EXPRESSION OF CHEMOKINES AND CHEMOKINE RECEPTORS IN THE PLACENTAL INTERVILLOUS SPACE DURING PLACENTAL MALARIA

by

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(Under the Direction of Julie M. Moore)

ABSTRACT

Placental malaria (PM) causes 75,000-200,000 infant deaths and up to 500,000 cases of maternal anemia yearly. Effects of PM include maternal anemia, premature and/or low birth weight babies, and fetal mortality and are often more prevalent in paucigravid women. Given the profound immunological changes in a woman during pregnancy, the occurrence of malaria infection during pregnancy confounds the maternal immune system even further. In this study, chemokine levels and soluble ICAM-1 were determined via enzyme-linked immunosorbent assay and correlated with chemokine receptor expression on maternal infiltrate and pathogenic factors of PM and pregnancy outcomes. Higher placental parasitemia, malaria pigment deposition and lower birth weights are associated with increased levels of MIG, IP-10, MIP-1 alpha, MIP-1 beta and suppressed levels of RANTES in the placenta. The presence of cells responding to FKN and the increased levels of soluble ICAM-1 during PM infection may contribute to the protection against PM infection.

INDEX WORDS: Placental malaria, *Plasmodium falciparum,* chemokine, immunology

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CHAPTER 1

INTRODUCTION

Placental malaria (PM) is a disease that causes between 75,000-200,000 infant deaths and afflicts up to 500,000 women with anemia during pregnancy. Most of these cases occur in tropical and sub-tropical environments and developing countries, where pre-natal care may be hard to find. In areas of high malaria transmission, where immunity to malaria is acquired throughout constant exposure, women carrying their first or second pregnancy are more susceptible to suffering from the various effects of PM, whereas in areas of low transmission, pregnancy in general leaves a woman susceptible to the effects of PM. These effects include maternal anemia, premature and/or low birth weight babies (<2500 grams), and maternal and fetal mortality. Given the profound immunological changes in a woman during pregnancy, the occurrence of malaria infection during pregnancy confounds the immune system even further.

The immunological environment of the placenta during pregnancy is biased towards T helper 2 (TH2) type responses to prevent the maternal cell-mediated immune system from attacking the developing 'foreign' fetus, which displays paternally-derived antigens on the fetal syncytiotrophoblast (ST) cells. Malaria infection causes this TH2 bias to shift towards a cytotoxic TH1 environment within the placenta, inviting a maternal immunological response capable of harming the fetus and comprimising the pregnancy. As stated above, this response is strongest in women carrying their first or second pregnancies and often results in poor pregnancy outcomes and severe illness in the mother. Interestingly, in women carrying their third (or more) pregnancy, immunity to this PM phenomenon develops, presumably because of exposure to malaria during previous pregnancies, and the pregnancy can be carried to term, usually with little or no deficiencies. This immune response that causes harm to the fetus and mother during early pregnancies is capable of providing protection in women carrying multiple pregnancies, and the reasons for this shift between harm and protection are not known.

SIGNIFICANCE

Although there has been a vast amount of research on the factors contributing to PM, including but not limited to studies of the cytoadherence interaction between ST cells and the malaria-infected erythrocytes, gene expression of the parasite during PM, the maternal cell infiltrate responding to infected erythrocytes in the placenta, and cytokine and chemokine detection within the placental intervillous space, there are still many gaps in the knowledge of the immune response to PM, both in the factors contributing to the pathology of and the protection against this disease. It is important to understand the full spectrum of biological events of malaria infection during pregnancy, which occur despite the current use and benefits of malaria prophylaxis for pregnant women. By understanding the associations and sources of various immunological and pathogenic factors, understanding of the disease will increase, as well as the ability to develop new and pertinent treatments for malaria infection during pregnancy. This is especially critical with the increasing occurrence of anti-malarial drug resistance. This study aims to contribute to the present body of knowledge concerning the immunological environment of the placenta during placental malaria infection.

HYPOTHESIS

The purpose of this study was to determine chemokine and chemokine receptor expression in the intervillous space of the placenta to contribute to the present knowledge of the placental environment during placental malaria (PM) infection and how maternal immunological factors contribute to the pathology of or aid in the immune protection against PM. The hypothesis guiding this research was that the presence of particular chemokines in the intervillous space of the placenta during malaria infection will influence the recruitment of certain types of maternal immune cells, capable of responding via cellular expression of

corresponding chemokine receptors. In addition, based on the delicate balance between protection and pathogenesis that is swayed by immunological factors, it is expected that the presence of these chemokines will be associated with either aspects of pathogenesis or protection against PM. This relationship seen between the chemokine and responding immune cells associated with pathogenesis will be absent in PM negative pregnancies.

To address this hypothesis, the following specific aims were addressed:

1. To determine the chemokine protein levels and corresponding chemokine receptor expression on maternal immune cells in the peripheral and placental blood of women living in a malaria endemic area. This was addressed by conducting enzyme-linked immunosorbent assays (ELISAs) on plasma samples to detect chemokine levels, and by staining maternal leukocytes with antibody for detection via flow cytometry.

2. To determine the contribution of chemokines and corresponding maternal cell infiltrate to the pathogenesis or protection of PM. This was addressed by comparing the chemokines and cell infiltrate with known pathogenic factors of PM (i.e. placental parasitemia and pigment deposition in the placenta) as well as pregnancy outcome (birth weight).

CHAPTER 2

LITERATURE REVIEW

MALARIA

There are four species of the *Plasmodium* genus of intracellular parasites that cause malaria infection in humans: *P. vivax, P. malariae, P. falciparum, and P. ovale*. Of the four species, *P. falciparum* causes the most severe disease due to the ability of these parasites to sequester within the vascular endothelium (Reviewed in 39). There are between 300-500 million new cases of malaria per year, and 1-2 million deaths associated with this disease, mostly occurring in tropical environments and developing nations.

The life cycle of *Plasmodium* parasites consists of the transmission of malarial sporozoites to humans via the infectious bites of female *Anopheles* mosquitoes. After sporozoites are released into the human, they migrate to the liver, and upon invasion of hepatocytes and first round of asexual reproduction, erupt as merozoites to infect red blood cells (iRBC). The free merozoites invade new RBCs to undergo subsequent rounds of reproduction and maturation into trophozoites and schizonts, eventually bursting out of the cell to invade other cells. This erythrocytic stage of the parasite's life cycle is associated with the symptoms of malaria infection, including cerebral malaria and impaired consciousness, hypoglycemia (41), metabolic acidosis (43), intermittent fevers and anemia (41, 42).

The parasites bind to the membrane of RBCs through the expression of several essential proteins, such as erythrocyte binding antigens (EBA), merozoite surface proteins (MSP), and in some species of *Plasmodium*, Duffy binding proteins (DBP) and apical membrane antigens (AMA) that interact with the plasma membrane of the RBC (Reviewed in 37). In *P. falciparum* specifically, some of the essential proteins for survival within the human host include MSP-1 and EBA-175, which are required for RBC invasion. Though the erythrocyte ligand for MSP-1 is unknown, it is known that EBA-175 binds to glycophorin A expressed on RBC membranes (36).

AMA-1 is also needed for apical orientation of the parasite to the RBC for proper invasion to take place.

A unique feature of *P. falciparum* is the redundant ability to bind and invade all RBCs, regardless of RBC protein or the age of the cell. *Plasmodium vivax* requires RBC expression of the Duffy blood group antigen for invasion, and is therefore restricted to invading only certain RBCs. Also, *P. vivax* only invades reticulocytes, which greatly limits its potential parasitemia. The ability of *P. falciparum* to bind to many receptors and invade all erythrocytes aids in the parasite's ability to escape host immune detection (Reviewed in 37).

Within sixteen hours of invasion, the asexually mature parasite, residing in a vacuole within the erythrocyte, presents parasitic adhesion proteins on the surface of the RBC. These proteins are capable of adhering to various molecules expressed on vascular endothelial cells which enable the parasite to evade immune clearance in the spleen. Specifically, the highly polymorphic protein *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), which is encoded by approximately 60 *var* genes (1), is responsible for binding to host molecules. The *var* genes are expressed one at a time, and the host molecules subject to adherence depend on the particular *var* gene expressed at that point in time. Several of these host molecules include, but are not limited to, CD36 (2, 83), intercellular adhesion molecule-1 (ICAM-1) (3, 18), E-selectin, P-selectin, vascular adhesion molecule (VCAM)-1 (4), thrombospondin, and membrane-bound fractalkine (FKN) (7). Infected erythrocytes can also bind to chondroitin sulfate A (CSA) (5, 6, 82) within the placental intervillous space. The switching of *var* gene expression by the parasite, a means of antigenic variation, allows it to escape detection by the host immune system (59) as well as bind to different host molecules.

Malaria parasites use hemoglobin as the major nutrient source from within the red blood cell (19). The parasite cannot digest the iron that is left after the parasite extracts the essential amino acids from the hemoglobin. Because the parasite cannot handle the toxic form of iron (20), malaria parasites form hemozoin, or malaria pigment, which consists of heme polymer units. When merozoites rupture out of the erythrocyte, this pigment is released into the blood and can be phagocytosed by mononuclear cells. Hemozoin-laden macrophages have been detected in the spleen, liver, bone marrow (22), and placenta (21). The inclusion of hemozoin by macrophages has been linked to an increased production of pro-inflammatory chemokines during malaria infection (24), suggesting that parasite by-products may modulate the host response to infection.

After multiple cycles of asexual reproduction and eruption and invasion of RBCs, *P. falciparum* parasites eventually mature into male and female gametocytes, which are then ingested during feeding by female *Anopheles* mosquitoes. Within the mosquito, the temperature change and environment of the mosquito midgut induces male gamete exflaggellation which allows for sexual reproduction. The newly formed ookinetes invade the mosquito's midgut and encyst on the outer midgut wall. Each oocysts produces hundreds of sporozoites which eventually migrate to the salivary glands where maturation to the sporozoite stage occurs. Once in the salivary glands, it is possible for the sporozoites to be injected into a host during the mosquito's next blood meal, thus completing the life cycle of malaria parasites.

The life cycle of *Plasmodium falciparum* is complex and multi-faceted. Depending on both human and mosquito, the parasite has evolved into an intricate enemy of much of the world's tropical population. Although immunity can develop in highly endemic areas, children and pregnant women are at higher risk for developing severe disease (33).

MALARIA IMMUNOLOGY

The human immune system is composed of innate, humoral, and cell-mediated immune responses. The induction and type of response is controlled by cell recognition of pathogens and the production of cytokines capable of activating the appropriate cells for producing protection and control of disease.

Malaria parasites are intracellular pathogens and elicit the production of cytokines that activate the response of TH1-type cells (34). These cytokines are produced by cells of the innate and adaptive immune systems, such as mononuclear phagocytic cells, T cells (61, 62) and natural killer cells (60), which recognize iRBCs and produce tumor necrosis factor (TNF)-alpha and interferon (IFN)-gamma *in vitro*. These pro-inflammatory cytokines activate macrophages to phagocytose iRBCs and produce nitric oxide which acts to destruct the infected cell and inhibit parasite growth (56). Although necessary to induce protection against malaria, these pro-inflammatory cytokines have been associated with the pathology of malaria, such as high fevers, aches, hypoglycemia and coma (58, reviewed in 40). Specifically, the production and levels of TNF-alpha have been associated with severe malaria pathology (49, 50), and higher levels of interleukin (IL)-1, IL-6 and TNF-alpha have been associated with the pathology in malaria infection (70). Although suggested to be crucial for protection against malaria (71), it has been demonstrated in murine models that overproduction of IFN-gamma can also contribute to the pathology of disease (72), again demonstrating that a balance between beneficial and harmful immune responses during infection is likely to be required to achieve parasite clearance without damaging host tissues.

The cell-mediated immunity branch of the immune response is capable of eliciting different types of responses, depending on the type of pathogen introduced into the body. For

example, T helper cells differentiate into CD4+ TH1 or TH2 cells, responses which are important for different types of pathogens, and each works to down-regulate the other response (Reviewed in 63). For protection and control of malaria infection, a strong TH1 response is important (34) and induced by cytokines, such as TNF-alpha, IFN-gamma, IL-6, which also stimulate macrophage engulfment and killing of parasites (56). The induction of a TH1 response is important for B cell activation and the production of antibodies specific for parasitic proteins, to both block invasion of new RBCs by merozoites, and to aid in iRBC opsonization and recognition by macrophages (8-10). Cytotoxic CD8+ T cells may be important for lysing and killing infected hepatocytes during the liver stage of malaria infection, and IFN-gamma produced by CD8+ T cells has been associated with protection against malaria infection (Reviewed in 64). However, RBCs lack major histocompatibility complex (MHC) expression, which is an important surface ligand for CD8+ T cells to detect infection. Therefore these cells cannot destroy iRBCs although they are important in producing memory cells to aid in immunity to subsequent malaria infections.

The role of B cells in producing antibodies is also important in blocking invasion and neutralizing parasitic infection (8, 9). Antibodies specific for molecules expressed on free parasites can prevent the parasite from invading erythrocytes and render the parasite vulnerable to attack by innate and adaptive immune cells. Antibodies can also bind parasite proteins expressed on the surface of the RBCs and aid in macrophage recognition of infected cells through interaction with Fc receptors expressed on the macrophage (10, 57). Specifically, immunoglobulin (Ig) G1 and IgG3 subtypes contribute to macrophage opsonization of infected erythrocytes *in vitro* (57). Several parasite proteins has been detected as antibody targets and the presence of antibodies specific for these expressed proteins have been associated with protection.

Some of these proteins include rhoptry-associated membrane antigen (25), and apical membrane antigen-1 (AMA-1) (28). It should be noted that in a recent study antibody responses to AMA-1, while previously associated with protection, were not associated with the production of memory B cells during infection of children in a malaria endemic area (65). This suggests that antibodies may have a protective role in malaria infection but it should be noted that the presence of antibodies to specific *P. falciparum* antigens is not indicative that memory B cell responses have been produced simultaneously. It is clear that other factors contribute largely in the development of immunity to malaria infection.

Parasite byproducts, such as the release of hemozoin when the merozoites rupture the RBC, can also elicit host immune responses. It has been shown that hemozoin stimulates the production and release of IL-1 beta, and TNF-alpha (54, 55). The chemokines MIP-1 alpha and MIP-1 beta are also released by malaria-exposed PBMCs *in vitro* (66). The ingestion of hemozoin by phagocytic cells proves to be protective, via stimulation of cytokine secretion and induction of immune responses, but it should be noted that hemozoin has the ability to impair function of these cells by decreasing antigen presentation (68) and possibly altering IFN-gamma release by other immune cells (67).

Some of the adhesion molecules that iRBCs can bind to escape clearance by the spleen are known to be up-regulated on endothelial cells in response to pro-inflammatory cytokines, such as TNF-alpha, IFN-gamma and IL-1 beta (69), thus suggesting that the very immune factors needed to induce protection against malaria, also aid in the sequestration of the iRBCs and prolonged parasite survival. Specifically, ICAM-1 is a transmembrane glycoprotein that is expressed constitutively on many cells types, including some vascular endothelial cells, lymphocytes and monocytes (Reviewed in 92). Malaria infection is associated with an increase

in ICAM expression on the endothelium (77, 78), although there are conflicting results between ICAM expression and severity of malaria infection. Immunohistochemistry has revealed up-regulation of ICAM expression in fatal cerebral malaria infection, and, along with CD36, has shown localization of iRBCs with these molecules (79). Malaria infection has also been associated with increased levels of soluble adhesion molecules, sICAM-1, sVCAM-1 and sE-selectin (80, 81).

PLACENTAL MALARIA

Malaria infection during pregnancy is a huge public health concern. Accounting for the deaths of 75,000-200,000 infants and illness of up to 500,000 women, it is important to understand the immunological response during pregnancy to malaria. There are current and efficient treatments available to ward off malaria infection in use, although the cost and feasibility of maintaining health care in developing countries is often difficult. Some of these treatments include the use of insecticide-treated bed nets to prevent the infective bite of the mosquito, as well as the administration of drugs, such as sulphodoxine-pyrimethamane, proguanil or mefloquine, that are safe for use during pregnancy (Reviewed in 94). Despite the development of these drugs, it is often difficult to distribute the drugs widely, especially in rural areas of developing countries where malaria infections are rampant, and the development of drug resistance has served as another set back in providing care and treatment to pregnant women. Efforts are currently underway to develop vaccines to generate protection against the pregnancy specific *var* gene that confers binding in the placenta. Due to the polymorphism of the *var2csa* gene, and the chance that other genes may be expressed during PM, it is challenging to develop a vaccine targeting epitopes that are recognized by maternal antibodies and that would be protective across populations, furthering the need to fully understand the development of natural

immunity to PM (Reviewed in 95). Based on these challenges, it is imperative to study and understand the immune response during PM infection, not only to increase understanding of how the disease progresses and how the body naturally responds, but also to aid in the development of new and effective treatments for treating those infected, particularly those infected during pregnancy.

Malaria infection during pregnancy is associated with the accumulation of asexual mature parasite infected erythrocytes within the intervillous space of the placenta (13, 44, 45, 75). The parasites that adhere in the placenta are different than parasites isolated from non-pregnant adults and children (38, 51). This suggests that the occurrence of malaria infection during pregnancy is associated with a unique subpopulation of *Plasmodium falciparum* parasites. This accumulation is mediated by a novel parasite adhesion protein, expressed on the surface of the red blood cell which binds to glycosaminoglycans expressed on the surface of placental trophoblast cells. In response to this sequestration of iRBC, maternal lymphocytes and monocytes infiltrate the intervillous space (IVS) (47) and are associated with PM, contributing to the detrimental effects of the disease, including abortion (33), premature births and low birth weight babies (47), maternal anemia (46), maternal hypertension (17) and fetal and/or maternal death (12, 46).

The *var2csa* encodes a variant form of the *Plasmodium falciparum* PfEMP-1 protein gene that is expressed on the surface of iRBC. The *var2csa*-derived expression of PfEMP-1 enables the parasite to adhere to CSA which is expressed by fetal trophoblast cells (38). Specifically, the parasites isolated in PM infections preferentially bind to CSA, though other cell surface molecules may also contribute to this adherence, such as the glycosaminoglycan hyaluronic acid (16). The adherence of iRBC to CSA is dependent on the distinct pattern of sulfation of placental CSA (23). As well as preventing the clearance of iRBCs from the blood by

the spleen, it is possible that this cytoadherence of iRBCs to CSA decreases the occurrence of phagocytosis by host macrophages in the placenta (93). In addition to binding CSA, another mechanism of sequestering occurs when non-immune immunoglobulins bind iRBCs which then interact with surface molecules and possibly Fc receptors expressed on trophoblast cells (73, 74).

