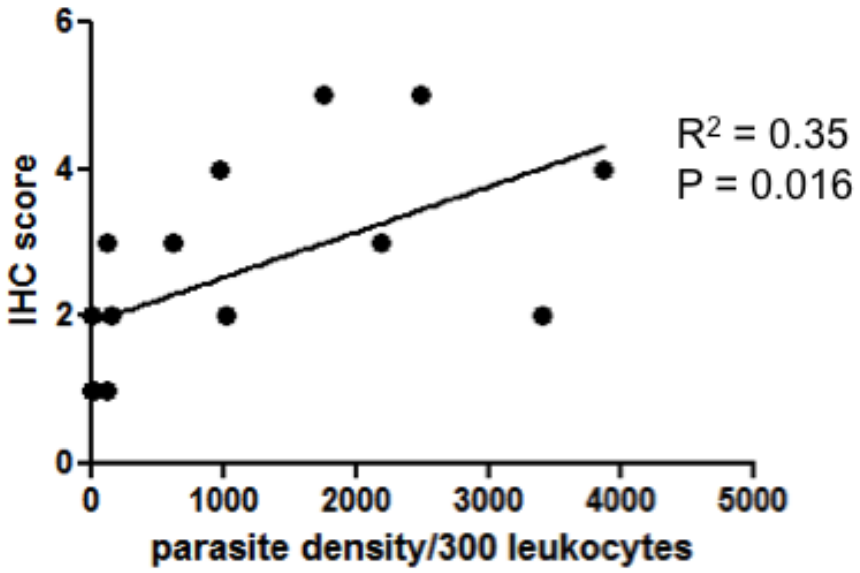
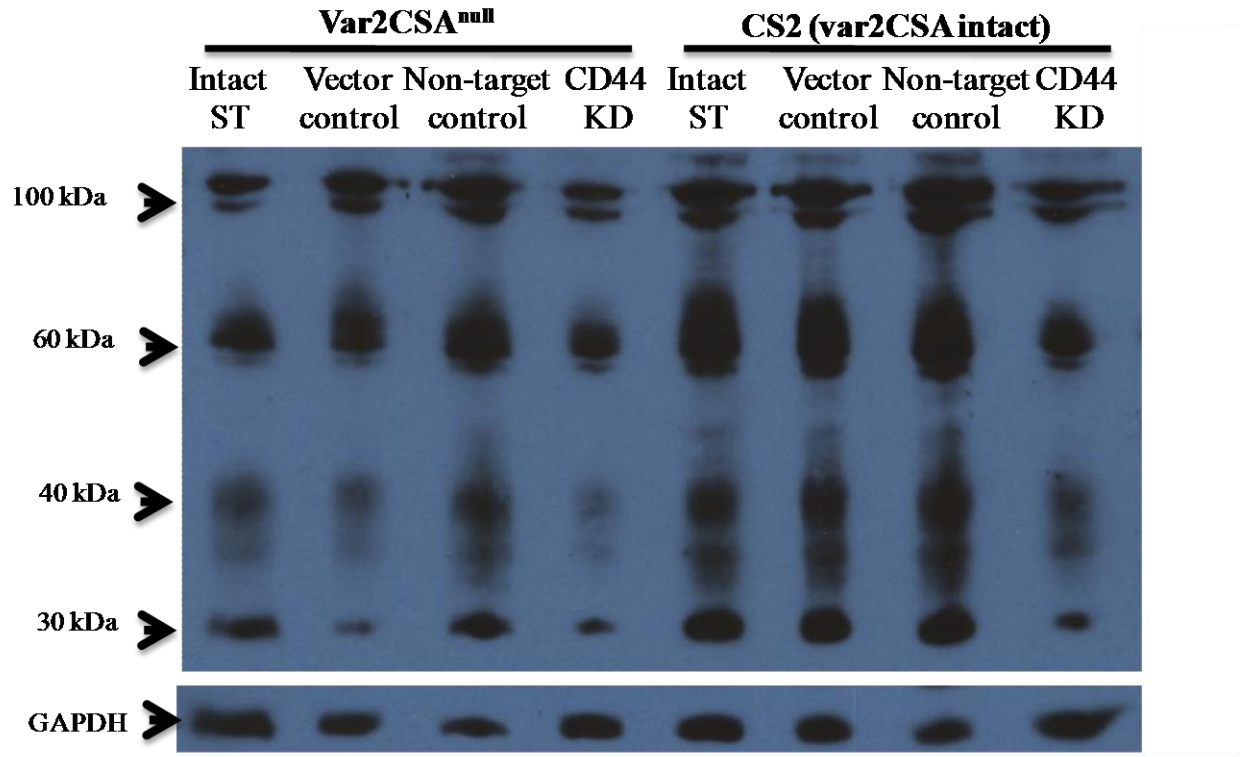
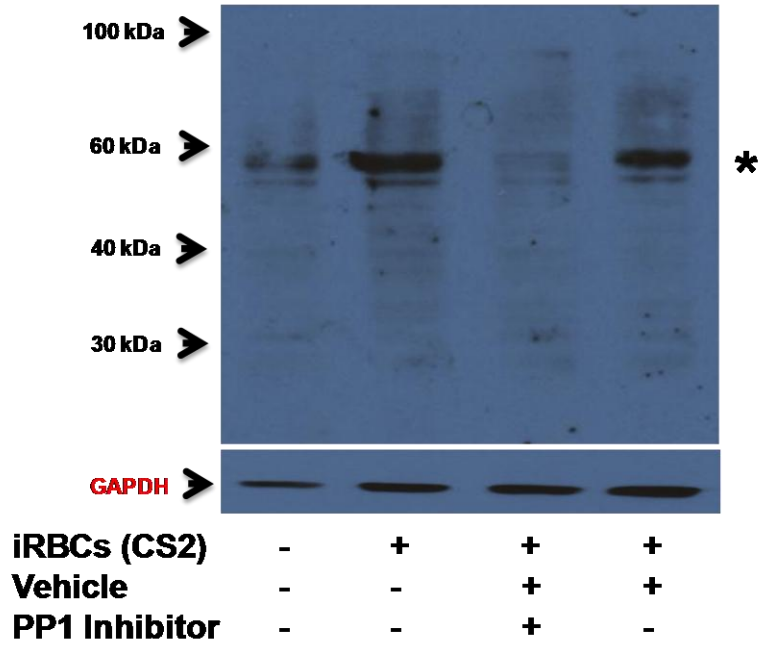


