PLACENTAL MALARIA: UNDERSTANDING THE MATERNAL/FETAL INTERFACE

by

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(Under the Direction of Julie M Moore)

ABSTRACT

Pregnant women, especially those in their first pregnancy, appear to be more susceptible to malaria infections even in endemic areas where by adulthood substantial acquired immunity to malaria is apparent. This often results in what is referred to as placental malaria (PM). PM is a major public health problem associated with poor fetal outcomes such low birth weight and maternal anemia. It is characterized by the sequestration of malarial-infected red blood cells (iRBCs) in the placental intervillous spaces and infiltration of maternal immune cells. While the accumulation of iRBCs is known to be mediated by the binding of iRBCs to syncytiotrophoblast (ST), fetal cells in direct contact with maternal blood, little is known about how this binding influences ST immune function. This has been due in part to the lack of an appropriate system to perform the necessary experiments. In this study, such a system was developed using both primary trophoblasts cells and a placental choriocarcinoma cell line, BeWo. This system was used to assess the biochemical and immunological changes induced in ST upon specific binding of ST-adherent iRBCs and malarial components such as hemozoin (the malaria pigment) and crude malaria antigen. For comparison, ST responses to

lipopolysaccharide (LPS) were assessed. The binding of $iRBC^{ST}$ led to an increase in the tyrosine phosphorylation of at least two ST proteins. The mitogen activated protein kinase (MAPK), JNK pathway, involved in many cellular activation processes and gene expression of most immune factors, was also activated. Stimulation with LPS, malarial antigens and hemozoin all led to an increased phosphorylation of ERK1/2 MAPKs. Stimulation of the ST cells with LPS led to increases in the gene expression and protein secretion of TNF-α, MIP-1α and MIP-1β and IL-8. Only modest increases in expression of TNF-α, TGF-β and IL-8 mRNA were observed with iRBC binding and this did not result in the secretion of these cytokines. However, binding of iRBC led to the secretion of macrophage migration inhibitory factor (MIF) and to the chemotaxis of peripheral blood mononuclear cells. Treatment with hemozoin stimulated the secretion of IL-8.

Taken together, these results suggest that during PM, the binding of iRBCs and interaction with malarial components stimulates intracellular signaling and gene expression changes in the ST. This leads to the secretion of proinflammatory chemokines that influence the local immunological milieu, making the ST an active immunologic player in the placental environment during PM.

INDEX WORDS: Placenta, Malaria, Syncytiotrophoblast, MAPK, Gene-expression, ICAM-1, Hemozoin, LPS, Activation, Pregnancy

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DEDICATION

I dedicate this work to Alberto and Simona Lucchi. I did not have to think on this one. It goes without saying that if I have finally finished the race, it is all because of the caring support given to me by Alberto Lucchi, my husband and best friend. To Simona, my precious one, you are the best a mother can ask for. For all those questions about ‘mum’s cells’ and placentas. Finally, we can ‘rest the cells in peace’. This was a family business and I am greatly indebted. ASANTE SANA!

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CHAPTER 1

INTRODUCTION

Epidemiological studies have shown that pregnant women are more susceptible to malaria infection even in endemic areas where people acquire partial immunity to malaria. The mature late stages of the most lethal causative agent of malaria, *Plasmodium falciparum,* are known to sequester in the host microvasculature where they adhere to the host endothelium, escaping clearance in the spleen. This cytoadherence phenomenon is believed to be responsible for the development of much of the pathology associated with malaria. Malaria infection during pregnancy results in the sequestration of malaria infected red blood cells (iRBCs) in the intervillous space (IVS) of the placenta and binding of these iRBCs to the syncytiotrophoblast cells (ST; fetal cells in direct contact with maternal blood). This is commonly accompanied by inflammatory cell infiltrates in the IVS, and placental lesions such as focal syncytial necrosis. This phenomenon is referred to as placental malaria (PM). PM is an important public health problem. It is estimated that up to 200,000 infants die annually due to adverse consequences of PM. It is associated with maternal anemia, premature delivery, and low birth weight (LBW; <2500 g) babies. The underlying biological basis for this susceptibility to malaria during pregnancy, and the associated poor birth outcomes, remain poorly understood.

P. falciparum resident in RBCs exports proteins to the RBC membrane. These proteins can serve as ligands for cytoadherence; *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is implicated in this cytoadherence. This protein is encoded by highly polymorphic members of the *var* gene family. Only one *var* gene is expressed on the surface of an iRBC at any given time, making it possible to select for parasites expressing one particular adhesion phenotype. So far, the only uncontroversial receptor for iRBCs adherence in the placenta via PfEMP1 is the glycosaminoglycan chondroitin sulfate A

(CSA) expressed by the ST. The demonstrated sequestration of iRBCs in the IVS of the placenta and their cytoadherence to ST raises several questions:

1. Does the binding to ST induce intracellular activation of the ST?
2. If so, how is this activation manifested and how can it be experimentally detected?

In order to decipher the role of the ST in contributing to the local immune responses during PM, a reliable *in vitro* system is required. To date, most studies of CSA-iRBC binding have involved in vitro adherence assays with commercially available CSA or chondroitin sulfate proteoglycans (CSPG) from different sources coated onto plastic dishes. However, these CSPGs differ widely in the structural features of their glycosaminoglycan (GAG) chains and therefore exhibit differential adherence characteristics. Recently, sections of cryopreserved placenta have been used for adhesion studies. While this method appears to provide a suitable *in vitro* system for the study of the adhesion of iRBCs to CSA, it is inappropriate for time course experiments or any functional studies interested in ST responses to iRBC binding.

Studies exist which demonstrate that adhesion molecules can serve as receptor-signaling molecules capable of transducing extracellular signals leading to cellular activation. Adhesion of iRBCs to endothelial cells via CD36 was shown to induce intracellular signaling in the endothelial cells. It is not known whether the binding of iRBCs to CSA on the ST during PM leads to ST intracellular signaling or if the suggested receptors merely serve as attachment points for the parasites but have no cellular signal transduction consequences in the ST.

Cytokines and chemokines are known to play major roles in shaping the immune responses. The role of local cytokine and chemokine responses in the placenta during PM

has been demonstrated. These have mainly been shown to be secreted by maternal immune cells. For example, interleukin-8 (IL-8), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β, macrophage migration inhibitory factor (MIF) and monocyte attractant protein-1 (MCP-1) were all shown to be elevated during PM. Elevated production of IFN-γ and TNF-α in the IVS of the placenta was associated with protection but also pathology while elevated mRNA levels for MCP-1, MIP-1α and IL-8 were associated with enhanced monocyte infiltration to the IVS and with LBW and intrauterine growth retardation. The role of the ST cells in contributing to this cytokine/chemokine immune response during PM has not been elucidated. The ST cells have been shown to be capable of secreting both pro- and anti- inflammatory cytokines in response to pathogenic bacteria and other intrauterine infections. It is, therefore, potentially capable of influencing the local immunological environment during PM.

The influence of the ST on the accumulation of maternal leukocytes during PM is still not known. It has previously been shown that activated lymphocytic cells bind to ST *in vitro*. Adhesion of these cells to ST was shown to be stimulated by pretreatment of the ST cultures with several cytokines either alone or together. Maternal leukocyte-ST adhesion may have implications for normal trophoblast function and recruitment and/or retention of these effector cells in the placenta. LPS-activated monocytes were shown to adhere to ST in an IFN-γ-dependent manner and this adhesion resulted in focal ST apoptosis. Several receptors/ligands have been implicated in this interaction. It is known that activated monocytes adhere to the ST via receptors such as lymphocyte function-associated antigen-1 (LFA-1) on the monocytes to intracellular adhesion molecule 1 (ICAM-1) on the ST. Both TNF-α and IFN-γ are known to upregulate the expression of

ICAM-1 on ST. High levels of IFN-γ and TNF-α are elaborated during PM and it is therefore possible that during PM the expression of ICAM-1 on the ST may be upregulated. It is not known if direct binding of iRBCs can upregulate ICAM-1 or other adhesion molecules on ST. Enhanced expression of ICAM-1 during PM may impact the attachment, recruitment and retention of maternal leukocyte cells in the IVS and the ST, thus explaining the increased monocytic infiltration observed during PM.

Significance

There is accumulating evidence indicating that the host's immunological response, particularly proinflammatory cytokines and chemokines, play a major role in malaria pathophysiology. To date, little is known about how the binding of iRBCs to the ST may influence this immunological response in the placenta. Without detailed knowledge of how the host (i.e., ST) and parasite interact and respond to each other, especially in terms of development, maintenance and control or modulation of immune responses in the pregnant uterus (placenta), therapeutic methodologies for the management of malaria during pregnancy cannot be fully evaluated. An understanding of this interaction of iRBCs with ST cells is only attainable if an appropriate model system exists. The system developed in this study provides a means to study the nature and implications of iRBC binding in the placenta. Only through characterization of iRBC binding phenotypes, how these binding phenotypes are selected for, the parasite binding ligands involved, the iRBC receptors on the ST, and the functional changes induced in the ST by iRBC binding, will a complete understanding of the biology of the maternal/fetal interface in the context of PM be achieved. This study assessed the extent to which ST function is influenced by cytoadherent iRBCs or interaction with malarial

components such as hemozoin (malarial pigment) and malarial antigen through a series of biochemical and immunological investigations. The findings from this study demonstrates that the ST is not a passive component of the placenta during PM but is capable of responding to interaction with iRBCs and malarial components via signal transduction and production of chemokines. Therefore any intervention strategies for malaria in pregnancy will have to consider this immunological contribution of the ST. This study also enhances our understanding of the general biology of the maternal/fetal interface which has important implications for reproductive biology in general and for other infectious diseases such as HIV and intrauterine bacterial infections.

Hypothesis:

The central hypothesis of this study was that the ST contributes to the local immune environment in the IVS during PM by responding to the binding of the iRBCs and to stimulation with malarial components. To test the proposed hypothesis, four specific aims were addressed:

1. To determine the impact of iRBCs binding on the activation of the ST. This was addressed by assessing signal transduction changes in the ST upon the binding of iRBC and on stimulation with malarial components.
2. Secondly, to assess the mRNA expression levels of known cytokines and chemokines genes by real-time PCR and the protein secretion of these cytokines and chemokines by standard ELISA methods following binding of $iRBC^{ST}$ or interaction with malarial components.
3. To determine the ability of the response of ST to iRBCs to recruit maternal immune cells (peripheral blood mononuclear cells) using chemotactic assays.

4. To determine the impact of iRBC binding on expression of cell adhesion molecules such as ICAM-1 by ST.

An *in vitro* system using normal human placental ST cells and a choriocarcinoma cell line (BeWo) together with iRBCs selected for ST-binding was developed. This system was used for iRBCs adhesion studies and functional studies. Results from this study demonstrate for the first time that adhesion of iRBCs to ST activates ST cells by inducing tyrosine phosphorylation of several proteins and the enhanced phosphorylation of the mitogen activated protein kinase (MAPK) pathway. This adherence also led to the secretion of MIF and the chemotaxis of PBMCs but did not lead to upregulation of ICAM-1. The interaction of the ST cells with crude malarial antigens led to the phosphorylation of ERK1/2. Stimulation with hemozoin led to an early phosphorylation of ERK1/2 and a time-dependent secretion of IL-8.

CHAPTER 2

LITERATURE REVIEW

MALARIA

Causative Agents and Life Cycle

The causative agents of the disease malaria are protozoan parasites of the genus *Plasmodium,* family Plasmodiidae. Only four of the over 100 species of plasmodia are infectious to humans, causing malaria in its various forms: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae.* Of these four, *P. falciparum* is by far the most common and the most virulent. This work focused only on *P. falciparum.* The malaria parasite is transmitted from one person to the other by female mosquitoes of the genus *Anopheles*. The males do not transmit the disease as they feed only on plant juices. While feeding on its host, the female mosquito injects the sporozoite forms of the parasite into the bloodstream that find initial abode in the host's hepatocytes. In the hepatocytes, the sporozoites differentiate and divide mitotically into massive numbers of liver merozoites. The merozoites are then released into circulation and subsequently infect host red blood cells (RBCs), thus beginning the asexual blood-stage lifecycle of the parasites (erythrocytic cycle). In the RBCs, they mature and divide for 48–72 h (depending on the *Plasmodium* species) through three distinct stages. The ring stage accounts for about half of the intraerythrocytic cycle, but it is metabolically nondescript (1). It is followed by the trophozoite stage, a very active period during which most of the RBC cytoplasm is consumed. Finally, parasites undergo 4–5 rounds of binary divisions during the schizont stage, producing a new brood of merozoites that reinvade fresh RBCs and resume the cycle. It is this erythrocytic cycle that is associated with the clinical manifestations of the disease such as bouts of fever and anemia. The merozoites can also mature into the sexual forms of the parasites giving rise to male and female

gametocytes. These stages are infective for the mosquito that ingests them during its next blood meal and may continue the cycle on the next blood meal.

Epidemiology

Malaria is one of the most prevalent human infections worldwide and continues to be one of the main global health problems of our time. Its social and economic burden in endemic countries is immense. Over 40% of the world's population live in malaria-endemic areas (2). It is estimated that annually, about 2.2 billion people are exposed to the threat of *P. falciparum* malaria, and that between 300–600 million clinical attacks are attributable to this parasite yearly (2, 3). Ninety percent of deaths occur in sub-Saharan Africa, the majority involving children less than 5 years of age. In addition to children, pregnant women (particularly those in their first pregnancy (primigravidae)) (4) and non-immune people e.g., travelers (5, 6) are at highest risk of severe disease. However, during malaria epidemics and in non-malarious regions, all age groups may be at risk of severe disease. This can occur either when changes in the physical environment (caused by climatic variation, agricultural projects or mining, for example) increase the capacity of mosquitoes to transmit the disease or when population displacements (natural disasters, war) expose non-immune populations to infection (7, 8).

Clinical Manifestations of Malaria

In non-immune individuals, the erythrocytic stage of the infection is associated with a range of host pathologies, from mild fever to fatal multiorgan disease. While destruction of RBCs, during the parasite's life cycle, contributes significantly to disease

(mainly anemia), the rupture of iRBCs triggers a cascade of inflammatory responses which, if not controlled, can lead to death (9). At the time of schizont rupture, many parasite antigens are released into the blood stream stimulating the production of cytokines such as tumor necrosis factor alpha (TNF-α) and other factors (10) from cells of both the innate and adaptive immune systems. These then trigger the classical symptoms of fever, rigors and nausea, but also contribute to control of parasite replication (reviewed by Richards et al.(11)). Other nonspecific symptoms include chills, malaise, headache, myalgias, cough and gastrointestinal symptoms. Fever and splenomegaly are the most frequent physical findings on examination. Less often, hepatomegaly, jaundice and abdominal tenderness are noted. At highest risk of complications from malaria are non-immune people, children less than five years of age, and pregnant women. Complications generally involve the central nervous, pulmonary, renal and hematopoietic systems (12). Severe malaria may manifest itself as severe anemia (hemoglobin < 50 g/L or hematocrit < 15 %), cerebral malaria and, in pregnant women, placental malaria (PM; discussed later). Cerebral malaria is manifested by altered level of consciousness, focal neurologic findings and seizures (13). Mortality is high (15% to 25%), and survivors may have residual neurologic deficits (14). Although semi-immune people and those living in endemic regions tend not to experience severe malaria, they may still experience complications from recurrent infections. In young children, severe anemia is the most common complication of chronic malaria (15).

BIOLOGY OF *PLASMODIUM FALCIPARUM*

The survival and transmission of malaria parasites depends on the ability of the invasive stages of the parasite to recognize and invade the appropriate host cell types. The exoerythrocytic stage in the liver release merozoites that invade the human mature RBCs, a niche within the most highly terminally differentiated host cell. This choice of niche by the parasite appears 'clever' at the beginning as life inside this normally quiescent cell offers the parasite protection from the host's immune system since the RBCs express few if any major histocompatibility complex (MHC) antigens and cannot be directly recognized by T cells. However, this cell provides very little in the way of cellular infrastructure for the parasite. In fact, the mature human RBC has been referred to as a 'floating corpse' (16) because it has no nucleus, no protein synthesis capability and no protein-trafficking machinery. Therefore, in order to survive and replicate in the RBC, the parasite has to orchestrate survival tactics involving several ingenious mechanisms.

RBC Invasion

The initial hurdle for the parasite is to invade the host RBC that does not normally undergo endocytosis. Merozoite invasion of RBCs is a complex, multistep process. After exiting the hepatocytes, the free merozoites recognize, attach, and enter erythrocytes via a very rapid process. A number of merozoite surface proteins (MSP) have been identified, but their role in invasion is still under investigation (17-19). These MSP proteins are exposed to the host's immune system just before reentry and are capable of eliciting antibody responses. In fact, MSP-1 is a major candidate for vaccine development

(reviewed in Holder et al. (20)). It is therefore imperative that the invasion process is fast and highly efficient in order to escape immune attack. Proteins such as MSP-1 also seem to play a role in the initiation of invasion since antibodies directed against MSP-1 subunits appear to block erythrocyte invasion in vitro and in vivo (21-23). Invasion involves an initial "long-distance" recognition of surface receptors followed by a reorientation process whereby low-affinity contacts are maintained (24). Upon reorientation, the merozoite forms an irreversible tight junction between its apical end and the RBC membrane. This initial recognition and junction forming steps have been extensively studied, and are mediated by multiple receptor/ligand interactions (25-28). The tight junction then moves from the apical to posterior pole (29) and this is thought to be powered by the parasite's actin-myosin system (30, 31). The parasite's surface coat is shed at the moving junction by a serine protease, or "sheddase" (32, 33). Upon reaching the posterior pole, the adhesive proteins at the junction are also proteolytically removed facilitating resealing of the membrane (34). By this process, the parasite does not actually penetrate the membrane but invades in a manner that creates a parasitophorous vacuole, which surround the parasite. The malaria parasites remain within the parasitophorous vacuole throughout their intraerythrocytic life.

Hemoglobin Degradation

Another obstacle the parasite faces in the RBC is nutrient uptake. During this intraerythrocytic stage, the parasite utilizes hemoglobin as the major nutrient source (35) proteolytically degrading enormous amounts of hemoglobin (about 60-80%) during a short period of their life cycle (36, 37). This process involves ingestion of RBC

cytoplasm (38), delivery to acidic digestive vacuoles and sequential, efficient proteolysis by a set of specific enzymes (39-41). The generated amino acids are utilized for the growth and maturation of the parasite (35, 42). However, the process of hemoglobin degradation generates toxic oxygen radicals (43) and free heme. Free heme can damage cellular metabolism by the inhibition of enzymes (41, 44), the peroxidization of membranes (45) and the production of oxidative free radicals in the acidic environment of the digestive vacuole (43). Therefore, to survive this, the parasite detoxifies these by-products by dismutation (46) and polymerization, respectively.

Hemozoin Formation

This reactive free heme is polymerized into an insoluble, indigestible crystalline substance called malaria pigment or hemozoin (Hz) (35). The pigment is visible microscopically in stages that are actively degrading hemoglobin, such as trophozoites, schizonts, and gametocytes. Hz has been demonstrated to be a heme polymer with a coordinate bond from the central ferric iron of one heme to the propionate carboxylate group of the next heme (47). It is also associated with different parasite proteins (48, 49). Hz polymerization has been demonstrated to be an enzymatic activity involving several proteins and enzymes such as heme polymerase. For example, in vitro, *P. falciparum* histidine-rich protein II (PfHRPII) was shown to bind to heme and initiate polymer formation (50). However, once Hz is formed, extension has been shown to proceed even in the absence of proteins (50). Apart from proteins, nonphysiological lipids have also been shown to initiate polymer formation (51). Hz has been used as a biomarker for malaria infections as it is present in large amounts in the reticulo-circulation during

infections. Hz is easily phagocytosed by the immune cells and has been shown to have stimulatory activity on some cell functions. Accumulation of Hz-laden phagocytes in spleen, liver, bone marrow (52, 53) and placenta (54) has been reported. this process of Hz formation is the target of such current antimalarials such as quinine and chloroquine (55).

RBC remodeling

The invasion of RBCs by *P. falciparum* elicits major morphologic changes in the composition of the RBCs, essentially remodeling them. One of these is the establishment of protein-trafficking pathways that target parasite-encoded proteins to the RBC cytoplasm and cell surface (56-58). These modifications play a major role in the survival of the parasite as they mediate the uptake of nutrients from the host. Studies have shown that these processes are probably accomplished through elaboration of the parasitophorous vacuole into extensive membranous structures that extend to the RBC surface (59). These structures have been visualized as either a continuous 'tubulovesicular' membrane network or a heterogeneous mixture of cisternae-shaped vesicles called Maurer's clefts (59, 60). The details of how proteins are targeted specifically to this secretory pathway are not well understood but recent studies have identified trafficking motifs that may regulate the transport of parasite-encoded proteins across the parasitophorous vacuole (57, 61) and along the Maurer's clefts (62) into the RBC cytoplasm and membrane.

Perhaps the most obvious modification to the host RBC, and one that is involved in malaria pathogenesis, is the development of several hundred knob-like protrusions

about 80-100 nm in diameter on the surface membrane (63, 64). These are electron-dense protrusions displayed on the surface of iRBCs and act as attachment points to host vasculature. Knobs consist of a number of parasite-encoded proteins, which can be divided into two major classes: the submembranous structural proteins that are placed toward the cytoplasmic side in the knobs and the adherent proteins present at the surface of the knobs. Several submembranous structural proteins have been identified and include the knob-associated histidine-rich protein (KAHRP), *P. falciparum*-infected erythrocyte membrane protein-2 (PfEMP-2) and PfEMP-3. Examples of known surface proteins include PfEMP-1, sequestrin, pfalhesin. Apart from parasite-encoded proteins, several host proteins, e.g. spectrin, actin, and band 4.1, are also known to interact with the knob proteins to produce functional knobs (65, 66).

Some surface proteins, such as PfEMP1, exhibit a high rate of antigenic variation (67) and comprise the variant surface antigens (VSA) expressed on iRBCs. VSA is a collective term for clonally variant antigens that are expressed on the surface of iRBCs (68). The only well-characterized VSA is the PfEMP1 family which is encoded by the highly polymorphic members of the *var* gene family (69-72). Every *P. falciparum* isolate/line/clone has a repertoire of approximately 50–60 *var* genes per haploid genome and only one *var* gene is expressed on the surface of an iRBC at any given time (73). The *var* genes vary in molecular size from 6 to 15 kb and are extremely divergent in sequence. Members of the *var* gene family share a similar organization: a large 5′ exon with high sequence variability separated from a smaller highly conserved 3′ exon by a predicted transmembrane sequence (74-76). The *var* genes show high sequence diversity

(71) and the switching of expression from one variant to another (67, 77) brings about the antigenic variation that is characteristic of malaria parasites (78, 79).

The Cytoadherence Phenomenon (Malaria Pathogenesis)

Sequestration, the adherence of iRBCs containing late developmental stages of the parasite to the host vasculature, is characteristic of *P. falciparum* infections. This is mediated, as mentioned above, by ligands expressed on the surface of iRBCs binding to receptors on host cells. Different adhesion phenotypes are observed and are associated with the expression of distinct VSAs on the surface of iRBCs (reviewed in (68)). PfEMP1 is implicated in both endothelial and epithelial cytoadherence (80-82). It binds to host receptors such as CD36 (83), intercellular adhesion molecule-1 (ICAM-1) (84), E-selectin, vascular cell adhesion molecule 1 (VCAM-1) (85), and chondroitin sulfate A (CSA) (82, 86) the latter being highly expressed in the placenta. Under physiologic flow conditions, iRBCs have been shown to interact synergistically with the different adhesion molecules on microvascular endothelium in a shear-dependent manner (87), mimicking the adhesive events of the leukocyte recruitment cascade (88). Cytoadherence may protect the parasite from splenic destruction (89) but is the basis of malaria pathogenesis. Sequestration in cerebral postcapillary venules through receptors such as CD36 is a characteristic feature of one of the most fatal forms of malaria, cerebral malaria (90, 91). The resulting sequestration of iRBCs also leads to microcirculatory obstruction and has been proposed to be the basis of the multiorgan dysfunction seen during severe malaria infection (92). Cytoadherence has also been observed in the placenta (93), kidney and lung (94).

During cytoadherence, the host cell receptors not only provide points of attachment for the parasites but also play more dynamic roles. Adhesion of iRBCs has been shown to lead to modulation of cellular functions and/or induction of signal transduction. For example, the initial attachment of iRBCs to CD36 on endothelium under flow conditions was shown to trigger a Src-family kinase-dependent intracellular signal that was responsible for increasing subsequent adhesion of iRBCs to CD36 by means of an ectoalkaline phosphatase (95). The binding of iRBCs to monocytes via the CD36 ligand resulted in a respiratory burst, suggesting that this may play a role in the induction of malaria pathophysiology via the reactive oxygen intermediates produced by monocytes at the sequestration point (96). The adherence of iRBCs to CD36 on dendritic cells, the primary antigen-presenting cells, was shown to inhibit dendritic cell maturation (97, 98), interfering with the normal function of these cells. Therefore, cytoadherence is a crucial phenomenon for the parasite; it plays majors roles in the initiation of response to the infection, it is the basis of malaria pathogenesis, and may play a role in dampening the immune response to the parasite.

MALARIA AND THE IMMUNE SYSTEM

The parasite has evolved several mechanisms to escape the host immune response and ensure its survival. One of these is its location in the RBC which does not express the MHC antigens. *P. falciparum* has been associated with incredible antigenic variation, always keeping a step ahead of the host immune system. Interaction of iRBCs with immune cells has been shown to lead to cellular modulations. The maturation of dendritic cells, the host antigen presenting cells, was shown to be impaired in the presence of

iRBCs (97, 98) and ingestion of Hz by monocytes was shown to severely impair their functions (99). On the other hand, some reports have demonstrated that Hz induces monocytes/macrophages to secrete inflammatory mediators such as TNF-α and chemokines (100-102) and to induce dendritic cell maturation (103). It has been shown previously that heat-stable exoantigens in the supernatants of blood-stage parasite cultures induced the release of TNF in vitro from activated macrophages and behaved like toxins in vivo (104-106). Bate et al (1994) observed the same activity using sonicated iRBC and uninfected RBC (uRBC), with the exception that the TNF-inducing activity was approximately 200 times higher in iRBCs than in uRBCs (107). The majority of the parasite proteins expressed on the surface of the parasite are anchored via glycosylphosphatidylinositol (GPI) (108) and several studies identified this malaria toxin to be these GPI anchors (109-111).