In response to iRBC adherence to syncytiotrophoblast cells, there is an influx of monocytes and macrophages within the placenta (75). The presence of these cells in the placenta during PM has been associated with poor pregnancy outcomes. This may be due to expression of immune factors by maternal cell infiltrate which are detrimental for the fetus and mother, resulting in decreased blood flow within the placenta (48), hypoxia, and hindrance of maternal IgG transfer across the placental barrier (84). It is also hypothesized that fetal defects caused by PM infection are due to decreased nutrient and oxygen exchange between the mother and the fetus (85). Ultimately, these factors are believed to contribute to premature births, low birth weight babies, maternal anemia and death. In addition to the immune response, PM is histologically associated with syncytial necrosis and loss of microvilli, cytotrophoblast proliferation and the irregular thickening of the trophoblast membrane (75), suggesting that malaria infection results in physiological changes of the placenta that interfere with healthy maternal-fetal exchanges and can be harmful to the pregnancy.

Cytokines, originally defined as soluble molecules that effect cellular functions, more recently have been defined as soluble glycoproteins that directly function to activate cells or recruit cells of the immune system to certain areas of the body. With many roles, including the activation of cells, regulation of cellular migration, and regulation of angiogenesis, the expression of these molecules and their respective receptors on target cells is crucial to immunological processes (Reviewed in 76).

The environment of a non-infected placenta usually favors a bias for type 2 cytokines, which aid in fetal protection from the maternal immune system (86, 87). Fetal cells are capable of displaying 'foreign' paternal major histocompatibility complex molecules, which may initiate a harmful immune response from the mother to the fetus, resulting in abortion and/or difficulty in future pregnancies fathered by the same man. Cytokines associated with the TH2 immune response, such as IL-4, IL-6, IL-10, are necessary for skewing the local immune response away from a cytotoxic environment which would aid in fetal rejection and towards a tolerant environment.

Malaria infection, however, shifts the cytokine milieu within the placenta from a TH2 towards a TH1 bias (34). The TH1 cytokine response, consisting of TNF-alpha, IFN-gamma, IL-1 and IL-6, is required for immune cell activation against malaria infection. The levels of these cytokines have been associated with both disease severity and poor pregnancy outcomes in women experiencing their first malaria-exposed pregnancy. Specifically, TNF-alpha and IL-8, produced by placental macrophages containing hemozoin are associated with intrauterine growth retardation (14). Whereas some cytokines have been associated with the detrimental effects of PM, some have been identified with protection against PM. Specifically, elevated IFN-gamma has been detected in higher levels of PM negative multigravid women than in PM positive women (35).

Placental malaria is associated with maternal monocyte infiltrate, with stark correlations between the density of monocytes and low birth weight (88). These cells are suggested to be attracted to the placenta through expression of beta-chemokines, such as MIP-1 alpha and MIP-1 beta (14, 90, 91), and it has been shown that the cellular expression of the ligand to these

chemokines, CCR5, is up-regulated in PM (89). However, further studies are required as these assumptions have not yet been demonstrated concretely.

Women carrying their first or second pregnancy are more susceptible to PM infection (33). It has been suggested that the susceptibility of these women is linked to the absence of pre-existing antibodies specific for proteins expressed by CSA-binding placental parasites (26, 27, 30). The presences of these antibodies are associated with decreased PM infection (27) and are predominantly composed of IgG1 and IgG3 antibody subtypes (29).

It is obvious that the interaction between the malaria parasite and human immune system is complex, and that this interaction is made even more complex by pregnancy.

HUMAN PREGNANCY

Pregnancy is established by the invasion of the uterine wall by fetal cytotrophoblast cells. These cells are important in the formation of the maternal-fetal interface, and must migrate near the maternal uterine arteries in order for nutrient and waste exchange to develop efficiently (15). Pregnancy is maintained by the establishment of maternal immune tolerance to the semi-allogeneic fetus, which expresses paternal major histocompatibility complex antigens. Without immune tolerance at the maternal fetal interface, the maternal immune system can reject the implanted fetus, resulting in abortion.

Cytokines and chemokines are required for the establishment and maintenance of pregnancy, and in the instance of placental infection, the recruitment of maternal immune cells. After blastocyst implantation, fetal trophoblast cells must migrate into the uterine wall to form both a concrete place of attachment to the mother and set up an interface at which nutrients and wastes can be exchanged between the fetus and the mother. The chemokines involved in the migration of fetal trophoblast cells into the uterine wall are produced by decidual stromal cells

and cause migration through the corresponding expression of chemokine receptors on the trophoblast cells. Specifically, mRNA for fractalkine, HCC-1 and MIP-1 beta has been detected during the implantation window and has been suggested that these chemokines are associated with early trophoblast migration (31).

In addition to aiding in trophoblast invasion, chemokines are also responsible for recruiting and maintaining a specific subset of maternal immune cells within the placenta. These cells are necessary for establishing maternal tolerance to the semi-allogeneic fetus, which expresses paternal antigens. The subset of maternal leukocytes capable of establishing fetal tolerance consists of TH2- type cells. TH2 cells differentiate via cytokine influence and hormone influence. During pregnancy, fetal cells of the placenta produce hormones that are known immunosuppressants, such as progesterone, estrogen, and chorionic gonadotropin.

CHEMOKINE/CHEMOKINE RECEPTOR INTRODUCTION

Chemokines, short for chemotactic cytokines, are small polypeptides (7-10 kilo Daltons) that can induce leukocyte adhesion, transmigration, and maturation, and angiogenesis. Produced by a variety of cells, chemokines produce gradients that actively recruit cells expressing chemokine receptors until the receptor is desensitized with the high concentration of chemokine present. The highest chemokine gradient may end at sites of inflammation, or it may end in areas where other chemokines are expressed, allowing cells to migrate further into the site of inflammation where other chemokines may be expressed. Because chemokines only attract specific subsets of cells bearing the corresponding receptor, it is thought that the chemokine milieu is at least partially responsible for the resulting cell phenotype found in areas of inflammation (as reviewed in 32).

There are four families of chemokines classified based on the number of cysteine residues in the amino-terminus. These families are: C, CC, CXC and CX3C, with the X representing any other amino acid. The N-terminus of the chemokine appears to be important for receptor ligation whereas the C-terminus binds glycoasminoglycans and may be utilized for binding to the surface of the endothelial cells that did not produce that particular chemokine. By binding to the surface molecules of the endothelium or epithelium, chemokines can arrest leukocytes from the blood flow and begin the tethering and rolling process required for cell migration out of the blood and towards areas of inflammation.

Cells that respond to the chemokine gradients express receptors that are specific for the chemokines. The receptors are seven-transmembrane spanning receptors, coupled to a GTP-binding protein that, once the receptor is ligated, induces intracellular changes through activating intracellular signaling cascades, usually involving an influx of intracellular calcium and mitogen-activated protein (MAP)-kinase family activation. Changes are made in the cell cytoskeleton that allow the cell to migrate. In areas of very high chemokine expression, the receptors become saturated and insensitive to that particular chemokine. It is here that the cell may carry out its function or continue migrating towards other chemokines through the expression of other chemokine receptors.

CONCLUSION

It is apparent that the immunological response to malaria infection is complex and involves the interaction and balance of multiple branches of the immune system. Malaria infection during pregnancy causes an even greater immunological challenge to the mother, and often results in poor pregnancy outcomes and severe illness. It is crucial to understand the

natural immune response during PM infection, as the understanding of the disease will increase and allow for the development of better treatments.

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CHAPTER 3

EXPRESSION OF CHEMOKINES AND CHEMOKINE RECEPTORS IN THE PLACENTAL INTERVILLOUS SPACE DURING PLACENTAL MALARIA[1]

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ABSTRACT

Placental malaria (PM) causes 75,000-200,000 infant deaths and up to 500,000 cases of maternal anemia yearly. Effects of PM include maternal anemia, premature and/or low birth weight babies, and maternal and fetal mortality and are often more prevalent in paucigravid women. Given the profound immunological changes in a woman during pregnancy, the occurrence of malaria infection during pregnancy confounds the maternal immune system even further. In this study, placental chemokine levels and soluble intercellular adhesion molecule (ICAM)-1 were determined via enzyme-linked immunosorbent assay and correlated with chemokine receptor expression detected via flow cytometry on maternal T cells and monocytes and pathogenic factors of PM determined via immunohistochemical analysis and pregnancy outcomes. Higher placental parasitemia, malaria pigment deposition and lower birth weights are associated with increased levels of monokine-induced by interferon-gamma (MIG), interferon-gamma inducible protein (IP)-10, macrophage inflammatory protein (MIP)-1 alpha, MIP-1 beta and suppressed levels of regulated upon activation, normal T-cell expressed and secreted (RANTES), suggesting that these chemokines may contribute to the pathogenesis of PM. The presence of cells responding to fractalkine (FKN) and the increased levels of soluble ICAM-1 during PM infection may be associated with the protection against PM infection.

Keywords: Placental malaria, *Plasmodium falciparum,* chemokine, immunology

INTRODUCTION

Of the four species of malaria parasites that infect humans, *Plasmodium falciparum* is the most virulent. The culprit of approximately two million deaths worldwide per year, this parasite has a large impact on the health and economy of tropical and developing nations. In areas of high transmission, it is possible for humans to develop immunity to *P. falciparum*, though infection by this parasite can still result in severe illness and death for children and pregnant women. It is known that women carrying their first and second pregnancy are more susceptible to the ill effects of placental malaria (PM) (2), whereas women carrying at least their third pregnancy develop a protective immunity. Despite the vast amount of research on this immunological phenomenon, the differences contributing to the pathogenesis of disease or the protection remain unknown.

A unique feature of *P. falciparum* is the ability to cytoadhere to the microvasculature of the host through expression of adhesion proteins encoded by the *var* gene family and found on the surface of the erythrocyte (1). This cytoadherence phenomenon aids in the evasion of immune defense responses. Several molecules that *P. falciparum* binds include intercellular adhesion molecule (ICAM)-1, CD36, E-selectin, vascular cell adhesion molecule (VCAM)-1, hyaluronic acid (HA) and chondroitin sulfate A (CSA) (3-7), the latter which is highly expressed in the placenta and is a known adhesion molecule during PM.

During PM, malaria infected erythrocytes expressing the *var2csa* gene product bind to fetal cells in the placenta expressing CSA (13), although there may be other parasite genes expressed that can mediate this binding. It is possible that maternal immune cells infiltrate into the placenta in response to this binding, presumably through the expression of cytokines and chemokines, although the actual mechanism that initiates cell recruitment is not known. Certain

aspects of this immune response, such as the presence of maternal monocyte infiltrate, have been associated with poor birth outcomes and maternal anemia (14). Immunological factors contributing to the pathogenesis of PM of susceptible pregnancies, such as a bias towards Th1 cytokine response (8) have been detected. There have also been reports that other immunological factors, such as interferon (IFN)-gamma (9), contribute to the protection against PM infection, showing that an important balance of immune factors must exist within the placenta that influences the outcome of the pregnancy. It is important to note, however, that there are conflicting reports about the contributions of IFN- gamma and its contribution to the pathology or the protection against PM infection (42) further demonstrating the ambiguous environment of the placenta during PM. While there has been a substantial amount of research pertaining to the immunological response during PM, there likely remain other factors that contribute to the environment of the placenta that may sway the response away or towards protection against PM.

This study aims to further define the chemokine milieu, the maternal cellular infiltrate and relationships of these immune factors with PM pathology. The expression of chemokines, detected via ELISA, and associations between the chemokines and corresponding chemokine receptors on maternal immune cells, detected by flow cytometry, were conducted. In addition, the associations between levels of chemokine expression and the presence of maternal immune cells expressing the corresponding chemokine receptors were examined with placental parasitemia, malaria pigment deposition, and the birth weight of babies.

MATERIALS AND METHODS

Study Populations

Blood, plasma and placental tissue samples were collected from women residing in malaria endemic areas of western Kenya. Women were recruited from Nyanza Provincial General Hospital in Kisumu, Kenya and at the Siaya District Hospital in Siaya, Kenya. Kisumu and Siaya are located in western Kenya. The original study site, Nyanza Provincial General Hospital in Kisumu, is the referral hospital for the Nyanza Province. The study site was moved to rural Siaya district due to a decrease in malaria transmission in Kisumu, probably due to a frequent administration of anti-malarial drugs and education, though this has never been studied. The Siaya District Hospital serves the people of Siaya District, one of the poorest districts in Kenya and home of a high rate of malaria transmission, with an estimated entomological inoculation rate of 300 infective bites per person per year (32). Of the women recruited in this study in Siaya, the prevalence of PM is 29.5% primigravidae, 15.1% in secundigravidae (women carrying their second pregnancy) and 7.3% in multigravidae (Moore, personal communication). Samples and data from a subset of these women were used for the present study (clinical parameters are summarized in Table 3.1).

The areas of subject recruitment offer favorable environments for the transmission of *Plasmodium* malaria parasites, transmitted by the *Anopheles gambiae* mosquito. The *Plasmodium* species in this region are *P. falciparum, P. malariae, and P. ovale*, with the most prevalent species causing infections being *P. falciparum* (38).

Informed consent was obtained and involvement of human subjects was approved by University of Georgia and Centers for Disease Control and Prevention Institutional Review Boards and Kenya Medical Research Institute (KEMRI) Ethical Review Committee.

For some experiments, fresh human placentas were obtained from women delivering by elective caesarean section at St. Mary's Hospital or Athens Regional Hospital in Athens, Georgia. Written, informed consent was obtained and study design and involvement of human subjects were approved by the University of Georgia and St. Mary's Health Care System IRBs. Placentas used for western blotting assays were malaria and HIV negative as determined by subject questionnaire information.

Sample Collection

Syncytiotrophoblast cells were isolated from fresh human placenta, delivered by elective caesarean collection, as described previously (27).

Placental blood and plasma samples, collected in Kenya, were collected via prick and perfusion methods (25, 26). Prick samples were collected within five to ten minutes of placental expulsion via placental prick with a needle to allow maternal intervillous blood to be collected in tubes containing heparin or EDTA anti-coagulants. Platelet-free plasma was separated via centrifugation and samples were frozen in liquid nitrogen. Perfused blood samples were collected by pumping phosphate-buffered saline solution into the maternal intervillous space via peristaltic pump. Maternal blood was collected as it was washed out of the intervillous space (26).

Peripheral blood and plasma samples, collected in Kenya, were obtained via venous puncture. Platelet-free plasma was isolated via centrifugation and stored in liquid nitrogen.

Blood smears were made by a trained lab technician. Briefly, thick and thin blood smears were made on one slide. The thick smear made by allowing a thick drop of blood to drop to the slide and smearing lightly with another slide. Thin smears were made by smearing a smaller drop of blood with the blunt end of another microscope slide. Parasitemia was

determined by observing thin blood smear slides under oil immersion calculated using the following equation: (number of infected erythrocytes / total number of erythrocytes) x 100 with a minimum of three hundred cells counted.

Malaria pigment score was determined by thick blood smear. Briefly, blood smears were observed under a light microscope and number of white blood cells (WBC) were counted in each field. Percent of pigmented WBC was determined by the following equation: (number of pigmented WBC / number of total WBC) x 100. Scoring was determined as 0 = no pigmented WBC, 1 = <10% pigmented WBC, 2 = 10-25% pigmented WBC, 3 = 26-50% pigmented WBC, and 4 = >50% pigmented WBC (23). A minimum of 300 WBC were scored.

Protein Array

To determine population expression of cytokines and chemokines, eight sets of ten samples were chosen based on infection status and gravidity. Two groups of primigravid samples and two groups of multigravid samples were chosen based on infection status: malaria non-infected and malaria infected. It should be noted that in several groups sample number was limited due to the amount of plasma collected, the volume difference was corrected with assay buffer, as per manufacturer's instructions. Samples were tested using the Proteome Profiler™ antibody array, Human Cytokine Array Panel A Array Kit from R & D Systems. Briefly, samples were pooled and incubated with antibody cocktail and added to a membrane containing antibodies for thirty-six cytokines, chemokines and soluble adhesion molecules, according to manufacturer's instructions. The thirty six factors are: C5a, CD40 ligand, G-CSF, GM-CSF, GRO-alpha, I-309, sICAM-1, IFN-gamma, IL-1 alpha, IL-1 beta, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32 alpha, IP-10, ITAC, MCP-1, MIF, MIP-1 alpha, MIP-1 beta, Serpin E1, RANTES, SDF-1, TNF-alpha, sTREM-1.

Final detection of cytokine levels were determined by enhancement with chemiluminescence (ECL, Amersham) on Kodak film using an automated film developer. Results were compared to PM- multigravid samples, and increases in immune factor expression were denoted +, ++, +++ accordingly.

Enzyme-linked Immunosorbent Assay

For detection of soluble fractalkine (FKN/CX3CL1), regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5), soluble intercellular adhesion molecule (ICAM-1/CD54), macrophage inflammatory protein (MIP)-1 alpha (CCL3) and MIP-1 beta (CCL4) in plasma samples, ELISA DuoSet kits were purchased from R & D Systems, Inc. and used according to manufacturer's instructions. For detection of soluble monokine-induced by IFN-gamma (MIG/CXCL9) and IFN-gamma inducible protein (IP) -10 (CXCL10), antibodies and recombinant proteins were purchased from PharMingen for used in standard sandwich ELISAs and used according to manufacturer's instructions.