Figure 3.5.

A)


Var2CSA^null
CS2 (var2CSA intact)
Intact ST
Vector control
Non-target control
CD44 KD
Intact ST
Vector control
Non-target conrol
CD44 KD
100 kDa
60 kDa
40 kDa
30 kDa
GAPDH

B)


100 kDa
60 kDa
40 kDa
30 kDa
GAPDH
*
iRBCs (CS2) - + + +
Vehicle - - + +
PP1 Inhibitor - - + -

CHAPTER 4

DIFFERENTIAL RESPONSE OF HUMAN TROPHOBLAST AND MONOCYTIC CELLS TO CHONDROITIN SULFATE A- ADHERENT *PLASMODIUM FALCIPARUM* INFECTED ERYTHROCYTES AND THE TLR2 LIGAND, PAM3C SK4

[2]

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To be submitted

ABSTRACT

The human immune response to malarial infection is known to be multifactorial, involving aspects of both innate and adaptive immune mechanisms. However, the complexity of innate immune responses that are engaged simultaneously by *Plasmodium falciparum* infected red blood cells (iRBCs) and malarial components are not well understood, especially during pregnancy. In the infected placenta, iRBCs sequester in the maternal blood space, inducing immune activation of the fetoplacental epithelium, the syncytiotrophoblast (ST), resulting in recruitment of monocytes and local secretion of a number of pro-inflammatory cytokines and chemokines. While evidence shows that both iRBCs and hemozoin (Hz), the catabolic byproduct of hemoglobin metabolism by the intraerythrocytic parasite, both activate immunologic function in ST, the ability of this cell to respond to other malarial components like glycosylphosphatidylinositols (GPIs), which act through Toll-like receptor (TLR) 2, has not been examined. Likewise, how this stimulation may affect the responses of recruited monocytes in the placental milieu and their participation in placental pathogenesis remains understudied. In *in vitro* experiments, both ST and a monocytic cell line, THP-1, when stimulated with a known TLR2 ligand Pam3C SK4, exhibit immune activation, marked by secretion of interleukin (IL)-8. Neither cell type produces IL-8 following exposure to iRBCs. However, the presence of iRBCs augments Pam3C SK4-induced IL-8 secretion from ST, while the same exposure suppresses IL-8 secretion by THP-1 cells. In addition, whereas iRBC exposure induces upregulation of CD44, a putative CSA-adherence iRBC receptor, on ST, this response is induced only by Pam3C SK4 in THP-1 cells. Together, these results suggest that ST and THP-1 responses to dual exposure to iRBCs and a TLR2 ligand are divergent, with complex exposure to malarial products promoting

ST innate immune responses but suppressing those from monocytes. Thus, as previously proposed, ST likely plays an important contributing role in placental malaria pathogenesis.

Key words: Syncytiotrophoblast, Monocytes, THP-1, Pam3C SK4, Glycosylphosphatidylinositols, Toll-like receptor, Interleukin-8

INTRODUCTION

Pregnant women are at an elevated risk of malaria infection, more pronounced in earlier pregnancies with better outcomes in subsequent ones [1]. The increased risk in primigravidae is due to sequestration of infected red blood cells (iRBCs) in the placenta via a specific parasite/host receptor/ligand interaction within the intervillous space and at the syncytiotrophoblast (ST), fetal cells in contact with maternal circulation [2]. The presence of iRBCs in the intervillous space together with parasite components released during iRBC destruction by mature late trophozoites prompt immune activation of ST, marked by secretion of pro-inflammatory cytokines and chemokines [3-5]. These immune responses are thought to participate in the inflammatory environment in the infected placenta, marked by excessive accumulation of inflammatory cells [3-6].

In the placenta, chondroitin sulfate A (CSA) on ST interacts with the parasite protein VAR2CSA, a product of the *P. falciparum var2csa* gene, on the iRBC surface [7, 8]. Recently, we established CD44 proteoglycan, a cell adhesion molecule, as a functional receptor for CSA-adherent iRBCs on ST [S. Owino et al, unpublished data]. Because CD44 is a signaling molecule, its engagement may be responsible for the immunologic activation of primary ST that is elicited by VAR2CSA-bearing iRBCs [3, 4]. Aside from the iRBC itself, malarial toxins (GPI and Hz), released by mature parasites exiting iRBC, interact with host cells and induce inflammatory responses [9-13]. Three pattern-recognition receptors, TLRs, have been identified as recognizing these parasite-derived molecules: TLR2 and TLR4 respond to malarial GPIs and TLR9 responds to DNA complexed with natural Hz [14-16]. Studies in term placenta have demonstrated expression of TLR-1–10 at the mRNA level [17, 18]. TLR2 and -4 have been detected at the protein level in term trophoblast cells and with expression restricted to the ST, intermediate trophoblast, and extravillous trophoblast cell populations [17, 19-21]. We have

shown TLR9 protein expression in primary ST (N. Lucchi et al, unpublished). Activation of TLR2 or -4 on trophoblast using bacterial lipoproteins, peptidoglycan (PDG), has been shown to drive secretion of IL-6 and IL-8 [17]. Our studies have shown that the ST mounts a pro-inflammatory response to Hz [5]. The impact of engagement of TLR-2 in the context of placental malaria (PM) has not been explored.