Both innate and adaptive immune systems regulate infection with malaria. In many areas where malaria is endemic, it appears that activation of innate responses by blood-stage parasites allows effective priming of anti-blood-stage immune responses that eventually confer very significant levels of humoral and cellular immunity (112). The parasite induces a specific immune response, stimulating the release of cytokines from the host's immune cells which might play an important function in activating the host's monocytes, neutrophils, natural killer (NK) cells (113) and T- cells.

Innate immunity

Mononuclear phagocytes, NK cells and gamma delta T cells ($\gamma\delta^+$ T cells) all appear to play a role in innate immune responses observed early in malaria infections.

This is mainly via the secretion of several proinflammatory cytokines that can derive from either the innate or the adaptive arm of the immune response. The innate cytokine response involves cytokines such as IL-12, IFN-γ, and TNF-α. These are essential mediators of protective immunity to erythrocytic malaria as they induce parasitocidal macrophage activation (114). The rapid induction of cytokines may enable the infected host to effectively control the exponential replication of blood stage parasites until the adaptive immune response can take over. The numbers of NK cells were shown to increase during malaria infections and their role in immunity to malaria was demonstrated by their ability to lyse iRBCs (113). *P. falciparum*-infected RBCs induced a rapid production of IFN-γ from NK cells in vitro (115). IFN-γ and TNF-α act synergistically to optimize nitric oxide (NO) and reactive oxygen intermediate production from macrophages, which is involved in parasite killing (116, 117). During early malaria infections, the numbers of $\gamma\delta^+$ T cells were shown to expand (118) indicating their possible role in the early response against the parasite. Hensmann et al. (119) demonstrated that naïve $\gamma\delta^+$ T cells produced both TNF and IFN-γ within 18 h of exposure to intact iRBCs in vitro and other studies showed that $\gamma\delta^+$ T cells from malaria-naïve donors inhibited parasite replication (120, 121). One of the major roles of innate immune response appears to be the production of the immunoregulatory cytokines, which are critical for the development of type 1 immune responses involving CD4+ Thl cells, B cells, and effector cells which mediate cell-mediated and antibody-dependent adaptive immune responses.

Humoral immunity

Malaria induces both polyclonal and specific immunoglobulin production (122). The protective role of antibodies was demonstrated by the ability of passively transferred antibodies from immune adults to protect against natural and challenge infections with *P. falciparum* (123-125). Many of the malaria-specific antibodies produced are species- and stage-specific, reacting with a wide variety of asexual stage antigens. The major mechanism for antibody-mediated parasite neutralization is thought to involve monocytes or other leukocytes as effector cells (123, 126). However, by reacting with parasite-derived antigens expressed on the surface of iRBCs, antibodies can inhibit the intraerythrocytic development of the parasite (127, 128) thereby promoting elimination by the spleen. Antibodies can also inhibit merozoite invasion either by the neutralization of the free merozoites via antibodies against some of the MSP or by interference with the merozoite invasion process (129, 130). Moreover, opsonization of the iRBCs with antibodies significantly increases their susceptibility to phagocytosis, cytotoxicity and parasite inhibition by various innate effector cells such as neutrophils and monocytes (123, 131). Cytophilic IgG antibodies, IgG1 and IgG3 isotype (132, 133 4082) primarily mediate antibody-dependent protection.

Cell-Mediated Immunity

T cells play a crucial role in both induction and maintenance of cell-mediated immunity to malaria (134, 135). This cellular response to malaria is characterized by the secretion of an array of immune factors such as cytokines (136, 137) and NO (135) induced by the interaction of opsinized iRBCs with host effector T-cells. Protective T-

cell responses against the blood stage of infection have been demonstrated following low-dose vaccination with iRBCs (138). In vitro studies have demonstrated that $CD4^+$ T cells respond to malaria antigens by proliferation and secretion of IFN-γ or IL-4 (136, 137). They are required to help B cells produce antibodies that are essential for parasite clearance (135). Proinflammatory cytokines such as IFN-γ, IL-1, IL-6 and TNF have been shown to be protective by inducing parasite killing by monocytes/macrophages. In contrast, anti-inflammatory cytokines such as IL-10 counteract the production and possible cytopathic effects of these pro-inflammatory cytokines (139). As human RBCs do not express MHC antigens, lysis of iRBCs by cytotoxic T lymphocytes, $CD8^+$ T cells, has no role in the defense against erythrocytic stages of the parasite. However, $CD8^+$ T cells play important effector roles in pre-erythrocytic immunity (140-142) and contribute to protection against severe malaria (143, 144). $CD4^+$ cells have been shown to also play a pivotal role in the induction of protective memory $CD8^+$ T cell responses against the exo-erythrocytic stages (145).

Acquired Immunity to Malaria

It is clear that in endemic areas, a degree of immunity to severe, life-threatening malaria is achieved (146). The death toll by malaria in endemic areas is primarily borne by young children under the age of five whereas the disease is relatively mild in adults, implying that protective immunity is acquired with age (147). Whereas immunity to some pathogens is long-lived after a single infection, immunity to malaria is probably never complete as evidenced by the repeated infections experienced by people living in endemic areas (146, 148, 149). The clinical presentation of malaria among such semi-

immune people is typically less severe than in non-immune people. In fact a significant proportion (up to 80%) of immune people with parasitemia may be completely asymptomatic (150). Acquisition of this protection is a slow process that takes years to develop (151). Its maintenance appears to require continuous exposure to the parasite (152). One reason for this slow acquisition is the capacity of the parasite to switch its VSAs, the major targets for protective antibodies, resulting in extensive antigenic variation (153). Several recent reports have supported the idea that naturally acquired immunity develops through the piecemeal acquisition of a repertoire of specific VSA antibody responses and that clinical episodes of malaria correspond to gaps in this developing repertoire (148, 154, 155).

BIOLOGY OF HUMAN PREGNANCY

Pregnancy causes a number of physiological changes that affect the way the pregnant woman interacts with the developing fetus. During pregnancy a special disc-shaped multifunctional organ, the placenta, develops. The placenta is an ephemeral organ present only during pregnancy and delivered after the newborn's birth, hence its common name, the afterbirth. It is indispensable for the proper development of the fetus as it serves as the interface between the mother and the developing fetus and also as the conduit through which nutrients and waste products are exchanged (156). The placenta is also an endocrine organ producing numerous hormones (157) necessary for pregnancy maintenance. One of the classic hormones produced early in pregnancy (thus commonly used in pregnancy tests) is the human chorionic gonadotropin (hCG), produced by the placenta cells. hCG is important as it stimulates trophoblastic invasion during

implantation (158, 159). Progesterone, which is produced in large amounts, has important roles in the maintenance of pregnancy (160, 161). Others such as placental lactogen act together with human placenta growth factor to increase the amount of glucose and lipids in the maternal blood resulting in increased transfer of these nutrients to the fetus and thus promoting fetal growth (162, 163).

Soon after fertilization, the zygote moves along the fallopian tube while undergoing massive proliferation to form the morula. The morula is covered in the zona pellicula which makes it 'roll' its way into the uterus. In the uterus, the zona pellicula is lost and the morula undergoes further differentiation into the blastocyst. The blastocyst consists of an inner cell mass, an internal cavity, and an outer layer known as trophoectoderm that consist of cells known as trophoblasts. Here the blastocyst attaches to the uterine wall (endometrium) of the mother and begins the first stage of placenta development, implantation (164). A receptive endometrium is necessary for proper implantation to occur and it is believed that a cross talk exists between the endometrium and the embryo which orchestrates successful implantation (165). As reviewed by Giudice, (1999), some of the soluble factors involved in this 'conversation' include hormones such as progesterone, integrins, proteases, cytokines such as IL-1 and growth factors such as epidermal growth factor (EGF) and colony-stimulating factor-1 (165). Other essential factors are the leukemia inhibitory factor (LIF) produced by the endometrium and its receptor expressed by the blastocyst (166).

Placentation (The maternal-fetal interface)

Successful human reproduction depends on formation of the placenta (placentation) (156). Placentation entails a highly unusual differentiation process of specialized undifferentiated mononuclear fetal cells, of epithelial origin, known as cytotrophoblast (CT) cells. The chorionic villi are the basic building blocks of the human placenta that are covered by two trophoblastic cell layers, one of which is the CT. Two different specialized chorionic villi exist: floating and anchoring villi. Some of the CT cells retain an undifferentiated phenotype throughout pregnancy and provide a reservoir of trophoblast cells. The remaining CT cells undergo either of two differentiation pathways leading to the formation of two distinct trophoblast phenotypes; invasive and fusion phenotypes (167). In the anchoring villi (invasive phenotype), the CT aggregate into cell columns that attach to the uterine wall and anchor the placenta. These proliferative CT cells differentiate and invade deeply into the endometrium reaching the first third of the adjacent myometrium. This population of CT cells, also known as extravillous trophoblasts, remodels the endometrium and its vasculature (168, 169). This endovascular invasion within the uterine wall is necessary, since the remodeling of the maternal spiral arteries generates a high volume, low resistance vascular system that permits continuous and adequate blood flow to the placenta and growing fetus (170, 171). The implantation process is highly localized and is more akin to tumorigenesis than to organogenesis (as reviewed in (172)). In the floating villi (fusion phenotype), the trophoblast cells fuse and differentiate to form the multinucleated syncytiotrophoblast (ST) layer, which functions as the natural physical barrier between maternal and fetal blood (173, 174). This layer undergoes continuous renewal via the fusion of the

mononucleated CT cells found immediately beneath the ST. This dynamic process, also known as trophoblast turnover, is necessary, as the multinucleated ST is highly differentiated and, as such, unable to regenerate (173, 174). Between the floating villi of the placenta, in the intervillous space (IVS), maternal blood circulates in continuum with peripheral circulation. The maternal and fetal circulations are uniquely juxtapositioned in order to facilitating physiological functions such as nutrient and gaseous exchange.

Syncytiotrophoblast

ST is a continuous, uninterrupted layer covering the floating villi and lining the IVS. It is directly bathed in maternal blood and therefore mediates the nutrient, gas, and waste exchange between the developing fetus and the mother. In vivo, the ST is indeed a continuous structure and where it is interrupted by degeneration, the gap has been shown to be filled by fibrin-type fibrinoid (175). The ST surface is almost completely covered by microvilli that highly increase the maternal fetal contact zone. The microvillus surface has been reported to express polysaccharides such as chondroitin sulfate A (CSA) (176) which has relevance in malaria during pregnancy (discussed later). The ST also serves as a hormonal and immunological interface between the mother and the fetus. ST has been shown to be capable of secreting cytokines /chemokines important for a successful pregnancy (177) and in response to intrauterine pathogenic bacteria and viruses such as cytomegalovirus, in which case both pro- and anti- inflammatory cytokines were shown to be produced (178, 179). Bacterial infections in the placenta have been shown to result in adverse pregnancy outcomes due to the immunoactive nature of the bacterial lipopolysaccharide (LPS), which leads to a proinflammatory state in the fetoplacental unit

(180, 181). In addition to cytokines, chemokines have also been shown to be potent proinflammatory mediators induced by LPS in first trimester trophoblasts (182). All these imply that the ST is unlikely to remain inert during intrauterine infections.

Immunology of Pregnancy

During pregnancy, the mother is exposed to non-self antigens (fetus). How this semiallogeneic fetus escapes rejection by the mother remains a biologic enigma that has intrigued immunologists since antiquity. Several mechanisms have been put forward to explain this paradox. In their review, Thellin et al.(183), highlight 'ten ways to support a child for nine months' which summaries the known mechanisms through which the 'semi-allogeneic graft' is maintained during pregnancy. The fetal trophoblast cells appear to play the major role in evading recognition by the maternal immune system. Both systemic and locally produced factors play a role in protection of the fetus. Systemic effects are due to immuno-active hormones such as progesterone. This hormone is synthesized in large quantities by the placenta and is able to lower the immune response, e.g. inhibition of lymphocyte activation and the generation of cytotoxic T lymphocytes (reviewed in (161)). Other hormones, such as the placental growth hormone which replaces progressively the pituitary growth hormone during pregnancy (184), might also help to modulate the immune system (183). Locally at the placental level, many different mechanisms are involved, working alone or in interaction with each other. Major histocompatibility complex (MHC) class I expression is reduced on the ST (185, 186). However, there is a specific expression of the non-classical MHC class I, human leukocyte antigen-G (HLA-G) by extravillous trophoblast (187, 188) which may act to

inhibit the cytolytic activity of NK cells by binding to the killer-cell immunoglobulin-like receptor expressed by the NK cells (189). Indoleamine 2, 3 dioxygenase (IDO), which catabolizes the essential amino acid tryptophan, is produced by the ST and thus reduces close-range immune cell reactions protecting the fetus from immune cell attack (190). The Fas/Fas-ligand system involved in cellular turnover, tumor cell elimination, antiviral responses or protection of tissues against activated lymphocytes is though to be active in controlling fetal rejection too. Trophoblast cells synthesize and express Fas ligand (191, 192), which is capable of inducing maternal immune cell apoptosis since the latter are known to express Fas. Fas present on the trophoblasts do not seem to transduce apoptotic signals in these cells (193). LIF and its receptor, which play a major role in implantation (166), are also thought to help in trophoblastic growth and differentiation (166), thereby promoting fetal well being. Dichotomous T helper cell 1 (Th1- non-inflammatory) and Th2 (proinflammatory) cytokine/growth factor responses are proposed to be involved in pregnancy loss and success, respectively (194-199). Th1 and Th2 cells exert different actions with Th1 cells synthesizing proinflammatory cytokines such as IL-2 and IFN-γ whereas Th2 cells preferentially synthesize the anti-inflammatory cytokines IL-4, IL-5, IL-6, and IL-10 (200, 201). Uterine decidual and both CT cells and ST produce a huge array of cytokines which, in part, also contribute to the deviation of the immune response from Th1 to Th2 (202, 203). However, this bias may leave the mother more open to infections whose control is Th1-dependent (204).

As can be perceived from the above discussion, many different mechanisms acting locally or at a distance, ensure survival of the fetus and the balance of influence leading either to tolerance or fetal demise is under control of internal (maternal and fetal)

and external (environmental) factors. Under certain circumstances, failure of one or more of the tolerance mechanisms may occur and usually recurrent abortions ensue (197, 205, 206) or in some cases, poor fetal outcomes such as preterm deliveries and low birth weight (LBW) babies are observed. As reviewed by Kanellopoulos-Langevin (207), current evidence suggests that inflammation, complement activation and/or leukocyte infiltration precede abortion and are associated with poor fetal outcomes. Common external factors that appear to induce imbalance in the mechanisms involved in pregnancy success include intrauterine infections. These infections, especially bacterial, are associated with poor fetal outcomes which are suspected to be caused by defects in placental development and adverse effects on the trophoblast cells in association with proinflammatory responses evoked by the infection (208, 209).

MALARIA IN PREGNANCY

The underlying biological basis for susceptibility to malaria during pregnancy remains poorly understood. One theory proposes that high levels of steroid hormones in pregnant women may impair anti-malarial immunity (210), thereby allowing parasites to expand unfettered by anti-parasitic immune mechanisms. A second theory highlights the modulations of cell-mediated immunity during pregnancy which favors the survival of the fetoplacental allograft (211). However, these changes are not thought to result in significant immune impairment and do not explain the increased susceptibility to malaria by primigravidae compared to multigravidae women. More recently it has been proposed that the glycosaminoglycan, CSA, expressed by the ST cells in the placenta provides a receptor for the parasite ligand, PfEMP1 allowing a rare subpopulation of iRBCs to

cytoadhere to the placenta (212). This cytoadherence allows these parasites to proliferate in this anatomical location while avoiding the host's spleen-mediated killing mechanisms (89, 213). This sequestration of iRBCs is one of the hallmarks of malaria during pregnancy (93, 214, 215).

Epidemiology

Malaria during pregnancy poses substantial risk to the mother, the fetus, and the neonate. However, the clinical features of malaria in pregnancy depend to a large extent on the immune status of the woman, which in turn is determined by previous exposure to malaria (216-219). In low or non-endemic regions, where the women have little or no pre-existing immunity to malaria, pregnant women are two to three times more likely to develop severe disease than are non-pregnant adults in the same area (4, 220). In these areas malaria during pregnancy is associated with extremely high risks of maternal and perinatal mortality (221), women of all parities are affected, and any of the manifestations of severe malaria may occur (220). In high transmission areas, women have developed considerable immunity to malaria by childbearing age, yet numerous epidemiological studies have shown that these women, upon becoming pregnant, are more susceptible to malaria infection than their non-pregnant counterparts (222-224). However, even in the pregnant woman, malaria infections in these regions are frequently asymptomatic, and severe disease is rare (4, 222) but may lead to maternal anemia and poor fetal outcomes like LBW babies and prematurity (225, 226). The gravidity of the woman has been shown to have an effect on the susceptibility to malaria infection in these areas. The prevalence rates of peripheral and placental parasitemias, as well as

parasite densities, are highest in primigravid women in high endemic areas (4, 222) with subsequent pregnancies demonstrating improved prognosis (4, 222). The increased incidence of LBW associated with malaria in pregnancy is a leading cause of death, because LBW infants are vulnerable to many life-threatening ailments (227). It is estimated that 75 000–200 000 infant deaths occur annually because of malaria in pregnancy (228).

Placental Malaria

The deleterious effect of malaria in pregnancy is mainly due to the sequestration of iRBCs in the placenta and the subsequent local immunological responses (229, 230) that are triggered, a phenomenon referred to as placental malaria (PM) (figure 2.1). PfEMP1/CSA interactions are thought to be critical for the development of PM. Several studies have confirmed the role of CSA in mediating the binding of iRBCs in the placenta (212, 231-235). Other receptors have been suggested to play minor roles in this cytoadherence. For example, Beeson et al. (236) showed that hyaluronic acid (HA) was capable of mediating this binding. Sartelet et al. (237) observed higher expression of ICAM-1 localized on the ST in malaria-infected placentas, and they suggested that this receptor might play a role in the sequestration of the iRBCs in the placenta. *In vitro* studies have confirmed that iRBCs are capable of binding directly to the ST via CSA and receptors like ICAM-1 (238). Currently, however, it appears that CSA is the principal iRBC receptor in the placenta (212, 231-235). CSA is a glycosaminoglycan linked to the cell surface via a membrane-associated protein. In general, it consists of a heteropolymer of alternating glucuronic acid and 4-sulfated *N*-acetylglucosamine residues (239). CSA

from different sources may differ substantially in their sulfation patterns with placental CSA having a low sulfation pattern (240). The adhesion of iRBC is strongly dependent on sulfation in the fourth position as well as the specific saccharide chains (241-243).

Apart from the sequestration of iRBCs in the IVS and on the ST, malaria-associated placental pathology has been demonstrated to accompany PM; this includes placental lesions such as focal ST damage, loss of syncytial microvilli, and marked irregular thickening of trophoblastic basement membranes (54, 244). PM can, on some occasions, be accompanied by massive accumulation of inflammatory immune cells in the IVS of the placenta (226, 245). Histological observations have revealed the presence of the malaria pigment, Hz, deposited in the IVS and also within macrophages found there (214, 246). Many of the adverse effects of malaria in pregnancy are associated with PM. The sequestration of iRBCs in this anatomical site has been shown to correlate with LBW, maternal anemia, and mortality (222, 225, 247). The accumulation of maternal immune cells has also been indicated in poor fetal outcomes (229, 230) especially via the secretion of proinflammatory cytokines and chemokines.

Immune Responses during Placental Malaria

Cytokines are known to play major roles in immune responses. The role of local cytokine responses in the placenta during PM has been demonstrated (230, 248-250). Elevated production of IFN-γ by intervillous blood mononuclear cells (IVBMC) was associated with protection in an area of high transmission of malaria (250). In this study, IVBMC from PM-negative multigravidae women produced higher levels of IFN-γ compared with PM-positive multigravidae women or primigravidae/secundigravidae

women implying that this cytokine may be important in protection from PM. Elevated levels of TNF-α and IFN-γ were observed in placentas collected from women with PM infection (249). Fievet et al., (248) demonstrated that PM infection induced immune responses that involved both the Th1 (IFN-γ, TNF-α, IL-1β) and Th2 (IL-6, IL10) cell activation with a bias towards the Th1. Production of IL-10 by IVBMC was also shown to be increased in PM (230, 250) and this was hypothesized to be important in the control of the negative effects of Th1 cytokines on pregnancy.

Apart from cytokines, production of several chemokines has also been observed in association with PM (230, 251, 252) and recently also with monocytic infiltration (229). Macrophage inhibitory protein-1β (MIP-1β) was shown to be significantly upregulated in the IVB plasma during PM, and high MIP-1α levels in placenta were associated with high-density PM infections (251). Elevated mRNA levels for monocyte attractant protein-1 (MCP-1), MIP-1α and IL-8 were associated with PM and with monocyte infiltration in the IVS (229, 230). Increased levels of IL-8 in association with PM were also associated with intrauterine growth retardation (IUGR) (229, 230). Increased levels of the immune factor macrophage migration inhibitory factor (MIF) were observed in women with PM (252). MIF may play a role in immune responses to malaria during pregnancy by virtue of its ability to activate macrophages and to overcome the immunosuppressive effect of glucocorticoids (253), therefore helping in the clearance of the parasites. The infiltration of immune cells in the IVS observed during PM (245, 254) has been associated with the unwanted effects mentioned earlier, such as premature deliveries and LBW (229); these in turn are associated with an increased risk for neonatal mortality (227, 255). These immune cells appear to be the main source of the

protective immune response against PM via secretion of cytokines like TNF-α and chemokines by macrophages and IFN-γ by T cells. Fetal stromal cells were also shown to participate in this response during PM by the secretion of MCP-1 (229). However, the role of the ST cells, which are in contact with iRBCs and their products, in contributing to this local immune response to PM has not been elucidated.

Acquired immunity to PM

As mentioned earlier, primigravid women are at a higher risk of PM regardless of living in malaria endemic regions but become less susceptible to the infection with subsequent pregnancies (4). The differential susceptibility to malaria in pregnancy as a function of gravidity has been attributed to the lack, in primigravid women, of anti-adhesion antibodies against malaria in pregnancy specific VSA, the CSA-binding phenotype (256-260). Apart from this apparent gravidity-dependent immunity to PM, the binding phenotype of placental parasite isolates was shown to be gender-specific; often placental parasite isolates bound to CSA, in vitro, but did not bind to CD36, whereas parasites from men and non-pregnant women did not bind to CSA but bound CD36 (212, 261). In vitro and field studies on CSA-mediated binding of iRBCs demonstrated that sera from multigravidae women contain antibodies capable of agglutinating placental parasite isolates or inhibiting their binding to CSA (261, 262). Salanti et al. (263) reported a marked upregulation of a single var gene in several *P. falciparum* parasite isolates after selection for adhesion to CSA in vitro and after isolation from the placenta. The gene was shown to belong to a highly conserved and common var gene subfamily referred to as *var2csa*. Later, several other studies confirmed the role of *var2csa* gene in

CSA-binding parasites of the placenta (264-266). Complimenting these studies were others which demonstrated that anti-VAR2CSA IgGs were found in a sex-specific, parity-dependent manner, were involved in clinical protection against malaria in pregnancy (259, 267-271), and were shown to be conserved across geographic regions (270). These findings have caused a lot of excitement at the possibility of developing a vaccine for malaria in pregnancy.

A Vaccine for Malaria in Pregnancy (Not Quite Yet)

The PfEMP1 family of proteins is encoded by the highly polymorphic members of the *var* gene family (69-72). Given the known extreme diversity of this family, some authors have questioned how the identification of VAR2CSA as the CSA ligand explains the conserved epitopes indicated by sero-epidemiological data (270, 272). In fact, Cox et al. (273) reported a rapid acquisition of isolate-specific antibody responses to VSA(CSA). These results indicated that different CSA-adherent parasite lines may express antigenically distinct VSA and thus may not be as antigenically conserved as has been previously suggested. In the same study, levels of anti-VSA (CSA) were not significantly associated with PM infection indicating that primary immune responses to VSA (CSA) may not be sufficient to eradicate placental parasitemia in primigravidae. Yet others have wondered if more conserved parasite antigens may be responsible for the CSA-binding phenotype. So far, most evidence available in the literature concerns the biological relevance of PfEMP1 (reviewed in (272, 274)). However, a few other variant proteins expressed on the surface of iRBCs including the rifins, stevor, clag, and sequestrin, could also contribute to the induction of an immune response, which may have

different implications in the adhesion process in the placenta and in the evolution of the infection as reviewed by Sherman (274). Research over the past few years has provided partial answers to these concerns. However, definitive proof of the identity of the placental parasite CSA-binding ligand remains elusive.

In vitro System to Study CSA/PfEMP1 Interactions

One of the reasons for this lack of deeper knowledge on the PfEMP1/CSA interaction and role of ST during PM is the unavailability of a good in vitro system to study these issues. To date, most studies of CSA-iRBC binding have involved in vitro adherence assays with CSA coated onto plastic dishes (241-243, 261, 275). In these studies, commercially available CSA and chondroitin sulfate proteoglycans (CSPG) from different sources have been used. However, these CSPGs differ widely in the structural features of their glycosaminoglycan chains and therefore exhibit differential adherence characteristics (240). Making the use of placental CSPGs a better option to most accurately assess CSA/iRBCs interactions. Recently, sections of cryopreserved placenta (276, 277) have been used for adhesion studies. Freshly isolated placental parasites and laboratory parasite strains selected for CSA-binding ability were shown to bind to the ST in these placenta cryosections (276). This method appears to provide a suitable in vitro system for the study of the adhesion of iRBCs to CSA, in that placental receptors (CSA and others?) are present in a native and thus molecularly and structurally relevant form. The caveat is that cryopreserved tissues are inappropriate for time course experiments to study the dynamics of selection of placenta-adherent iRBC phenotypes, and they cannot

be used for functional assessment of the consequences of cytoadherence on trophoblast function.