Immunohistochemistry

Tissue samples from Kenya were fixed in Streck Tissue Fixative (Streck Laboratories) and paraffin embedded. Slides containing 5-6 micrometer placental tissue samples were placed in a 58°C water bath overnight or for at least 2 hours. The slides were then placed in Declere® solution (Cell Marque) and heated at full pressure in a pressure cooker for 10 minutes. Slides were transferred to fresh Declere® solution for 5-minute hot rinse. Samples were washed in PBS, quenched in 10% hydrogen peroxide for 30 minutes at room temperature in a moist chamber and then washed again in PBS. Tissue samples were blocked in 3.5% goat serum in PBS for 1 hour at room temperature in a moist chamber. Slides were washed with PBS and incubated with the primary antibody overnight at 4°C in a moist chamber. Test slides were

incubated with mouse anti-fractalkine antibody in blocking buffer (4 μg/ml), and negative controls were incubated with mouse IgG antibody in blocking buffer (4 μg/ml). Slides were washed in PBS and incubated with biotinylated anti-mouse secondary antibody in blocking buffer (4 μl/ml) at room temperature in a moist chamber for 30 minutes. During the secondary antibody incubation, the Avidin-Biotin (AB) solution was prepared (2.5 ml PBS, 50 μl Avidin, 50 μl Biotinylated Horse Radish Peroxidase (HRP; Santa Cruz). Tissues were washed with PBS and incubated with the AB solution for 30 minutes at room temperature in a moist chamber. After washing, the enzyme reagent was added to the tissue samples (3.6 ml distilled water, 5 drops 10X substrate buffer, 1 drop 50X DAB chromagen, and 1 drop 50X peroxidase substrate; Santa Cruz). The reagent was left on the tissue samples for 4 minutes, and then the slides were washed with distilled water for 5 minutes. Samples were counterstained with hematoxylin for 10 seconds, and the slides were rinsed 2 times with tap water. The tissue samples were dehydrated using a gradient of ethanol (5 minutes in 70%, 20 minutes in 95%, 20 minutes in 100%), mounted in Flow Tex and observed using a light microscope. Scoring was determined by visual analysis of tissue sample slides, conducted by Dr. Carlos Abramowsky of Egleston Children's Hospital in Atlanta, Georgia.

Western Blot Assay

Syncytiotrophoblast cells were incubated with *Plasmodium falciparum*-infected RBCs and uninfected RBCs from a human donor, as control. Supernatants were collected at 0, 2, 4, 6, 8, 12, and 24 hours and frozen at -20°C. Supernatants were thawed and separated (20 ug protein/lane) on a 10% sodium dodecyl sulphate polyacrylamide gel (BioRad) and transferred to nitrocellulose membrane (BioRad) for one hour. Membranes were blocked in 3% non-fat milk PBS buffer and incubated with anti-FKN antibodies (R&D Systems) for one hour at room

temperature or overnight at 4°C. Final detection was with goat anti-mouse horseradish peroxidase labeled (HRP) secondary antibody (Sigma) in blocking buffer for one and a half hours at room temperature. FKN protein was visualized by enhanced chemiluminescence (ECL, Amersham) after three washes with distilled water on Kodak film using an automated film developer.

Flow Cytometry

Cell counts were calculated from maternal placental blood. Briefly, % CD3+ and % CD14+ were detected via anti-CD3 and anti-CD14 antibodies (PharMingen) by flow cytometry. T cells and monocyte counts were calculated by the equation: %CD3 (%CD14) x total white blood cell count, as determined by a Becton Coulter cell counter.

Peripheral and perfused leukocytes were stained with anti-CCR4 and anti-CCR5 antibody (PharMingen), anti-CX3CR1 antibody and control Ig antibody (MBL International). Cells were acquired with a FACSCalibur flow cytometry machine and data analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems).

Statistical Analysis

Statistics was performed using SAS Statistical software, version 8.02, for analyzing correlations between chemokines, chemokine receptors, and PM pathogenesis factors. Pearson and Spearman correlation tests were used for analyzing associations between chemokines, receptors, and pathogenic factors for normal and non-normally distributed data, as determined by Brown and Forsythe's Analysis of Variance test. All chemokine and chemokine receptor correlations were analyzed via Pearson correlation test, with the exception of MIP-1 alpha and MIP-1 beta, which were analyzed via Spearman's correlation test. The Kruskal-Wallis (KW) test was utilized for comparing peripheral and placental and comparing paucigravid and

multigravid levels of immune factor expression. Significant findings from the KW test were then tested via PROC MULTTEST to determine the adjusted p value for those factors.

RESULTS

Clinical Parameters of Study Subjects

Table 3.1 shows the clinical parameters of the women recruited in this study. There is a significant difference in age between PM+ multigravid and PM- multigravid women (proc multtest p=0.0006) and a slight difference between PM+ paucigravid and PM- multigravid women (proc multtest p=0.06). It is important to note that PM+ paucigravid women suffered higher placental parasitemia than PM+ multigravid women (proc multtest p=0.0004. Also of note is a tendency for PM+ paucigravid women to have lower birth weight babies than PM- multigravid women, though this is not a statistically significant difference. It should be noted that age can influence the maternal immunity to malaria (30).

Soluble Chemokine Detection in Placental Plasmas

Protein arrays were used to detect levels of cytokines, chemokines, and soluble adhesion molecules between infection status groups. Because the arrays contained antibodies for thirty-six different soluble molecules, ten samples per infection status and gravidity (PM- primigravid, PM- multigravid, PM+ primigravid and PM+ multigravid) were chosen and pooled to obtain population levels of cytokine expression in placental intervillous blood. Cytokine expression was scored by comparing PM+ groups with the PM- multigravid group (Table 3.2). The PM- multigravid group was chosen as the comparison group because in having given birth multiple times and being malaria negative, their placentas have achieved immunologic protection, a conclusion that is supported by an abundance of epidemiological data. The purpose of this array was to observe approximate expression of soluble immunological molecules in each infection

status to select appropriate factors for further study. There were several observed differences between expression in the grouped PM+ samples, compared to the PM- multigravid reference group. There was an elevated expression of C5a, GRO- alpha, soluble ICAM-1, IL-1 beta, IL-6, MIF, MIP-1 alpha, MIP-1 beta and SDF-1 in PM+ samples. It should also be noted that sICAM-1 is also increased in the PM- primigravid samples. Within the PM+ samples, the primigravid group expressed higher levels of GRO- alpha, soluble ICAM-1, IL-6, IP-10, ITAC, MIP-1 alpha, MIP-1 beta, serpin and RANTES than the PM+ multigravid group. This is of interest due to the detrimental effects of PM infection seen most frequently in primigravid women (2), that may be related to immunological differences between PM+ primigravid women and PM+ multigravid women (9). Based on these results, soluble ICAM-1, IP-10, MIP-1 alpha, MIP-1 beta, and RANTES were chosen for more in depth detection via ELISA. In addition, MIG was chosen for detection via ELISA because of the similarity between MIG and IP-10, described below, and where a previous report detected IP-10 in the intervillous space of PM+ samples (22), there have been no studies reporting MIG expression in context of PM infection.

Briefly, chemokines chosen to study in further detail in this study are produced by activated immune cells and attract other immune cells bearing the corresponding chemokine receptor. Monokine-induced by IFN- gamma (MIG) and IFN- gamma Inducible Protein (IP)-10 are chemokines produced by IFN-gamma activated macrophages that activate immune cells expressing the CXCR3 receptor. Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES) is produced by T cells, platelets, and epithelial cells and recruits T cells, monocytes, dendritic cells and natural killer cells that express receptors CCR1, CCR3, CCR5, or CCR9. Macrophage Inflammatory Protein (MIP) - 1 alpha and MIP-1 beta are both produced by macrophages and recruits T cells, monocytes, dendritic cells and natural killer cells and bind to

CCR5. Intercellular Adhesion Molecule -1 (ICAM-1) is expressed on leukocytes, epithelial and endothelial cells and serves as an adhesion molecule for immune cells expressing lymphocyte function associated antigen (LFA)-1 (CD11a/CD18) and the leukocyte integrin MAC-1 (CD11b/CD18). Up-regulated expression by IFN-gamma and TNF-alpha stimulation, ICAM-1 can be cleaved from the surface to serve as a regulatory molecule by competing for previous mentioned ligands and preventing cellular adhesion. Fractalkine (FKN) is a surface-bound adhesion molecule expressed on endothelial and epithelial cells that can be cleaved as a chemokine that recruits T cells and monocytes expressing the CX3CR1 receptor, specific solely for FKN.

Peripheral and placental chemokine levels often differ (40), given the unique placental environment required for successful pregnancy. In context of PM infection, there are several differences between PM- and PM+ samples as well as differences between peripheral and placental samples. As shown in Figure 3.1A, FKN levels differ significantly between the periphery and the placenta in PM- (proc multtest p=<0.0001) and between PM- placenta and PM+ periphery (proc multtest p=<0.0001), with the former being higher. MIG levels differ between the periphery and the placenta in PM+ samples (proc multtest p=<0.0001), as shown in Figure 3.1B. There is also a significant difference in MIG expression between PM- and PM+ placental samples (proc multtest p=<0.0001) and between PM- peripheral and PM+ placental samples (proc multtest p=<0.0001), with the higher expression observed in PM+ samples. The levels and pattern of expression seen with MIG expression are similar for IP-10 expression. As shown in Figure 3.1C, there is a significant difference in IP-10 expression between the PM+ periphery and PM+ placenta (proc multtest p=<0.0001). There is a significant difference in IP-10 expression between PM- peripheral and PM+ placental samples (proc multtest p=<0.0001)

and a slight difference between PM+ and PM- placental levels (proc multtest p=0.059). The levels of RANTES expression (Figure 3.1D) are also increased in the placenta, regardless of infection status (KW p=0.0008), though after further statistical testing, the only statistical significance was found between PM+ peripheral and PM-placental samples (proc multtest p=0.002). Similar to FKN (Figure 3.1A), there is a trend toward increased RANTES expression in the placentae of PM- women, when compared to the periphery of PM+ women (proc multtest p=0.002). There is a significant difference of MIP-1 alpha expression (KW p=<0.0001), as shown in Figure 3.1E, though no statistical significance was found between groups with further statistical tests. The expression pattern of MIP-1 beta is shown in Figure 3.1F and is similar to that of MIP-1 alpha. While MIP-1 beta is significantly different within the four groups (KW p=<0.0001), the difference is not significant after performing more rigorous tests. These statistical results suggest a trend, seen clearly in the graphs, that the expression of these chemokines is different within the placenta. Lastly, Figure 3.1G shows the expression of sICAM-1, which is slightly increased in the placenta of PM+ samples when compared to the PM+ peripheral expression (proc multtest p=0.08).

Due to the immunological differences in chemokine expression that we observed with the protein array between paucigravidae and multigravidae, chemokine levels in placental samples were detected and grouped according to gravidity to determine differences in chemokine levels that may exist between the new and experienced gravid woman. As shown in Figure 3.2A, fractalkine (FKN) protein levels appear decreased with the occurrence of PM infection in paucigravid women when compared to PM- women, though this is not a statistically significant difference. The increased expression of MIG in paucigravid PM+ women is significantly higher than PM- samples (PM- paucigravid: proc mullets p=0.002, PM- multigravid: p=0.0009) and

PM+ multigravid samples (proc multtest p=0.0001; Figure 3.2B). There is no significant difference in expression of IP-10 between the four groups, though there does appear to be a marginal increase in the PM+ paucigravid women (Figure 3.2C). It should be noted that there is a slight increase, though not statistically significant, in MIG and IP-10 expression in PM- paucigravidae when compared to PM- multigravidae. In this study, RANTES expression is different between groups (KW p=0.04), although none of these differences are robust enough to withstand the rigor of pairwise comparisons with correction for multiple testing. Interestingly, the apparent expression pattern observed in the graph is different from previous reports, which detected no statistically significant changes in RANTES expression in the placenta (21, 22). Also detected in this study are different levels of MIP-1 alpha (KW p=0.005) and MIP-1 beta (KW p=0.02) between groups (Figure 3.2E, F). It should be noted that the increase of MIP-1 beta in PM+ samples appears lower in paucigravid samples than in multigravid samples; although for all parameters tested the latter were of limited sample size, making robust analysis difficult. Lastly, Figure 3.2G shows the marginal increase in soluble ICAM-1 in PM+ paucigravid women compared to PM+ multigravid (proc multtest p=0.08). It should be noted that there appears to be increases in FKN and MIG in PM+ paucigravid samples, when compared to PM+ multigravid samples, that lack statistical significance, possibly due to the insufficient sample size of the latter group.

Overall, PM+ paucigravid samples express lower levels of FKN and RANTES and higher levels of MIG, IP-10, and MIP-1 beta when compared to PM+ multigravid samples and PM- samples. These findings reveal more differences between the PM+ paucigravid women, PM+ multigravid women, and PM- women that may contribute to the pathogenesis and susceptibility of paucigravid women to PM infection.

Chemokine Receptor Expression on Maternal Leukocytes within the Intervillous Space

In agreement with previous reports (10, 11, 21-23), this study shows that there are several significant increases in placental expression of several chemokines during malaria infection. To determine the chemokine contribution to maternal cell infiltrate within the intervillous space, the CX3CR1, CCR4 and CCR5 chemokine receptors were detected via flow cytometry.

Fractalkine (CX3CL1) is the only known chemokine to bind to the CX3CR1 chemokine receptor that is expressed on T cells and monocytes (39). There is no statistically significant difference in the percent of T cells expressing CX3CR1 in PM+ placentas, compared to PM+ periphery and there is also no statistical difference in the percent of T cells expressing CX3CR1 observed between PM+ placental expression and PM- placental expression, as shown in Table 3.3 However, there is a lower percent of monocytes expressing CX3CR1 in the PM+ peripheral samples when compared to PM- placental levels (proc multtest $p=0.01$) and a lower percentage in PM+ placental samples when compared to PM- peripheral samples (proc multtest $p=0.02$). It should also be noted that there is a significant difference between PM- peripheral and placental samples (proc multtest $p=<0.0001$). There is also a significantly different intensity of CX3CR1 expression on monocytes (KW $p=0.001$). Also shown in Table 3.3 is the percentage of placental T cells and monocytes expressing CCR5 and the intensity of CCR5 expression. The percentage of T cells expressing CCR5 is not elevated during PM infection when compared to non-infected samples (Table 3.3). The expression of CCR5 on T cells is similar to CCR5 expression in monocytes, though the intensity of CCR5 expression on monocytes is much higher than on T cells.

Interestingly, it appears that the percentage of CX3CR1 expressing T cells and monocytes are increased in PM+ multigravid women compared to all other gravidity groups,

though this difference is not statistically significant (Table 3.4). Though this result may be influenced by a limited sample size of PM+ multigravidae, it suggests that cells with this phenotype are detected in women developing protective immunity against PM infection and is especially interesting in light of the decrease in soluble FKN levels detected in the placental plasma of PM+ multigravid women. It is possible that the apparent decrease of soluble FKN in PM+ multigravid women is a result of FKN binding to the CX3CR1 receptor of maternal immune cells, which renders it undetectable via ELISA. Table 3.4 also shows the expression of CCR4 and CCR5 on T cells and monocytes grouped via gravidity and infection status. Although not statistically significant, there appear to be decreased percentages of T cells expressing CCR5 and intensity of T cell expression of CCR5 in PM+ paucigravid women when comparing this group to PM- paucigravid or PM+ multigravid women. Interestingly, there also appear to be increased percentages of monocytes expressing CCR5 in PM+ paucigravid women, when compared to PM- multigravid women, and the intensity of monocyte CCR5 expression in PM+ paucigravid women is highest among all groups of gravidity and infection status. It should be noted that the expression of CCR5 on T cells is positively associated with CCR5 expression on monocytes (Pearson's $r=0.7$, $p=0.04$) in PM+ paucigravid women. There is no change in the percentage of T cells or monocytes expressing CCR4. Interestingly, there is a marginal increase in the intensity of CCR4 expression on these cells in both multigravid groups when compared to paucigravid groups, regardless of infection status, suggesting that cells expressing higher levels of CCR4 may be beneficial to healthier pregnancy outcomes. It is important to note that cell expression of CCR4 is related to Th2 immune responses, which are important to successful pregnancies.

Associations between Chemokine and Chemokine Receptors in the Intervillous Space

Figure 3.3 shows correlations between chemokine expression within the intervillous space, and corresponding chemokine receptor expression on T cells and monocytes. Data shown represent results from PM+ paucigravid placental samples (filled circles, solid line) and PM- multigravid placental samples (clear circles, dashed line). These two groups are compared in these correlations because PM+ paucigravid women are susceptible to the detrimental effects of PM infection, and presumably will have a different placental immunological environment when compared to non-infected, "protected" multigravid women.

Soluble FKN and CX3CR1-expressing T cell and monocyte correlations are shown in Figures 3.3A and B, respectively. There appears to be a positive correlation between FKN expression and CX3CR1 expressing T cells in PM+ paucigravid samples ($r=0.4$, $p=0.1$). Interestingly, there is a trend for decreasing CX3CR1-expressing T cells in samples with higher soluble FKN expression in PM- multigravid women. There appears to be no clear association between FKN expression and CX3CR1-expressing monocytes in PM+ paucigravid samples ($r=0.3$, $p=0.3$) or PM- multigravid samples ($r=0.05$, $p=0.8$). It should be noted that there is a small sample size for flow cytometry data on CX3CR1 expression on T cells and monocytes in PM+ paucigravid women due to logistical problems in Kenya which may influence the outcome of the results.

There is no association between RANTES expression and the percentage of T cells expressing CCR5 PM+ paucigravid women and a tendency for a negative association between RANTES expression and percentage of T cells expressing CCR5 in PM- multigravid women, (Pearson's $r=-0.1$, $p=0.8$ and $r=-0.1$, $p=0.8$, respectively, Figure 3.3C). Interestingly, there is a negative association between RANTES and CCR5 expressing monocytes in PM+ paucigravidae

(r=-0.7, p=0.04, Figure 3.3D), whereas there is no clear association with CCR5 expressing cells and RANTES in PM- multigravid samples (r=-0.3, p=0.2).

There appear to be slightly positive, though not significant, correlations between MIP-1 alpha and MIP-1 beta and CCR5 expression on T cells and monocytes in PM+ paucigravid women (Figure 3.3E-H). It should be noted that the intensity of T cell expression of CCR5 is weakly associated with MIP-1 alpha (Spearman's r=0.48, p=0.07) and more strongly associated with MIP-1 beta (Spearman's r=0.54, p=0.04) in paucigravid PM+ women (Figure 3.3I, J, respectively).