Signaling pathways for all of the pattern recognition receptors are complex, and a study of simultaneous activation is rare. Recently, CD44 was found to associate with TLR2 and negatively regulate *in vivo* inflammation mediated by TLRs [22]. Here, we looked at differential responses with single and simultaneous engagement of TLR2 and activation of monocytic THP-1 cells and primary ST with CSA-adherent iRBCs. Pam3C SK4 was used as a model TLR2 ligand to understand TLR2 signaling in the context of PM. Results from this study highlight to what extent components of the malarial parasites differentially stimulate ST and monocytes during PM.

MATERIALS AND METHODS

Placental samples. Informed consent to obtain fresh, term placenta from elective Cesarean sections was sought from women attending pre-operative counseling at Athens Regional Medical Center, Athens, Georgia USA, using forms pre-approved by both Institutional Review Boards of University of Georgia and Athens Regional Medical Center. Immediately post-Cesarean delivery, placentae were collected into a sterile container with heparin anticoagulant and samples transported to the laboratory for processing. Placentae were processed following our previously published protocol [3].

Cultivation of P. falciparum and isolation of trophozoite-infected iRBCs

P. falciparum CS2 (CSA-binding; WT) laboratory isolates were kept in continuous culture as described [3]. Cultures were maintained at 4% hematocrit in 75 cm^2 tissue culture flasks at 37°C in 25 mM HEPES, 0.05 mg/mL hypoxanthine, 2.2 mg/mL $NaHCO_3$, 0.5% Albumax, and 0.01 mg/mL gentamicin RPMI medium. Cultures were confirmed to be mycoplasma negative by routine PCR analysis. Synchronous cultures were achieved by weekly/biweekly treatment with 5% D-Sorbitol.

To purify trophozoites, sorbitol synchronized cultures were enriched over a 35% to 70% percoll step gradient and centrifuged at 2000 x *g*. Cells at the interface were harvested and washed three times in PBS supplemented with 2% fetal bovine serum (FBS). Cell numbers were determined and concentration adjusted to 1 x 10^6 cells per ml

Cell culture

Cytotrophoblast cells purified from fresh placentae were cultivated in 6 and 24 well cell culture-treated plates (Corning, New York. USA) at 3.0 x 10^5 cells/cm^2 and syncytium formation allowed to proceed, normally by day 4 of culture. Monocytic THP-1 cells were obtained from American Tissue Culture Corporation (ATCC). Cells were maintained in 24 well tissue culture treated plates in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), according to provider specifications. In each well, 1.0 x 10^4 THP-1 cells were plated and then rested for 24 hours. Both Primary ST and THP-1 cells were then stimulated for 12 hours with Pam3C SK4 (a kind gift of Dr. Geert-Jan Boons, Complex Carbohydrate Research Center, University of Georgia) at 100 ng/ml, 10 ng/ml and 1 ng/ml only with or without iRBC before supernatants were collected.

Flow cytometry

To assess CD44 expression following exposure to iRBCs and Pam3Cys 4K, THP-1 cells were recovered from cultures by centrifugation and then stained with monoclonal mouse anti-CD44 antibody (IgG2a k; MCA89, AbD Serotec, Raleigh, NC) or appropriate isotype control antibody (IgG2a k; cat# 14-4724-85, eBiosciences, San Diego, CA) followed by signal amplification for 30 minutes using a polyclonal biotin anti mouse IgG (cat # 13-4013-85), strepavidin FITC (cat # 11-4317-87) (eBioscience San Diego, CA, USA). Data were acquired on a FACSCalibur Becton Dickinson (BD Biosciences) cytometer and analyzed using FloJo software (Version 9.5.2, Tree Star, Inc.,).

Enzyme Linked Immunosobant Assays (ELISA)

Sandwich ELISAs were used to detect cytokine levels in supernatants following manufacture's protocols with slight modification. For both IL-8 and IL-6 samples were diluted 1:1 in reagent diluents. Both ELISA kits, including matched antibody pairs and recombinant standard were from R&D Systems.

Statistical analysis

Graphing and statistical analysis were done using GraphPad software (version 5; GraphPad Sofware Inc., CA)

RESULTS

Pam3C SK4 stimulates secretion of IL-8 by ST and THP-1 cells in a concentration dependent manner.

Trafficking of cells within a microimmune environment is mainly orchestrated by chemokines, molecules secreted by immune and other cell types that form a chemoattractive gradient [24]. Here, secretion of IL-8, a proinflammatory chemokine responsible for immune activation of macrophages, epithelial cells and many other cell types and potent chemoattraction of neutrophils, was examined [25]. Following exposure to increasing concentrations of Pam3C SK4, a known TLR2 ligand, dose-dependent secretion of IL-8 by THP-1 cells (Figure 1A) and ST (Figure 1B) was observed.