SUMMARY AND GAPS IN KNOWLEDGE

Our understanding of placental cytoadherence has increased significantly in the past decade. This has, however, relied on the use of commercially available CSA and the use of different CSPGs for the binding assays. Several important unanswered questions remain, especially if the development of novel chemotherapeutics and immunoprophylaxis for pregnant women is to be achieved. So far, it has proven difficult to determine whether malaria in pregnancy results in impaired placental development and function. It is clear that both the cytoadherence and the proinflammatory immune responses elicited during PM play major roles in the pathogenesis of PM, but if the ST plays any role in this response as it does during a bacterial infection is still not known. Adhesion molecules can serve as receptor-signaling molecules capable of transducing extracellular signals resulting to cellular activation (278, 279). It is not clear whether the binding of iRBCs to CSA expressed on the ST leads to ST intracellular signaling or if the suggested receptors merely serve as attachment points for the parasites. The role of Hz, found in the placenta during PM, has received little attention, yet Hz has been used as a biomarker of placental parasitization during PM (280, 281) and is known to be immunostimulatory (100-102). Although placental Hz load per se was not associated with poor fetal outcomes (280, 281), some results suggest that Hz-laden macrophages produce potentially harmful proinflammatory responses in the placenta which have been

associated with LBW and/or IUGR (229, 230). It is not known whether the ST responds to Hz by either secretion of cytokines or chemokines.

The interaction of the ST with the maternal leukocytes that accumulate during PM is not well understood. It has previously been shown that lymphocytic cells can bind to ST in vitro, resulting in focal ST apoptosis (282). Adhesion of these cells to ST was shown to be stimulated by pretreatment of the ST cultures with several cytokines such as TNF-α, granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-1β and IFN-γ either alone or together (283). It is known that activated monocytes adhere to the ST via receptors like leukocyte function-associated antigen 1 (LFA-1) on the monocytes to ICAM-1 on the ST (282) and the very late activation antigen-4 (VLA-4; $\alpha_4\beta_1$ integrin) was shown to mediate the attachment of lymphocytic cells to an unidentified receptor on the ST (283). TNF-α and IFN-γ are known to upregulate the expression of surface receptors like ICAM-1 on ST (238, 284) and high levels of these cytokines are elaborated during PM (248-250). It is therefore possible that during PM the expression of ICAM-1 on ST may be enhanced and thus affect the binding of maternal immune cells to the ST. It is not known if the ST plays a role in the recruitment and retention of the maternal immune cells in the IVS by the secretion of chemokines in response to iRBC.

In order to answer some of these questions, an in vitro system needs to be established which allows investigation of the responses elicited in the ST on interaction with iRBC and other malarial components such as Hz. In this study, we developed such a system, which we have used to attempt to shed some light on the possible outcomes of ST interaction with iRBC and malarial components.

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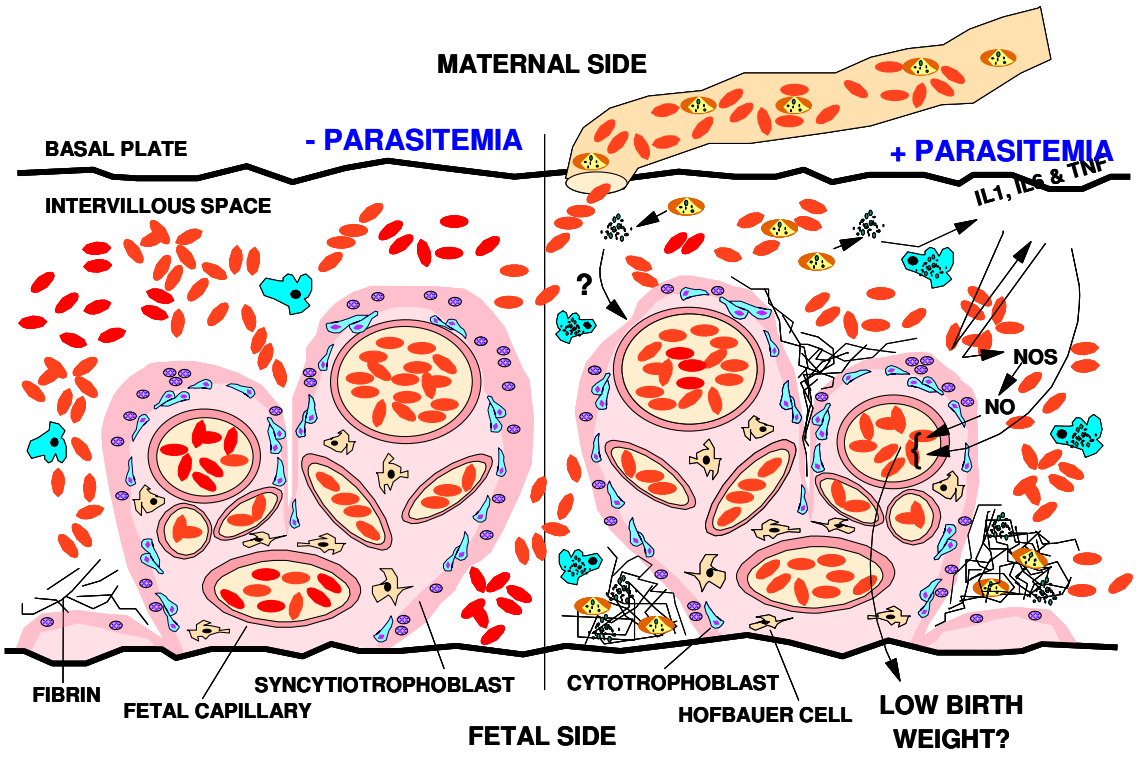
Figure 2.1: Pathogenesis of placental malaria.

The right panel depicts a cross-section of the maternal–fetal interface showing the CT cells beneath the ST that lines the fetal villi. The ST is in direct contact with maternal blood. During malaria in pregnancy, the iRBCs sequester in the IVS of the placenta (left panel). This results in the deposition of malarial pigment, Hz (black dots) within the circulating phagocytes and within the fibrin and in a local immune response characterized mainly by proinflammatory cytokines (IL-1, TNF) and chemokines (IL-8). PM is associated with LBW, which is a risk factor for early childhood mortality.

Figure courtesy of Julie M. Moore



CHAPTER 3

PLASMODIUM FALCIPARUM-INFECTED RED BLOOD CELLS SELECTED FOR BINDING TO CULTURED SYNCYTIOTROPHOBLAST BIND TO CHONDROITIN SULFATE A AND INDUCE TYROSINE PHOSPHORYLATION IN THE SYNCYTIOTROPHOBLAST[1]

[1] Lucchi, NW, Koopman, R, Peterson, DS, and Moore, JM. 2006. Placenta. Apr-May; 27(4-5):384-94. Reprinted here with permission from publisher.

ABSTRACT

An important pathogenic complication of malaria during human pregnancy is sequestration of Plasmodium-infected red blood cells (iRBCs) in the placental intervillous spaces. This sequestration is thought to be mediated in part by binding of the iRBCs to receptors expressed on the syncytiotrophoblast (ST) membrane. We report here the use of a dynamic system to study the consequences of this cytoadherence on ST function using human syncytiotrophoblast and the choriocarcinoma cell line, BeWo. Laboratory isolates of *Plasmodium falciparum* were selected for their ability to bind to ST and used to investigate binding-induced cellular changes in the ST. Treatment of the ST cells with chondroitinase ABC suggested that the selected parasites bind predominantly to chondroitin sulfate A, but other receptors for parasite binding may be involved. Intracellular signaling in the ST induced by iRBCs binding was investigated by assessing tyrosine phosphorylation of ST proteins following iRBC binding. We demonstrate for the first time that iRBC cytoadherence to syncytiotrophoblast enhances tyrosine phosphorylation of a series of proteins in these cells. This approach will be useful in further studies of ST function in the malaria-infected placenta, the dynamics of selection of syncytiotrophoblast-binding parasites, and the identification of new receptors for parasite cytoadherence in the placenta.

Key words: Placenta malaria; syncytiotrophoblast; cytoadherence; chondroitin sulfate A; activation; tyrosine phosphorylation

INTRODUCTION

Epidemiological studies have shown that pregnant women, especially primigravidae, are more susceptible to severe clinical malaria regardless of previous exposure-induced immunity [1, 2]. Malaria during pregnancy is an important problem because it can lead to maternal anemia and poor fetal outcomes like low birth weight (LBW) babies and prematurity [3, 4]. One of the hallmarks of malaria during pregnancy is the presence of Plasmodium-infected RBCs (iRBCs) in the intervillous spaces (IVS) of the placenta [5-7], often at densities much higher than those found in the periphery [2, 8]. This phenomenon is referred to as placental malaria (PM), and is characterized also by inflammatory cell infiltrates in the IVS and placental lesions such as focal syncytial necrosis, loss of syncytial microvilli, and marked irregular thickening of trophoblastic basement membranes [7, 9]. The underlying biological basis for this susceptibility to malaria during pregnancy, placental malaria, and poor birth outcomes remains poorly understood. One theory proposes that high levels of steroid hormones in pregnant women may impair anti-malarial immunity [10], thereby allowing parasites to expand unfettered by anti-parasitic immune mechanisms. A second, more recent theory proposes that a rare subpopulation of iRBCs that adheres to receptors on fetal villous tissue is the underlying cause for accumulation of malarial parasites in the placenta [11], and has been proposed to directly contribute to maternal anemia and low birth weight [12].

RBCs infected with the mature late stages (trophozoites) of *Plasmodium falciparum* are known to sequester in the host microvasculature where they adhere to the host endothelium, escaping clearance in the spleen. PM is unique in that it is the only

known example of iRBC cytoadherence to epithelial rather than endothelial cells. Numerous reports suggest that chondroitin sulfate A (CSA), a glycosaminoglycan expressed on the syncytiotrophoblast (ST) and in the IVS, serves as a major receptor for *Plasmodium falciparum* in the placenta[11, 13, 14]. However, hyaluronic acid [15, 16] and ICAM-1 [17, 18] may also play a role.

P. falciparum resident in RBCs export proteins to the RBC membrane. These proteins can serve as ligands for cytoadherence; *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is implicated in both endothelial and epithelial cytoadherence[19-21]. This protein is encoded by highly polymorphic members of the *var* gene family[22-25]. Only one *var* gene is expressed on the surface of an iRBC at any given time, making it possible to experimentally select for parasites expressing one particular adhesion phenotype[26]. PfEMP1/CSA interactions are thought to be critical for PM; however, some authors have suggested that other parasite ligands might be used [27, 28] and it is possible that other placental receptors are also involved.

Most studies of CSA-iRBC binding have involved in vitro adherence assays with CSA coated onto plastic dishes[13, 29, 30, 31, Fried, 2002 #324]. In these studies, CSA and chondroitin sulfate proteoglycans (CSPG) from different sources have been used. However, these CSPGs differ widely in the structural features of their glycosaminoglycan (GAG) chains and therefore exhibit differential adherence characteristics[32], demonstrating the need to use placental CSPGs to most accurately assess CSA/iRBCs interactions that are specific to the placenta. Recently, sections of cryopreserved placenta [33, 34] have been used for adhesion studies. Freshly isolated placental parasites and

laboratory parasite strains selected for CSA-binding ability were shown to bind to the ST on these placenta cryosections [33]. This method appears to provide a suitable in vitro system for the study of the adhesion of iRBCs to CSA, in that placental receptors (CSA and others?) are present in a native and thus molecularly and structurally relevant form. However, cryopreserved tissues are inappropriate for time course experiments to study the dynamics of selection of placenta-adherent iRBC phenotypes, and they cannot be used for functional assessment of the consequences of cytoadherence on trophoblast function.

To circumvent these limitations, we chose to use normal human placental ST and a choriocarcinoma cell line (BeWo) for iRBCs adhesion studies in vitro. Maubert *et al.* reported use of this model system to show that the primary human ST expresses CSA and supports binding of iRBCs isolated from human placenta[18]. We demonstrate here that syncytialized BeWo ($BeWo^{ST}$) also expresses CSA. We have used these cells to select for $BeWo^{ST}$ and primary ST-adherent iRBC ($iRBC^{ST}$) by sequential panning of two laboratory strains of *P. falciparum*. These $iRBC^{ST}$ appear to bind predominately to CSA, as we have shown that pretreatment of the ST with chondroitinase ABC enzyme significantly reduced iRBC binding. We further used this model system to investigate whether iRBC binding has an impact on ST function. We demonstrate for the first time that adhesion of iRBCs to ST induces tyrosine phosphorylation of several proteins. Thus, it will be possible to use this system to characterize the molecular dynamics of *P. falciparum* binding and potential mechanisms of selection for a placenta-adherent phenotype, and to investigate additional effects of iRBCs/ST interactions on ST function.

MATERIALS AND METHODS

Study participants

Placentas were obtained from women delivering by elective caesarean section at St. Mary's Hospital, Athens. Written, informed consent was obtained. Two placentas were used in this study from women aged 31 (secundigravida) and 34 years (primigravida). The study design and involvement of human subjects were approved by University of Georgia and St. Mary's Health Care System IRBs. These were both malaria and HIV negative as determined from information obtained from the study subjects by questionnaire.

Isolation of human primary trophoblasts from placenta

Primary placental cytotrophoblast cells were isolated from the obtained fresh human placentas (used within 30 minutes to 2 hours post removal) essentially as described [35] with some minor modifications. The chorionic villous tissue was removed from the placenta using a sharp sterile scalpel. The tissue was then minced and subjected to four rounds of 30-minute enzymatic digestion using trypsin and DNase I (Sigma). The released cells were then washed and layered on a 5-75% freshly prepared percoll (Sigma) gradient to separate the cells and obtain trophoblast preparations. In order to obtain a pure trophoblast preparation, these cells were incubated with mouse-anti human CD9 antibody (Pharmingen) (which binds all cells except the trophoblasts [35, 36]) and anti-major histocompatibility complex (MHC) class 1 antibodies (W6/32, ATCC; antibodies purified from murine ascites). The unbound cells were then immunopurified by negative

selection over a column containing glass beads coated with goat anti-mouse polyclonal antibodies [35]. The immunopurified cells were cryopreserved in liquid nitrogen until use.

Trophoblast cell culture

Primary cytotrophoblasts were cultured as described [18]. Briefly, cells were thawed quickly in a 37^{o}C water bath and then washed in Iscove's Modified Dulbecco's medium (IMDM, Cellgro) containing 10% fetal bovine serum (FBS; Hyclone), 10 ng/ml recombinant human epidermal growth factor (rhEGF; Sigma), 100 units/ml of penicillin and 100 μg/ml streptomycin (Gibco) (complete IMDM). Cells were then plated in 60 mm and 100 mm tissue culture plates at a 1 x 10^6 cells/ml and placed in a 37^{o}C incubator with an atmosphere of 5% CO_2. After 4 hours in culture the non-adherent cells were washed off and the plates filled with complete IMDM medium. The medium was changed every day for five days after which the cells were grown in EGF-free medium. The ST began to form on day 6 after plating and experiments were performed on culture day 10. The purity of the ST was determined by staining the ST with anti-vimentin monoclonal antibodies (Sigma, clone # V9) to ascertain that there was no contamination with fibroblasts or other vimentin-positive cells. Preparations that had more than 1% fibroblast contamination were discarded.

Choriocarcinoma cell culture

The human choriocarcinoma cell line BeWo was obtained from American Type Tissue Culture (CCl-98, ATTC). These cells were maintained in Ham's F12-K culture medium

(#30-2004, ATTC) supplemented with 10% FBS as single mononuclear cytotrophoblasts (CT) cells. For experiments, the cells were induced to form ST by the addition of 40 μM forskolin (Sigma) to the culture medium for 48 hours with daily medium change [37]. On the third day, the medium was replaced with forskolin-free medium. The cells formed syncytiotrophoblast ($BeWo^{ST}$) by day 4 of culture at which time they were used for experiments. The cell cultures were routinely tested for mycoplasma contamination by staining with Hoechst dye (Sigma).

Malaria parasites

Three laboratory strains of malaria parasites were used: 3D7 (NIH clone of NF54), FCR3 (Malaria Research and Reference Reagent Resource Center (MR4)) and CS2 (MR4). These were maintained in culture using human blood group O+ blood from local donors, in RPMI-HEPES medium supplemented with 25mmol/L sodium bicarbonate, 20 μg/ml gentamicin and 10% filtered human serum (Red Cross). Culture flasks were maintained in a 37^{o}C incubator and gassed with a mixture of 5% oxygen and 5% carbon dioxide blood gas. Cultures were made synchronous by freezing and thawing cycles that result in mostly ring stage forms since this procedure tends to kill most of the mature late-stage parasites. Experiments were conducted using cultures consisting largely of mature trophozoite and schizont stages. The parasite cultures were routinely tested for mycoplasma contamination by PCR.

Selection for cytoadhesion to human ST by panning and cytoadhesion assays

Selection of cytoadherent iRBCs was performed as described [38] with some modification. Late stage trophozoites of both the 3D7 and FCR3 strains were washed 3 times with buffer containing RPMI-1640 (Cellgro), 25mM HEPES (Sigma) and 10% human serum, pH 6.8 (binding medium) and then resuspended at 2-4 % hematocrit. The ST cell monolayer was washed once with binding medium before the washed iRBCs were overlaid on the syncytium and incubated for 1.5 hours at room temperature with gentle rocking every 15 minutes. The cells were then washed 3 times with medium to remove unbound iRBCs and uninfected RBCs. Fresh RBCs were added to the flask at a 5% hematocrit and incubated as above for parasite culture. The following day the bound iRBCs with mature parasites ruptured and the parasites infected the freshly added RBCs which were collected and transferred into a T-25 flask (Corning) and the culture continued. This was repeated 5-7 times to obtain ST-adherent iRBCs ($iRBC^{ST}$: $3D7^{ST}$ and $FCR3^{ST}$). The $iRBC^{ST}$ were frozen in liquid nitrogen until use in cytoadhesion assays. For the cytoadhesion assays, iRBC containing mature stage trophozoites of the $3D7^{ST}$, $FCR3^{ST}$ and CS2 parasites were used at a 5% parasitemia and 2-4% hematocrit. The iRBCs were resuspended in binding medium and overlaid on either $BeWo^{ST}$ or primary ST and incubated for 1.5 hours at 37^{o}C. Normal uninfected RBCs (uRBCs) were used as a negative control. Unbound cells were washed off by three repeated washes with medium. The presence of iRBCs was verified by pre-staining the iRBCs with 0.1 μg/10^5 iRBC ethidium bromide (BioRad) for 15 minutes before the cytoadhesion assay.

Immunoflourescence staining of CSA on the surface of BeWoST

BeWoST cells were grown on cover slips and induced to form ST. The monolayer was fixed using 1:1 acetone: methanol fixative for 1 minute and then blocked for 1 hour at room temperature using 3% bovine serum albumin in phosphate buffered saline. The monolayer was washed and incubated with a mouse anti-CSA monoclonal antibody (Sigma clone # CS-56, diluted 1:200) or an isotype control antibody (Sigma). Staining to identify CSA bound to antibody was revealed by use of fluorescein isothiocyanate (FITC)-conjugated anti-mouse polyclonal secondary antibody (Sigma # F 5262, diluted 1:400) and observation under a fluorescence microscope.

Cytoadhesion inhibition assays

To block the ability of iRBCs to bind to CSA, both primary ST and BeWoST were pre-incubated with 0.5 U/ml of chondroitinase ABC enzyme (Fluka) for 45 minutes at 37^{o} C. The cell monolayers were then washed twice with binding medium and the iRBCST or uRBCs controls added. The co-culture was incubated for 1.5 hours as described above. The cells were observed under an inverted microscope and the numbers of iRBCST bound per mm^2 of ST was determined by counting 10 random fields under high power magnification. Percentage inhibition was determined as follows: 100-[(number of cells bound /mm2 in the presence of inhibitor ÷ number of cells bound/mm2 in the absence of inhibitor) X 100].

Protein lysate preparation and immunoblotting for tyrosine phosphorylated proteins

BeWoST were co-cultured with the selected iRBC (iRBCST) or uRBC controls 15 and 30 minutes or left unstimulated. Cells were scraped from plates with 1 mL PBS/SOV (sodium orthorvanadate (1mM) Sigma) and pelleted at 7000 x *g* for 5 minutes. The cells were lysed for 15 minutes on ice with 300 µl lysis buffer (50mM Tris-HCl, pH 7.4, 1% NP-40; 0.25% sodium deoxycholate; 150 mM sodium chloride; 1 mM EDTA; 1 mM PMSF; 1 µg/ml aprotinin, leupeptin and pepstatin; and 1 mM sodium orthovanadate (all from Sigma). Lysates were pelleted by centrifugation at 14000 x g for 15 minutes and the pre-cleared lysates were collected and stored at -80^{o} C. Thawed protein samples (20 µg/lane) were separated electrophoretically on a 10% gradient sodium dodecyl sulphate-polyacrylamide gel (BioRad) and transferred to nitrocellulose membrane (BioRad) for 1 hour. Membranes were incubated at 37° C in blocking solution (3% non-fat milk in PBS) followed by incubation with anti-tyrosine phosphorylation antibodies (1 µg/ml; 4G10, Upstate) in blocking buffer for 1 hour at room temperature. Final detection was with goat anti-mouse–horseradish peroxidase-labelled (HRP) secondary antibody (1:2000; Sigma) in blocking buffer for 1.5 hour at room temperature. Tyrosine phosphorylated proteins were visualized by enhanced chemiluminescence (ECL, Amersham) after four washes with distilled water on Kodak film using an automated film developer. Blots were stripped in buffer (2% SDS; 62.5 mM Tris–HCl, pH 6.7; 100 mM 2-mercaptoethanol) and reprobed in a similar fashion using monoclonal antibodies against β-actin (Sigma, clone # AC-15). The latter was used as a loading control for the densitometry analysis which was done using QuantityOne software (BioRad).

Statistics

Student's T-test was performed for the inhibition assay to determine significant differences between the bound iRBCs in the presence of enzyme compared to controls.

RESULTS

Isolation of primary cytotrophoblast cells and induction of BeWoST formation

The BeWo cells were induced to form ST with forskolin, an activator of cAMP [37]. The BeWo cytotrophoblasts consisted of single mononucleated cells (Figure 1A) which formed multinucleated cells after 3 days of forskolin treatment (Figure 1B; note grouping of nuclei). BeWoST typically contained 10-46 nuclei. Primary ST formation was induced in isolated cytotrophoblasts by addition of rhEGF; mature ST was obtained after 6 days in culture. These cells contained 10-50 nuclei per syncytium (Figure 1C). The purity of the cytotrophoblast cells was monitored using anti-vimentin antibody (an endothelial cell and fibroblast marker). The preparations contained fewer than 20 vimentin-positive cells per 10^6 seeded cells, indicating that the preparations were highly purified cytotrophoblasts (data not shown). The primary ST cultures were grown for up to 21 days without fibroblast growth or degradation.

Selection of malaria parasites capable of binding to ST

Cultured BeWoST cells were used to select for iRBC that preferentially bind to ST. ST-adherent iRBC (iRBCST) from two different laboratory strains of *P. falciparum*, 3D7 and FCR3 were selected. Strong binding was evident after 6 and 4 rounds of panning with

the 3D7 and FCR3 strains respectively (seen as small, bright dots on and around the ST in figure 2). The $3D7^{ST}$ tended to lose its binding phenotype when put in continuous culture, necessitating 5-6 times repanning. In contrast, the $FCR3^{ST}$ strain maintained its ST-binding phenotype through several rounds of cryopreservation and thawing. CS2 parasites, known to bind to CSA having been selected for CSA-binding from the FAF-EA8CHO-5 strain [39] by panning twice on immobilized CSA [40], were also included in the binding assays. As expected, the CS2 parasites bound to both primary ST and $BeWo^{ST}$ without prior selection for binding to these cells (figure 2). Normal uninfected RBCs used as a negative control did not bind to either primary ST or $BeWo^{ST}$ (figure 2) nor did unselected 3D7 and FCR3 iRBCs (data not shown). To ensure that cells observed to bind on the ST were indeed iRBCs, $iRBC^{ST}$ were pre-stained with ethidium bromide before the cytoadherence assay. Fluorescence microscopy revealed the presence of red fluorescence dots on and around the ST (figure 3A), confirming that the bound erythrocytes (figure 3B) were iRBCs. The selected iRBCs were shown to bind to $BeWo^{ST}$ and to a lesser extent, mononuclear cytotrophoblast, but not to a kidney epithelial cell line, 293T cells (data not shown). The selected $3D7^{ST}$ and $FCR3^{ST}$ iRBCs were also shown to bind to primary ST cells without prior selection on primary ST cells (figure 2).

iRBC binding is mediated by CSA as demonstrated by inhibition of binding by chondroitinase ABC

While in vitro cultured primary ST is known to express CSA ([18]; data not shown), its expression on the $BeWo^{ST}$ membrane has not been demonstrated. Immunocytochemistry

with a monoclonal antibody specific for CSA shows that BeWoST does express CSA (figure 4A). To identify to what extent the binding of 3D7ST and FCR3ST is mediated by CSA, BeWoST and primary ST cells were treated with 0.5 U/ml of chondroitinase ABC before addition of iRBCST. Figure 5 shows that this treatment significantly inhibited 3D7ST and CS2 binding to both BeWoST and primary ST (Table 1). However, the chondroitinase ABC treatment did not completely abrogate iRBCST binding.

Binding of iRBCs to ST leads to cellular activation of the ST

Host cell receptors for iRBC binding, particularly CD36 and ICAM-1, are cell surface molecules that are capable of mediating the delivery of intracellular signals. In fact, iRBC engagement of CD36 on endothelial cells has been shown to induce signaling through the ERK1/2 and p38 pathways via activation by src-family kinases [41]. To assess the ability of iRBCST to induce a CSA-mediated intracellular response in ST, proteins were isolated from the ST after the binding of iRBCST and subjected to western blotting using a pan phosphotyrosine antibody. The binding of iRBCs led to an increase in the tyrosine phosphorylation of ~93kDa and 85kDa ST cellular proteins after 15 and 30 minutes incubation with iRBCST whereas incubation with normal uninfected RBC did not lead to any such changes (Figure 6). Densitometric analysis showed 1.6 and 2.6 fold increases in the tyrosine phosphorylation of the 93kDa protein and 2 and 2.4 fold increases for the 85kDa protein after 15 and 30 minutes, respectively.