Surface Fractalkine Expression on Syncytiotrophoblast Cells

Surface expression of FKN was determined in placental tissues obtained from women living in malaria endemic areas of western Kenya by immunohistochemistry. Trophoblast cell surface FKN expression was up-regulated in placentas with placental malaria when compared to non-infected placental samples (Figure 3.4 and Table 3.5). Table 3.5 compares the presence of surface bound FKN on syncytiotrophoblast cells from PM- and PM+ placental samples, the presence of IVS maternal inflammatory cells and parasite counts. There is a small but not statistically significant increase in surface bound FKN in PM+ placentas (Mean ± standard deviation: 3.6 ± 0.6) compared to PM- placentas (3.3 ± 0.9). The expression of FKN is slightly increased in paucigravid placental samples (3.6 ± 0.8) compared to multigravid samples (0.9 ± 0.5), though this increase is also not statistically significant. There is a tendency for higher FKN expression in samples with IVS inflammatory cells compared to samples without inflammation (3.7 ± 0.6 and 3.3 ± 0.9, respectively). There is a significant increase in syncytiotrophoblast FKN expression in placental samples with high density parasite loads, classified as having parasite counts >15,000 per microliter of blood (2.2 ± 0.6), compared to low density parasite

loads, classified as having parasite counts 0-15,000 per microliter of blood (3.4 ± 0.5, p=0.044). There is a slightly higher level of soluble FKN detected in the IVS of samples with lower surface bound FKN (59 ng ± 45) than samples with higher surface bound FKN (53 ng ± 28), with lower surface bound FKN indicating a FKN score of 0-3.5 and higher surface bound FKN indicating a FKN score of 2-3. Additional testing will be required to confirm, but if FKN is being cleaved from the surface of syncytiotrophoblast cells, it is logical to expect a higher amount of soluble FKN in placental plasma samples derived from placentae with lower syncytiotrophoblast surface-bound FKN expression.

Fractalkine Secretion from Syncytiotrophoblast Cells

As shown in Figure 3.5, syncytiotrophoblast cells exposed to infected erythrocytes produced soluble FKN whereas very little soluble FKN was detected in supernatants collected from syncytiotrophoblast cells exposed to uninfected erythrocytes. Also of note is that FKN in supernatants from cells exposed to cytoadherent infected erythrocytes is time dependent, decreasing after eight hours of exposure to infected erythrocytes. One reason that there may be a decrease in soluble FKN detected after eight hours is instability of the protein. The stability of soluble FKN in solution has not been determined.

Placental Chemokine Levels and Placental Malaria Pathogenesis

The immunological environment of the placenta is capable of influencing the outcome of the pregnancy, especially in the context of infection. Due to the occurrence of PM+ pathogenesis seen most frequently in PM+ paucigravid women, chemokine levels were correlated with pathogenic factors of PM infection to determine if any correlations exist between the PM+ paucigravid placental immunological environment and the detrimental effects of PM+ infection. The chemokine levels and placental parasitemia of PM+ paucigravid women are

shown in Figure 3.6. While there is a significant increase in surface bound FKN in samples with high parasitemia (Table 3.5), there appears to be no association between soluble FKN and placental parasitemia (r=0.06, p=0.7, Figure 3.6A). In addition to a significantly higher level of MIG protein expression in PM+ paucigravid women when compared to PM+ multigravid and PM- women (Figure 3.2B), MIG is positively associated with placental parasitemia (Pearson's r=0.35, p=0.02). Although there is no association between IP-10 expression and placental parasitemia (r=0.05, p=0.7, Figure 3.6C), IP-10 is positively associated with CD3+ T cell infiltrate (Pearson R=0.86, p=0.01) and monocyte count (Pearson's r=0.7, p=0.003). There is a trend for a negative association between RANTES and soluble ICAM-1 and placental parasitemia, as shown in Figure 3.6D and G, respectively (Pearson's r=-0.1, p=0.4 and r=-0.33, p=0.06). Also of note are weak negative associations between MIP-1 alpha (Spearman's r=-0.29, p=0.05; Figure 3.6E) and MIP-1 beta and parasitemia (r=-0.31, p=0.06; Figure 3.6F).

Pigment deposition is one of the hallmark signs of PM. Figure 3.7 shows the association between chemokine levels and pigment deposition, as determined via blood smear, of PM+ paucigravid women. Fractalkine expression is weakly negatively associated with pigment deposition (Pearson's r=-0.4, p=0.06; Figure 3.7A). Figures 3.7B shows a positive trend towards MIG expression and the presence of pigmented cells (Pearson's r=0.39, p=0.07), a trend interestingly not observed with IP-10 expression (r=0.38, p=0.07, Figure 3.7C). As shown in Figure 3.7D, there appears to be no association between RANTES expression and pigment deposition (r=-0.1, p=0.5). There is a positive association between MIP-1 alpha and MIP-1 beta and pigment deposition (Spearman's r=0.2, p=0.05 and r=0.38, p=0.02, Figures 3.7E and F). There is also a trend for increased sICAM-1 expression and higher pigment deposition (Figure 3.7G), though this is not a statistically significant association (r=0.1, p=0.3).

Figure 3.8 shows chemokine level associations with birth weight. Fractalkine and MIP-1 alpha levels (paucigravid: Pearson's r=0.2, p=0.3 and r=0.2, p=0.3, respectively) do not appear to be associated with the birth weight of babies born to PM+ paucigravid women (Figure 3.8 A, E, respectively). Interestingly, there does appear to be a positive association for FKN in PM- multigravid women (multigravid: r=0.2, p=0.3, Figure 3.8A). There is a tendency for decreased MIG levels and IP-10 levels and birth weight in PM+ paucigravidae (Pearson's r=-0.3, p=0.1, Figure3.8B and r=-0.26, p=0.2, Figures 3.8B, C, respectively) although these associations are not statistically significant. It should be noted that there is also no statistical association between MIG and IP-10 and birth weight in PM- multigravid women (r=-0.1, p=0.6 and r=-0.1, p=0.5, respectively). Interestingly, whereas there does not seem to be an association between RANTES (Pearson's r=0.3, p=0.1) and birth weight in PM+ paucigravid women, there does appear to be a positive tendency in the PM- multigravid women (r=0.2, p=0.3, Figure 3.8D). Though not statistically significant, there seems to be a positive association between MIP-1 beta and birth weight for PM+ paucigravidae (Pearson's r=0.02, p=0.9, Figure 3.8F), a trend that is negative in the PM- multigravidae (r=-0.1, p=0.5). Figure 3.8G shows the association between sICAM-1 and birth weight in PM+ paucigravidae and PM- multigravidae (Pearson's r=0.5, p=0.03 and r=-0.4, p=0.07, respectively). While the best fit line for sICAM-1 in PM+ paucigravid women appears to have a negative slope, the statistical analysis reveals a positive association between sICAM-1 and birth weight in these women. It is possible that the line appears to show a negative association due to the high variability present in human studies. Though sICAM-1 is the only factor statistically associated with birth weight, the negative expressions of MIG and IP-10 and positive expression of MIP-1 beta with increasing birth weight suggest that these chemokines may contribute birth outcomes, and require further investigation.

Chemokine Receptor Associations with Placental Malaria Pathogenesis

Associations between chemokine receptor expression on maternal T cells and monocytes isolated from the placenta and the placental parasitemia of PM+ paucigravid women are shown in Figure 3.9. There is a tendency for decreased expression of CX3CR1 on T cells with increasing placental parasitemia (Pearson's r=-0.37, p=0.2, Figure 3.9A) and a significantly positive association between CX3CR1 expression on monocytes and placental parasitemia (Pearson's r=0.63, p=0.02, Figure 3.9B). While there is one outlier that influences the best fit line of the correlations in Figures 3.9C and D, there appears to be an increased expression of CCR5 on T cells (Pearson's r=0.2, p=0.4) and a decreased expression of CCR5 on monocytes (r=-0.1, p=0.5) with increasing placental parasitemia. The CCR4 expression on T cells shows a decreasing tendency with increasing parasitemia, and CCR4 expression on monocytes shows a slightly increasing tendency with increasing parasitemia (Pearson's r=0.51, p=0.2, Figure 3.9E and r=0.09, p=0.7, Figure3.9F, respectively). It should be noted that in Figure 3.9E and F there are outliers that may influence the outcome of the best fit lines showing the associations between receptor expression and placental parasitemia.

Chemokine receptor expression is also associated with pigment deposition in PM+ paucigravid women. There appears to be a tendency for higher percentage of T cells expressing CX3CR1 (Pearson's r=0.2, p=0.5) in samples with higher pigment deposition (Figure 3.10A), although there is a significantly negative correlation between CX3CR1 expression on monocytes and pigment deposition (r=-0.64, p=0.02, Figure 3.10B). Pigment deposition is not correlated with intensity of CCR5 expression on T cells (r=0.04, p=0.8, Figure 3.10C) but is strongly correlated with the intensity of CCR5 expression on monocytes (r=0.85, p=0.003, Figure 3.10D). The correlation between T cell CCR4 expression and pigment deposition is inconclusive (r=0.2,

p=0.6), given a small sample size and high variability between samples that may influence the data. There seems to be a decrease in CCR4 expression on monocytes with increasing pigment deposition (r=-0.4, p=0.09, Figure 3.10F).

Chemokine receptor expression and birth weight associations are shown in Figure 3.11. Figures 3.11A and B show a striking, though not statistically significant, tendency for positive CX3CR1 expression on T cells and monocytes with increasing birth weight in PM+ paucigravid women (Pearson's r=0.3, p=0.5 and r=0.06, p=0.9, respectively). This tendency for increased percentage of CX3CR1 expressing T cells is seen also in PM- multigravidae (r=0.2, p=0.4) though the trend is negative in PM- multigravid monocytes (r=-0.4, p=0.1). Interestingly, there is a negative association between percent of T cells expressing CCR5 and birth weight in PM+ paucigravid and PM- multigravid women, although the association is stronger in PM+ paucigravid (paucigravid: Pearson's r=-0.62, p=0.03, multigravid: r=-0.45, p=0.04, Figure 3.11C). There is a negative trend that is also seen in with the percentage of monocytes expressing CCR5 and birth weight, though this is not significant (paucigravid: r=-0.5, p=0.1, multigravid: r=-0.3, p=0.1, Figure 3.11D). Lastly, there appears to be no association between CCR4 expression on T cells and birth weight (paucigravid: r=0.03, p=0.9, and multigravid: r=0.1, p=0.5, Figure 3.11E) though CCR4 expression on monocytes is slightly negatively correlated with birth weight (paucigravid r=-0.6, p=0.059 and multigravid: r=0.5, p=0.02, Figure 3.11F).

DISCUSSION

In placental malaria, it is thought that the immune response in the intervillous space may serve in a protective role by controlling malaria infection, but if dysregulated may also contribute to the detrimental effects of the infection. The detection of immune factors and corresponding

immune cell presence in the intervillous space of the placenta may provide insight into the contributions of these factors towards protection or pathogenesis of PM.

Within this study population, women participating in this project were divided into groups based on infection status (PM- or PM+) and gravidity (paucigravid or multigravid). Women carrying their first or second pregnancy are faced with a more difficult immunological challenge during infection given that the parasites capable of adhering to the syncytiotrophoblast cells express a protein rarely seen in the periphery of non-pregnant women, men, or children (13). Because PM+ multigravid women in this study population have almost certainly been exposed to malaria during previous pregnancies and have begun building immunity to placental malaria, the resulting immunological environment within the placenta is unlikely to reveal associations between immunological factors and the pathogenesis of PM infection. Based on this and in the context of this study, it was decided that comparisons between PM+ paucigravid women and PM- multigravid would provide a stronger observation of the contributions that immunological factors may have in the pathogenesis of placental malaria. Also, because the exposure to malaria during pregnancy is expected to be constant for all groups, the multigravid women have developed a protective immune response against malaria infection. Additionally, within this population of women, PM+ paucigravid women birthed babies of lower weight than the PM- multigravid women and suffered higher parasite loads than the PM+ multigravid women, confirming the susceptibility of this group of women to the detrimental effects of PM infection.

The placenta is an organ with unique characteristics, requiring an immunologically different environment for pregnancies to be carried to term successfully. In general, chemokine levels in this study were shown to be elevated in the placental intervillous space, regardless of

infection status. Of particular interest, MIG, IP-10 and sICAM-1 levels were significantly increased in the placentae of PM+ women, compared to PM- women. There also appear to be slight increases of MIP-1 alpha and MIP-1 beta in the placentae of PM+ women. These data suggest that, though these factors are generally increased in the placenta, the occurrence of PM infection further influences the expression levels of these factors and therefore influences the delicate placental immunological environment. Also of note are a significant decrease in FKN expression and RANTES expression in the placentae of PM+ women, compared to PM- women, again suggesting that PM infection is altering the levels of chemokines present in successful, 'protected' pregnancies. These results agree with previous reports illustrating the immunological difference in the placenta, compared to the periphery, in context of PM infection (9, 41) and provide a rationale for studying immunological events in the placenta in more detail.

Given that the immunological environment of the placenta needed to maintain healthy and full term pregnancies is biased toward a Th2 response, infection with malaria causes complex change, shifting this response to a Th1 bias in order to control the infection. This shift can result in damage to the placenta and the pregnancy, and is typically more frequent in paucigravid women. In the context of PM infection, there were significantly higher levels of MIG and soluble ICAM and slight increases in IP-10, MIP-1 alpha and MIP-1 beta in the placentae of PM+ paucigravid women, when compared to PM+ multigravid and PM- women. Although it has been previously shown that IP-10 is increased during PM infection (22), the increase of MIG protein is a novel finding. Due to the high homology between MIG and IP-10 (33), it is perhaps not surprising that the two chemokines are elevated in PM infection similarly. What is surprising, however, is the novel finding that MIG and IP-10 may be contributors to the immunological pathology of PM infection, shown in the significant increases in these

chemokines with pigment deposition, the increased expression with increasing placental parasitemia and higher expression with lower birth weights. Produced in higher levels during PM infection, it is reasonable to assume that these two chemokines may attract maternal cells to the intervillous space that contribute to the pathogenesis and poor birth outcomes of this disease. These chemokines are produced by macrophages activated by IFN- gamma and attract T cells and a small percentage of monocytes expressing the CXCR3 receptor. Qin et al have shown that the expression of CXCR3 is commonly found on activated and memory T cells and is frequently found in conjunction with CCR5 expression and increased adhesion molecule expression. That report also suggests that CXCR3 is expressed on activated T cells in a similar pattern to CCR5 and that cells expressing both receptors are often associated with migration to sites of inflammation (34). Clearly, it is critical to examine CXCR3 expression on maternal immune cells within the intervillous space, and studies are currently underway to detect CXCR3 via immunohistochemistry that may later be correlated with the expression of these chemokines. Studies are also underway to investigate associations with adhesion molecule expression and the presence of CD4+ memory T cells.

There have been conflicting reports of the levels of MIP-1 alpha and MIP-1 beta in PM infection (21-23), though it has been shown that there is some association between the MIP chemokines and monocyte accumulation (21) and pigment deposition (23). The MIP chemokines are closely related, and share close to 50% homology with RANTES (Reviewed in 36), another chemokine included in this study to be described below. These three chemokines attract cells expressing the CCR5 receptor, a receptor that is expressed only on activated T cells of the Th1 immune response and on mature macrophages, both of which are seen in the placenta during PM infection. Produced by activated lymphocytes and mononuclear cells, the expression

of both MIP chemokines is greatly enhanced by IFN-gamma (Reviewed in 35), another immunological factor present in PM infection (9). This study showed a slight increase of MIP-1 alpha and MIP-1 beta expression in PM+ infection, though the differences between gravidity groups were not significant. In agreement with Chaisavaneeyakorn et al, the present study shows an increased level of expression of both MIP chemokines with higher pigment deposition in PM+ infection, and furthers these findings by showing a significantly positive correlation with pigment deposition in PM+ paucigravid samples. It has also been shown that monocytes in PM+ placentae have an increased CCR5 expression (24), which is a chemokine receptor for the MIP chemokines and RANTES. In this study, both MIP chemokines were positively associated with CCR5 intensity of expression on T cells and revealed a trend for increased CCR5 expression on monocytes in PM+ paucigravid women, suggesting that these chemokines are contributing to the recruitment of CCR5 expressing maternal cells. In addition, there was also a highly significant association between CCR5 expression on monocytes and a trend for increased CCR5 expression on T cells and pigment deposition. Taken all together, the data suggest that the MIP chemokines may recruit CCR5 expressing cells to the placenta which in turn contribute to the pathogenesis of PM in paucigravid women. Interestingly, the expression of CCR5 on both T cells and monocytes show convincing trends for decreased expression with increasing birth weights, suggesting that the absence of cells expressing CCR5 is associated with improved pregnancy outcomes. Although there appears to be no association with these chemokines and increasing birth weights, the data show that the MIP chemokines are elevated in context of malaria infection and may contribute to the recruitment of CCR5 expressing T cells and monocytes. That the presence of cells expressing CCR5 is associated with pigment deposition and lower birth weights in PM+ paucigravid women further suggests that these cells are contributing to the pathology of

PM infection. Furthermore, there is a slight increase in the intensity of CCR4 expression on T cells in multigravid women when compared to paucigravid women, regardless of infection status, suggesting that cells expressing CCR4 are relevant to experienced and 'protected' pregnancies. Also of note is a decreased expression in the percentage of CCR4 expressing T cells with increasing placental parasitemia in PM+ paucigravid women. The percentage of monocytes expressing CCR4 in PM+ paucigravid women slightly decreases with increasing presence of pigmented leukocytes, and appears to increase with increasing birth weight. These data further suggest that the occurrence of PM alters the Th2 environment and that the absence of cells expressing CCR4 may be linked to the pathology of infection.

This study has shown an up-regulation in the placental levels of the chemokines MIG, IP-10, MIP-1 alpha and MIP-1 beta, all of which are capable of attracting monocytes and T cells. Interestingly, the phenotype of activated T cells expressing CXCR3 and CCR5 is associated with inflammatory responses (34). It is possible that, with the initial influx of maternal cells infiltrate recruited to the placenta in response to PM infection, cytokines and chemokines are produced in higher levels to activate and recruit more cells to ward off the infection. Chemokines such as MIG, IP-10 and the MIP chemokines, may be produced by activated macrophages within the placenta to recruit more macrophages and T cells. The newly arrived immune cells, activated and functioning, may produce additional chemokines to recruit more immune cells, resulting in a superfluous amount of maternal cells within the intervillous space. This compaction of cells can contribute to decreased nutrient exchange between the mother and fetus, due to physiologically limited area of the intervillous space.