P. falciparum iRBCs influence Pam3C SK4 dependent secretion of IL-8 secretion by monocytic cells (THP1) and primary ST cells

CD44 is crucial for influencing a number of cellular functions, ranging from adhesion, inflammation and phagocytosis [26, 27]. Recently we observed that CD44 also serves as a receptor for chondroitin sulfate A-adherent iRBCs on ST, and influences ST immunological responses to iRBCs (S. Owino et al, submitted; see Chapter 3). Interestingly, engagement of CD44 by its common ligand, hyaluronan, simultaneous with activation of TLRs on mouse bone marrow derived macrophages was shown to inhibit TLR signaling and subsequent cellular activation [22]. To assess if engagement of CSA proteoglycans on THP-1 cells by iRBCs has a similar impact, cells were co-incubated with CS2 iRBCs and Pam3C SK4. Under these conditions, secretion of IL-8 post-TLR-2 stimulation with either 10 or 100 ng/mL of Pam3C SK4 was suppressed by contact with iRBCs (Figure 2A). In contrast, secretion of IL-8 by primary ST

cells was enhanced by dual exposure to iRBCs and sufficient Pam3C SK4 (100 ng/mL) of (Figure 2B). Overall, ST cells were three fold more robust at IL-8 secretion compared to monocytic THP1 cells

Stimulation of monocytes by a TLR-2 ligand changes surface expression of CD44 proteoglycan

Following the observation that CD44 suppresses TLR2 mediated immune activation on mouse bone marrow derived macrophages [22] and its role as a functional receptor for CSA-adherent iRBCs on ST (S. Owino et al, submitted; see Chapter 3), we assessed the impact of exposure of THP-1 cells to Pam3C SK4 on CD44 expression. Our results indicate a dose-dependent upregulation of CD44 on these cells (Figure 3).

DISCUSSION

In general, immune activation and cellular dysfunction in most organs during malaria infection are consequences of iRBC cytoadhesion [28, 29]. Other parasite products or "toxins" released during rupture of iRBC and exit of mature parasites are immunogenic, including Hz and GPIs [13, 30]. To the host, Hz is toxic [31], and induces proinflammatory activity in antigen presenting cells [32-37] and ST [5]. Endosomal TLR9 engages parasite DNA in association with Hz [38] and stimulates the inflammatory secretory response [39]. As a salient feature in chronic PM, Hz is found ubiquitously within phagocytic cells and becomes embedded in IVS fibrin [29]. TLR2/1 heterodimers interact with parasite GPIs released from rupturing mature iRBCs [13, 40-44], an interaction that enhances signal for adhesion molecule expression on macrophages and endothelium, and promote inflammatory responses [45-49]. In PM, theories of GPIs promotion

of inflammation have been advanced, spanning from disruption of angiogenesis to impairment of placental function via activation of complement component C5 [9]. Trophoblasts have been shown to respond to a variety of TLR ligands confirming expression of TLR1-4 and 6 [50], and we have demonstrated TLR9 in primary ST (N. Lucchi et al, unpublished). Thus, pathogenesis of PM is likely a contribution of sequestering iRBCs, Hz and GPIs, eliciting both maternal and ST-derived inflammatory responses. Indeed, our recent observations demonstrated that Hz-exposed ST can secret chemokines and immune factors that determine the fate of peripheral blood mononuclear cells with regards to homing and phenotype of primary human monocytes [5]. Given the nature of ST to respond to broad array of pathogens [50], and the observation that ST functional changes are unique in the face of iRBC versus Hz exposure [3-5], this study was undertaken to examine the nature of the response of ST to an analog of malarial GPI, Pam3C SK4. Work by other groups has shown that TLR9 is a receptor for Hz-associated parasite DNA [38]. Because GPIs (and Pam3C SK4) act via TLR2, we anticipated that the ST response to such stimulation might be unique. Although the data are still preliminary, the results show that similar to stimulation with Hz [5], Pam3C SK4 elicits a robust IL-8 response from ST and could account for much of IL-8 secreted in the intervillous space, given the three fold difference in the amounts produced by monocytic THP1 cells. This response is similar to other epithelia (namely, airway epithelial cells), which also produce IL-8 in response to this TLR2 ligand [51].

A common pathological change in PM is intervillositis, massive maternal inflammatory infiltrate, composed predominantly of monocytes. This response is strongly associated with fetal compromise [29]. For this reason, understanding how malarial toxins influence monocytic function is also relevant for defining mechanisms that underlie poor birth outcomes associated with PM. Additionally, *in vitro* studies in mice show a TLR2 dependent secretion of

inflammatory cytokines when macrophages are stimulated with *P. falciparum* GPI, and lipopeptide Pam3CysK4 [52]. In human studies, parasite GPI resulted in immune activation and increased expression of complement C5a receptor by the monocytes [9, 10, 13]. In this study, THP-1 cells were also found to release IL-8 in response to Pam3C SK4 in a dose-dependent manner. Thus, as suggested by others, monocyte responses to malarial toxins like GPIs are likely to play a critical role in the inflammatory responses associated with poor birth outcomes in PM [53]. An unexpected result of TLR2 ligation by Pam3C SK4 was increased expression of CD44 on THP-1 cells. In addition to its potential role as an activating iRBC receptor on ST, CD44 has also been shown to function as a phagocytosis receptor on murine macrophages in an antibody-independent manner [54] as well as interacting with TLR2, an interaction that leads to suppression of TLR-mediated inflammation [22]. This is potentially exciting, since CD44 bears CSA moieties and was previously shown to be a receptor for CSA-adherent iRBCs on endothelium [55] and could explain reduced TLR-2 mediated secretion of IL-8 in THP-1 cells. Thus, during PM, binding of iRBCs to CD44 on monocytes may promote phagocytic uptake of iRBCs with a CSA adherence phenotype without the assistance of opsonizing antibodies, which are notably absent in primigravid women, the most PM-vulnerable group [56, 57]. These cells may also become less inflammatory during this process due to downmodulation of TLR2 activity as shown here. Expression levels of TLR2 were not examined in this work, but confirmation of unchanged levels would suggest that functional changes in signaling account for the observed reduction in IL-8 secretion.