DISCUSSION

While significant progress has been made towards improved understanding of the interaction of *P. falciparum*–infected RBCs with the placenta (in IVS and directly with ST) with the use of commercially available CSA and different cell culture systems, these have not provided a fully suitable means to evaluate the nature and consequences of iRBC adherence to ST. The present study has made use of two cell types, primary trophoblasts and BeWo choriocarcinoma cells, to provide such a system. As mentioned above, highly purified primary ST have been isolated and used by other researchers to study cytoadherence of malaria-infected erythrocytes in vitro [18]. BeWo cells are known to resemble primary ST cells in many aspects, including the ability to produce signature placenta-derived hormones [42], to form ST [37] in culture, and, as we demonstrate, to express CSA at the cell membrane. In contrast to primary ST, the BeWo cells can be propagated indefinitely as mononuclear cells in vitro and greatly expanded to provide copious material for functional studies such as that reported here. Both BeWo cells and primary trophoblasts have proven to be equally useful in the present model system for exploring the biology of trophoblast/*Plasmodium* interactions.

The processes leading to the accumulation of iRBC in the placenta are poorly understood. The most prevailing theory is that the placenta provides an ideal environment for sequestration of iRBC by providing optimal conditions for their multiplication as they adhere to specific host receptors like CSA [11] and HA [16]. In this report, we show that it is possible to select for iRBCs that bind to primary ST and $BeWo^{ST}$. These laboratory *P. falciparum* strains were selected for ST-binding by

panning on BeWoST. With the exception of CS2 which is a known CSA-binder [39], the parasite strains used were not pre-selected to bind to plastic bound CSA or CSPGs. Nonetheless, the iRBCST bound to CSA, as chondroitinase treatment significantly abrogated their binding to ST. That chondroitinase treatment did not completely abrogate binding is in keeping with previous studies which demonstrated that the placental parasite populations do not adhere in a uniform manner to immobilized CSA or CS receptors, implying that other factors may be confounding this interaction or other receptors are involved [28]. The latter idea is supported by the recent finding that the ability of antibodies from pregnant women to block binding of iRBCs to placental CSPGs does not directly correlate with their ability to recognize variant surface antigens on placenta-derived iRBCs [28]. We propose that in the absence of natural placental parasite isolates, iRBCST populations such as those generated in this study are preferable for investigations of placental cytoadherence, because they are selected for binding to receptors (CSA and perhaps others) that are naturally present on the ST. Thus, the system could be used to identify other potential host receptors for placental iRBC cytoadherence.

This model system might also be used to assess the molecular dynamics of the trophoblast binding phenotype in *P. falciparum.* Until recently, it was widely assumed, based on gene expression studies of *P. falciparum* lines that were selected to bind to CSA, that the placental selection of iRBCs produced unique parasite populations that cytoadhere via PfEMP1 expressed by the *var1CSA* [43] and *FCR3varCSA* genes [21]. However, the *var1CSA* gene is only rarely transcribed in parasites derived from naturally infected placentas [44]. It is therefore not clear which PfEMP1 molecules represent CSA-binding ligands or if other parasite antigens can confer the CSA-binding phenotype.

Panning for iRBCST on primary ST should allow for the dynamics of *var* gene expression to be explored, and help to reveal other potential parasite ligands that are necessary and sufficient for binding to placental tissue.

It is known that adhesion molecules can serve as receptor-signaling molecules capable of transducing extracellular signals resulting in cellular activation [45]. Several studies have hinted that iRBCs adhesion might also induce intracellular signaling: the binding of iRBCs to monocytes via CD36 resulted in a respiratory burst [46], and cross-linking of CD36 on monocytes activated the extracellular signal regulated kinase 1/2 (ERK 1/2) and p38 MAP kinase pathways in a src-family kinase-dependent manner [41]. The adhering of iRBCs has also been shown to inhibit dendritic cell maturation through the interaction with cell surface CD36 [47, 48]. These results imply that cytoadherence can lead to modulation of cellular functions. Like CD36, ICAM-1 also signals through MAP kinases, as well as src-family kinases [49]. Signaling though CSPGs has not been well defined, although the chemokine RANTES was recently show to bind to a glycosaminoglycan (heparin sulfate and/or chondroitin sulfate) on the CD44 molecule on Chinese Hamster Ovary (CHO) cells and to stimulate ERK1/2 signaling in those cells [50, 51]. Also, the chemokine CXCL4 has been proposed to signal through CSPGs on neutrophils, monocytes, and lymphocytes [52]. The nature of the proteoglycans that express CSA on the ST membrane have not been determined, although thrombomodulin is one possibility [53]. Based on the results from the current study, binding of iRBCs to BeWoST (and primary ST; N. Lucchi et al., unpublished data) induces kinase activity in the ST. This is a novel finding, showing for the first time that cellular activation is induced in ST by iRBC binding. Identification of the phosphorylated proteins and the

kinases responsible, studies which are currently under way, will be necessary to fully characterize the functional significance of this activation.

While the functional effects of binding of iRBCs to ST during PM remain to be investigated fully, the ST is known to be highly immunoactive, and is capable of secreting many cytokines [54-57]. While this activity is important for successful pregnancy [58], both pro- and anti-inflammatory factors were shown to be produced by trophoblasts in response to pathogenic bacteria [59]. We have recently demonstrated that $BeWo^{ST}$ secretes macrophage migration inhibitory factor in response to $iRBC^{ST}$ binding (Chaisavaneeyakorn *et al*., in press) and are currently exploring other malaria-induced gene expression changes in ST using this model system. These studies will have important implications for the understanding of the biology of placental malaria, as they will now add to the equation the contribution of the fetus. The ability of the trophoblast to manipulate the local immune environment, and thus influence local maternal immune responses to placental malaria, potentially participating in both protective and pathogenic immune mechanisms, has significant implications for the prevention and control of this important public health problem.

Like all in vitro systems this cell culture model has some limitations. For example, there is a continuous flow of blood in the IVS of the placenta and therefore the static conditions used in this model may not reflect the true in vivo situation. It should be possible in future work, however, to perform these studies in a flow cell. Also, in vivo, the ST is in direct contact not only with iRBC during PM but also with maternal immune cells and their secreted soluble factors, including cytokines and chemokines. These

factors may have profound effects on the ST in combination with the cytoadherence of iRBCs. We are currently investigating how these other parameters may influence the system, and aim to further develop it to reflect as much as possible the in vivo environment.

Without detailed knowledge on how the host (ST) and parasite interact and respond to each other, especially in terms of development, maintenance and control or modulation of immune responses in the placenta, therapeutic methodologies for the management of malaria during pregnancy cannot be fully evaluated. This is only attainable if an appropriate model system exists. The system described here provides the best means described to date to study the nature and implications of iRBC binding in the placenta. Only through characterization of iRBC binding phenotypes, how these binding phenotypes are selected for, the parasite binding ligands, the iRBC receptors on the ST, and the functional changes induced in the ST by iRBC binding, will a complete understanding of the biology of the maternal/fetal interface in the context of placental malaria be achieved.

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Figure 3.1

Induction of syncytium formation in BeWo and primary cytotrophoblast cells.

BeWo cytotrophoblast cells (A) were induced by forskolin treatment to produce syncytium (B). Primary cytotrophoblast cells were induced to syncytialize by culturing in medium containing rhEGF (C) (x 200 magnification).

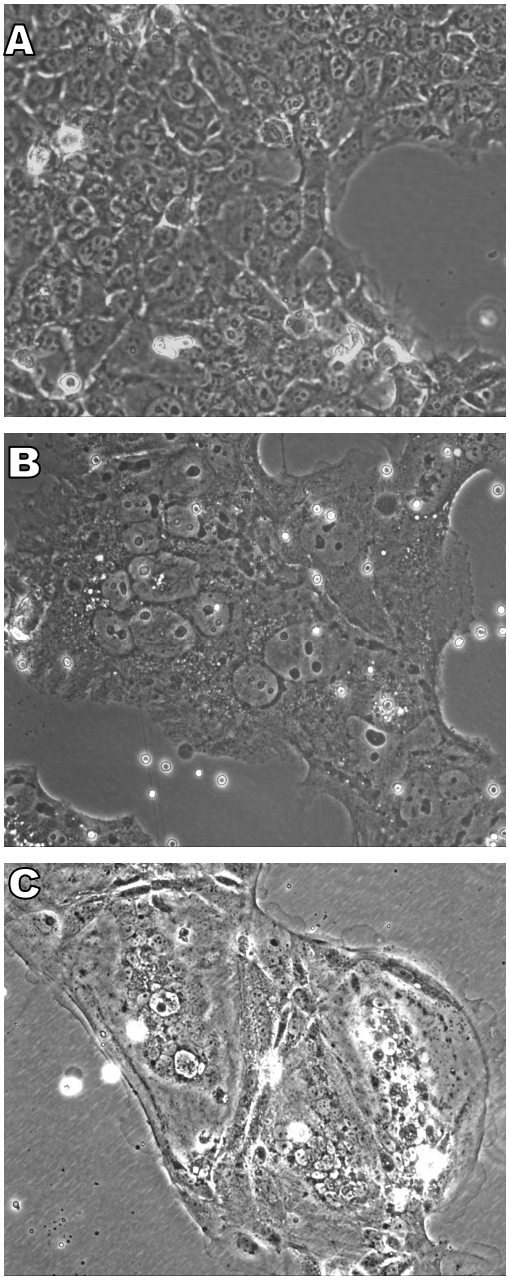
A
B
C

Figure 3.2.

Selected and CSA-binding malaria parasites cytoadhere to BeWoST and primary ST.

Normal uninfected RBCs (uRBCs) do not bind to either primary ST or BeWoST, whereas 3D7ST and FCR3ST parasite strains panned on BeWoST for this phenotype bind robustly, seen as small, bright dots. CS2 parasites, known to bind to CSA, also bind to primary ST and BeWoST. (X200 magnification for all left panels and primary ST. The rest are x340).

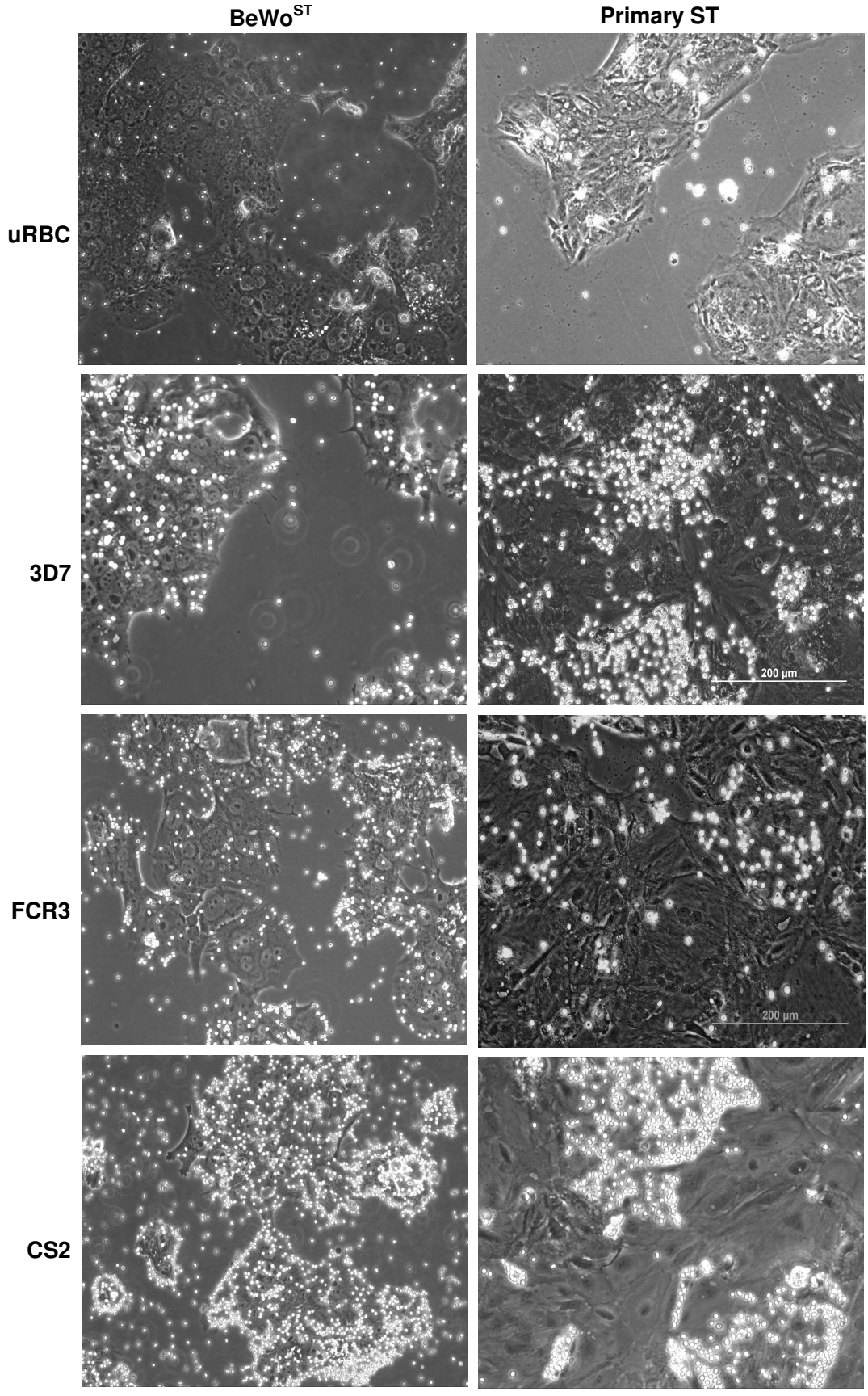
BeWo^ST
Primary ST
uRBC
3D7
FCR3
CS2
200 µm
200 µm

Figure 3.3.

Binding RBCs are parasitized.

Staining of iRBCST with ethidium bromide prior to the cytoadherence assay reveals that most bound cells (seen in phase contrast, B) are iRBCs, seen as bright red dots (A). (x200 magnification).

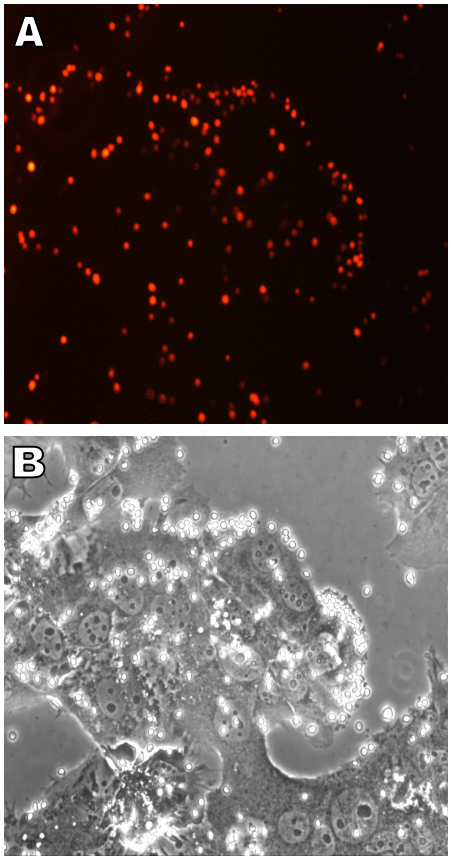
A
B

Figure 3.4.

BeWoST express membrane CSA.

BeWoST incubated with mouse anti-CSA antibody (A) or isotype control antibody (B) followed by an anti-mouse FITC-conjugated secondary antibody demonstrates CSA expression on BeWoST. (x 400 magnification).

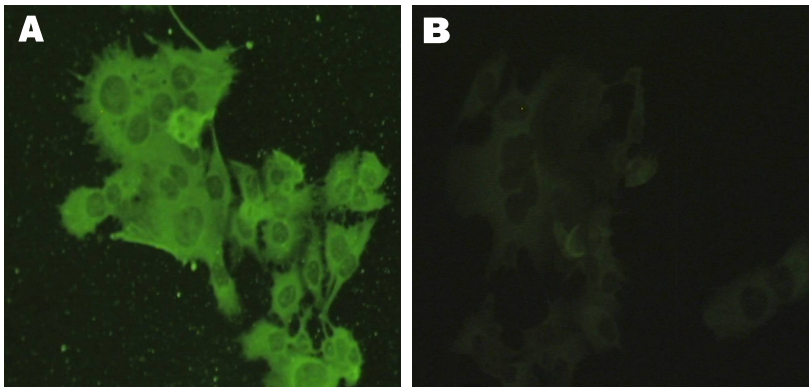
A
B

Figure 3.5.

Binding of selected iRBC to BeWoST and primary ST is mediated in part by chondroitin sulfate A.

BeWoST and primary ST cells were pre-treated with 0.5U/ml of chondroitinase ABC or medium only. The binding of 3D7ST was significantly inhibited by chondroitinase ABC pretreatment. (x200 magnification).

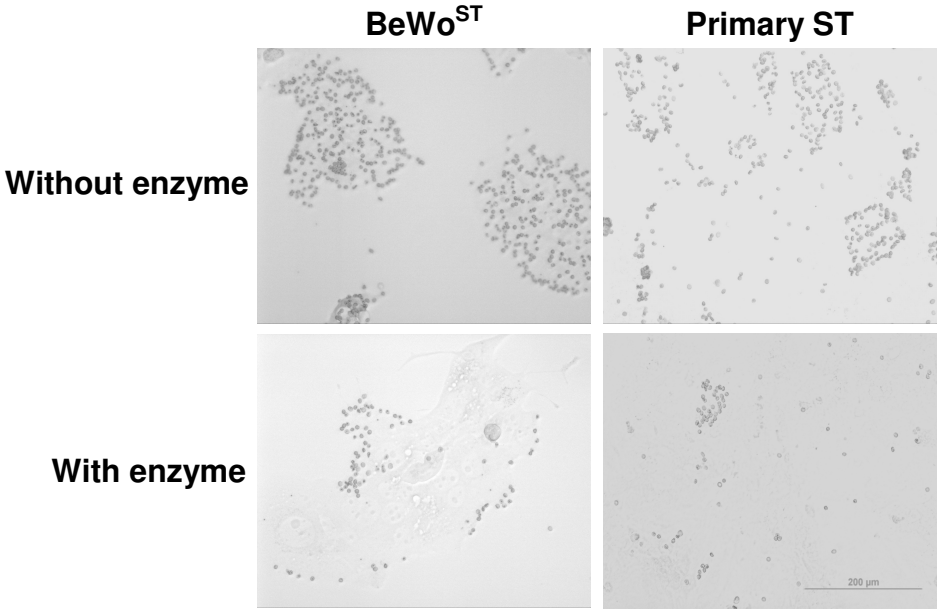
BeWo^ST
Primary ST
Without enzyme
With enzyme
200 µm

Figure 3. 6

Induction of tyrosine phosphorylation following iRBCST binding to ST.

Western blotting of tyrosine phosphorylated cellular proteins from BeWoST treated with iRBCST (left) or uninfected RBCs (right) for 15 and 30 minutes show specific enhanced phosphorylation of 85 and 93 kDa proteins in the iRBCST treated cells.

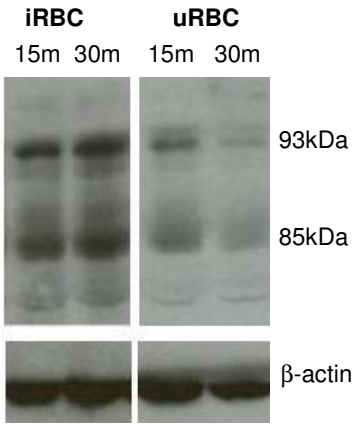
iRBC
uRBC
15m 30m 15m 30m
93kDa
85kDa
β-actin

Table 3.1: Binding of selected iRBC to BeWoST and primary ST is abrogated by pre-treatment with chondroitinase ABC.

BeWoST and primary ST cells were pre-treated with 0.5U/ml of chondroitinase ABC or medium only before the adhesion assay was performed. The number of binding iRBC per mm^2 of cell surface was counted for 10 random fields. Binding of the selected iRBCST and CS2 parasites was significantly inhibited by chondroitinase ABC pretreatment with p values much less than 0.05. Values shown are mean ± standard error.

Table 3.1

Parasite strain	**BeWoST**			
	Number of iRBC per mm^2		% inhibition	P value
	chondroitinase ABC			
	-	+		
3D7ST	2906 ± 231	300 ± 150	89	$P < 0.001$
FCR3 ST	2842 ± 235	400 ± 139	86	$P = 0.002$
CS2	1837 ± 174	225 ± 37	87	$P < 0.001$
Parasite strain	**Primary ST**			
	Number of iRBC per mm^2		% inhibition	P value
	chondroitinase ABC			
	-	-		
3D7 ST	3050 ± 309	972 ± 147	74	$P = 0.010$
FCR3 ST	1527 ± 331	435 ± 39	71	$P = 0.003$
CS2	3820 ± 224	630 ± 136	84	$P < 0.001$

CHAPTER 4

IMMUNOLOGIC ACTIVATION OF HUMAN SYNCYTIOTROPHOBLAST CELLS BY *PLASMODIUM FALCIPARUM*[1]

[1] Lucchi NW, Peterson, DS, and Moore, JM. To be submitted to Infection and Immunity.

ABSTRACT

An important pathogenic complication of malaria during human pregnancy is sequestration of malaria-infected red blood cells (iRBCs) in the intervillous spaces (IVS) of the placenta. This sequestration is mediated by binding of iRBCs to receptors expressed on the syncytiotrophoblast (ST). How the ST responds to this assault remains poorly understood. In previous studies, selection of ST-adherent iRBCs ($iRBC^{ST}$) was performed and the interaction of $iRBC^{ST}$ was shown to lead to tyrosine phosphorylation of at least two ST proteins and secretion of macrophage migration inhibitory factor (MIF). In the current study, primary ST cells were used to further assess intracellular signaling and characterize gene expression changes found in ST following $iRBC^{ST}$ binding or interaction with crude malarial antigen. The binding of $iRBC^{ST}$ led to an increase in the phosphorylation of c-Jun N-terminal kinase (JNK) mitogen activated protein kinase (MAPK), while stimulation with malarial antigen led to an increased phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) proteins. To assess if this signaling led to any cytokine gene expression changes, mRNA expression levels of selected cytokines and chemokines was assessed by real-time PCR. The binding of $iRBC^{ST}$ to the ST cells led to modest (1.9-3.3 fold) increases in the expression of TNF-α, TGF-β and IL-8 mRNA but not to the measurable secretion these cytokines. While this interaction did not upregulate cellular expression of ICAM-1 on ST cells, it enhanced the migration of peripheral blood mononuclear cells towards stimulated ST. These results suggest that the ST may participate in shaping the local immunological milieu during placental malaria and in the recruitment of maternal immune cells to the IVS.

Key Words: syncytiotrophoblast, placental malaria, gene expression, chemotaxis, chemokines, activation, MAPK, Plasmodium falciparum

INTRODUCTION

It is estimated that annually about 2.2 billion people are exposed to the threat of *P. falciparum* malaria, and that between 300–600 million clinical attacks are attributable to this parasite yearly (1, 47). Ninety percent of deaths occur in sub-Saharan Africa, the majority involving children less than 5 years of age. In addition to children, pregnant women (particularly those in their first pregnancy (primigravidae) (6, 29) are at highest risk of severe disease. Sequestration, the adherence of iRBCs containing late developmental stages of the parasite to the host vasculature, is characteristic of *P. falciparum* infections. A hallmark of malaria during pregnancy is the sequestration of these malaria-infected red blood cells (iRBCs) in the intervillous space (IVS) of the placenta (7, 34, 54). This is usually accompanied by the infiltration of maternal leukocytes in the IVS (36, 42) and placental pathology including placental lesions such as focal ST damage (8, 20). This is referred to as placental malaria (PM). PM poses substantial risk to the mother, the fetus, and the neonate. In high transmission areas, women have developed considerable immunity to malaria by childbearing age and the infection is frequently asymptomatic with severe disease occurring rarely (6, 29). However, PM may lead to maternal anemia and poor fetal outcomes such as low birth weight (LBW) babies and prematurity (30, 42). The increased incidence of LBW associated with PM is a leading cause of death, because LBW infants are vulnerable to many life-threatening ailments (28). It is estimated that 75 000–200 000 infant deaths occur annually because of PM (48).

The sequestration of iRBCs in the placenta is thought to be mediated by the cytoadherence of iRBCs to receptors on the syncytiotrophoblast (ST). ST cells are

epithelial fetal cells that are in direct contact with maternal blood within the IVS. Parasite-encoded surface ligands are expressed on the membrane of iRBCs and these are thought to mediate this adherence. The only well-characterized cytoadherence protein is the *P. falciparum*-infected erythrocyte membrane protein-1 (PfEMP1) encoded by the highly polymorphic members of the *var* gene family (3, 38, 46, 49). Currently, it appears that chondroitin sulfate A (CSA) is the principal iRBC receptor in the placenta (12, 14, 18, 39-41). CSA is a glycosaminoglycan linked to the ST cell surface via a membrane-associated protein and is highly expressed on the ST cells.

The host's immunological response, particularly proinflammatory cytokines and chemokines, has been implicated in protection against PM (17, 19, 31, 32). Elevated production of interferon gamma (IFN-γ) by intervillous blood mononuclear cells (IVBMC) was associated with protection (31). Fievet et al. demonstrated that PM infection led to both Th1 (IFN-γ, TNF-α, IL-1β) and Th2 (IL-6, IL-10) cell activation with a bias towards the Th1 (17). Production of IL-10 by IVBMC was also shown to be increased in PM (31, 32) and was hypothesized to be important in the control of the negative effects of Th1 cytokines on pregnancy. Apart from cytokines, several proinflammatory chemokines have also been observed in association with PM including IL-8 (2, 32), MCP-1 and beta chemokines (2, 10, 11, 32). Massively increased levels of macrophage migration inhibitory factor (MIF) were observed in the placental plasma from women with PM (11). The proinflammatory immune responses during PM have been associated with the ensuing monocytic infiltration (2) observed in PM (36, 37). However, there is accumulating evidence that these same responses play a major role in malaria pathophysiology and contribute to morbidity (53). These accumulating immune

cells appear to be the main source of the proinflammatory response although other placental cells such as fetal stromal cells (2) and ST cells may contribute to this response.