In this study, RANTES expression was detected in increased amounts in placental samples, regardless of infection status. Abrams et al failed to observe an increase in RANTES

expression or any association between RANTES expression and monocyte infiltrate during PM infection. This study supports those findings, detecting significantly lower expression of RANTES in PM+ paucigravid women when compared to PM- women and revealing a negative trend between RANTES expression and CCR5 expression on monocytes. It should be noted that there appears to be no statistically significant association between RANTES and birth weight in PM+ paucigravidae samples, an association that appears convincingly positive in PM- multigravid women. In a study of malaria infection in children, the suppression of RANTES production was observed in children having suffered from severe malaria infection, compared to children having only experienced mild malaria infection, suggesting that the suppression of RANTES may contribute to disease severity (31). It is possible, given the decreased levels of RANTES expression in PM+ paucigravidae, that the suppression of RANTES contributes to poor birth outcomes, and the acquisition of more samples may be required to study this possibility. The suppression of RANTES may contribute to a decrease in memory T cell recruitment and activation, resulting in inefficient parasite clearance. Without efficient parasite clearance, the maternal cells within the intervillous space will continue to produce chemokines in response to the parasites to recruit more cells, resulting in the accumulation of immune cells that are harmful to the pregnancy, as discussed previously. Interestingly, this study shows that PM- paucigravid women express the highest levels of RANTES. As mentioned above, a possible suppression of RANTES in context of malaria infection may explain the difference observed between the paucigravid groups. The increase observed in these women when compared to multigravid women may be explained simply through the number of pregnancies. Paucigravid women in general may be considered ‘new’ to the changes pregnancy warrants, and therefore may produce more of this chemokine than multigravid women.

Fractalkine is a novel chemokine, the only member of the CX3C chemokine family, and the only known ligand for the CX3CR1 receptor (39). In addition to a soluble form, as was detected in placental plasma, fractalkine is also expressed as a surface-bound molecule capable of adhering and attracting leukocytes, and has also been detected as a possible adhesion molecule for infected erythrocytes in cerebral malaria (10). Scantly expressed on the surface of endothelial and epithelial cells, membrane bound FKN is up-regulated in response to pro-inflammatory cytokines TNF-alpha and IFN-gamma, cytokines that are also prevalent in placental malaria infection. Novel findings in the present study include an up-regulation of surface-bound FKN expression in PM samples with high parasitemia and an increase in FKN cleavage from syncytiotrophoblast cells *in vitro* in response to cytoadherent *Plasmodium falciparum*- infected erythrocytes, suggesting that, as in cerebral malaria, FKN may contribute to the immunological response of PM. For example, it is possible that surface-bound FKN may serve as additional adhesion molecule within the placenta and the cleavage of surface-bound FKN into the soluble form may be one response of the syncytiotrophoblast to PM infection, though the details and methods of studying this phenomenon require further investigation. Interestingly, FKN was detected at marginally lower levels in PM+ paucigravid plasma samples when compared to PM- women in this study. Given the positive association between FKN expression and T cell expression of CX3CR1, and a slight increase in CX3CR1 expression on monocytes in PM+ multigravid women, it may be plausible to assume that the lower levels of FKN detected in PM+ women are due to ligation with the CX3CR1 receptor, thus failing detection via ELISA. It should be noted, however, that there is not a significant increase in the percent of cells expressing CX3CR1 in PM+ paucigravid women and the decreased FKN levels in PM+ paucigravidae are most likely not due to receptor ligation. Taking this into

consideration, along with the absence of an association between FKN and placental parasitemia or birth weight, and a negative association between FKN and pigment deposition in PM+ paucigravid women, it seems unlikely that FKN is attracting cells associated with the pathological aspects of PM infection in PM+ paucigravid women. It is also plausible to speculate that the cells expressing a higher CCR5 and lower CX3CR1 phenotype, as detected in PM+ paucigravid samples in this study, may contribute to the pathogenic factors of PM infection, whereas cells expressing a lower CCR5 and higher CX3CR1 phenotype, as detected in PM+ multigravid samples, may contribute to the immunological protection against PM+ effects. It should be noted, however, that detection of CCR5 and CX3CR1 was not simultaneous in these samples, and it would be worthwhile to simultaneously study the expression patterns of CCR5 and CX3CR1 within the same cell population and in context of PM infection. While surface-bound expression of FKN is up-regulated in placentae with high parasitemia and could theoretically serve as an additional adhesion molecule for infected erythrocytes, it appears that the expression and presence of CX3CR1 expressing immune cells is associated with the development of protective immunity to PM infection observed in PM+ multigravid women.

In addition to the chemokines mentioned above, increased levels of sICAM-1 were detected in the placentas of PM+ paucigravid women in this study. Soluble ICAM (sICAM)-1 has been detected previously in increased levels in context of cerebral malaria (16) and in placental malaria (15), though the latter increase reported by Jakobsen et al was not statistically significant. The present study shows a statistically significant increase in the level of sICAM-1 in PM infection of paucigravid women, an increase which is absent in PM negative women and in PM+ multigravid women. Activated immune cells, such as T cells and monocytes, can display increased expression of surface-bound ICAM-1 and is sometimes released from the cell

surface via cleavage by tumor necrosis-alpha converting enzyme (TACE) (17). This shedding may be necessary for immune regulatory functions (Reviewed in 18). The cytokines TNF-alpha and IFN-gamma induce the shedding of ICAM-1 in localized inflammation sites where there are several functions that sICAM-1 may fulfill. One is that sICAM-1 becomes a soluble competitor for ligands LFA-1 and Mac-1 expressed on lymphocytes and mononuclear cells. By binding to those ligands, sICAM-1 prevents those cells from adhering to surface-bound ICAM-1 and therefore decreases cellular adhesion. This is plausible in context of PM , as the cytokines that induce ICAM-1 shedding are prevalent in the placenta during infection and the shedding of ICAM-1 may contribute to a reduction in the maternal cell load within the intervillous space. Another function for this shedding is to decrease the actual surface expression of ICAM-1 on lymphocytes and mononuclear cells, therefore preventing those immune cells from binding to surface bound adhesion molecules expressed by other immune cells, epithelial or endothelial cells. It has also been shown in murine cell lines that the presence of sICAM-1 can induce the production of cytokines (19). It is possible, considering the possible regulatory roles of sICAM-1, that this molecule may be involved in the regulation of the maternal inflammation observed in PM by preventing the adhesion of maternal immune cells. Sugiyama et al reported an increased ICAM-1 expression on monocytes in PM that is correlated with maternal leukocyte infiltrate and suggest that this surface-bound ICAM-1 expression aids in maternal cellular adhesion to infected erythrocytes and fibrin deposits in PM infection. In this report, the increased sICAM-1 levels in PM+ paucigravid women suggest that sICAM-1 is shed from maternal cells expressing surface bound ICAM-1 in an immunoregulatory attempt to decrease cellular adhesion and aggregation within the intervillous space. Interestingly, there is a slightly positive association between sICAM-1 and placental parasitemia, and sICAM-1 and pigment deposition, though neither is

significant, which may imply that cleavage of sICAM-1 is one mechanism that can regulate the maternal infiltration responding to malaria infection in PM+ paucigravid women.

Given that, to some degree, a heightened immune response is necessary to clear malaria infection, there are situations when the over expression or possibly the suppression of immunological factors contribute to the pathogenesis of the infection. This study describes the associations between pathology of placental malaria infection and the increased expression MIG, IP-10, MIP-1 alpha, MIP-1 beta and the suppression of RANTES. Also shown are associations of CCR5 expression on maternal immune cell infiltrate with pathogenic factors of PM infection. It also describes possible associations of FKN and cells expressing CX3CR1 with improved pregnancy outcomes and a possible regulatory role for sICAM-1 in PM infection. These findings contribute to the determination of immunological factors and their contribution to the pathogenesis or protection of PM infection.

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Table 3.1

Clinical parameter table

The clinical parameters of subject age, birth weight (grams), hemoglobin levels (g/dL), % placental parasitemia, parasite counts per uL of blood, and pigment scores are given in Table 3.1. Women with placental parasitemia are considered PM+. Data shown represent the mean ± standard deviation for each gravidity group of PM- and PM+ infection status groups. Statistical analysis was conducted via Kruskal-Wallis test, and significant findings were further analyzed via PROC MULTTEST. The age is slight difference between PM- multigravid and PM+ paucigravid groups (KW p=0.008, proc multtest p=0.06), and PM- multigravid and PM+ multigravid groups (p=0.0006). There is a significant difference in parasite counts between PM+ paucigravid women and PM- paucigravid women (proc multtest p=0.001), PM- multigravid women (p=0.001) and PM+ multigravid women (p=0.0004). The pigment score, described in the materials and methods, is significantly different between PM+ women and PM- women (proc multtest, p=<0.0001 for paucigravidae and multigravidae) and between PM+ paucigravid women and PM+ multigravid women (p=<0.0001).

Table 3.1

	PM- Paucigravid	PM- Multigravid	PM+ Paucigravid	PM+ Multigravid
Age	20 ± 3 N=90	28 ± 6 N=65	20 ± 3 N=55	127 ± 5 N=8
Birth Weight (g)	3218 ± 417 N=71	3370 ± 434 N=51	3090 ± 492 N=43	3500 ± 166 N=8
Hemoglobin (g/dL)	12 ± 2 N=78	11 ± 3 N=59	10 ± 3 N=50	10 ± 3 N=8
% Placental Parasitemia	0 ± 0 N=89	0 ± 0 N=63	9 ± 16 N=54	3 ± 7 N=8
PlacentalParasite Count (per uL blood)	0 ± 0 N=89	0 ± 0 N=63	39 ± 87 N=54	15 ± 34 N=8
Pigment Score	0 ± 0.1 N=89	0 ± 0 N=63	1 ± 1 N=54	1 ± 1 N=8

Table 3.2

Protein array for cytokine, chemokine, and adhesion molecule detection in placental intervillous blood

Plasma samples were pooled according to infection status (PM- or PM+) and gravidity (primigravid, women carrying their first pregnancy, and multigravid, women carrying their third or more pregnancy) to obtain population cytokine, chemokine, and soluble adhesion molecule expression. Results of the assay were determined by comparing all infection status groups and gravidities to the PM- multigravid group. Data from this reference group are not shown and are denoted with – in the table. Only cytokines with clear increases or decreases are shown. Small increases, one fold higher than the reference group, are indicated in the table by +, ++ indicates a two fold increase and +++ indicate a three fold increase, and no increase is indicated by 0.

Table 3.2

Cytokine	PM- primigravid	PM- multigravid	PM+ primigravid	PM+ multigravid
C5a	+	Reference group	++	++
GRO- alpha	++	-	+++	+
sICAM-1	++	-	++	+
IL-1 beta	+	-	++	++
IL-1 ra	++	-	+	+
IL-6	+	-	++	+
IL-8	++	-	+	+
IL-16	+	-	+	+
IP-10	0	-	++	0
ITAC	0	-	+	0
MIF	++	-	++	+
MIP-1 alpha	0	-	++	+
MIP-1 beta	+	-	++	0
Serpin	++	-	++	+
RANTES	++	-	++	+
SDF-1	0	-	+	+

Table 3.3

Chemokine receptor expression on maternal leukocytes within the intervillous space

The percent of T cells and monocytes expressing chemokine receptors and the intensity of receptor expression were detected via flow cytometry. The CX3CR1, CCR5, and CCR4 receptors were detected. Statistics were calculated using Kruskal-Wallis test, with significant findings further tested for pairwise comparisons using PROC MULTTEST. Data are presented as median (interquartile range) for % receptor expression on T cells and monocytes, and for intensity of receptor expression (MFI). There is a significant difference in PM- peripheral percentage of monocytes expressing CX3CR1 when compared to PM- placental (proc multtest p=<0.0001) and PM+ placental (p=0.02) monocyte percentages. There is also a difference between PM+ peripheral and PM- placental percentages of CX3CR1 expressing monocytes (p=0.01). Though the difference failed to withstand further rigorous analysis, there is a difference in the intensity of CX3CR1 expression on monocytes (KW p=0.001). There is also a difference in the percentage of monocytes expressing CCR4 (KW p=0.03).

Table 3.3

	Peripheral		Placental	
	PM-	PM+	PM-	PM+
T cell %CX3CR1	23 (16-31) N=47	20 (14-23) N=15	20 (9-30) N=48	21 (7-46) N=14
T cell CX3CR1 MFI	14 (12-22) N=47	21 (14-43) N=15	14 (11-19) N=48	21 (12-38) N=14
Monocyte %CX3CR1	34 (20-56) N=47	23 (12-45) N=15	7 (4-13) N=48	8 (5-21) N=14
Monocyte CX3CR1 MFI	30 (26-60) N=47	79 (35-186) N=15	46 (34-66) N=48	50 (43-104) N=14
T cell %CCR5	26 (1-38) N=40	13 (1-24) N=13	21 (2-43) N=48	5 (2-38) N=17
T cell CCR5 MFI	70 (44-105) N=40	71 (40-114) N=13	55 (36-94) N=48	60 (32-107) N=17
Monocyte %CCR5	20 (11-31) N=33	13 (4-24) N=18	20 (5-41) N=34	13 (3-49) N=19
Monocyte CCR5 MFI	156 (98-200) N=33	146 (82-196) N=18	111 (83-141) N=34	165 (99-269) N=19
T cell %CCR4	4 (1-13) N=30	2 (1-3) N=8	2 (1-5) N=48	2 (1-4) N=17
T cell CCR4 MFI	26 (23-45) N=30	46 (29-56) N=8	28 (22-53) N=48	36 (25-39) N=17
Monocyte %CCR4	1 (0-1) N=33	1 (0-1) N=18	1 (0-1) N=34	1 (0-1) N=19
Monocyte CCR4 MFI	48 (29-111) N=33	68 (41-112) N=18	51 (29-68) N=34	57 (38-82) N=19

Table 3.4

T cell and monocyte counts and CX3CR1 expression on maternal T cells and monocytes within the intervillous space

Chemokine receptor expression was analyzed via flow cytometry. The data shown represent the median %CX3CR1 on T cells and monocytes and the intensity of CX3CR1 expression on T cells and monocytes, with the interquartile range in parenthesis. T cell counts per microliter of blood (KW p=0.06) and monocyte counts per microliter of blood (KW p=0.01) are also included, with statistical significance, determined by the Kruskal-Wallis test.

Table 3.4

	PM- Paucigravid	PM- Multigravid	PM+ Paucigravid	PM+ Multigravid
T cell %CX3CR1	22 (12-33) N=23	14 (6-25) N=25	12 (6-36) N=12	43 (36-51) N=2
T cell CX3CR1 MFI	14 (12-16) N=23	16 (11-21) N=25	19 (12-38) N=12	49 (43-55) N=2
Monocyte %CX3CR1	7 (6-12) N=23	7 (3-14) N=25	8 (5-12) N=12	51 (43-60) N=2
Monocyte CX3CR1 MFI	40 (34-55) N=23	50 (39-70) N=25	48 (43-90) N=12	87 (70-103) N=2
T cell %CCR5	36 (4-42) N=21	14 (1-41) N=27	3 (3-28) N=14	37 (27-43) N=4
T cell CCR5 MFI	67 (40-101) N=21	54 (36-82) N=27	54 (34-104) N=14	79 (52-116) N=4
Monocyte %CCR5	25 (5-36) N=16	9 (3-43) N=19	13 (3-22) N=15	20 (5-38) N=5
Monocyte CCR5 MFI	112 (94-145) N=16	158 (105-257) N=19	219 (111-229) N=15	111 (84-141) N=5
T cell %CCR4	2 (1-6) N=16	2 (1-4) N=15	2 (1-5) N=8	1 (1-1) N=1
T cell CCR4 MFI	23 (22-33) N=16	40 (25-55) N=15	34 (23-42) N=8	38 (38-38) N=1
Monocyte %CCR4	0.2 (0-0.4) N=15	0.3 (0-0.5) N=19	0.5 (0.2-0.5) N=14	0.2 (0-0.4) M=5
Monocyte CCR4 MFI	45 (29-59) N=15	54 (34-71) N=19	54 (41-70) N=14	64 (49-82) N=5
Placental T Cell Count	8266 (6995-9451) N=15	10683 (8414-12072) N=12	10402 (7339-13578) N=7	4649 (4561-14999) N=3
Placental Monocyte Count	1856 (1302-2751) N=26	1987 (1333-2893) N=24	3003 (1923-3948) N=26	1957 (1655-4787) N=8

Table 3.5

Histology reports indicating strength of fractalkine expression on syncytiotrophoblast cells

Histology reports documented malaria infection status, gravidity, inflammation status, inflammatory cell infiltrate, and parasite counts in placental tissue samples. Fractalkine staining intensity was semi quantitatively scored by a placental pathologist on a scale from 0-4, with 0 indicating no fractalkine staining and 4 indicating intense fractalkine staining. Soluble FKN was detected via ELISA, ND= not determined.

Table 3.5

Sample ID	Malaria	Gravidity	FKN Score	Soluble FKN (ng)	Inflammation	Cell Infiltrate	Parasite Count
UGA1.124	-	1	1	0	no		0
UGA1.553	-	1	3	0	no		0
UGA1.121	-	1	1.5	0	no		0
UGA1.0070	-	2	2.5	56	no		0
UGA1.542	-	2	0	0	no		0
UGA1.597	-	2	2.5	ND	no		0
UGA1.145	-	2	0	0	no		0
UGA1.194	-	2	2	0	no		0
UGA1.223	-	2	1.5	121	no		0
UGA1.184	-	2	1	0	no		0
UGA1.389	-	3	1	ND	no		0
UGA1.402	-	3	1	0	no		0
UGA1.471	-	3	1	52	focal	neutrophils	0
UGA1.369	-	4	0	70	no		0
UGA1.261	-	5	1.5	0	no		0
UGA1.0041	+	1	1	0	no		187
UGA1.595	+	1	2.5	80	scant	neutrophils	187
UGA1.507	+	1	1.5	ND	yes	monoyctes	187
UGA1.549	+	1	1.5	ND	scant	neutrophils	320
UGA1.394	+	1	1	ND	focal	monoyctes	560
UGA1.398	+	1	1.5	15	focal	monoyctes	12240
UGA1.263	+	1	0	27	focal	monoyctes	131067
UGA1.558	+	2	1	47	scant	neutrophils	107
UGA1.358	+	2	1.5	ND	no		267
UGA1.407	+	2	2.5	ND	focal	neutrophils	102907
UGA1.395	+	2	1.5	18	scant	monoyctes	135947
UGA1.579	+	2	2.5	24	focal	monoyctes	136267
UGA1.544	+	4	1	ND	no		347

Figure 3.1

Chemokine expression in the periphery and placentae of PM- and PM+ women

Peripheral and placental plasma chemokine levels were detected via ELISA. Peri indicates peripheral samples and Plac indicates placental samples. Non-infected samples are denoted PM- and placental malaria infected samples are denoted PM+. Statistics reported are PROC MULTTEST results, or Kruskal-Wallis results as noted. A. Fractalkine levels: PM- periphery (n=52) versus PM- placenta (n=68), p=<0.0001. PM- placenta (n=68) versus PM+ periphery (n=42), p=<0.0001. B. MIG levels: PM- periphery (n=52) versus PM+ placenta (n=36), p=<0.0001. PM- placenta (n=55) versus PM+ placenta (n=36), p=<0.0001. PM+ periphery (n=42) versus PM+ placenta (n=36), p=<0.0001. C. IP-10 Levels: PM- periphery (n=52) versus PM+ placenta (n=37), p=<0.0001. PM+ periphery (n=42) versus PM+ placenta (n=37), p=<0.0001. PM- placenta (n=56) versus PM+ placenta (37), P=0.059. D. RANTES levels: PM- placenta (n=55) versus PM+ periphery (n=38), p=0.002. E. MIP-1 alpha Levels: KW p=0<0.0001. F. MIP-1 beta levels: KW p=<0.0001. G. sICAM-1 levels: PM+ periphery (n=42) versus PM+ placenta (n=36), p=<0.08.