The present work provides additional evidence that understanding of the complexity of cellular responses to malarial ligands, including those that interact with TLRs, is essential to a complete understanding of malarial pathogenesis. In this context, it is noteworthy that

polymorphisms in several TLRs impact on malaria susceptibility and outcome. For example, a Ser180Leu mutation in TIRAP, the adaptor protein that transmits TLR2- and TLR4- initiated signaling, which is common in Africa and Europe, influences disease outcome [58-60]. Also, clinical data highlight the influence of TLR polymorphisms during PM. For example, the odds of malaria-associated anemia were more than doubled in TLR-1 S248N heterozygous women [61]. It is worth noting that TLR1 signaling is dependent on formation of a dimer with TLR2 [51, 62]; the TRL2/1 heterodimer is the functional receptor for GPI [13]. Other observations showed that TLR4 Asp299Gly mutation is associated with a higher risk of maternal anemia and TLR4 Asp299Gly and TLR9 T148C mutations increased risk of low birth-weight babies [63]. Based on the present work, as well as a large body of published data, it is clear that such polymorphisms, if functional, may act at both the level of maternal (monocyte) and fetal (ST) levels.

Our preliminary observations suggest the involvement of Src kinases in TLR2-mediated IL-8 secretion since inhibition of Src kinases negatively impacted IL-8 secretion by THP-1 monocytes (see appendix 1), but further work will be required to confirm this observation. The extent to which Src kinases are involved in the apparent enhancement of Pam3C SK4-induced IL-8 secretion by ST in the presence of CSA-adherent iRBCs should also be assessed in future studies.

In conclusion, this study provides provocative evidence indicating that TLR2 ligands like malarial GPIs may impose a significant impact on the progression of PM pathogenesis, but with differential impact of ST and monocyte function in the presence of CSA-adherent iRBCs. While this study employed a synthetic analog for GPIs, evidence suggests that the response observed here is consistent with that elicited by native *P. falciparum* GPIs [9, 13]. Expansion of these

studies to include either native or synthetic GPIs and examination of other inflammatory markers and functions promises to enhance our understanding of malarial pathogenesis, particularly at the placental level.

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Figure legends.

Figure 4.1. Pam3C SK4 elicits IL-8 secretion by monocytic cells (THP1) and primary ST.

A. THP-1 monocytic cells were cultured and stimulated for 12 hours as described in Materials and Methods. Shown are concentrations of IL-8 (pg/ml) secreted in response to varying concentrations of Pam3C SK4 and by unstimulated cells (Med). Data represent three independent experiments.

B. Placental cells were cultured and stimulated for 12 hour as described in Materials and Methods. Shown are concentrations of IL-8 (pg/ml) secreted in response to varying concentrations of Pam3C SK4 and by unstimulated cells (Medium). Data are representative of four independent experiments.

Figure 4.2. *P. falciparum* iRBCs influence Pam3C SK4 dependent secretion of IL-8 secretion by monocytic cells (THP1) and primary ST cells

A Monocytic cell line (THP1) were cultured and stimulated for 12 hour as described in Materials and Methods. Shown are concentrations of IL-8 (pg/ml) secreted in response to varying concentrations of Pam3C SK4 and by unstimulated cells (Medium), in the presence or absence of VAR2CSA competent (WT). Data are representative of two independent experiments.

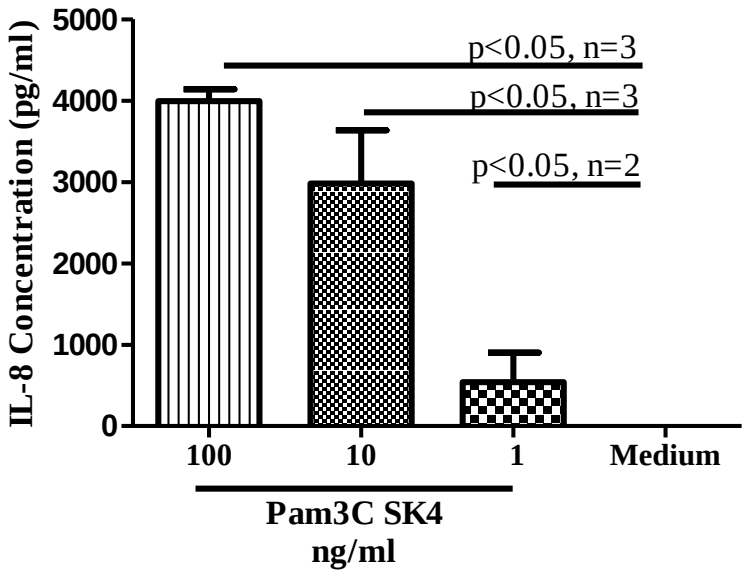
B. Placental cells were cultured and stimulated for 12 hour as described in Materials and Methods. Shown are concentrations of IL-8 (pg/ml) secreted in response to varying concentrations of Pam3C SK4 and by unstimulated cells (Medium), in the presence or absence of VAR2CSA competent (WT). Data are representative of two independent experiments.