It has been shown previously that heat-stable exoantigens in the supernatants of blood-stage parasite cultures induced the release of tumor necrosis factor (TNF) from activated macrophages in vitro and behaved like toxins in vivo (5, 51, 52). Bate et al. observed the same activity using sonicated iRBC and uRBC with the exception that the TNF inducing activity was about 200 times higher in iRBCs than in uRBCs (4). Later studies identified the glycosylphosphatidylinositol (GPI) to be a major malaria toxin (43, 44, 50). GPIs are known to anchor most of the parasite protein to the parasite surface (21). The sequestration of iRBCs in the IVS may result in high concentrations of these malarial toxins within the placenta creating a potentially harmful environment. It is therefore of interest to investigate if malaria toxins activate the ST.

The interaction of the maternal leukocytes and the ST during PM is still not completely understood. It has previously been shown that activated lymphocytic cells bind to ST in vitro via intracellular adhesion molecule-1 (ICAM-1) expressed by the ST. The impact of the iRBCs binding to ST on the expression of ICAM-1 had not been investigated. Given the proposed role of infiltrating maternal immune cells in mediating PM pathophysiology (2), it is important to determine if the ST cells contribute in the recruitment or retention of these cells either by secretion of chemokines or upregulation of ICAM-1.

Many investigations of immunologic responses during PM have focused on the maternal side of the maternal-fetal interface, but it is clear that fetally derived tissues are

active participants in maintaining the immunological milieu at the maternal-fetal interface (22). In this study, in vitro experiments using a placental cell line, BeWo, and primary trophoblast cells together with selected ST-adherent iRBC (26) were conducted to determine the impact of iRBC binding to ST and interaction with malarial products. Cellular activation of ST was assessed by observing activation of mitogen activated protein kinases (MAPK) upon stimulation with iRBC or crude malaria antigens. In addition, gene expression levels and secretion of selected cytokines and chemokines were examined using real-time PCR and standard ELISA, respectively. In addition, this study investigated the ability of $iRBC^{ST}$–stimulated ST to upregulate ICAM-1 expression on ST and to stimulate the migration of peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

Trophoblast culture

BeWo cells were grown in minimum essential medium and induced to form ST ($BeWo^{ST}$) by use of 40uM forskolin as previously described (26). Primary placental CT cells were isolated from fresh human placentas obtained from women delivering at Athens Regional Hospital, Athens as described (26). All protocols were approved by the University of Georgia Institutional Review board and written, informed consent was obtained. The immunopurified cells were used immediately or cryopreserved in liquid nitrogen until use.

Malarial antigen preparation

Crude malarial antigen was prepared from mycoplasma-free late-stage parasite cultures. The iRBCs were washed once in RPMI 1640 and resuspended to a 10%

hematocrit. These were then layered on cold 60% percoll and centrifuged for 20 minutes at 1200 X g for 15 minutes at 4^{o}C. The interphase, containing trophozoites and schizonts, was collected and washed 3 times with sterile, LPS-free PBS. These were sonicated at 25w for 2 minutes with short intervals to lyse the RBC. The mixture was then centrifuged to remove particulates, the protein content determined by Bradford assay and resuspended at a 1mg/ml concentration. The same procedure was followed to prepare control antigen from uninfected RBCs (uRBCs). These were stored at -80^{o}C until used.

Immunoblotting

The ST cells were stimulated with either 40ug/ml crude malarial antigen or incubated with iRBCST for given time points after which whole-cell lysate extracts were obtained as described (26). Unstimulated cells or cells incubated with uRBCs were also included in the assays as controls. Immunoblotting was carried out as described earlier (26) with a few changes. The membranes were probed with primary antibodies against phosphorylated ERK1/2, ERK, JNK and p38 (Cell signaling Technology Inc., Beverly, MA) as recommended by the manufacturer. Final detection was performed with appropriate horseradish peroxidase-labelled (HRP) secondary antibodies (Sigma St. Louis, MO) in blocking buffer for 1 hour at room temperature. Phosphorylated proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL). Each membrane was stripped with freshly prepared stripping buffer (2% SDS; 62.5 mM Tris–HCl, pH 6.7; 100 mM 2-mercaptoethanol) and reprobed in a similar fashion with antibodies against total (non-phosphorylated) forms of the relevant MAPK or beta actin. The latter were used as loading controls and for densitometric analysis performed using QuantityOne software (Bio-Rad, Hercules, CA). For ICAM-1 experiments, the ST cells

were stimulated with 10ng/ml of recombinant human tumor necrosis factor alpha (rhTNF-α , BD Biosciences, San Jose, CA): iRBCST: uRBCs: iRBC Ag or uRBC Ag. Total protein 920ug/lane) was separated on a 10% gradient SDS-PAGE. Detection was carried out using anti-ICAM-1 monoclonal antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) with the necessary secondary antibody. The membranes were stripped as described above and reprobed with anti-β-actin antibodies (Sigma, St. Louis, MO) to allow densitometric analysis.

Real-time PCR

Total RNA was isolated from trophoblast cells using the RNeasy Qiagen kit (Qiagen, Valencia, CA) following the manufacturer's protocol and stored at –85^{o}C. Contaminating genomic DNA (gDNA) was digested using RNAse-free DNase (Ambion Inc. Austin, TX) as recommended by the manufacturer. First strand cDNA was synthesized from 1μg of obtained total RNA using the Omniscript reverse transcription kit (Qiagen, Valencia, CA). Real-time PCR was carried out using specific primers for IL-8, IL-10, MIF, TNF-α TGF-β, MCP-1, IFN-γ and 18S ribosomal RNA (table 4.1) (all from MWG-Biotech Inc., High Point, NC). All the primers used were first validated for use in comparative real-time PCR. Real-time PCR was performed using the Mx3000P thermocycler and program (Stratagene, Cedar Creek, TX). No template controls and no reverse transcription controls were included. The 2^ΔΔ CT method of analysis was used with the 18S RNA gene as normalizing gene and unstimulated control cell RNA as the calibrator. Results are given as fold increase over unstimulated cells.

Cytokine and chemokine ELISA

ST cells were stimulated for 2, 4, 8, 12, and 24 hours with $iRBC^{ST}$, uRBCs, and 40μg/ml crude malarial antigen or left unstimulated. Supernatants were collected and a standard sandwich ELISA was performed according to the manufacturer's protocol (R&D Systems, Inc. Minneapolis, MN) for TNF-α, TGF-β, IL-10, MIF and IL-8.

Chemotaxis Assay

Peripheral blood was obtained from female volunteers by a trained phlebotomist. An equal part of sterile phosphate buffered saline (PBS) was added to the blood and this was layered on fico-lite LymphoH (Atlanta Biological, Atlanta, GA) in a 50ml centrifuge tube. To obtain PBMCs, the tube was centrifuged at 355xg for 30 minutes at 25°C. The interface, containing the PBMCs, was colleted and washed twice with sterile PBS. The cells were counted and used for the chemotaxis experiments.

ST cells were grown in 24-well plates (BD Biosciences, Franklin Lakes, NJ) and stimulated with $iRBC^{ST}$ or uRBCs for 12 hours or left unstimulated. PBMCs were used at 5×10^5 cells/ml. 3-μm pore size cells culture inserts (BD Biosciences, Franklin Lakes, NJ) were placed into each well and 350μl of PBMC were added and allowed to migrate for 12 hours. The inserts were removed and placed in new wells. Using a cotton swab the inserts were carefully wiped to remove cells that had not migrated. Migrated cells were stained with calcein-AM (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Stained cells were washed 3 times. The number of migrated cells was determined by counting five random fields under an inverted fluorescence microscope at X20 magnification.

RESULTS

Stimulation of JNK1 in ST cells by binding of iRBCST

An increased phosphorylation of JNK1 was evident after 30 minutes incubation with iRBCST (figure 4.1A and B). This remained high through the subsequent time points. Stimulation with uRBCs did not lead to any increased phosphorylation of JNK1 but appeared to result in a decline after 60 and 120 minutes (figure 4.1A). Adherence of iRBCST to ST cells or stimulation with uRBCs did not lead to changes in ERK1; it was constitutively phosphorylated (figure 4.1C). Phosphorylation of p38 in ST cells was not observed even with stimulation (figure 4.1D).

Stimulation of ERK1/2 MAPK by malarial antigens

An early activation of ERK1/2, as evidenced by a 3.2 fold increase in its phosphorylation 5 minutes after stimulation (figure 4.2A and B) was observed with crude malarial antigen treatment. This was sustained for the duration of stimulation time. Treatment with uRBC antigen appeared to lead to a slight increase (1.6 fold) in the phosphorylation of ERK1/2 5 minutes after stimulation but this returned to baseline levels after 30 minutes (figure 4.2A and B). Malarial antigen treatment of ST cells did not lead to changes in JNK1/2 or p38 (data not shown).

Gene expression changes in ST stimulated with cytoadherence iRBCST

Following in vitro stimulation of primary ST with iRBCST, marginal increases (1.5-~3 fold) were observed in the mRNA expression of TNF-α, TGF-β and IL-8, but not IL-10 or MIF (figure 4.3). IFN-γ and MCP-I gene expression were not detected even after stimulation (data not shown). Changes in gene expression were calculated relative to gene expression obtained upon stimulation with uRBCs.

Cytokine secretion upon stimulation with iRBCST

Supernatants from similarly stimulated ST cells were assayed for production of TNF-α, TGF-β, IL-10, MIP-1α, MIP-1β, MIF and IL-8. A massive secretion of MIF was observed upon stimulation with iRBCST but not with uRBCs (figure 4.4A). Constitutive expression of TGF-β, MIF and IL-8 was observed but secretion of TGF-β (data not shown) and IL-8 did not change upon stimulation (figure 4.4B). No secretion of TNF-α, MIP-1α, MIP-1β, and IL-10 was observed (data not shown).

PBMC migrate toward iRBCST -stimulated ST cells

There was an increased migration of PBMCs toward iRBCST-stimulated ST cells (mean number of 116 cells/8 high power fields) compared to uRBC-stimulated (mean number of 68 cells/8 high power fields) or unstimulated ST (mean number of 45 cells/8 high power fields) (figure 4.5). Binding of iRBCST resulted in a 2.8 fold increase in migration of PBMCs compared to spontaneous migration while uRBC stimulated a 1.7 fold increase. Stimulation with LPS was used as a positive control and as expected, this resulted in a migration of PMBC towards stimulated ST (mean number of 140 cells /8 high power fields) (figure 4.5A) and a 3.4 fold increase of cell migration compared to unstimulated cells.

Binding of iRBC does not led to ICAM-1 expression by ST cells

Stimulation of ST cell with rhTNF-α and LPS resulted in the upregulation of ICAM-1 expression as assayed by western blotting (figure 4.6). However, exposure to iRBCST, uRBC, iRBC Ag or uRBC Ag did not lead to the upregulation of ICAM-1 expression (figure 4.6).

DISCUSSION

Parasite cytoadherence protects malaria parasites from splenic destruction (16), and is the basis of malaria pathogenesis both in cerebral malaria (27, 45) and PM (54). Results from this study demonstrate that the ST is capable of responding to the cytoadherence of malaria-infected RBCs as evidenced by the induction of MAPK pathways by the binding of $iRBC^{ST}$ and in response to malarial antigen. Secretion of immune factors such as MIF by ST was observed upon binding of $iRBC^{ST}$ and this stimulation led to the increase migration of PBMC towards $iRBC^{ST}$-stimulated ST cells. In this study, upregulated expression of ICAM-1 by ST cells was not observed upon stimulation.

The activation of MAPK pathways via phosphorylation is initiated by a large variety of external signals and leads to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis (reviewed in (55)). For example, activated ERK dimers can regulate targets in the cytosol and translocate to the nucleus where they phosphorylate a variety of transcription factors regulating gene expression (25, 33). JNK also translocates to the nucleus where it can regulate the activity of multiple transcription factors (24). This study demonstrates that the binding of $iRBC^{ST}$ to ST cells leads to the activation of JNK pathway as evidenced by its phosphorylation post stimulation. In a previous study, we demonstrated that the binding of $iRBC^{ST}$ to ST cells resulted in the tyrosine phosphorylation of at least two ST proteins (26). Several studies have shown that during cytoadherence host cell receptors not only provide points of attachment for the parasites but also play more dynamic roles that result in the modulation of cellular functions and/or induction of signal transduction (56). The JNK

pathway has been implicated in the gene expression and secretion of cytokines such as TNF-α . Its activation in $iRBC^{ST}$-stimulated ST suggests that iRBC binding to ST cells may lead to secretion of immune factors.

The ST is known to be immunoactive, secreting cytokines and chemokines important for the maintenance of pregnancy (22) and in response to bacterial infections and products like LPS (13, 15, 22) and (unpublished data). To determine if the observed signaling led to the secretion of any immune factors, the gene expression profiles and secretion of selected cytokines and chemokines were investigated. Such treatment led to a massive secretion of MIF by ST cells. Previously we demonstrated similar results with $BeWo^{ST}$ (9). The levels of MIF were shown to be very high in the intervillous blood plasma compared to peripheral blood plasma and this level increased in the presence of PM infection (11). The findings from this study suggest that direct binding of iRBCs can stimulate ST to secrete MIF. Previous findings suggested that MIF plays an important biologic role during pregnancy, although the exact role remains to be determined (23). MIF is known to be involved in macrophage activation (35) and retention as it inhibits macrophage migration. Therefore, MIF may play a role in the protection against any organism threatening to invade the villi and in the retention of maternal immune cells in the IVS observed in some case of PM.

$IRBC^{ST}$ binding to ST did not lead to the secretion of TNF-α, TGF-β, MIP-1α MIP-1β, IL-10 and IL-8 although there was a marginal increase in the mRNA levels of TNF-α, IL-8 and TGF-β. It is possible that the observed mRNA upregulation was not biologically relevant because it did not result in a concomitant translation of the proteins, likely due to post-transcriptional control measures. During PM, elevated levels TNF-α,

IL-10 (17) or (2, 32) have been demonstrated in the placenta. These inflammatory immune factors were shown to be produced largely by macrophages and T cells. Thus, this study suggests that the ST cells do not contribute to the increased levels of these cytokines in the placenta. However, other as yet unidentified immune mediators may be induced by this stimulation. The activation of JNK1 upon $iRBC^{ST}$ binding and chemotaxis studies strongly support this notion; $iRBC^{ST}$-stimulated ST mediated the migration of PBMCs as did LPS-stimulated ST cell, whereas this was not observed with uRBC-stimulated ST cells or unstimulated cells. Further experiments will be necessary to identify the chemotactic factors involved in the induction of PBMCs migration by $iRBC^{ST}$-stimulated ST.

The current study did not observe any upregulation of ICAM-1 expression upon $iRBC^{ST}$ binding. This was not surprising given that ICAM-1 expression is upregulated by proinflammatory cytokines such as TNF-α and IFN-γ, and we did not observe either TNF-α or IFN-γ secretion by ST cells upon the binding of $iRBC^{ST}$. It can be speculated that in vivo, the elevated levels of both TNF-α and IFN-γ in the placenta during PM may lead to ICAM-1 expression by ST cells. However, even immunohistochemistry assays for ICAM-1 expression using PM-positive placenta sections from Kenya (a malaria endemic region) did not show any upregulation of ICAM-1 (data not shown).

During schizont burst, many immunostimulatory malarial antigens are released that are capable of inducing cellular activation (5, 51, 52). In the placenta, where the mature late stages of the parasite are sequestered, these 'toxins' are capable of interacting with the ST. In this study, activation of ERK1/2 upon stimulation with crude malarial antigen was observed. This is indicative of a response by the ST cells to the presence of

malaria parasite in the placenta. This study did not identify the ERK1/2-stimulatory factor in the crude malaria preparation although it is hypothesized that this is mainly mediated by the GPIs of the parasite. Indeed, several studies have shown that malarial GPIs can induce cellular activation of macrophages and result in secretion of proinflammatory cytokines such as TNF-α (43). It is possible that the amount of malarial antigen used here was not sufficient to induce secretion of immune factors or perpharps malarial GPIs do not stimulate ST to secrete the selected immune factors assayed here. Further investigation using purified GPIs is required to clearly identify the role of GPIs in stimulation of ST.

In conclusion, this study provides evidence that the interaction of malarial parasites and components with ST cells in the placenta induces immunologic changes in the ST cells as evidenced by the activation of the MAPK pathways, secretion of MIF and PBMC chemotaxis. Thus, ST cells play an active immunological role in response to malaria parasites in the placenta and are capable of influencing the local maternal immune environment. Findings from this study also imply that immune factors that remain to be identified are being secreted by the ST cells upon $iRBC^{ST}$ binding. Ongoing studies are trying to elucidate this by use of cDNA microarrays to study more extensive gene expression changes that may be induced by the binding of iRBCs to ST.

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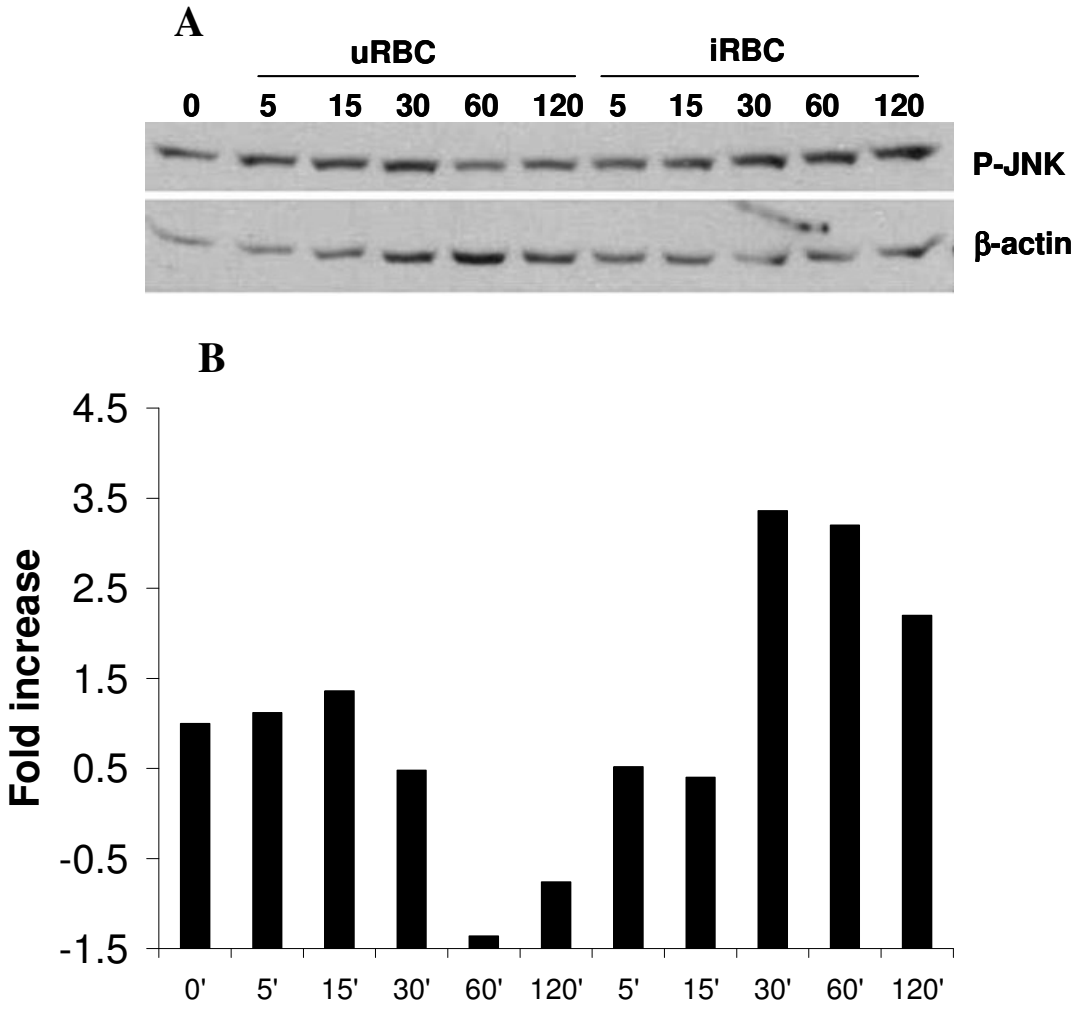
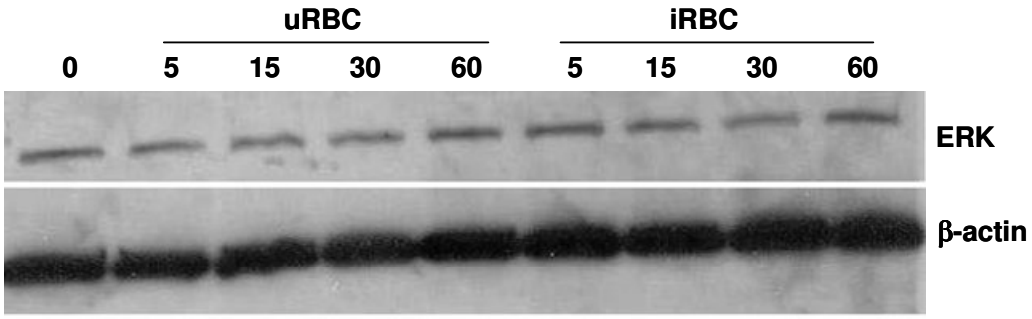
Figure 4.1

Activation of JNK but not ERK1 or p38 following iRBCST binding

ST cells were co-cultured with iRBCST or uRBC for the indicated time course or left unstimulated (0). Western blotting of cellular proteins from ST cells shows specific enhanced phosphorylation (3-fold) of JNK1 (p-JNK) after 30 minutes of co-culture with iRBCST which remained high even after 120 minutes (A and B). Co-culture with uRBCs did not lead to increased phosphorylation although there was a slight decline in the phosphorylation of JNK1 after 60 and 120 minutes. This treatment did not lead to changes in ERK1 (C), which was constitutively expressed. Activation of p38 was not detected even after stimulation (D). Data shown represent one of two comparable experiments.


A
uRBC
iRBC
0
5
15
30
60
120
5
15
30
60
120
P-JNK
β-actin
B
Fold increase
4.5
3.5
2.5
1.5
0.5
-0.5
-1.5
0'
5'
15'
30'
60'
120'
5'
15'
30'
60'
120'

C


uRBC
iRBC
0
5
15
30
60
5
15
30
60
ERK
β-actin

D

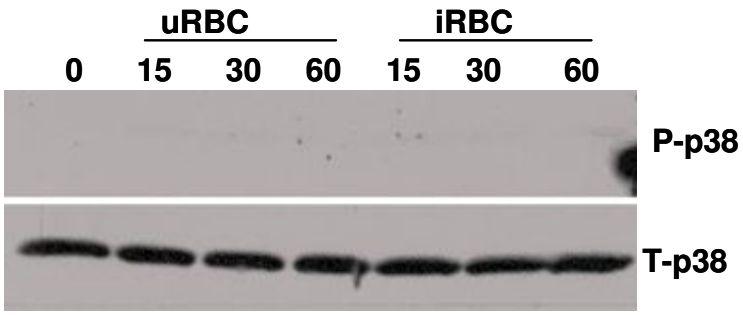

uRBC
iRBC
0
15
30
60
15
30
60
P-p38
T-p38

Figure 4.2:

Activation of ERK1/2 following stimulation with crude malaria antigen

ST cells were stimulated with 40ug/ml of crude malarial antigen or with uRBC antigen for the given time course. Western blotting of phosphorylated MAPK proteins was carried out. Cellular proteins from ST cells treated with crude malarial antigen show an early (5 minute post stimulation) enhanced phosphorylation of ERK1/2 (A). This activation was sustained through out the stimulation period. Stimulation with uRBC antigens resulted in a sight increase in ERK1/2 activation but this returned to baseline levels after 30 minutes (A). Figure B represents the densitometric analysis. This treatment did not lead to the phosphorylation of p38 nor JNK1/2 (not shown). Data represent one of two experiments.

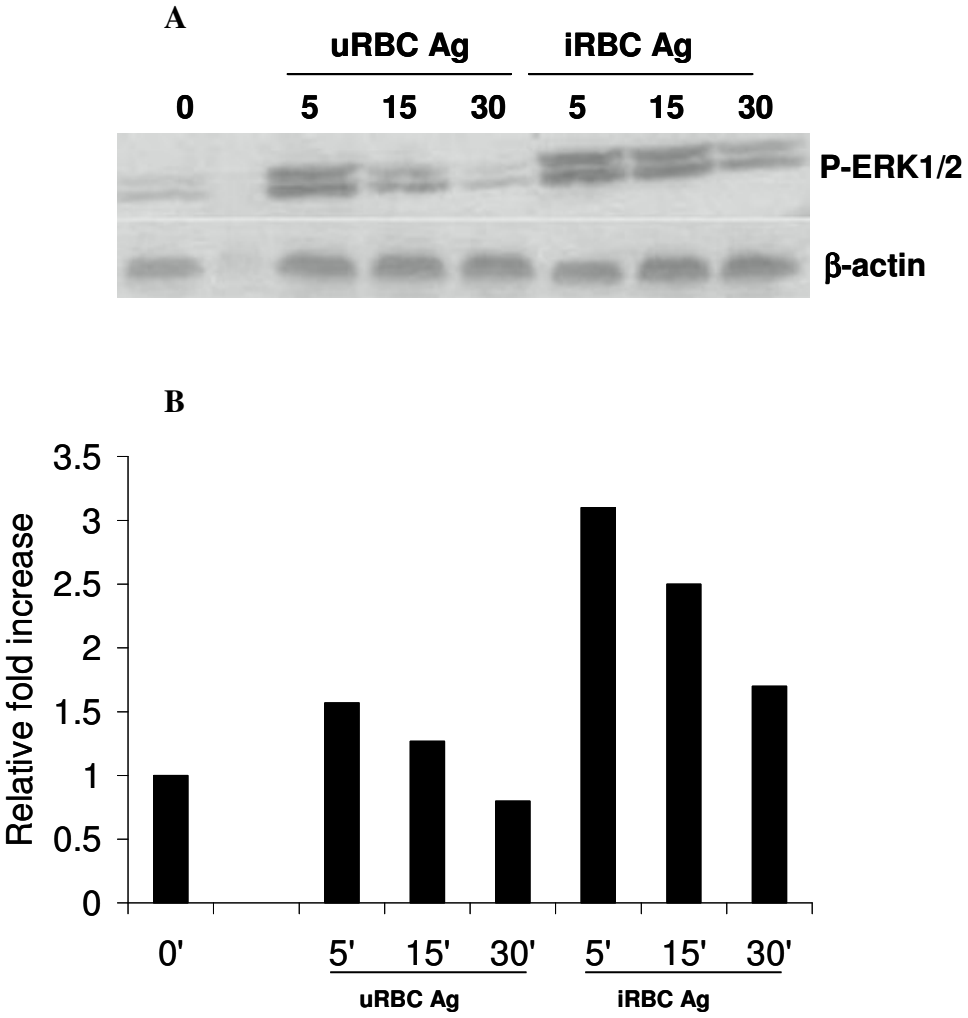

A
uRBC Ag
iRBC Ag
0
5
15
30
5
15
30
P-ERK1/2
β-actin
B
Relative fold increase
3.5
3
2.5
2
1.5
1
0.5
0
0'
5'
15'
30'
5'
15'
30'
uRBC Ag
iRBC Ag

Figure 4.3:

Gene expression changes in ST stimulated with iRBCST

ST cells were stimulated with either uRBC or iRBCST. RNA was extracted and gene expression changes assayed by real time PCR. This treatment of ST led to the marginal upregulation both TNF-α and TGF-β, which showed 1.8 and about 3 fold increases respectively at 2 hours post stimulation. There was a slight increase in the expression IL-8 at 2 hours stimulation which declined at 4 and 8 hours post stimulation and then increased at 12 hours. Both MIF and IL-10 mRNA did not change considerably with this treatment. Dotted line shows gene expression of unstimulated cells. Data shown represent one of 3 experiments.