Figure 3.1

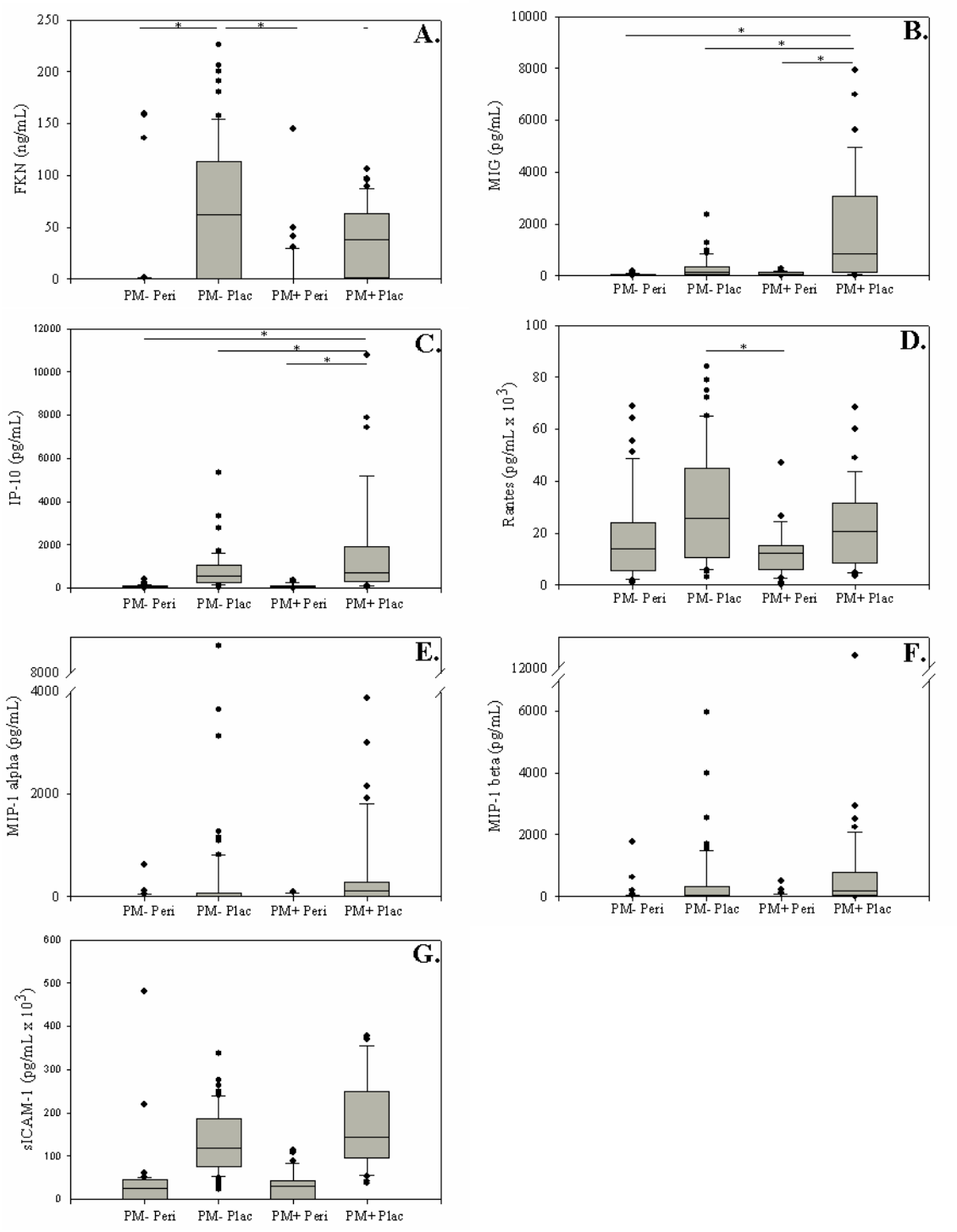

A.
FKN (ng/mL)
250
200
150
100
50
0
PM- Peri
PM- Plac
PM+ Peri
PM+ Plac
B.
MIG (pg/mL)
10000
8000
6000
4000
2000
0
C.
IP-10 (pg/mL)
12000
10000
8000
6000
4000
2000
0
D.
Rantes (pg/mL x 10^3)
100
80
60
40
20
0
E.
MIP-1 alpha (pg/mL)
8000
4000
2000
0
F.
MIP-1 beta (pg/mL)
12000
6000
4000
2000
0
G.
sICAM-1 (pg/mL x 10^3)
600
500
400
300
200
100
0

Figure 3.2

Placental plasma levels of chemokines and soluble ICAM-1

Placental plasma levels of chemokines and soluble adhesion molecule were detected via ELISA. Paucigravid samples are denoted Pauci, and multigravid samples are denoted Multi. Non-infected placenta samples are denoted PM- and malaria infected samples are denoted PM+. Statistics reported are PROC MULTTEST results, or Kruskal-Wallis results, as noted. A. Fractalkine Levels: not significant. B. MIG Levels: PM+ paucigravid (n=31) versus PM- paucigravid (n=29), p=<0.0001. PM+ paucigravid (n=31) versus PM- multigravid (n=25), p=0.0009. PM+ paucigravid (n=31) versus PM+ multigravid (n=5), p=0.0001. C. IP-10 Levels: not significant. D. RANTES Levels: KW p=0.04. E. MIP-1 alpha Levels: KW p=0.005. F. MIP-1 beta Levels: KW p=0.02. G. Soluble ICAM-1 Levels: PM+ paucigravid (n=32) versus PM+ multigravid (n=5), p=0.08.

Figure 3.2

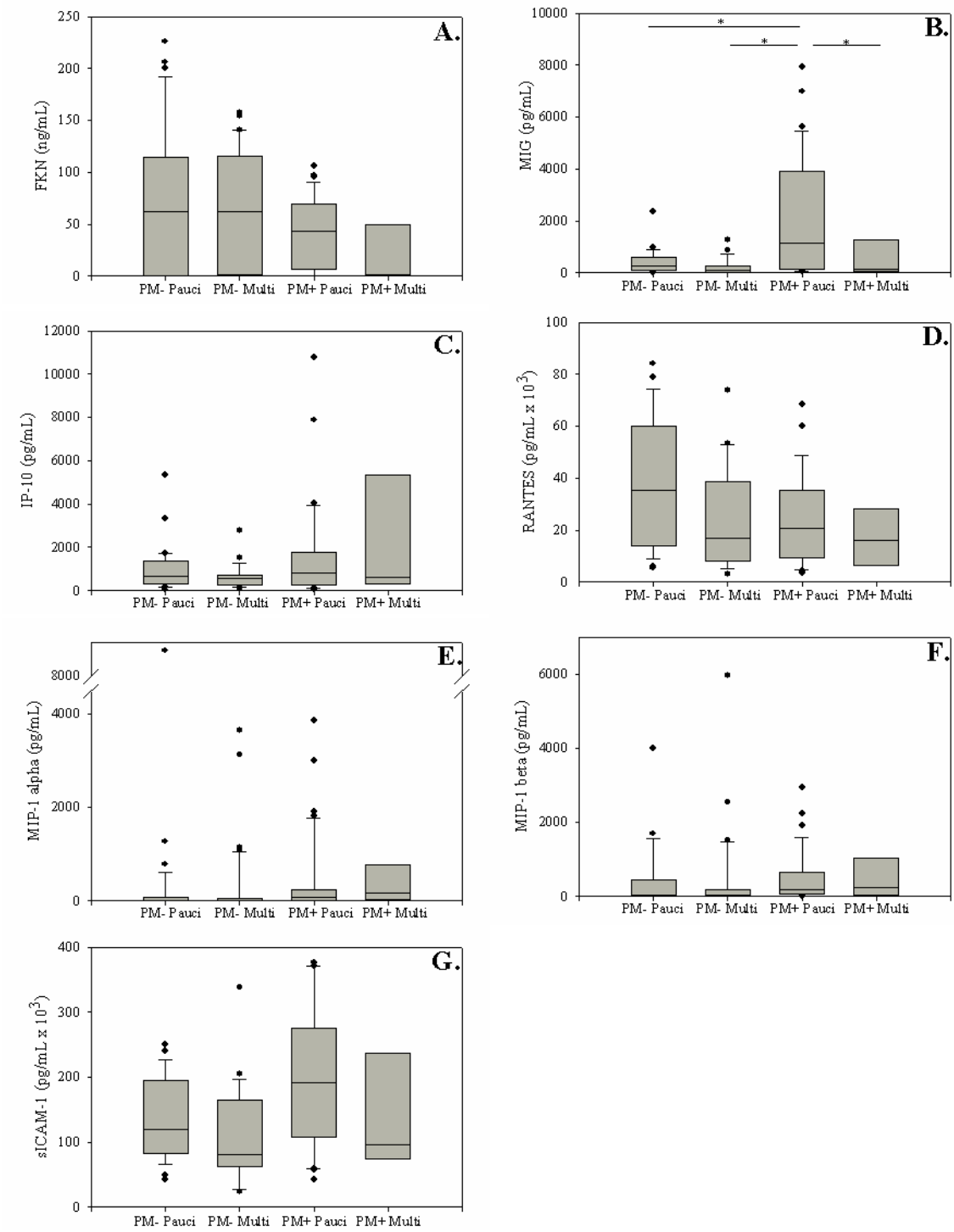
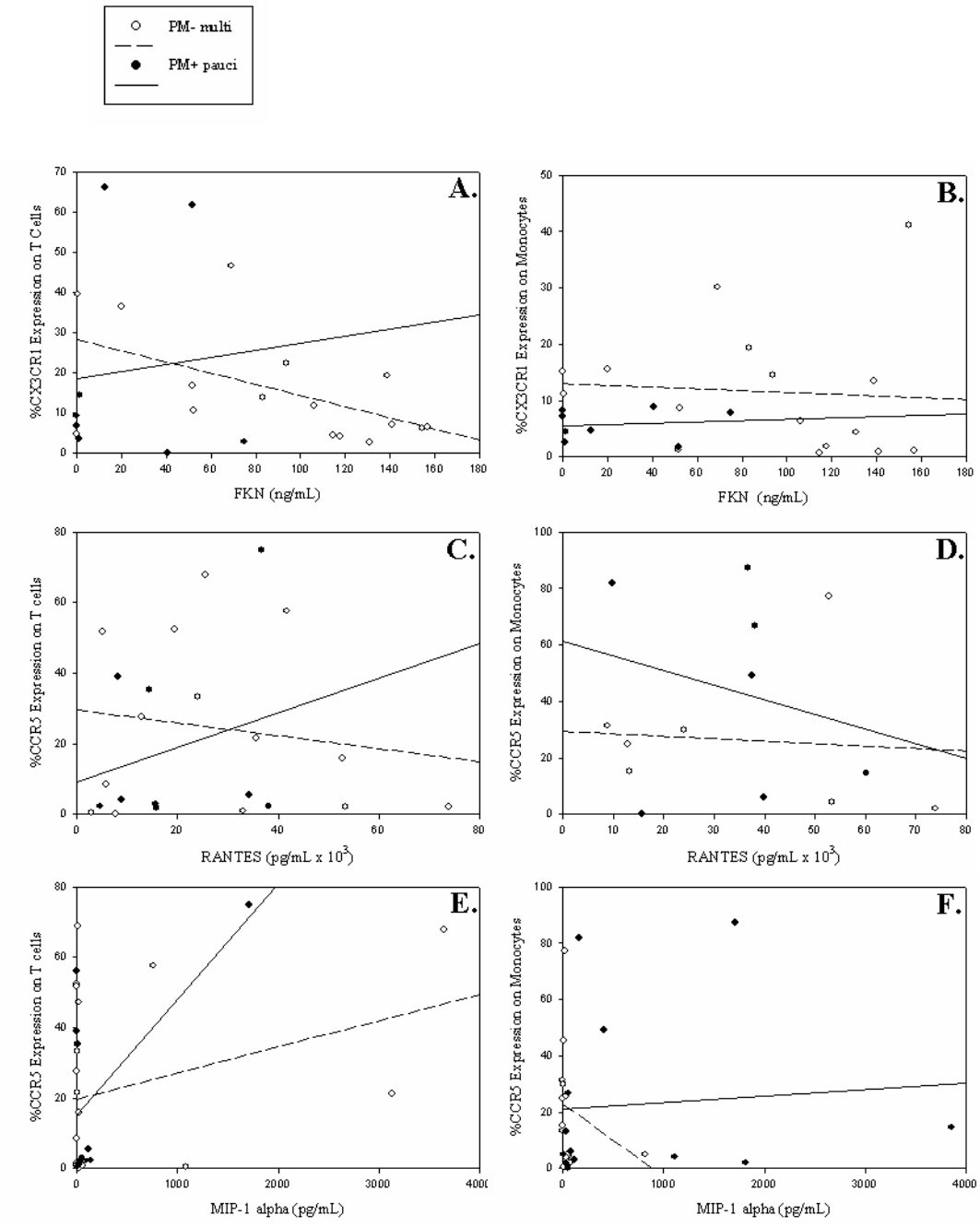

A.
FKN (ng/mL)
250
200
150
100
50
0
PM- Pauci
PM- Multi
PM+ Pauci
PM+ Multi
B.
MIG (pg/mL)
10000
8000
6000
4000
2000
0
*
*
*
C.
IP-10 (pg/mL)
12000
10000
8000
6000
4000
2000
0
D.
RANTES (pg/mL x 10^3)
100
80
60
40
20
0
E.
MIP-1 alpha (pg/mL)
8000
4000
2000
0
F.
MIP-1 beta (pg/mL)
6000
4000
2000
0
G.
sICAM-1 (pg/mL x 10^3)
400
300
200
100
0

Figure 3.3

Placental chemokine levels and chemokine receptor correlations

Placental blood chemokine expression was determined via ELISA. Placental chemokine receptor expression and intensity of expression was detected on maternal T cells and monocytes, determined via flow cytometry detection and analysis. Filled symbols indicate PM+ paucigravid samples and white symbols indicate PM- multigravid samples. All statistics were determined via Pearson's correlation test or Spearman's correlation test (MIP-1 alpha and MIP-1 beta), where indicated. A. FKN levels and %CX3CR1 expression on T cells: not statistically significant. B. FKN levels and %CX3CR1 expression on monocytes: not statistically significant. C. RANTES levels and % CCR5 expression on T cells: not statistically significant. D. RANTES levels and %CCR5 expression on monocytes (paucigravid: r=-0.7, p=0.04 and multigravid: r=-0.3, p=0.2). E. MIP-1 alpha levels and %CCR5 expression on T cells: not statistically significant. F. MIP-1 alpha levels and %CCR5 expression on monocytes: not statistically significant. G. MIP-1 beta levels and %CCR5 expression on T cells (paucigravid: r=0.2, p=0.2 and multigravid: r=0.45, p=0.02). H. MIP-1 beta and %CCR5 expression on monocytes: not statistically significant. I. MIP-1 alpha levels and intensity of CCR5 expression on T cells: not statistically significant. J. MIP-1 beta levels and intensity of CCR5 expression on monocytes (paucigravid: Spearman's r=0.54, P=0.04 and multigravid: r=0.06, p=0.7).