Figure 4.3. Pam3C SK4 induces increased CD44 expression on THP-1 cells.

THP-1 monocytic cells were cultured and stimulated for 12 hour as described in Materials and Methods. The plot shows median fluorescence intensity of cell surface CD44 in cells exposed to increasing concentrations of Pam3C SK4 and by unstimulated cells (Med), as well as for an antibody isotype. Data represent two independent experiments.

Figure 4.1.

A)


IL-8 Concentration (pg/ml)
5000
4000
3000
2000
1000
0
p<0.05, n=3
p<0.05, n=3
p<0.05, n=2
100
10
1
Medium
Pam3C SK4
ng/ml

B)

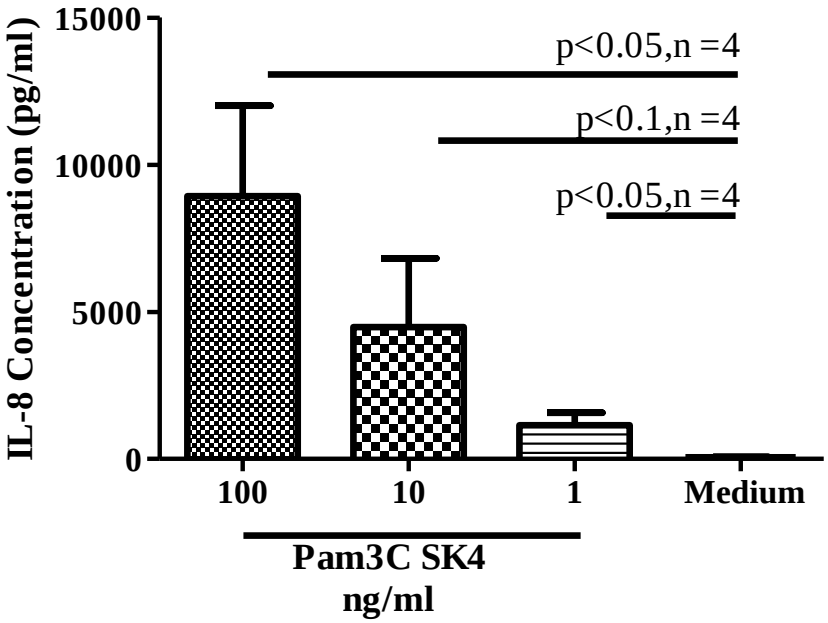
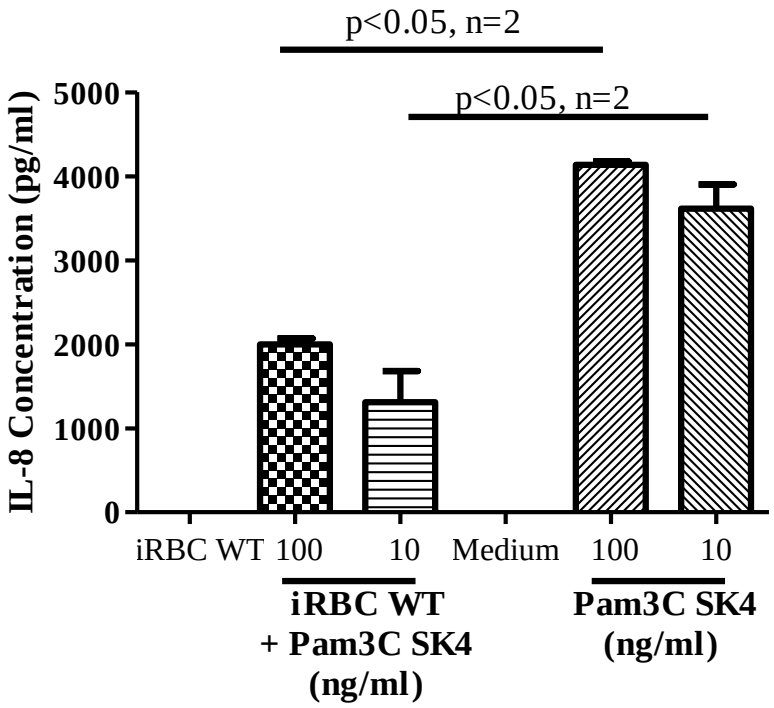

IL-8 Concentration (pg/ml)
15000
10000
5000
0
p<0.05,n =4
p<0.1,n =4
p<0.05,n =4
100
10
1
Medium
Pam3C SK4
ng/ml

Figure 4.2.

A)


p<0.05, n=2
p<0.05, n=2
IL-8 Concentration (pg/ml)
5000
4000
3000
2000
1000
0
iRBC WT
100
10
Medium
100
10
iRBC WT + Pam3C SK4 (ng/ml)
Pam3C SK4 (ng/ml)

B)