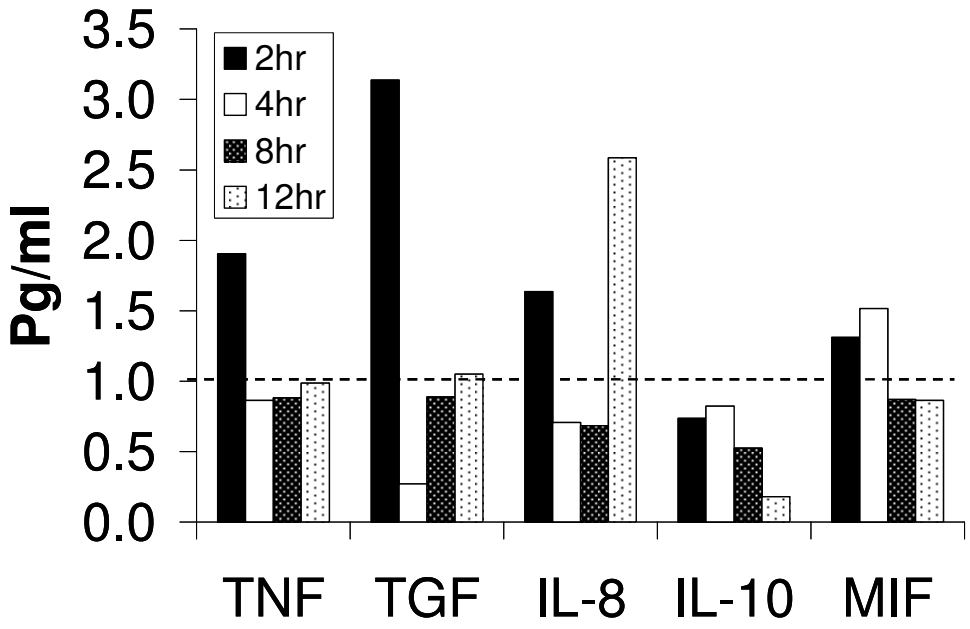
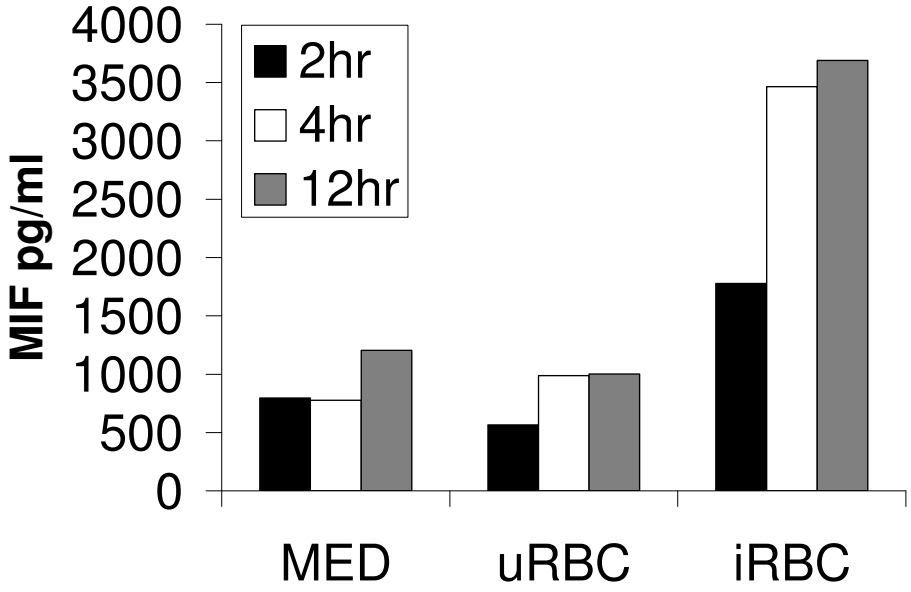

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pg/ml
2hr
4hr
8hr
12hr
TNF
TGF
IL-8
IL-10
MIF

Figure 4.4

Secretion of MIF and IL-8 by primary ST cells upon the binding of iRBCST

ST cells were stimulated with iRBCST (iRBC), uRBCs or left unstimulated (MED) for the time course shown in figure. (A) iRBCST binding led to increased secretion of MIF. Stimulation with uRBC did not lead to any increase in MIF secretion more than that observed for unstimulated ST (A). Results shown are one representative of two experiments. (B) There was a constitutive secretion of IL-8. Although IL-8 increased over time, iRBCST binding did not induce an additional increase compared to medium or uRBCs. Results shown are one representative of three experiments. No detectable amounts of TNF-α, TGF-β, MIP-1α, MIP-1β and IL-10 were observed (not shown). Stimulation with iRBC Ag or uRBC Ag did not increase the secretion of any of the tested cytokines and chemokines (not shown).

A


4000
3500
3000
2500
2000
1500
1000
500
0
MIF pg/ml
2hr
4hr
12hr
MED
uRBC
iRBC

B

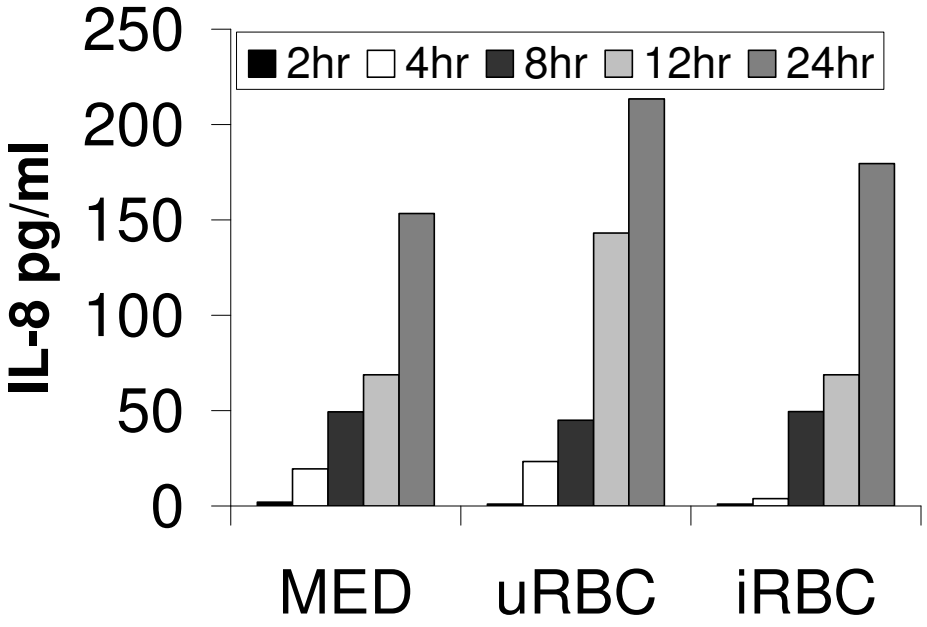
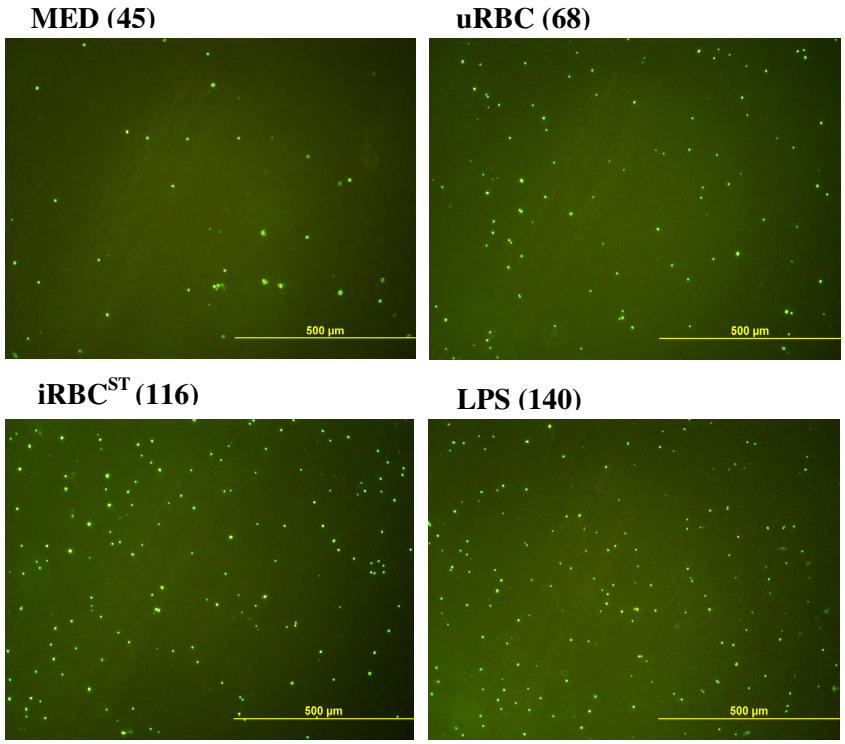

250
200
150
100
50
0
IL-8 pg/ml
2hr
4hr
8hr
12hr
24hr
MED
uRBC
iRBC

Figure 4.5

Migration of PBMC towards iRBCST -stimulated ST cells

ST cells were stimulated with iRBCST, uRBC, LPS or left unstimulated for 12 hours. 350µl of PBMCs (5 X 10^5 cells/ml) was seeded onto the 3-µm pore size cell culture inserts and the cells allowed to migrate for 12 hours. Migrated cells were stained with Calcein AM fluorescence stain and observed under an inverted fluorescence microscope. Migrated cells were quantitated by counting migrated cells in 8 random fields for each stimulants. (A) Panels are labeled with stimulant (mean migrated cells). (B) The mean of migrated cells was compared to cells migrated towards unstimulated ST (MED) and the fold increase of mean cells migrated upon stimulation over spontaneous migration of cell was plotted. There was an increase in the migration of PBMC towards iRBCST-stimulated cells, comparable to that observed with LPS but this was not observed with ST cells stimulated with uRBCs.

A


MED (45)
uRBC (68)
iRBC^ST (116)
LPS (140)
500 µm
500 µm
500 µm
500 µm

B

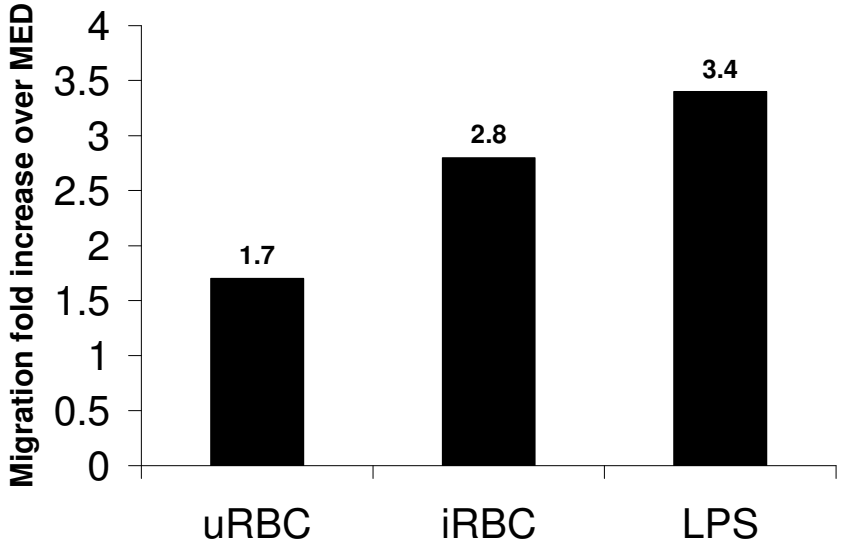

Migration fold increase over MED
4
3.5
3
2.5
2
1.5
1
0.5
0
1.7
2.8
3.4
uRBC
iRBC
LPS

Figure 4.6

The binding of iRBCST does not upregulate ICAM-1 expression by ST cells

ST cells were left unstimulated (lane 1) or co-culture with 10ng/ml of rhTNF–α (lane 2), 10μg/ml of LPS (lane 3) iRBC Ag (lane 4), uRBC Ag (lane 5), iRBCST (lane 6), or uRBCs (lane 7) for 24 hours. Whole cell lysates were prepared from cells and immunoblotting used to detect ICAM-1 protein. TNF-α and LPS led to 2.5 and 2.3 fold increases in the expression of the ICAM-1 protein, respectively, compared to unstimulated cells. The other stimuli did not lead to any changes in ICAM-1 expression. B-actin was used as a loading control.

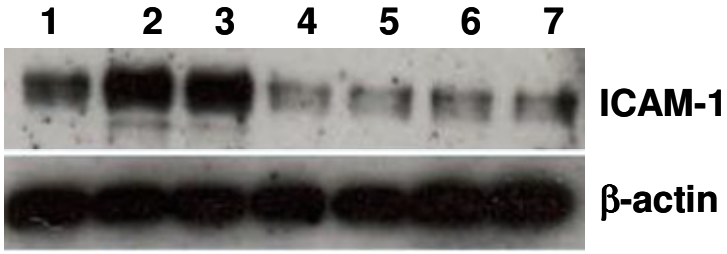

1
2
3
4
5
6
7
ICAM-1
β-actin

Table 4.1:

Oligonucleotide primers used in the amplification of the genes

Primer Name	**Sequence**
IL-8 Forward	5'-GCCAAGGAGTGCTAAAGAAC-3'
Il-8 Reverse	5'-TCCATCAGAAAGCTTTACA-3'
TNF-α Forward	5'- GAGCACTGAAAGCATGATCCG-3'
TNF-α Reverse	5'- AGCAGGCAGAAGAGCGTGGT-3'
MCP-1 Forward	5'-CAATCAATGCCCCAGTCACC-3'
MCP-1 Reverse	5'- GGAGTTTGGGTTTGCTTGTC-3
TGF-β Forward	5'-TACCAGAAATACAGCAACAAT-3'
TGF-β Reverse	5'-CTCCACGGCTCAACCACTG-3'
IFN-γ Forward	5'-GCATCGTTTTGGGTTCTCTTG-3'
IFN-γ Reverse	5'-TCCATTATCCGCTACATCTGAA-3'
IL-10 Forward	5'-GCACCCACTTCCCAGGCAA-3'
IL-10 Reverse	5'-GAAGGAATCATACTCACAAAGAAAG-3'
MIF Forward	5' CCACCGGCAAGCCCCCCCA -3'
MIF Reverse	5'-TGTAGGAGCGGTTCTGCG-3'
18 S Forward	5'-GTAACCCGTTGAACCCCATT-3'
18S Reverse	5'-CCATCCAATCGGTAGTAGCG-3'

CHAPTER 5

ACTIVATION OF SYNCYTIOTROPHOBLAST BY MALARIAL PIGMENT HEMOZOIN[1]

[1] Lucchi, NW and Moore JM. To be submitted to Infection and Immunity.

ABSTRACT:

Previous findings indicated that *Plasmodium falciparum* hemozoin (Hz), a parasite metabolite released during schizogeny, might be an important stimulator of proinflammatory mediators. Local proinflammatory responses in the placenta during malaria in pregnancy have been associated with the immunopathology related to malaria infection. This study investigated the secretion of chemokines and cytokines by ST cells upon Hz stimulation. Hz was found to stimulate the secretion of IL-8 from ST cells. This cellular response to Hz involved ERK1/2 phosphorylation since selective inhibition of the ERK1/2 pathway abolished IL-8 secretion. These findings provide a novel mechanism by which the ST cells responding to malaria parasite components such as Hz might modulate the immune response during placental malaria infection.

Key words: Hemozoin, syncytiotrophoblast, malaria, chemokines, IL-8, MAPK

INTRODUCTION

Regardless of the many years since its discovery, malaria continues to be a killer disease. In sub-Saharan Africa millions of deaths every year are attributable to this disease (1, 47). In malaria endemic regions the burden of the disease is shouldered by young children and pregnant women (32). The causative agents of malaria are protozoan parasites of the genus *Plasmodium*. The most common and most virulent of the four known species that are infectious to humans is *P. falciparum.* During its growth and replication in the host's red blood cells, the parasite utilizes hemoglobin as the major nutrient source (44), proteolytically degrading enormous amounts of hemoglobin in the process (38, 39). However, this also generates substantial amounts of free heme known to be toxic to the parasite (14, 50, 53). Heme detoxification is therefore a critical process for parasite survival. This is achieved by the polymerization of heme into an insoluble crystalline substance called malaria pigment or hemozoin (Hz) (44, 46). Crude unpurified Hz was found to be composed of many proteins of host and parasite origin, ferriprotoporphyrin-IX (hematin), and trace amounts of lipid (6, 15). This detoxification process is thought to be an enzymatic activity involving several proteins such as *P. falciparum* histidine-rich protein II (PfHRPII) (49) and nonphysiological lipids (7).

During malaria infections, Hz is visible microscopically, in stages that are actively degrading hemoglobin, such as trophozoites, schizonts, and gametocytes. It is present in large amounts in circulating phagocytic cells, monocytes and neutrophils (5, 43). Hz was believed to be an inert product that does not affect the underlying pathophysiological processes of malaria pathogenesis until the 1990s when ingestion of Hz by monocytes was shown to severely impair their functions (42). On the contrary,

other reports demonstrated that Hz was capable of inducing macrophage/monocyte chemokine and proinflammatory cytokine secretion (20, 22). It was shown to enhance the induction of macrophage nitric oxide (NO) synthesis by IFN-γ and to enhance the immune response of innate immune cells such as dendritic cells by promoting the production of cytokines, chemokines, and up-regulation of costimulatory molecules by these cells (10, 11). These findings suggested that Hz plays important roles in the modulation of the immune response during malaria infection.

Malaria infection during pregnancy results in the sequestration of infected red blood cells (iRBCs) in the intervillous space (IVS) of the placenta. Often this sequestration is accompanied by monocytic infiltration in the IVS (37) and malaria-associated placental pathology (8) resulting in a phenomenon referred to as placental malaria (PM). Placental histopathology during PM includes, among others, the presence of Hz both extracellularly in the IVS, deposited in fibrin, and in circulating maternal phagocytes (8, 31). Hz has been used as a biomarker of placental parasitization (30, 48) and its presence in the placenta has been used to categorize the intensity and longevity of PM infection (8); deposition of Hz in circulating cells was associated with active infections whereas Hz in fibrin was a feature of active-chronic infections (8). However, the potential immunological role of Hz in the placenta during PM has received little attention.

PM has been associated with maternal anemia and reduced birth weight (33); low birth weight (LBW) is a major determinant of infant mortality (29). At the interface between maternal blood and the fetal circulation in the placenta, is the syncytiotrophoblast (ST). In vivo, the ST is a continuous syncytium that lines the fetal

capillaries and mediates gaseous, nutrient and waste exchange. Both pro- and anti-inflammatory cytokines, in addition to interleukin 1 beta (IL-1β), IL-6, IL-8 and IL-10, can be produced by ST cells in response to pathogenic bacteria (16). Recently, we have demonstrated that in vitro, the binding of iRBCs to the ST cells induces tyrosine phosphorylation of ST proteins (27) and the secretion of macrophage migratory inhibitory factor (MIF) (9). It was shown that during PM, placental Hz load per se was not associated with poor fetal outcomes (30, 48). However, there is accumulating evidence indicating that apart from the parasite's virulence factors the host's immunological response, particularly proinflammatory cytokines and chemokines, participates in malaria pathophysiology (52). In PM, Hz-laden macrophages were shown to secrete proinflammatory cytokines and chemokines that were associated with placental monocyte recruitment (3, 35), a risk factor for LBW (37). Furthermore, Hz was shown to differentially regulate the secretion of proinflammatory cytokines by intervillous blood mononuclear cells (IVBMC) in HIV-1 seronegative and seropositive women in the context of PM (34). Thus, the immunologic impact of Hz in the placenta is potentially significant and needs to be further explored.

In this study, the ability of Hz to stimulate ST cells to secrete cytokines and chemokines was investigated. Possible involvement of the mitogen-activated protein kinase (MAPK) signaling pathway was also determined. Stimulation of ST cells with Hz resulted in the secretion of IL-8 in an ERK1/2-depedent manner.

MATERIALS AND METHODS

Isolation of human primary trophoblasts from placentas and cell culture

Term placentas were obtained from three women delivering by elective cesarean section at Athens Regional Health Center, Athens, after written informed consent was obtained. Primary placental CT cells were isolated from fresh human placentas and cultured as described previously (26). The immunopurified cells were used immediately or cryopreserved in liquid nitrogen until use. Primary CT cells ($1x10^6$) were matured to ST and then exposed to 50µg/ml of Hz (a kind gift from Dr. R. Bucala, Yale University) or left unexposed.

Protein lysate preparation and immunoblotting for MAPK proteins

Preparation of protein lysates was performed as previously described (26). Briefly, cells were scraped from plates and lysed on ice with 300 µl lysis buffer. Lysates were pelleted by centrifugation at 14000 x g and the pre-cleared lysates were collected and stored at -85^o C until needed. Protein assays were performed and 40 µg/lane of the lysates were separated on a 10% gradient sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membrane for 1 hour. Membranes were incubated at room temperature in blocking buffer (5% non-fat milk in Tris buffered saline with 0.05% Tween-20 (TBS-T) and then probed with primary antibodies against phosphorylated ERK1/2, JNK1/2 and p38 (Cell signaling Technology Inc., Beverly, MA) as recommended by the manufacturer. Final detection was performed with appropriate horseradish peroxidase-labeled (HRP) secondary antibodies (Sigma St. Louis, MO) in blocking buffer for 1 hour at room temperature. Phosphorylated proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL). Each membrane

was stripped with freshly prepared stripping buffer (2% SDS; 62.5 mM Tris–HCl, pH 6.7; 100 mM 2-mercaptoethanol) and reprobed in a similar fashion with antibodies against total (non-phosphorylated) forms of the relevant MAPK. The latter were used as loading controls for densitometric analysis performed using QuantityOne software (Bio-Rad, Hercules, CA).

Chemokine and Cytokine ELISA and Inhibition assays

ST cells were stimulated with 50μg/ml Hz over a 24h hour time-course. Supernatants were collected after 8, 12 and 24 hours and kept at -85^{o}C until used for ELISA. The cytokines assayed were, MIP-1α, MIP-1β, TNF-α, IL-10, TGF-β and IL-8. For the inhibition assays, the ST cells were first pretreated with 10uM of MEK1/2 inhibitor, UO126 (Cell signaling Technology, Inc. Beverly, MA) for two hours. The inhibitor was then washed off before stimulation of the cells with Hz. Standard sandwich ELISA was performed for IL-8 according to the manufacturer's protocol (R&D Systems, Inc. Minneapolis, MN). Percentage inhibition was calculated as follows; [(amount of secreted chemokine from cells stimulated with Hz - amount secreted from Hz-stimulated cells pre-incubated with inhibitor before stimulation)/ amount of secreted chemokine from cells stimulated with Hz)] *100.

Statistics

Student's t-test was used to compare mean secretion of the chemokines at the three time-points compared to secretion of unstimulated cells.

RESULTS

Secretion of IL-8 by ST cells upon Hz stimulation

A time-dependent secretion of IL-8 from the ST cells was observed when ST cells were exposed to 50μg/ml Hz (figure 5.1). A marginal constitutive secretion of IL-8 was observed which was upregulated upon stimulation. No secretion of MIP-1α, MIP-1β, TNF-α, TGF-β and IL-10 was detected (data not shown).

Activation of ERK1/2 by Hz

A 3-fold increase in the phosphorylation of ERK1/2 MAPK was observed when ST cells were exposed with Hz (figure 5.2A and B). This occurred at 5 minutes post stimulation and the activation appeared to return to baseline thereafter. Figure 5.2B demonstrates the densitometric analysis of the western blot. Phosphorylation of JNK1 appeared to be constitutive and not responsive to Hz stimulation, whereas phosphorylated forms of p38 were not detected (data not show).

IL-8 secretion is ERK1/2 Dependent

To examine the role of ERK1/2 phosphorylation in secretion of IL-8 from stimulated ST, cells were briefly exposed to MEK1/2 inhibitor before stimulation with Hz. MEK1/2 is found upstream of ERK1/2 and its role is to phosphorylated ERK1/2, leading to its activation. Therefore, inhibition of MEK1/2 inhibits the phosphorylation, and therefore, the activation of ERK1/2. Compared with cells not pretreated with inhibitor there was a 64% reduction in the production of IL-8 in pretreated cells (figure 5.3A). The concomitant reduction in ERK1/2 phosphorylation (figure 5.3A) confirms the importance of this MAPK in Hz-induced IL-8 secretion by ST.

DISCUSSION

The presence of Hz in circulating monocytes and neutrophils has been used as a marker for malaria severity (28, 36). In the placenta, it has been used to categorize malaria infection (8) and as a marker of placental histopathology during PM (8, 31). This study investigated the capacity of Hz to stimulate ST cells. Hz-stimulated ST cells were shown to secrete of IL-8 in an ERK1/2-dependent manner.