Figure 3.3


PM- multi
PM+ pauci
A.
%CX3CR1 Expression on T Cells
FKN (ng/mL)
B.
%CX3CR1 Expression on Monocytes
FKN (ng/mL)
C.
%CCR5 Expression on T cells
RANTES (pg/mL x 10^3)
D.
%CCR5 Expression on Monocytes
RANTES (pg/mL x 10^3)
E.
%CCR5 Expression on T cells
MIP-1 alpha (pg/mL)
F.
%CCR5 Expression on Monocytes
MIP-1 alpha (pg/mL)

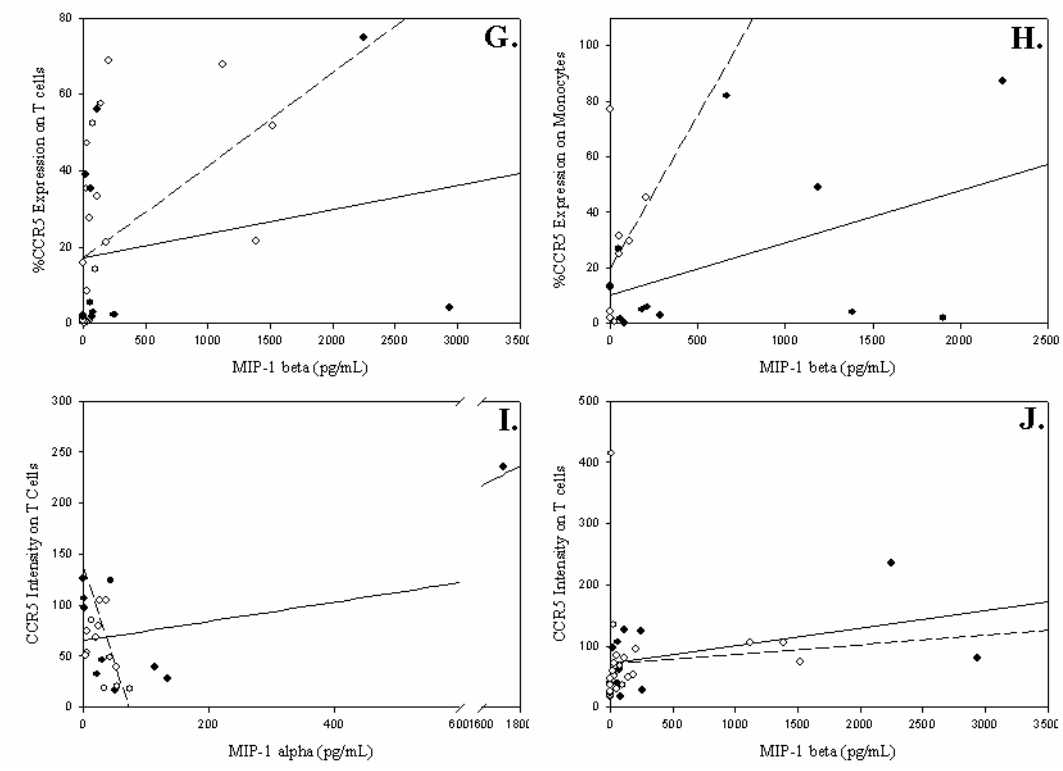

G.
%CCR5 Expression on T cells
MIP-1 beta (pg/mL)
H.
%CCR5 Expression on Monocytes
MIP-1 beta (pg/mL)
I.
CCR5 Intensity on T Cells
MIP-1 alpha (pg/mL)
J.
CCR5 Intensity on T cells
MIP-1 beta (pg/mL)

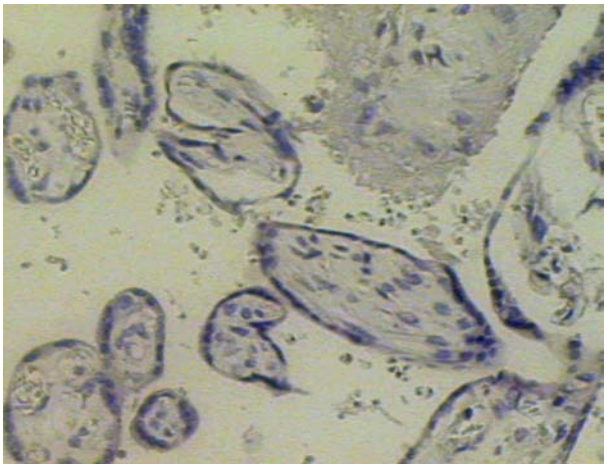
Figure 3.4

Immunohistochemical analysis of fractalkine expression in placental tissues

Placental tissues were fixed and primary anti-human fractalkine antibody was allowed to bind to the tissue thin sections. A secondary biotinylated antibody was allowed to bind to the primary antibody and activated by addition of avidin-biotinylated enzyme complex to stain tissues that contain membrane bound fractalkine. Anti-fractalkine staining of normal placental tissue and malaria-infected placental tissue. Brown staining is indicative of membrane-bound FKN and is present in *Plasmodium* infected placentas.

Figure 3.4

PM- Placenta



PM+ Placenta

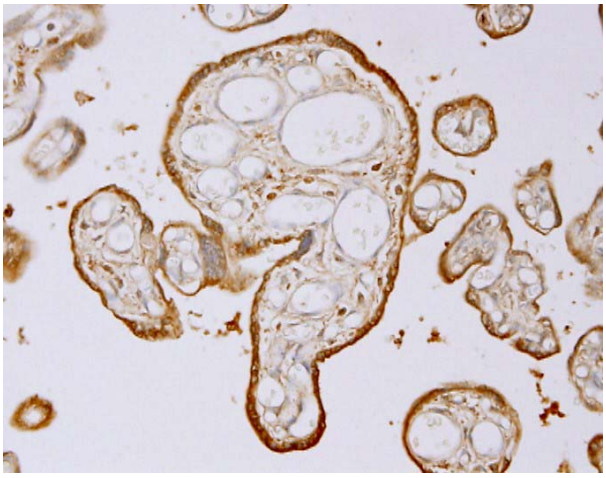


Figure 3.5

Soluble fractalkine secretion by syncytiotrophoblast cells

Primary syncytiotrophoblast cells were cultured and exposed to *Plasmodium falciparum*-infected erythrocytes and uninfected erythrocytes. Supernatants were collected after 2, 4, 8, 12, and 24 hours of exposure to erythrocytes and soluble FKN was detected via western blot assay.

Figure 3.5

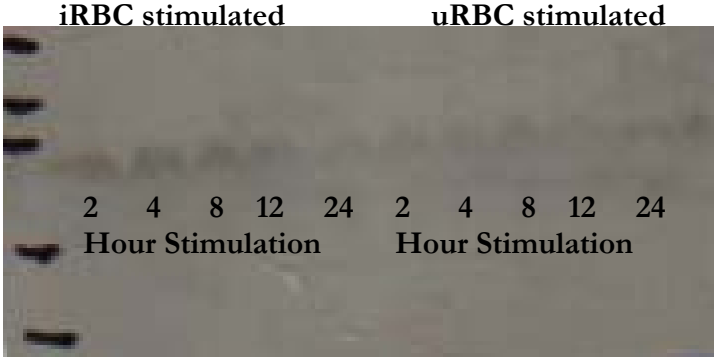

iRBC stimulated
uRBC stimulated
2 4 8 12 24 2 4 8 12 24
Hour Stimulation
Hour Stimulation

Figure 3.6

Placental chemokine expression associated with parasitemia

Data shown represent correlations between placental parasitemia and chemokine expression in paucigravid PM+ samples, as detected via blood smear and ELISA, respectively. Parasitemia was determined as describe in materials and methods. Significant differences, determined by Pearson's correlation, are indicated where applicable. A. FKN Levels: not statistically significant. B. MIG Levels ($r=0.35$, $P=0.02$). C. IP-10 Levels: not statistically significant. D. RANTES Levels: not statistically significant. E. MIP-1 alpha Levels ($r=-0.29$, $P=0.05$). F. MIP-1 beta Levels ($r=-0.31$, $P=0.06$) G. sICAM-1 Levels ($r=-0.3$, $P=0.06$).

Figure 3.6

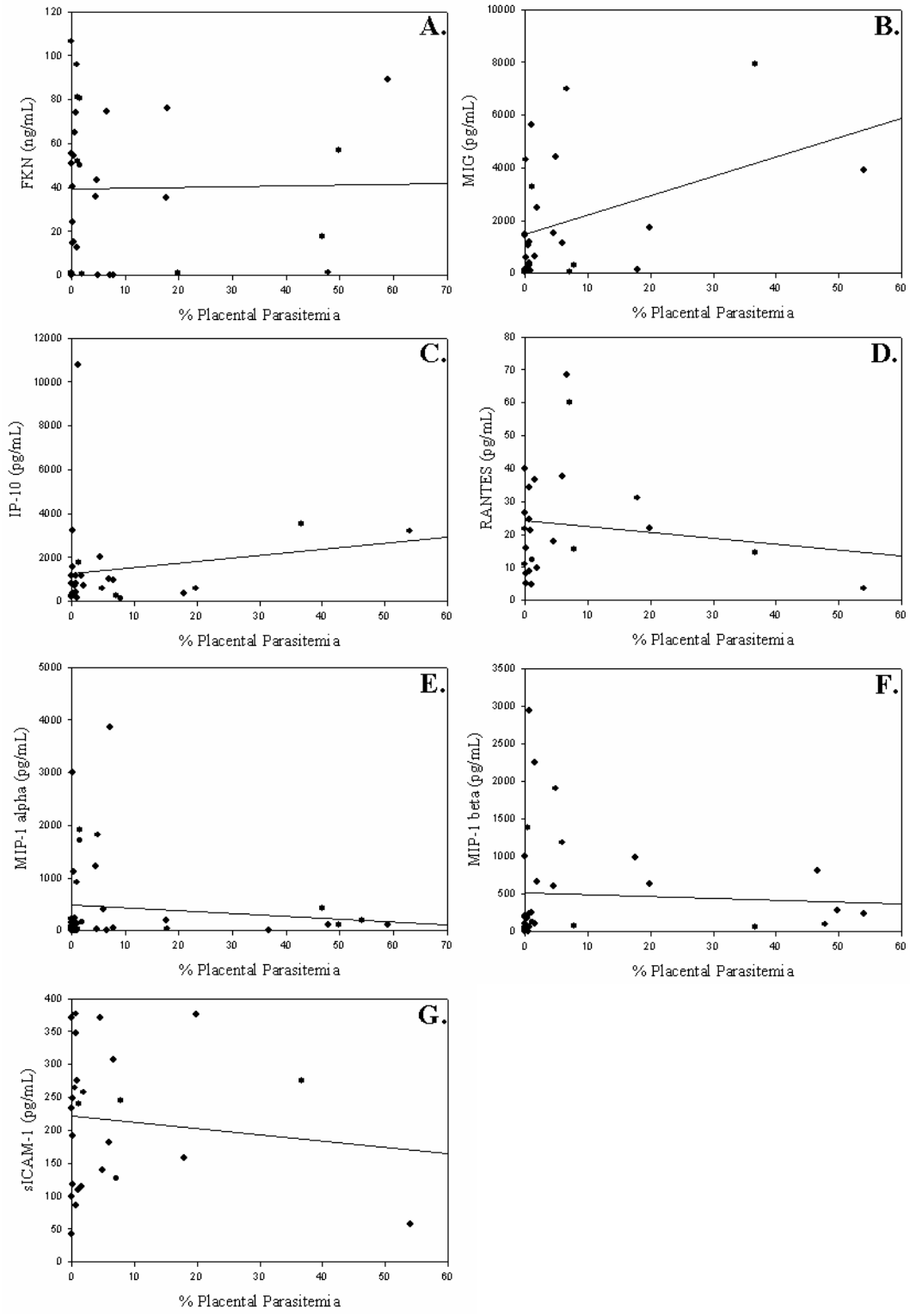

A.
FKN (ng/mL)
% Placental Parasitemia
B.
MIG (pg/mL)
% Placental Parasitemia
C.
IP-10 (pg/mL)
% Placental Parasitemia
D.
RANTES (pg/mL)
% Placental Parasitemia
E.
MIP-1 alpha (pg/mL)
% Placental Parasitemia
F.
MIP-1 beta (pg/mL)
% Placental Parasitemia
G.
sICAM-1 (pg/mL)
% Placental Parasitemia

Figure 3.7

Placental chemokine expression associated with pigment score

Placental chemokine levels, detected via ELISA, are associated with pigmented leukocytes in the intervillous space. Pigment score was determined as described in materials and methods. Data are representative of PM+ paucigravid women. Pigment score of 0 indicates no pigment deposition in white blood cells and pigment score of 4 indicates a strong presence of pigment in white blood cells (>50%). Statistical significance was determined by Pearson's correlation. A. FKN Levels (Pearson's r=-0.4, p=0.06). B. MIG Levels: not statistically significant. C. IP-10 Levels: not statistically significant. D. RANTES Levels: not statistically significant. E. MIP-1 alpha Levels (r=0.36, p=0.05). F. MIP-1 beta Levels (r=0.38, p=0.02). G. sICAM-1 Levels: not statistically significant.

Figure 3.7

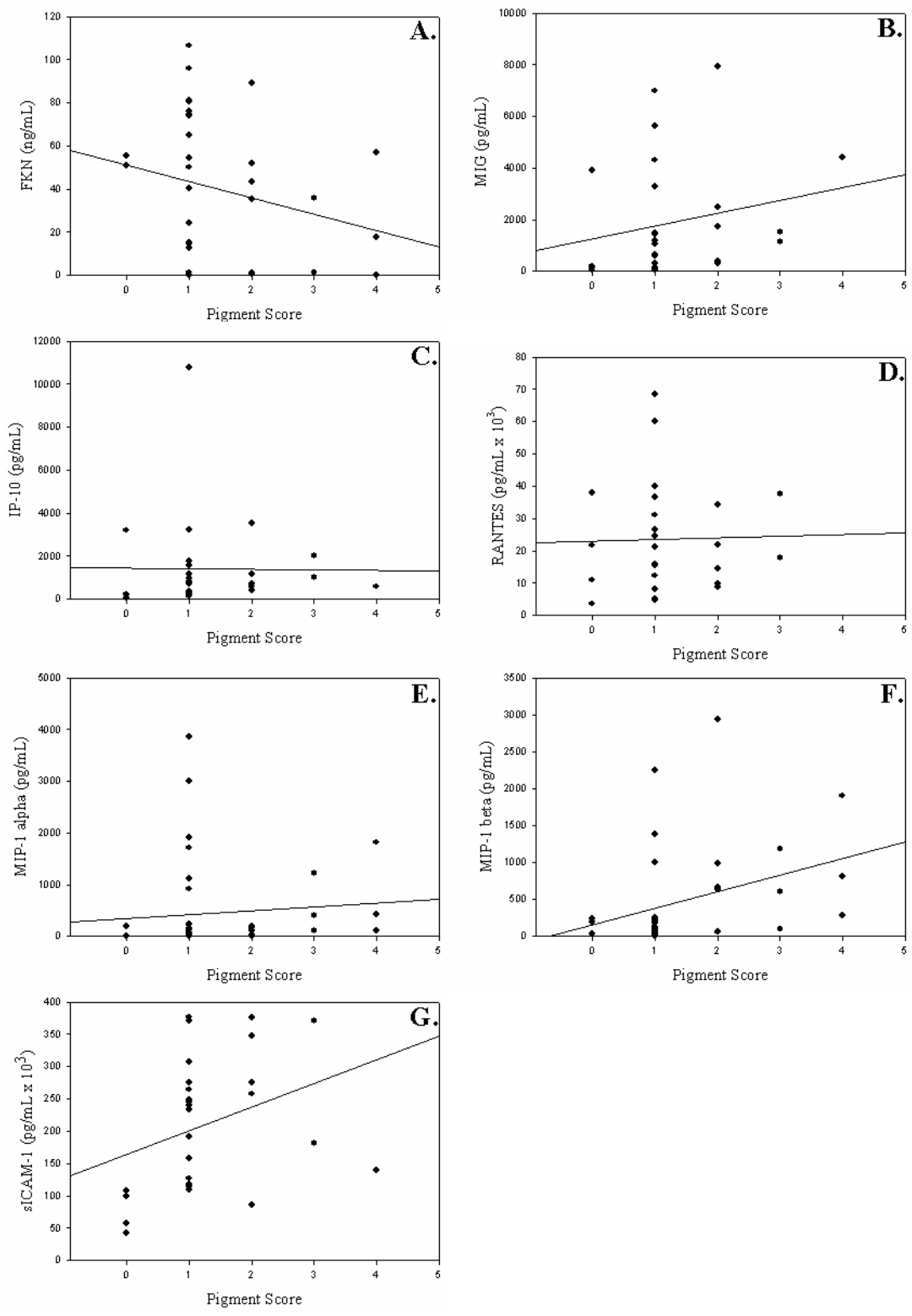
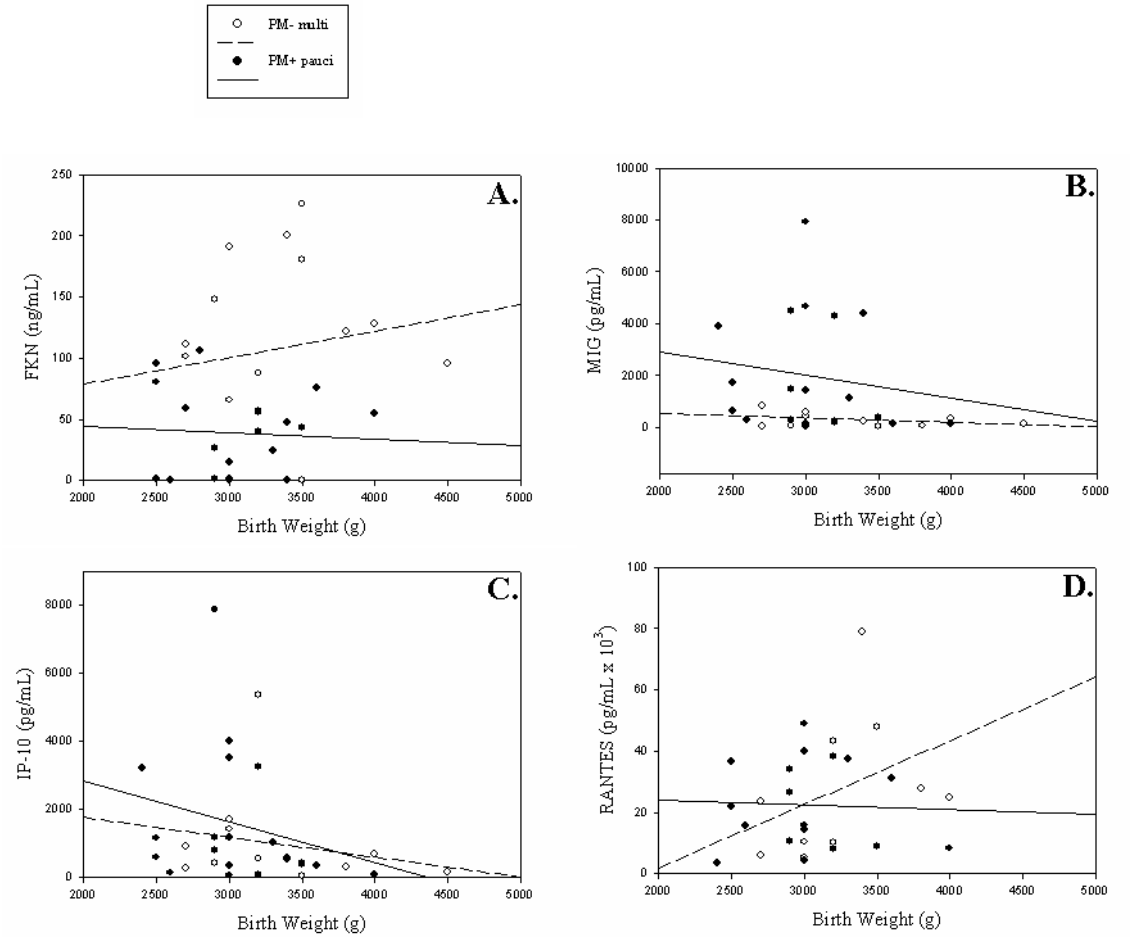

A.
FKN (ng/mL)
Pigment Score
B.
MIG (pg/mL)
Pigment Score
C.
IP-10 (pg/mL)
Pigment Score
D.
RANTES (pg/mL x 10^3)
Pigment Score
E.
MIP-1 alpha (pg/mL)
Pigment Score
F.
MIP-1 beta (pg/mL)
Pigment Score
G.
sICAM-1 (pg/mL x 10^3)
Pigment Score

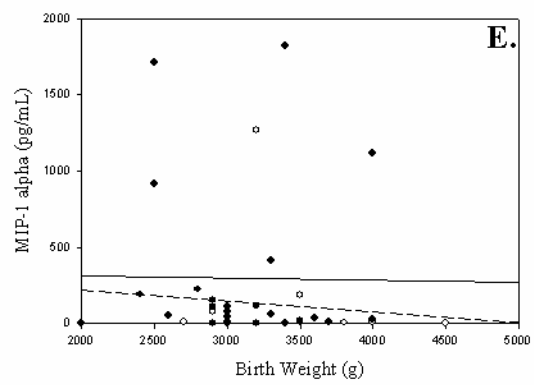
Figure 3.8

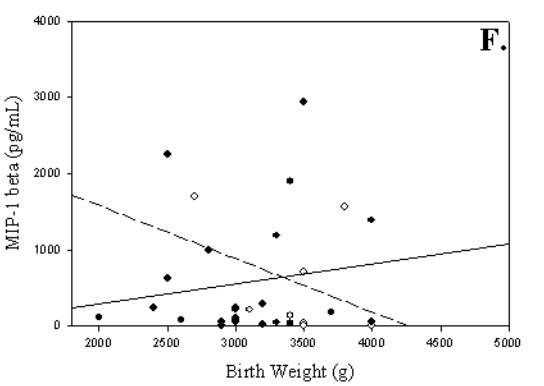
Placental chemokine expression associated with birth weight

Placental chemokine levels, detected via ELISA) of PM+ paucigravid women associated with birth weight. Statistics were determined using Pearson's correlation test. A. FKN levels: not statistically significant. B. MIG levels: not statistically significant. C. IP-10 Levels: not statistically significant. D. RANTES Levels: not statistically significant. E. MIP-1 alpha Levels: not statistically significant. F. MIP-1 beta Levels: not statistically significant. G. sICAM-1 (paucigravid: $r=0.5$, $p=0.03$ and multigravid: $r=-0.4$, $p=0.07$).