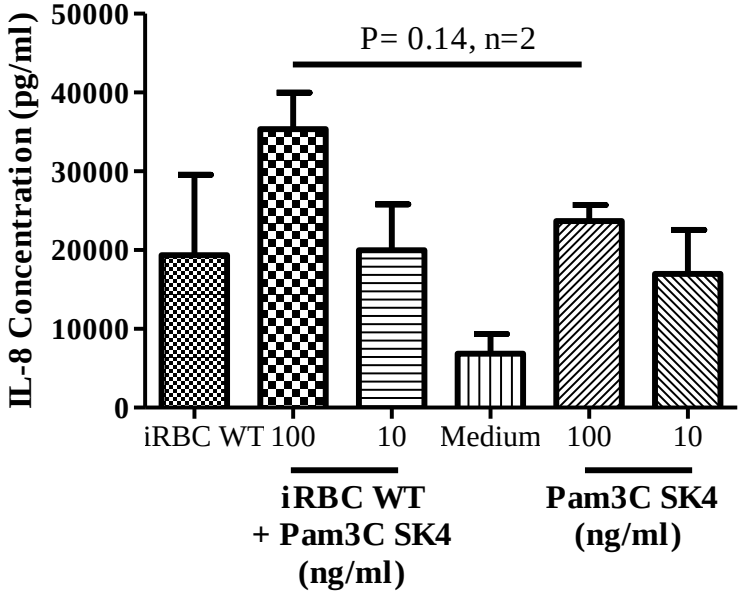
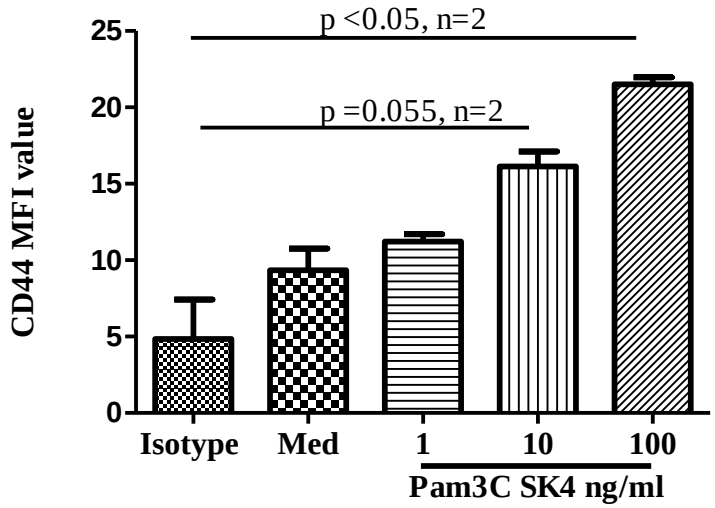

P= 0.14, n=2
IL-8 Concentration (pg/ml)
50000
40000
30000
20000
10000
0
iRBC WT
100
10
Medium
100
10
iRBC WT + Pam3C SK4 (ng/ml)
Pam3C SK4 (ng/ml)

Figure 4.3


p <0.05, n=2
p =0.055, n=2
CD44 MFI value
25
20
15
10
5
0
Isotype
Med
1
10
100
Pam3C SK4 ng/ml

CHAPTER 5

SUMMARY AND CONCLUSIONS

Malaria is an enigmatic disease and continues to be a leading public health problem requiring urgent solutions in terms of drugs and vaccine development. A basic understanding of the host/parasite interaction is paramount, and critical if effective control strategies to curb the disease are to be realized. Epidemiological studies indicate that, despite prior immunity due to parasite exposure, pregnancy renders women more susceptible to infection, and this is more pronounced during their earlier pregnancies. Malaria during pregnancy causes both maternal and newborn complications. The hallmark of malaria during pregnancy is the sequestration of infected red blood cells (iRBCs) in the placental intervillous space (IVS) often accompanied by the accumulation of maternal immune cells and several placental pathologies, giving rise to what has been referred to as placental malaria (PM). Complications associated with PM are; maternal anemia, premature delivery, low birth weight (LBW; <2500g), intrauterine growth retardation and in some cases abortion. Current annual estimations of infant mortality arising from complication of PM have been put at a staggering number of up to 200,000 infants. This together with the aforementioned consequences of PM justifies the need for a concise understanding of the parasite/host interactions occurring in pregnant women. A huge body of literature indicates that, apart from the parasite, the host's immunologic response, particularly proinflammatory cytokines and chemokines, are key in mediating PM pathology. For example, levels of IL-8 proinflammatory chemokine have been associated with LBW and intrauterine growth retardation. In addition, binding of iRBCs to syncytiotrophoblast (ST) cells and interaction with malarial components stimulate intercellular signaling and gene expression

changes in ST, marked by increased secretion of proinflammatory cytokine, IL-8 and MIF. Additionally, by secreting immune factors, ST cells seem to contribute to accumulation of maternal immune infiltrate.

The ST syncytium is in direct contact with maternal blood and severs as the interface between mother's blood and fetal circulation. During PM, a chondroitin sulfate A (CSA) glycosaminoglycan mediates binding to iRBCs associated parasite protein, *Plasmodium falciparum* erythrocyte membrane1 (PfEMP1). While the role of CSA is well established, a description of its protein backbone that could act as a putative signaling receptor on ST is lacking. In addition to the above, a concise knowledge into what and how components of malaria induce ST mediated immune modulation of the IVS milieu remains unclear. Noting these gaps in knowledge, the current study was undertaken with the aim of determining the role of CD44 molecule, a know CSA bearing proteoglycan and signal receptor on T cells. Also, these studies attempted to discern how a parasite derived component; gylcosylphophatidylinositols (GPIs) might influence the ST and its microimmune environment. Knowledge obtained here fits well with trying to define the components of ST cells involved in iRBC mediated activation of ST and furthers our basic understanding of parasite/host interaction.