Several lines of evidence strongly suggest that Hz could play an important role in cytokine and chemokine production during malaria infection. In vitro studies showed that both Hz and synthetic Hz (beta-hematin) induce the release of various proinflammatory mediators, including IL-1β, TNF-α (40) and beta chemokines such as MIP-1α and MIP-1β (20, 22, 45). Hz was also shown to potently enhance IFN-γ mediated inducible nitric oxide synthase (iNOS) mRNA and protein expression in macrophages leading to significant increases in nitric oxide (NO) production (21), as well as adhesion molecule expression and IL-6 production in human endothelial cells (51). Various signaling pathways are associated with cytokine or chemokine production by cells. Among these are the MAPK pathways. In humans, three main MAPK pathways have been well studied: the extracellular signal-regulated kinase-1/2 (ERK1/2), p38 and c-Jun N-terminal kinase1/2 (JNK1/2) (reviewed in(54)). The ERK1/2 pathway, together with NF-kappaB activation, have been implicated in the activation of murine macrophages with Hz. Hz was shown to: a) synergistically enhance the IFN-γ–inducible NO generation from macrophages via the ERK1/2- and NF-kβ– dependent pathways (21) and b) to enhance macrophage chemokine mRNA expression levels via similar pathways.

Due to their purported similarities with macrophages (2, 17), we hypothesized that exposure of ST cells to Hz might activate the MAPK pathway. Stimulation of ST cells with Hz resulted in enhanced phosphorylation of ERK1/2. That inhibition of this signaling pathway led to reduced secretion of IL-8 confirms a functional link between this pathway and IL-8 secretion in term ST cells. However, other pathways may be involved since ERK1/2 inhibition did not lead to complete abrogation of IL-8 secretion.

Signaling via the TLRs results in the activation of the MAPK pathway (4, 19, 24). So far, studies show that in murine spleen and dendritic cells Hz signals through the TLR9 to lead to the production of cytokines (10). Studies have reported mRNA expression of TLR-1 through TLR-10 (23, 55), as well as protein expression of TLR-2, TLR-4 and TLR-9, in term placenta (18). Therefore, it is possible that these molecules are involved in this activation. The cellular location of TLR9 on ST cells is still not clear. Some studies have shown that TLR9 has an intracellular location (25) while other have demonstrated its surface expression on peripheral blood mononuclear cells (12). If TLR9 were indeed the ligand mediating Hz activation of ST cells, it would have to be expressed on the surface since we did not observe any phagocytosis of Hz by the ST cells (unpublished data). We are currently conducting further investigations to show if indeed TLR9 is involved in this signaling.

During PM, the ST cells are continuously exposed to malarial components such as Hz and malarial antigen due to the sequestration of the iRBCs in the placenta. PM is associated with poor pregnancy outcomes, particularly LBW which is a known risk factor for neonatal mortality (29). Previous studies indicated that placental Hz loads per se did not correlate with these poor pregnancy outcomes (30, 48). However, chemokine and

cytokine production have been associated with the immunopathology related to PM (3, 35). Although proinflammatory cytokine and chemokine production during PM are well documented, the potential immunological role of Hz in the placenta during PM as it pertains to ST cells stimulation had remained largely unknown.

The current study demonstrates for the first time that fetal ST cells respond to stimulation with Hz by producing IL-8. In recent studies, elevated levels of IL-8 were demonstrated in the IVS of malaria-infected placentas; it was suggested this was produced by the infiltrating Hz-laden macrophages (3). This study shows that in addition to macrophages, ST cells are capable of contributing to the observed elevation of IL-8 in IVS during PM. IL-8 has been associated with recruitment of neutrophils to site of infection. In addition, studies have demonstrated that IL-8 may play a role in monocyte adhesion and activation (13). Due to their ability to recruit and induce activation of specific leukocyte subsets to the sites of infection, chemokines, such as IL-8, influence the immune system, thereby further inducing inflammatory conditions (41). In PM monocyte infiltration is a common feature and is associated with LBW (3, 35). The elevated levels of proinflammatory chemokines in the IVS observed during PM contribute to this infiltration and propagation of inflammatory responses. This study demonstrates that the ST also plays a role in this amplification by the production of IL-8 upon interaction with Hz.

In summary, this study demonstrates that in response to Hz, ST cells produce IL-8 in an ERK1/2 dependent manner. Elevated levels of IL-8 have been observed in the IVS during PM, and from this study, it is clear that the ST cells can contribute to these high levels of IL-8. Therefore, even in PM, as in the case of intrauterine bacterial infections,

the ST is an active immunological player and may be playing an important role in shaping the local immune milieu during PM.

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Figure 5.1:

Secretion of IL-8 by ST cells upon stimulation with Hz

ST cells were exposed to either 50μg/ml Hz or left unexposed over a 24-hour time course. Cell culture supernatants were collected and assayed for cytokine and chemokine protein by ELISA at time points shown. Compared to the unexposed ST, there was a time-dependent increase in the secretion of IL-8. These mean data are from three separate experiments (mean +/- SD). MIP-1α, MIP-1β, TNF-α, TGF-β and IL-10 were not detected (data not shown). * Compared to secretion by unexposed cells, $P<0.05$.

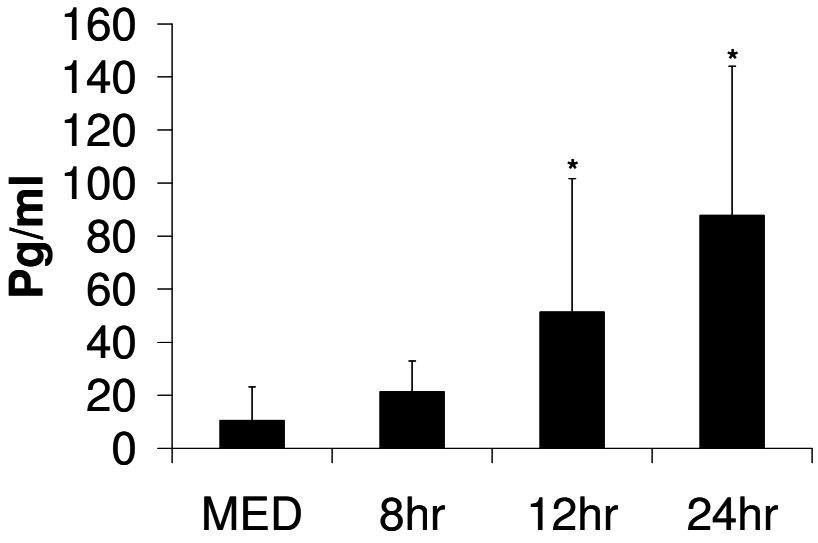

160
140
120
100
80
60
40
20
0
Pg/ml
*
*
MED
8hr
12hr
24hr

Figure 5.2:

Stimulation of ST with hemozoin results in an enhanced phosphorylation of ERK1/2

Western blotting of phosphorylated cellular proteins from ST cells treated with 50ug/ml of Hz for the indicated time course or left unstimulated (0) shows specific enhanced phosphorylation of ERK1/2 proteins which peaked at 5 minutes post stimulation (A and B). Figure B show the densitometric results of the western blotting for ERK1/2. There was no change in phosphorylation of JNK1/2, and phosphorylated p38 was not detected at all (data not shown). Data shown are from one representative experiment of two. P-ERK1/2 is phosphorylated ERK1/2 proteins and T-ERK1/2 is the unphosphorylated total ERK1/2 protein.

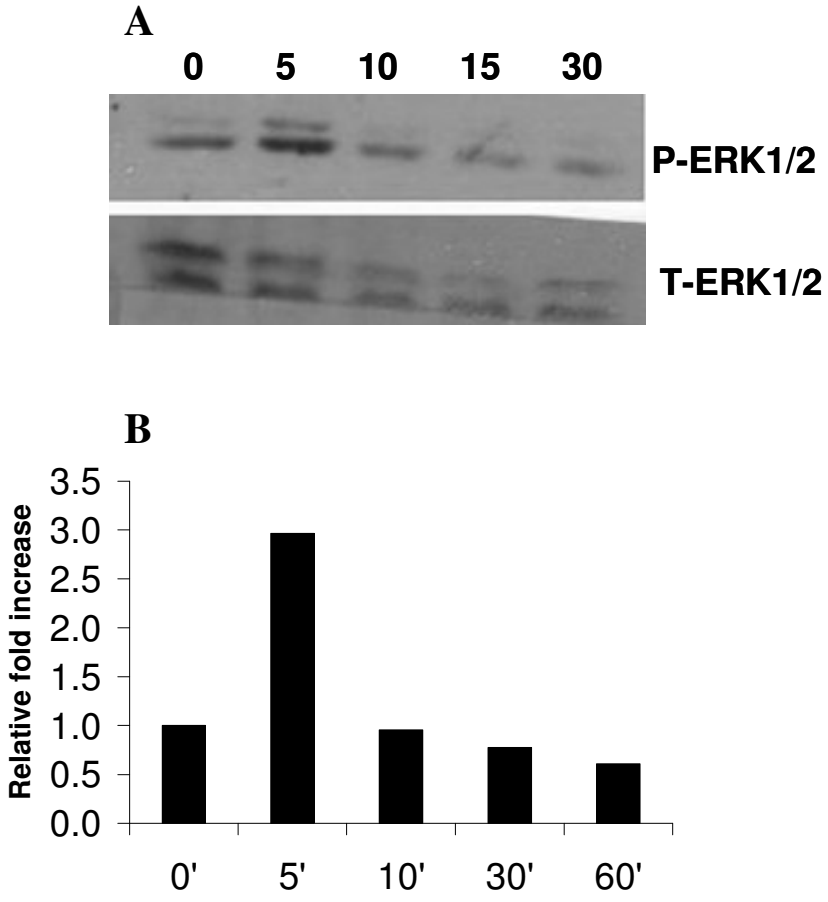
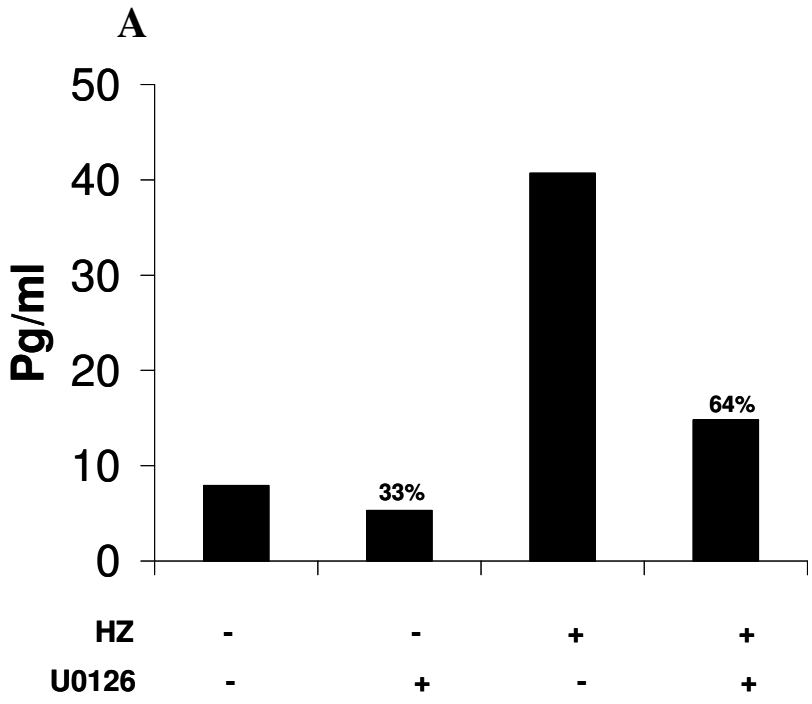

A
0
5
10
15
30
P-ERK1/2
T-ERK1/2
B
Relative fold increase
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0'
5'
10'
30'
60'

Figure 5.3:

Production of IL-8 by ST cells is ERK1/2 dependent

ST cells were pretreated with 10uM MEK1/2 inhibitor (U0126) for two hours, washed, and then stimulated with 5μg/ml Hz or left unstimulated for 8 hours. Supernatants were collected and assayed for the secretion of IL-8 using standard ELISA technique. Pretreatment with inhibitor led to a 64% inhibition of IL-8 secretion by the ST cells (A) and a concomitant reduction in the activation of ERK1/2 (B). Data represent one of two experiments.


A
50
40
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Pg/ml
33%
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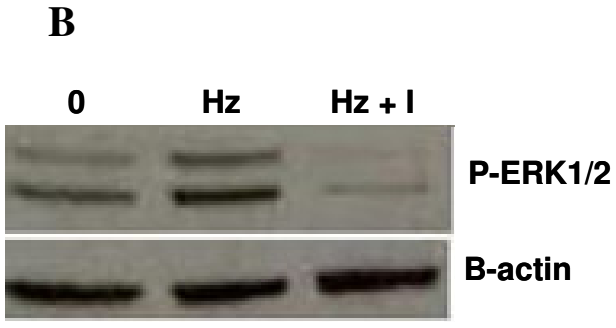
B
0
Hz
Hz + I
P-ERK1/2
B-actin

CHAPTER 6

LPS INDUCES SECRETION OF CHEMOKINES BY HUMAN SYNCYTIOTROPHOBLAST CELLS IN A MAPK-DEPENDENT MANNER.[1]

[1] Lucchi, NW and Moore, JM. Submitted to Journal of Reproductive Immunology, 3/15/06.

ABSTRACT

The maintenance of pregnancy depends on the nature and magnitude of the immune responses induced within the placenta. An elevated proinflammatory response in the intervillous space (IVS) is associated with adverse pregnancy outcomes. It is becoming more apparent that the syncytiotrophoblast (ST) cells, which are in direct contact with maternal blood, are capable of contributing to the local immune environment in response to maternal hematogenous infections or exposure to proinflammatory stimuli. In this study, we investigated mechanisms by which ST might recruit maternal immune effectors to IVS in response to bacterial infections. To assess this, primary ST cells were isolated from fresh term placentas and stimulated with lipopolysaccharide (LPS). LPS induced time-dependent expression and secretion of TNF-α, IL-8, MIP-1α and MIP-β from ST cells and an upregulation of ICAM-1. The stimulation also resulted in the activation of ERK1/2 mitogen-activated protein kinase (MAPK) but not p38 or JNK1/2. Inhibition of ERK1/2 lead to a reduction in the secretion of MIP-1β and IL-8 suggesting that their production is at least partly dependent on ERK1/2 activation. Results from this study reveal a potential mechanism by which differentiated ST cells modulate the local maternal immune responses during an intrauterine bacterial infection. Such responses could contribute to the clearance of the infection but also pathological features observed in intrauterine infections of the placenta.

Key Words: LPS, syncytiotrophoblast, placenta, ERK1/2, activation

1. INTRODUCTION

In the human placenta, the syncytiotrophoblast cells (ST) are in direct contact with maternal blood and can serve several roles: immunological, nutritional and endocrinological. Several studies indicate that the trophoblast cell is a pregnancy-specific component of the innate immune system (Abrahams and Mor, 2005, Guilbert et al., 1993, Guleria and Pollard, 2000), playing a key role in infection-associated changes in placental cytokine and chemokine expression. Bacterial infections in the placenta can result in adverse pregnancy outcomes due to the immunoactive nature of the bacterial lipopolysaccharide (LPS), which leads to a proinflammatory state in the fetoplacental unit. Recently, LPS was shown to lead to the secretion by ST cells of pro-inflammatory cytokines (Holmlund et al., 2002, Ma et al., 2006, Shimoya et al., 1999). In addition to cytokines, chemokines have also been shown to be potent proinflammatory mediators induced by LPS in macrophages (Fernandez et al., 2002, Harrison et al., 2005, Wang et al., 2000) and first trimester trophoblasts (Abrahams et al., 2005). Of interest are the beta chemokines, macrophage inflammatory protein (MIP)-1α and MIP-1β, which play major roles in the recruitment of leukocytes to sites of infection. In addition, these chemokines also activate these immune cells, resulting in an enhanced local inflammatory response (Luster, 2002, Taub, 1996). In the placenta, the accumulation of maternal immune cells in the intervillous space (IVS) is commonly observed during bacterial infections (Benirschke and Kaufmann, 2001) and is associated with increased risk of perinatal morbidity and mortality such as low birth weight, pre-term delivery and intrauterine growth retardation (Kim et al., 2005), reviewed in (Dietl, 2000). The secretion of IL-8, a chemokine that attracts neutrophils, and, to a lesser degree, monocytes, by ST cells in

response to LPS stimulation has been demonstrated (Holmlund et al., 2002, Ma et al., 2006, Shimoya et al., 1999). However, it is not known if this same stimulation leads to the production of MIP-1α and MIP-1β, chemokines that could further contribute to the intervillous inflammatory infiltrates associated with placental bacterial infections.

Another proinflammatory mediator demonstrated in the placenta during inflammatory responses is the adhesion molecule intracellular cell adhesion molecule -1 (ICAM-1; CD54). This is an inducible cell surface glycoprotein expressed at a low level on a subpopulation of hematopoietic cells, vascular endothelium, fibroblasts, and certain epithelial cells. However, its expression is dramatically increased at sites of inflammation, providing important means of regulating cell-cell interactions and thereby the cellular recruitment required for inflammatory responses (Rothlein et al., 1991, Rothlein and Wegner, 1992). ICAM-1 binds to the integrin leukocyte function antigen-1 (LFA-1), which is found on all leukocytes, including neutrophils and monocytes (Min et al., 2005). The upregulation of ICAM-1 expression on the ST cells during various infections such as toxoplasmosis (Chan et al., 2004) or placental villitis of unknown origin (Labarrere et al., 2005) and by proinflammatory cytokines (Chan and Guilbert, 2005, Xiao et al., 1997) has been demonstrated. More importantly, the binding of activated monocytes to ICAM-1 expressed by the ST was shown to induce trophoblast apoptosis, which could initiate placental villitis (Chan and Guilbert, 2005). These results suggest that elevated expression of ICAM-1 by villous trophoblast occurs during inflammatory reactions, and aberrant expression of this molecule may be an important pathological feature in inflammatory disorders of the placenta characterized by an

excessive accumulation of leukocytes in the IVS. Thus it is important to assess whether LPS stimulation leads to an increased expression of ICAM-1 by ST cells.

The innate immune recognition of bacterial products is mediated by a family of transmembrane receptors known as Toll-like receptors (TLRs). Messenger RNA derived from all of the known TLR genes have been observed in the placenta (Klaffenbach et al., 2005, Zarember and Godowski, 2002). Furthermore, the presence of functional TLR-2 and TLR-4 in the ST cells has been confirmed (Holmlund et al., 2002). The functionality of TLR-4 was demonstrated when stimulation of first trimester trophoblasts with LPS led to the activation of extracellular signal-regulated kinase (ERK) and c-jun NH_2-terminal kinase (JNK) mitogen activated protein kinase (MAPK) (Klaffenbach et al., 2005). However, it is not known if LPS also activates this same pathway in fully differentiated ST cells derived from term placenta.

In this study, stimulation of term primary ST cells with LPS was found to result in the elaboration of TNF-α, IL-8, MIP-1α and MIP-1β and enhancement of ICAM-1 expression. These functional changes were shown to be dependent on ERK1/2 activation as inhibition of this activation pathway resulted in a partial reduction of IL-8 and MIP-1β secretion.

2. MATERIALS AND METHODS

2.1. Isolation of human primary trophoblasts from placentas and cell culture

Term placentas were obtained from three women delivering by elective cesarean section at Athens Regional Health Center, Athens, after written informed consent was obtained. Primary placental cytotrophoblast cells were isolated as described previously (Lucchi et

al., 2005, Yui et al., 1994). Briefly, the chorionic villous tissue was removed, minced and subjected to four rounds of 30-minute enzymatic digestion using trypsin and DNase I (Sigma, St. Louis, MO). The released cells were then immunopurified using mouse-anti human CD9 antibody (Pharmingen, San Diego, CA) (which binds all cells except the trophoblasts) and anti-major histocompatibility complex (MHC) class 1 antibodies (W6/32, American Type Culture Collection, Manassas, VA; antibodies purified from murine ascites) over a goat anti-mouse glass bead column. Primary cytotrophoblasts were cultured as described (Lucchi et al., 2005). Briefly, cells were cultured in Iscove's Modified Dulbecco's medium (IMDM, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 10 ng/ml recombinant human epidermal growth factor (rhEGF; Sigma St. Louis, MO), 100 units/ml of penicillin and 100 µg/ml streptomycin (Invitrogen Corporation, Carlsbad, CA). Cells were plated at a 1 x 10^6 cells/ml density and placed in a 37^{o}C incubator with an atmosphere of 5% CO_2. The medium was changed every day for five days after which the cells were maintained in EGF-free medium. The purity of the ST cells was determined by staining with anti-vimentin monoclonal antibodies (Sigma, clone #V9) to ascertain that there was no contamination with fibroblasts or other vimentin-positive cells. Experiments were performed on day 8-10 old ST cells (Maubert et al., 1998, Chan and Guilbert, 2005).

2.2. Protein lysate preparation and immunoblotting for MAPK proteins

The preparation of the protein lysates was performed as previously described (Lucchi et al., 2005). Briefly, 1x10^6 primary cytotrophoblast were matured to ST and then stimulated with 10µg/ml of LPS (from *E. coli* 055:B5; Sigma St. Louis, MO) for a given

time-course or left unstimulated. Cells were scraped from plates and lysed on ice with 300 µl lysis buffer. Lysates were pelleted by centrifugation at 14000 x g and the pre-cleared lysates were collected and stored at -85^{o} C until needed. Protein assay was performed and 40 µg/lane of the lysates were separated on a 10% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane for 1 hour. Membranes were incubated at room temperature in blocking buffer (5% non-fat milk in Tris buffered saline with 0.05% Tween-20 (TBS-T) and then probed with primary antibodies against phosphorylated ERK1/2, JNK1/2 and p38 (Cell signaling Technology Inc., Beverly, MA) as recommended by the manufacturer. Final detection was performed with appropriate horseradish peroxidase-labelled (HRP) secondary antibodies (Sigma St. Louis, MO) in blocking buffer for 1 hour at room temperature. Phosphorylated proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL). Each membrane was stripped with freshly prepared stripping buffer (2% SDS; 62.5 mM Tris–HCl, pH 6.7; 100 mM 2-mercaptoethanol) and reprobed in a similar fashion with antibodies against total (non-phosphorylated) forms of the relevant MAPK. The latter were used as loading controls for densitometric analysis performed using QuantityOne software (Bio-Rad, Hercules, CA). For ICAM-1 experiments, the ST cells were stimulated with 10ng/ml of recombinant human tumor necrosis factor alpha (rhTNF-α ; BD Biosciences, San Jose, CA) or LPS. 20ug/lane of total protein was separated on a 10% gradient SDS-PAGE. Detection was carried out using anti-ICAM-1 monoclonal antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) with the necessary secondary antibody. The

membranes were stripped as described above and reprobed with anti-β-actin antibodies (Sigma, St. Louis, MO) to allow densitometric analysis.

2.3. Real-time reverse-transcription PCR

Total RNA was isolated from trophoblast cells using the RNeasy Qiagen kit (Qiagen, Valencia, CA) following the manufacturer's protocol and stored at –85^{o}C. Contaminating genomic DNA (gDNA) was digested using RNAse-free DNase (Ambion Inc. Austin, TX) as recommended by the manufacturer. First strand cDNA was synthesized from 1ug of obtained total RNA using the Omniscript reverse transcription kit (Qiagen, Valencia, CA). Real-time PCR was carried out using specific primers for MIP-1α (CCL3; # Hs 75703), MIP-1β (CCL4; # Hs 51407; Superarray Bioscience Corporation, Frederick, MD), IL-8 (forward: 5'-gccaaggagtgctaaagaac-3' and reverse: 5'-tccatcagaaagctttaca-3') and 18s RNA (forward: 5'-gtaacccgttgaaccccatt-3' and reverse: 5'-ccatccaatcggtagtagcg-3'; from MWG-Biotech Inc., High Point, NC). All the primers used were first validated for use in comparative real- time PCR. Real-time PCR was performed using the Mx3000P thermocycler and program (Stratagene, Cedar Creek, TX). No template controls and no reverse transcription controls were included. The 2^ΔΔ CT method of analysis was used with the 18S RNA gene as normalizing gene and unstimulated control cell RNA as the calibrator. Results are given as fold increase over unstimulated cells.

2.4. Chemokine ELISA and Inhibition assays

ST cells were stimulated with 10μg/ml LPS (from *E. coli* 055:B5; Sigma St. Louis, MO) over a 12h hour time-course. Supernatants were collected after 4, 8 and 12 hours and kept at -85^{o}C until used for ELISA. For the inhibition assays, the ST cells were first pretreated with 10uM of MEK1/2 inhibitor, UO126 (Cell signaling Technology, Inc. Beverly, MA)

for two hours. The inhibitor was then washed off before stimulation of the cells with LPS. Standard sandwich ELISA was performed according to the manufacturer's protocol (R&D Systems, Inc. Minneapolis, MN) for MIP-1α, MIP-1β and IL-8 chemokines. Percentage inhibition was calculated as follows; [(amount of secreted chemokine from cells stimulated with LPS - amount secreted from LPS-stimulated cells pre-incubated with inhibitor before stimulation)/ amount of secreted chemokine from cells stimulated with LPS)] *100.

2.5. Statistics

Student's t-test was used to compare secretion of the chemokines at the three time-points compared to secretion of unstimulated cells.

3. RESULTS

3.1. Upregulation of IL-8, MIP-1 α and β gene expression and protein secretion in LPS-stimulated ST cells.

Following in vitro stimulation of term ST with LPS, an increase in the mRNA expression of IL-8, MIP-1 α and MIP-1β, relative to unstimulated cells was observed (figure 1A). All the chemokine genes were upregulated within 2 hours of stimulation. Il-8 and MIP-1α decreased slightly thereafter whereas MIP-1β peaked at 4 hours then declined at 8 hours. Supernatants from similarly stimulated ST cells were assayed for IL-8, MIP-1 α, MIP-1β and TNF-α proteins. All these chemokines were secreted in a time-dependent manner from the ST upon exposure to LPS (figure 1B). Unstimulated ST cells did not secret detectable levels of TNF-α (figure 1B).