The in vitro binding of iRBCs to CD44 on ST cell lysates in a CSA dependent fashion and the overall presences of CD44 on placental histological sections confirmed the role CD44 plays with regard to mediating iRBCs binding onto ST. Furthermore, changes in tyrosine-phosphorylated pattern on a large array of proteins on ST stimulated with iRBC in a CD44 dependent manner. This was an interesting finding since some of the proteins, of approximately 60 kDa coincide with known Src kinases important for MAPK dependent cellular activation. Although the level of CD44 staining on placental ST remained unchanged in malaria positive

samples, the fact that malaria correlated with CD44 on leukocytes has great implications, since CD44 is known to participate in receptor mediated phagocytosis. It is possible that with added numbers of samples, a clear trend in CD44 levels on placental ST may be realized, however in our study and given limited samples no differences were seen.

The role of plasmodial GPI in activating both ST and human monocytes through toll-like receptor 2 (TLR2) an innate immune sensor has not been investigated. Here, by using a known TLR-2 ligand, PAM3C SK4, as GPI analog, we have shown an increased IL-8 secretion in both ST and monocyte. Additionally, we noted an increase on levels of CD44 on monocytic cell line post stimulation. Both of these observations are important since they start to shed light into what immunologic events might follow after iRBCs bursting and releasing parasite components such as GPI. Given that we have used GPI mimic and not the actual molecule, is be a potential limitation. As such, more experiments need to be done using actual GPI compounds. We are currently working with our collaborators towards this.

In conclusion, results from the current study suggest that during PM, binding of iRBCs to ST is to some degree mediated by CSA on CD44 and that this molecule influences phosphorylation events within the ST cells in a Src kinase family dependent manner. In addition, we show that malaria infection alters the CD44 profile on placental leukocytes. Furthermore, it is shown here that ST and monocyte immune responses is to some extent due TLR-2 ligation, and leads to increase of CD44 on monocyte.

APPENDICES

Appendix 1: Assessment of chondroitin sulfate biosynthesis by glycan mRNA array

Glycan array developed by Kelley Moremen and colleagues at Complex Carbohydrates Research Center (CCRC) targeting 68 relevant transcripts was used. Isolation of mRNA from primary ST exposed for 24 hours to CSA-adherent iRBCs, uninfected RBCs or hemozoin followed and were subjected to array analysis; analyzing changes for iRBCs relative to uRBCs and hemozoin relative to unstimulated ST

Gene type and function		**Treatment**		
Gene	Function	CSA-adherent iRBC	Hemozoin (Hz)	Potential implications for malaria
Heparan sulfate 6-O-sulfotransferase 3 (hs6st3)	Sulfation of heparan	Increase 1000%	Unchanged ___	Increased CSA
D-glucuronyl C5-epimerase	Promote synthesis of dermatan sulfate from CS	Unchanged __	Increased 240%	Decreased CS
Xylosetransferase I XYLTI	Initial enzyme in the biosynthesis of chondroitin sulfate (CS)	Unchanged __	Decreased -64%	Decreased CS
Glucosaminyl N-deacetylase/N-sulfotransfarase 4 NDST-4 (dual function enzyme	Sulfation of N-acetylglucosamine residues of heparan sulfate at N- position	Decreased -85%	Decreased -72%	Increased CS

Appendix 2: Summary of cytokine secretion by BeWo cells

Given that ST cells produce macrophage migration inhibitory factor (MIF) following activation by CSA-adherent iRBCs [1] the importance of CD44 in regulating the ST secretory response to iRBCs was also examined. Supernatant fluids collected from intact and CD44 KO BeWo cells at 12 and/or 24 hours post-iRBC exposure were subjected to sandwich ELISA to detect MIF and IL-6. Contrary to expectation, the absence of CD44 was associated with increased secretion of MIF, with the highest levels of secretion seen for responses to CSA-adherent iRBCs by CD44 KO cells. Slight increases overall for IL-6 secretion by CD44 KO cells relative to intact BeWos were also observed at 12 hours post-stimulation, but appeared to be independent of the stimulus. These observations suggest involvement of CD44 in regulation of the response of ST to iRBCs.

	Time post-stimulation	BeWo CD44 KO			BeWo intact/BeWo non-target control*		
		iRBC KO	iRBC WT	uRBC	iRBC KO	iRBC WT	uRBC
IL-6 (pg/mL)	12	15.7	10.4	14.5	3.7	3.0	2.9
IL-6 (pg/mL)	24	11.2	9.7	14.0	7.4	2.2	11.6
MIF (pg/mL)	12	85.6	839.7	0	100.8	17.6	0
MIF (pg/mL)	24	248.4	496.9	ND	64.6	183.2	ND

*Note: Control cells for data generated for IL-6 were intact BeWo cells; controls for MIF data were non-target lentivirus transduced BeWo cells. IL-6 data were generated in a single experiment; MIF data for 12 and 24 hours were generated in two separate experiments

Reference:

1. Chaisavaneeyakorn, S., et al., *Immunohistological characterization of macrophage migration inhibitory factor expression in Plasmodium falciparum-infected placentas.* Infect Immun, 2005. **73**(6): p. 3287-93.

Appendix 3 Src Kinase signaling is important in Pam3C SK4 induced IL-8 secretion by monocytes (THP1)

THP-1 monocytic cells were cultured and treated with 10μM/ml of a pan Src Kinase inhibitor (PP1, cat# 567809, EMD Millipore, Billerica MA, USA) or vehicle (DMSO) for 30 minutes then stimulated for 12 hours as described in Materials and Methods. Shown are concentrations of IL-8 (pg/ml) secreted in response to varying concentrations of Pam3C SK4, CSA-adherent iRBC and by unstimulated cells (Med). Data represent one experiment.