3.2. Induction of MAPK phosphorylation by LPS stimulation

To assess which signaling pathway was involved in the ST response to LPS stimulation, phosphorylated MAPK in ST cells was detected by western blotting. An early activation of ERK1/2 as evidenced by a transient 9-fold increase in its phosphorylation 5 minutes after addition of LPS was observed (figure 2A and B) which decreased to a 4-fold increase at 15 minutes post-stimulation and declined thereafter. LPS treatment of ST cells did not lead to major changes in JNK1/2 which was constitutively phosphorylated (figure 2C and D). In the experiment shown, there was a slight reduction in JNK1/2 phosphorylation levels at 15 and 30 minutes, but this was not consistently observed (not shown). Phosphorylated forms of p38 MAPK were not detected, although the total protein was easily detected (data not shown). There was an apparent preference for the phosphorylation of one of the isoforms of both ERK and JNK. In particular, ERK2 was more heavily activated than ERK1 following LPS stimulation. Densitometric analysis revealed a 2.5 fold increase for ERK1 and a 6.5 fold increase for ERK2 at 5 minutes post-stimulation with LPS. JNK1 was constitutively more phosphorylated than JNK2.

3.3. ERK1/2-dependent secretion of MIP-1β and IL-8

Because stimulation of ST cells led to the secretion of several inflammatory factors as well as phosphorylation of ERK1/2, the importance of this signaling pathway in the functional activation of ST was investigated. ST cells were pretreated with an inhibitor to MEK1/2, which is upstream of ERK1/2, for two hours before stimulation with LPS. This pretreatment led to a reduction in the secretion of IL-8 and MIP-1β compared to untreated cells (figure 3A). In three separate experiments, the percentage inhibition for the secretion of these cytokines ranged from 21% to 59% for IL-8 and 12% to 48% for

MIP-1β after 8 hours stimulation and 15% to 59% for IL-8 and 25% to 94% for MIP-1β after 12 hours. The expected reduction in phosphorylation of ERK1/2 was confirmed by western blotting (figure 3B).

3.4. Induction of ICAM-1 expression by ST upon LPS stimulation

To investigate the impact of LPS treatment on expression of ICAM-1 by ST cells, the cells were stimulated for 24 hours with LPS or recombinant human TNF-α (rhTNF-α). As expected, the latter resulted in the upregulation of ICAM-1 expression by the ST cells (figure 4). LPS treatment also strongly stimulated the expression of ICAM-1 expression to a level comparable to that induced by rhTNF-α (figure 4).

4. DISCUSSION:

Intrauterine bacterial infections and parasitic infections such as malaria during pregnancy can be accompanied by an intense infiltration of maternal immune cells, including monocytes and macrophages, into the placental IVS (Labarrere and Faulk, 1995, Beeson and Duffy, 2005). The expression of pro-inflammatory chemokines and cytokines within the IVS has been found in association with this cellular infiltration (Abrahams et al., 2004 (a), Abrahams et al., 2005, Abrams et al., 2003, Chan and Guilbert, 2005). However, elevated production of these immune factors (Klaffenbach et al., 2005, Zarember and Godowski, 2002) and aberrant distribution of maternal immune cells (Abrahams et al., 2004 (b), Wilczynski et al., 2003) at the maternal-fetal interface have been associated with increased risk of perinatal morbidity and mortality such as low birth weight babies, pre-term deliveries and intrauterine growth retardation (reviewed in (Dietl, 2000).The fact that the mRNA of at least 10 TLRs has been demonstrated in the placenta and the

protein expression of functional TLR-4 and TLR-2 is found in term ST cells (Holmlund et al., 2002) suggests that the ST cells are poised to respond to invading microorganisms. Recently, this potential immuno-active role of ST cells during intrauterine infections has become more apparent (Abrahams and Mor, 2005, Lucchi et al., 2005, Mor et al., 2005).

Chemokines mediate the migration of leukocytes to sites of inflammation and are important in the recruitment and activation of immune cells at the site of injury or infection. Since LPS has been shown to be a potent inducer of MIP-1α and MIP-1β in monocytes (Fernandez et al., 2002, Harrison et al., 2005, Wang et al., 2000) the expression of these chemokines in differentiated term ST stimulated with LPS was investigated in this study. An upregulation of mRNA levels of IL-8, MIP-1α and MIP-β was observed. This also led to a time-dependent secretion of these chemokines. Expression and secretion of IL-8 by term ST cells was previously demonstrated (Holmlund et al., 2002, Ma et al., 2006, Shimoya et al., 1999). The observation that LPS-stimulated ST cells produced elevated levels of IL-8, MIP-1α and MIP-β, all known to be chemotactic for leukocyte cells, suggests that in response to microbial infections, ST cells may promote the migration of maternal leukocytes to the intervillous space and in so doing contribute to the clearance of the microorganisms but also pathogenesis through leukocyte recruitment and activation. In a recent study, stimulation of first trimester trophoblast cells with LPS led to the secretion of IL-8 and MCP-1 which were capable of recruiting immune cells in *in vitro* chemotactic assays (Abrahams et al., 2005). Therefore, the production of chemokines by ST cells exposed to LPS may contribute to the pathologies, for example, placental villitis and preeclampsia, which are observed in the placenta during intrauterine infections.

The expression of ICAM-1 by villous trophoblast cells was previously shown to be elevated upon treatment with inflammatory cytokines such as TNF-α, interferon-γ, and interleukin-1α, and was suggested to occur only during an immune inflammatory reaction (Shrikant et al., 1994, Maubert et al., 1998, Xiao et al., 1997). Elevated expression of this adhesion molecule was associated with preterm labor and delivery, especially in the context of increased leukocyte infiltration (Marvin et al., 1999). In this study we demonstrate that stimulation of ST cells with LPS also results in an enhanced expression of the ICAM-1 protein. The upregulation of ICAM-1 was not surprising since this same treatment led to the secretion of TNF. Induction of both the secretion of chemokines and cytokines and upregulation of ICAM-1 on ST cells is a potentially undesirable combination for pregnancy outcome. For example, binding of activated monocytes to ST was shown to result in ST apoptosis (Chan and Guilbert, 2005). Therefore, these results suggest that the ST cells may play a more important role in the induction of immunopathologies and adverse pregnancy outcomes associated with intrauterine infections than was previously thought (Mor et al., 2005).

Signaling via the TLRs results in the activation of the MAPK pathway (An et al., 2002, Iankov et al., 2004, Kojima et al., 2004). It was hypothesized that this same pathway is evoked in ST cells by LPS stimulation and indeed, there was a very early increased phosphorylation of ERK1/2 MAPK but not p38 or JNK1/2 MAPK. In contrast to the JAR trophoblast cell line in which p38 is activated by LPS (Klaffenbach et al., 2005), primary ST cells did not activated this MAPK. This could be related to functional differences between primary and immortalized cells. In fact, in comparative functional studies using the trophoblast cell-line BeWo and primary ST cells, we have observed profound

differences between these cells in responses to malarial parasites (N. Lucchi et al., unpublished data). The observations reported here suggest that ligation of TLR-4 by LPS leads to the activation of ERK1/2 in term ST cells. There appears to be a preferential activation of ERK2 isoform compared to ERK1 in response to LPS and for constitutively higher phosphorylation of JNK1 compared to JNK2. There is paucity of information available in the literature on differential activation of the different MAPK isoforms, especially in ST cells. The two isoforms of ERK are about 90% homologous; knock out studies have shown that one isoform is capable of partially compensating for the absence of the other one, implying their roles are very similar. However, in the same study the ERK1 isoform was shown to be important in cellular activation of thymocyte maturation (Pages et al., 1999). In this study, the pattern of activation for both isoforms was very similar, occurring mainly at 5 minutes post-stimulation. This observation raises interesting questions about MAPK signaling in ST that will be the subject of future studies. Stimulation of ST cell with LPS also resulted in the secretion of IL-8, TNF-α, MIP-1α and MIP-1β. Secretion of IL- 8 by ST cells upon LPS stimulation has been reported previously (Holmlund et al., 2002, Ma et al., 2006, Shimoya et al., 1999) but the involvement of ERK1/2 in its secretion was not reported. That inhibition of this signaling pathway led to reduced secretion of MIP-1β and IL-8 confirms a functional link between TLRs and chemokine secretion in term ST cells. However, other pathways may be involved since ERK1/2 inhibition did not lead to complete abrogation of chemokine secretion.

In summary, this work confirms that ST cells are indeed pregnancy- specific innate immune cells capable of responding to immunologically to LPS and other

microorganisms. We have previously demonstrated that ST cells respond to malarial parasites by phosphorylating a series of proteins (Lucchi et al., 2005) and secreting the chemokine, macrophage migratory inhibitory factor (Chaisavaneeyakorn et al., 2005). Through secretion and expression of proinflammatory mediators in response to intrauterine infections, these cells are likely to play major immunological roles, perhaps protective by recruiting maternal immune effectors to the site of infection, but also pathogenic, by promoting damaging inflammatory responses that are known to lead to pregnancy complications. Overall, a fine balance of an inflammatory response is definitely required in order to control the infection and at the same time avoid immunopathology. Further studies examining the interplay between maternal immune cells and trophoblast will be required to elucidate how such a balance can be maintained.

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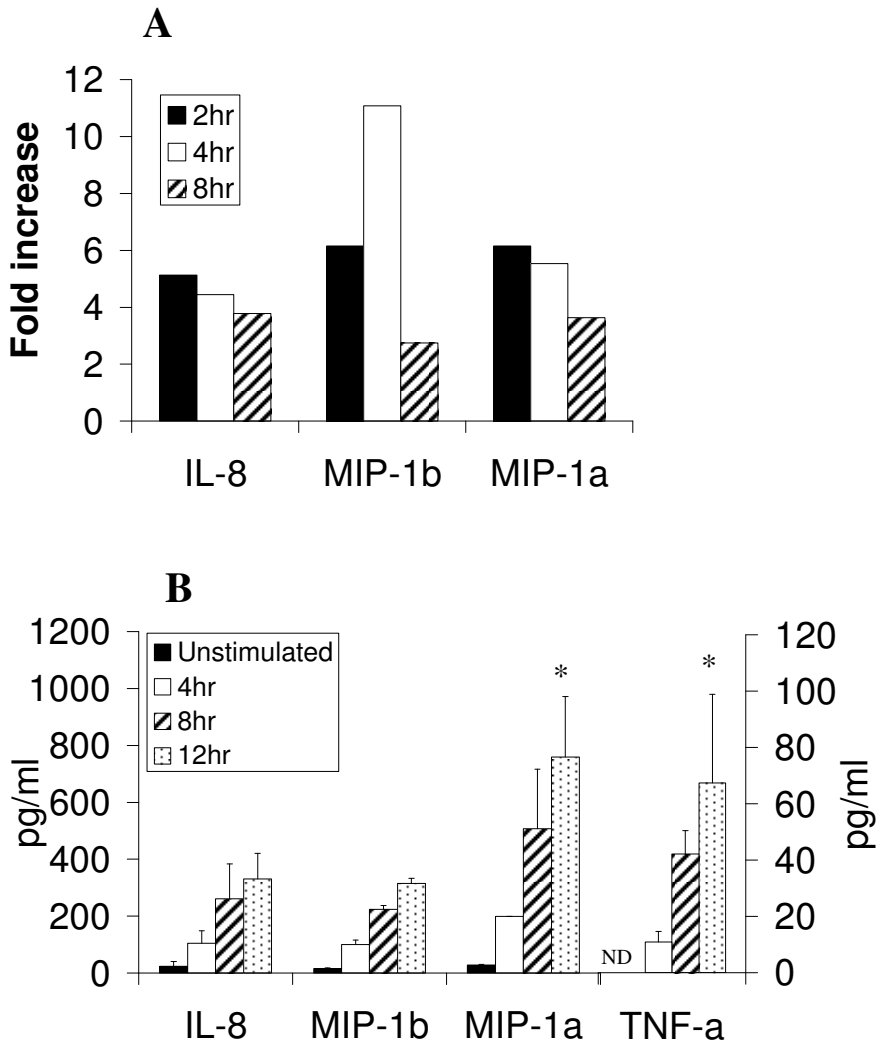
Figure 6.1:

Upregulation of IL-8, MIP-1 α and β gene expression and protein secretion in LPS-stimulated primary human ST cells.

ST cells were stimulated with either LPS or left unstimulated over a 12 hour time course. RNA was extracted and gene expression changes assayed by real time reverse-transcription PCR. This treatment of ST led to the upregulation of MIP-1α, MIP-1β and IL-8 genes (A). Cell culture supernatants were also collected and assayed for chemokine protein by ELISA. Compared to the unstimulated ST, there was a time-dependent increase in the secretion of MIP-1α, MIP-1β, IL-8, (primary Y axis) and TNF-α (secondary Y axis) (B). ND= not detected. Data represent three separate experiments for IL-8, MIP-1α and MIP-1β and two for TNF-α. * Compared to secretion by unstimulated cells, $P<0.05$.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IL-8
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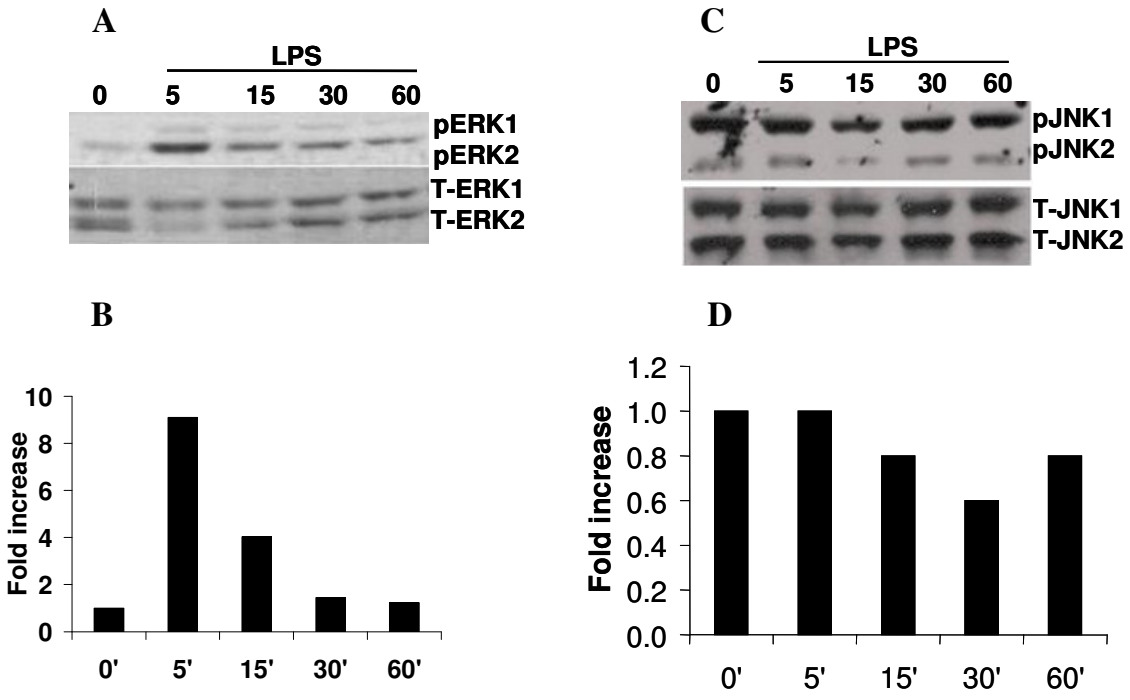
MIP-1a
TNF-a

Figure 6.2:

Activation of ERK1/2 MAPK following stimulation of ST with LPS.

Western blotting of phosphorylated cellular proteins from ST cells treated with 10ug/ml of LPS for the indicated time course or left unstimulated (0) shows specific enhanced phosphorylation of ERK1/2 proteins which peaked at 5 minutes post stimulation (A and B). There was no dramatic change in phosphorylation of JNK1/2 (C and D) although there was a slight decrease in activation after 15 and 30 minutes. Phosphorylated p38 was not detected at all (data not shown). B and D show the densitometric results of the western blotting for ERK 1/2 and JNK1/2 respectively. pERK1/2 = phosphorylated ERK1/2, pJNK1/2 = phosphorylated JNK1/2, T-ERK1/2 = total ERK1/2 and T-JNK1/2= total JNK1/2. Data shown are from one representative experiment of three for ERK1/2 and of two for JNK1/2.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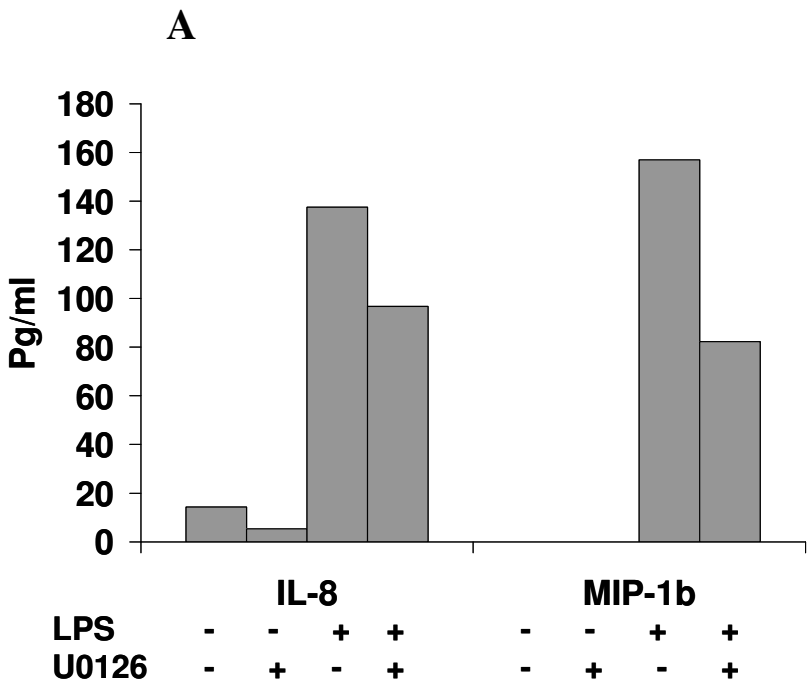
15
30
60
pJNK1
pJNK2
T-JNK1
T-JNK2
B
Fold increase
10
8
6
4
2
0
0'
5'
15'
30'
60'
D
Fold increase
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0'
5'
15'
30'
60'

Figure 6.3:

Inhibition of ERK1/2 partially reduces secretion of MIP-1β and IL-8.

ST cells were pretreated with 10uM MEK1/2 inhibitor (U0126) for two hours, washed, and then stimulated with 10ug/ml LPS for 8 hours. Supernatants were collected and assayed for the secretion of MIP-1β and IL-8 using standard ELISA technique. (A) IL-8 secretion decreased both in the presence (30%) and absence (60%) of LPS stimulation. MIP-1β decreased 48% following pretreatment with inhibitor and stimulation with LPS. (B) A concomitant reduction in the activation of ERK1/2 was observed upon pretreatment with the inhibitor and stimulation with LPS for 5 minutes (B). Data shown are from one representative experiment of three.


A
180
160
140
120
100
80
60
40
20
0
Pg/ml
IL-8
MIP-1b
LPS - - + + - - + +
U0126 - + - + - + - +

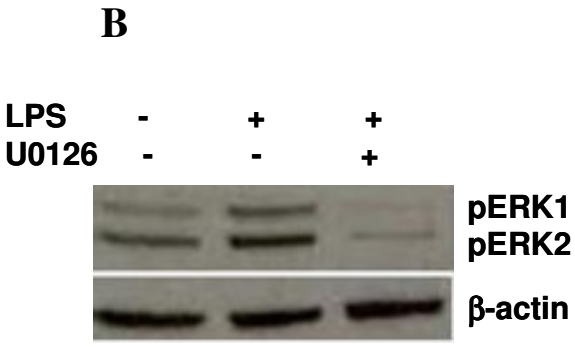
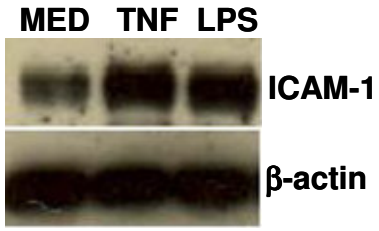

B
LPS - + +
U0126 - - +
pERK1
pERK2
β-actin

Figure 6.4

Figure 4: Enhanced ICAM-1 expression by ST upon LPS stimulation.

ST cells were incubated with 10ug/ml of LPS, 10ng/ml of rhTNF–α (TNF) or left unstimulated (MED) for 24 hours. Whole cell lysates were prepared from these cells and immunoblotting used to detect ICAM-1 protein. TNF-α and LPS led to 2.5 and 2.3 fold increases in the expression of the ICAM-1 protein, respectively, compared to unstimulated cells. B-actin was used as a loading control.


MED
TNF
LPS
ICAM-1
β-actin

CHAPTER 7

SUMMARY AND CONCLUSIONS

Malaria continues to be a leading public health problem needing urgent solutions in terms of drug and vaccine development. For these efforts to be successful, some basic understanding of the host/parasite interaction is necessary. Epidemiological studies indicate that pregnant women are more susceptible to malaria infections than their non-pregnant counterparts regardless of previous immunity to the disease. Malaria during pregnancy causes both maternal and newborn complications. It often leads to the sequestration of malaria-infected red blood cells (iRBCs) in the intervillous spaces (IVS) of the placenta. This is often accompanied by the accumulation of maternal immune cells and several placental pathologies, giving rise to what is commonly referred to as placental malaria (PM). PM is associated with maternal anemia, premature delivery, and low birth weight (LBW; <2500 g) babies. It is estimated that up to 200,000 infants die annually due to adverse consequences of PM. There is accumulating evidence indicating that, apart from the parasite's virulent factors, the host's immunological response, particularly proinflammatory cytokines and chemokines, participate in malaria pathophysiology. For example, elevated levels of IL-8 were associated with LBW and intrauterine growth retardation.

The fetal cells directly in contact with maternal blood in the placenta, the syncytiotrophoblast (ST), form a continuous cell layer (syncytium) which serves as the interface between mother's blood and fetal circulation. During PM, iRBCs are known to bind to ST cells via a glycosaminoglycan, chondroitin sulfate A (CSA). The parasite ligand mediating this adherence is the *Plasmodium falciparum* erythrocyte membrane protein1 (PfEMP1). While the role of the ST cells during intrauterine bacterial infections and some viral infections such as cytomegalovirus have been defined, its probable role

during PM has been largely ignored. This is partly due to the lack of a proper in vitro system needed to address the necessary questions, which due to ethical issues, can only be addressed by use of in vitro cell culture system or animal models. Noting this gap in knowledge, the current study was undertaken with the aim of developing an in vitro system to study the role of ST cells in PM. Once this system was established, the responses induced in ST cells upon interaction with iRBCs and malarial components such as hemozoin (Hz) and malarial antigen were examined. Application of this system will continue to help advance our knowledge on the response induced in the ST cells upon interaction with malaria parasites or malarial components and also with other infectious agents. Apart from providing a means to study the functional changes that may be induced in ST cells, this system can also be used to further investigate the *P. falciparum* cytoadherence phenomenon.

The binding of iRBCs selected to bind to ST cells ($iRBC^{ST}$) induced the phosphorylation of JNK MAPK but not of the other MAPK in the ST cells while incubation with both the Hz and malarial antigen led to the activation of ERK1/2. Activation of the MAPK pathway implies that the ST cells are capable of responding to the presence of malaria parasites. In this study, $iRBC^{ST}$ binding to ST cells induced the secretion of MIF but did not induce any changes in the secretion of the other cytokine and chemokines genes that were assayed. Stimulation with Hz led to the enhanced secretion of IL-8, as did stimulation with LPS. It is likely that ST secretes other unidentified factors upon iRBC binding since phosphorylation of JNK, which is involved in activation of several genes important in immune responses, was observed. This stimulation also led to the migration of PBMCs implying that other chemokines, not measured here, are being

secreted. Further research involving a larger set of genes or microarray analysis is needed to determine the identity of these factors.

Exposure to malaria antigen did not lead to the secretion of the selected cytokines and chemokines by ST cells although it led to the activation of ERK1/2. It is hypothesized that the stimulatory factor in this crude preparation is GPIs. Studies have demonstrated that GPIs induce cellular activation in immune cells mainly resulting in the secretion of TNF-α. During PM, the concentration of malarial GPIs in the placenta may be very high due to the sequestration of iRBCs in this location. It is possible that the amount used here was not enough to induce secretion of immune factors or simply malarial GPIs do not stimulate ST to secrete the selected immune factors assayed here. Again, microarray analysis will shed more light on what factors may be secreted, if at all, by ST cells upon malarial antigen (or purified GPIs) stimulation.

The role of Hz in the placenta had not been investigated despite the fact that Hz is present in the placenta during PM both extracellularly and in phagocytic cells. In this study, Hz induced the secretion of IL-8 in an ERK1/2 dependent manner. The levels of IL-8 in malaria-infected placentas have been shown to be higher compared to those in uninfected placentas. While the maternal monocytic cells that accumulate in the IVS during PM are capable of secreting this chemokine, this study shows that the ST cells could contribute to the enhanced levels of IL-8 in response to Hz. Increased levels of IL-8 may play a role in the recruitment of neutrophils to site of infection (IVS) but also in the activation of monocytes. Accumulation of maternal immune cells in the IVS is a potential danger to the fetus and in fact, one that has been associated with LBW. This study demonstrates that in response to $iRBC^{ST}$ binding the ST cells produce some yet

unidentified chemotactic factor(s) capable of mediating the migration of PBMC towards the stimulated ST cells. This implies that in vivo the ST cells may contribute to the observed monocytic infiltrate during PM, an important pathological factor. LPS is a well known potent inducer of proinflammatory responses from ST cells. As a positive control in this study, ST cells were stimulated with LPS. This stimulation induced the secretion of IL-8 and beta chemokines, MIP-1α and β in an ERK1/2 dependent manner. These results demonstrate that there is differential activation of ST cells by these stimuli (iRBCST, malarial components, hemozoin and LPS).

In conclusion, results from the current study suggest that during PM, the binding of iRBCs to ST cells and the interaction with malarial components stimulates intracellular signaling and gene expression changes in the ST. This leads to the secretion of the proinflammatory chemokine, IL-8 and MIF that may influence the local immunological milieu, making the ST an active immunologic player in the placental environment during PM. In addition, the ST cells appear to contribute to the maternal immune cell infiltrate observed during PM by secreting an as yet unidentified chemotactic factor(s).