Figure 3.8


PM- multi
PM+ pauci
A.
FKN (ng/mL)
Birth Weight (g)
B.
MIG (pg/mL)
Birth Weight (g)
C.
IP-10 (pg/mL)
Birth Weight (g)
D.
RANTES (pg/mL x 10^3)
Birth Weight (g)


E.
MIP-1 alpha (pg/mL)
2000
1500
1000
500
0
2000
2500
3000
3500
4000
4500
5000
Birth Weight (g)


F.
MIP-1 beta (pg/mL)
4000
3000
2000
1000
0
2000
2500
3000
3500
4000
4500
5000
Birth Weight (g)

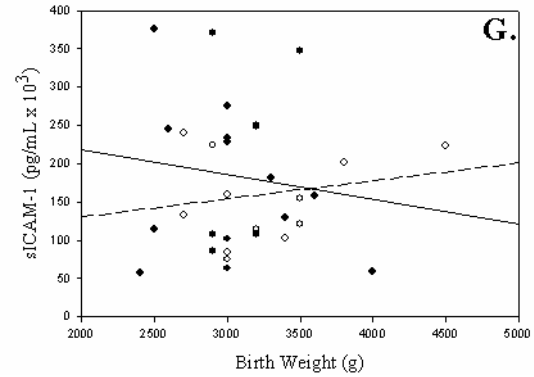

G.
sICAM-1 (pg/mL x 10^3)
400
350
300
250
200
150
100
50
0
2000
2500
3000
3500
4000
4500
5000
Birth Weight (g)

Figure 3.9

Placental chemokine receptor expression associated with placental parasitemia

Placental chemokine receptor expression of T cells and monocytes was detected via flow cytometry. Statistics were determined via Pearson's correlation analysis and are indicated where significant. A. CX3CR1 expression on T cells: not statistically significant. B. CX3CR1 expression on monocytes ($r=0.63$, $p=0.02$). C. CCR5 expression on T cells: not statistically significant. D. CCR5 expression on monocytes: not statistically significant. E. CCR4 expression on T cells: not statistically significant. F. CCR4 expression on monocytes: not statistically significant.

Figure 3.9

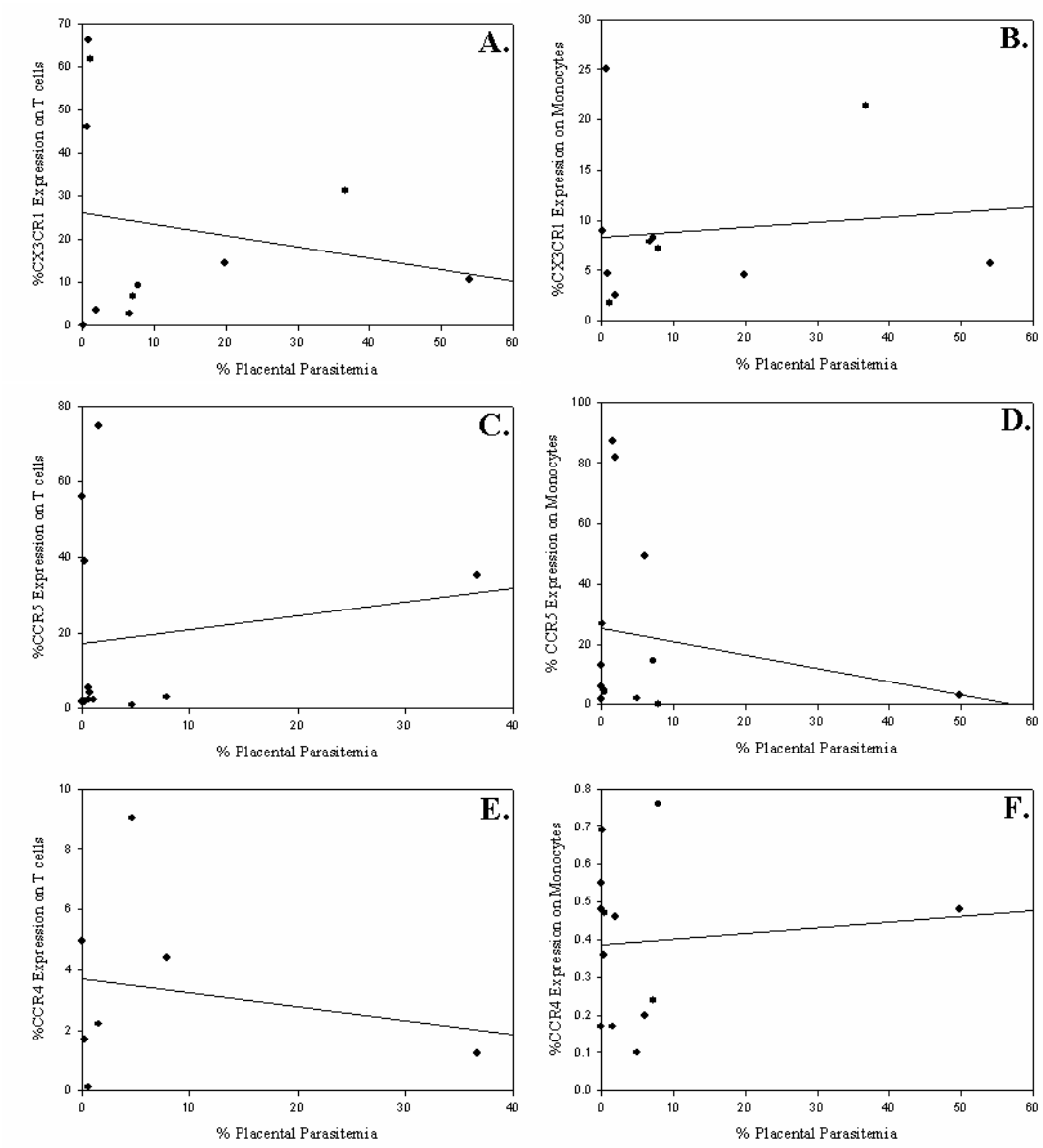

A.
%CX3CR1 Expression on T cells
% Placental Parasitemia
B.
%CX3CR1 Expression on Monocytes
% Placental Parasitemia
C.
%CCR3 Expression on T cells
% Placental Parasitemia
D.
% CCR5 Expression on Monocytes
% Placental Parasitemia
E.
%CCR4 Expression on T cells
% Placental Parasitemia
F.
%CCR4 Expression on Monocytes
% Placental Parasitemia

Figure 3.10

Placental chemokine receptor expression associated with pigment score

Chemokine receptor expression, detected via flow cytometry, is correlated with pigment score. Pigment score was determined as described in materials and methods. Data are representative of PM+ paucigravid women. Pigment score of 0 indicates no pigment in white blood cells and pigment score of 4 indicates strong pigment in white blood cells (>50%). Pearson's correlation is indicated where significant. A. CX3CR1 expression on T cells. B. CX3CR1 expression on monocytes (Pearson's r=0.64, p=0.02). C. Intensity of CCR5 expression on T cells (r=0.04, p=0.8). D. Intensity of CCR5 expression on monocytes (Pearson's r=0.85, p=0.003). E. CCR4 expression on T cells. F. CCR4 expression on monocytes.

Figure 3.10

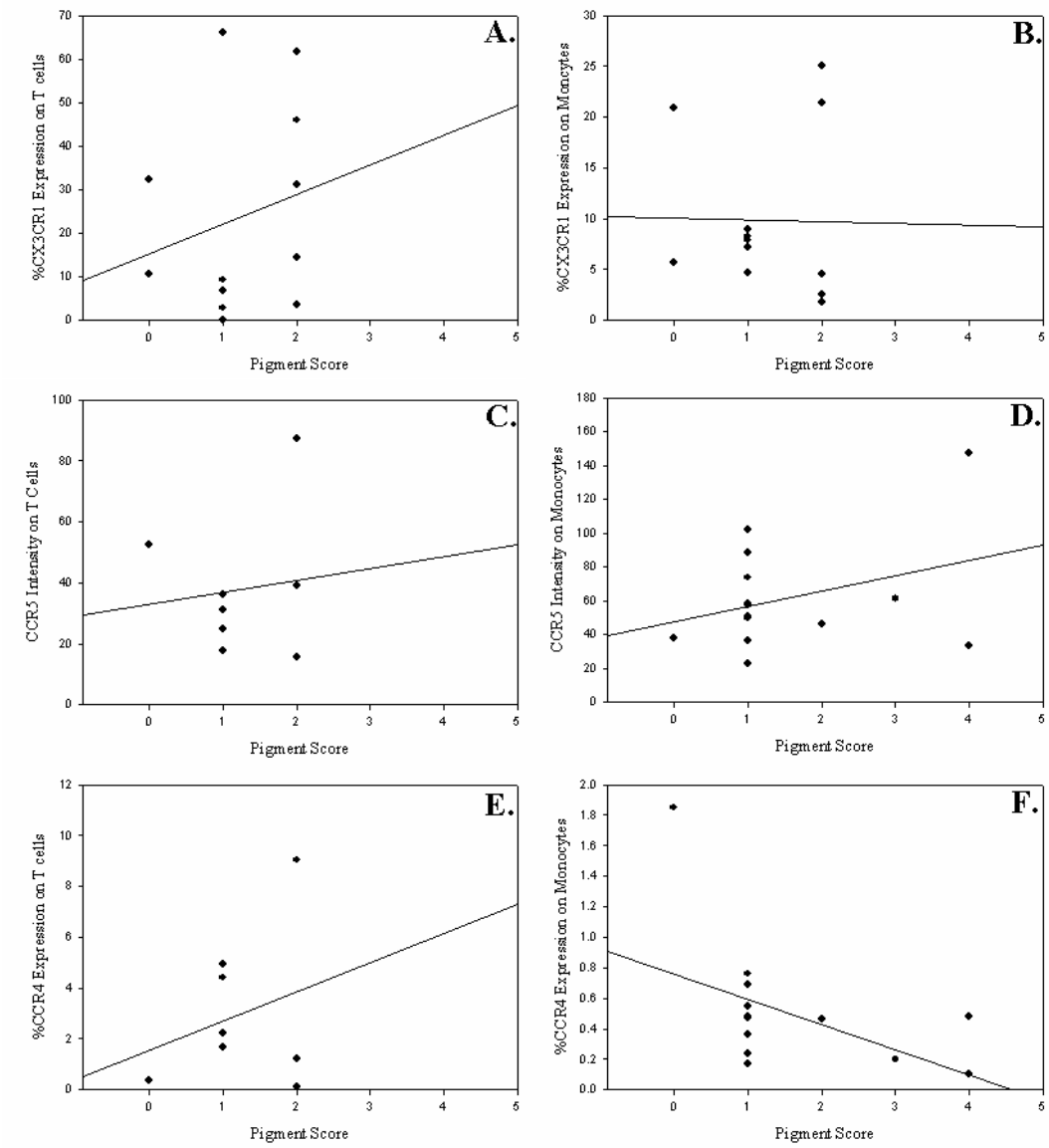
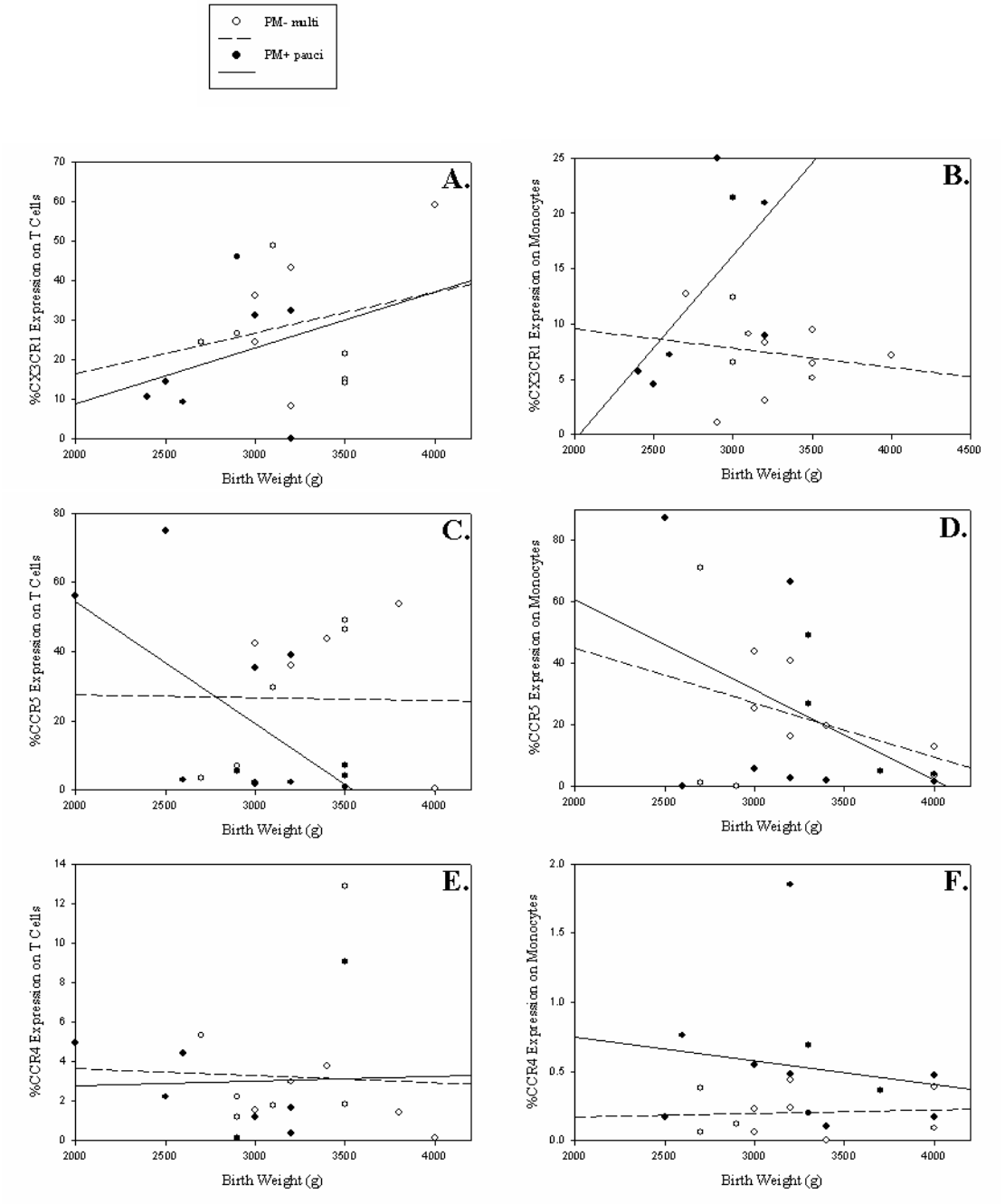

A.
%CX3CR1 Expression on T cells
Pigment Score
B.
%CX3CR1 Expression on Monocytes
Pigment Score
C.
CCR5 Intensity on T Cells
Pigment Score
D.
CCR5 Intensity on Monocytes
Pigment Score
E.
%CCR4 Expression on T cells
Pigment Score
F.
%CCR4 Expression on Monocytes
Pigment Score

Figure 3.11

Placental chemokine receptor and birth weight

Placental chemokine receptor expression of maternal T cells and monocytes, determined via flow cytometry, and associations with birth weight. Statistical analysis performed using Pearson's correlation test. A. %CX3CR1 expression on T cells: not statistically significant. B. %CX3CR1 expression on monocytes: not statistically significant. C. %CCR5 expression on T cells: (paucigravid: r=06, p=0.03 and multigravid: r=0.45, p=0.04). D. %CCR5 expression on monocytes: not statistically significant. E. %CCR4 expression on T cells: not statistically significant. F. %CCR4 expression on monocytes: (paucigravid: r=0.6, p=0.059 and multigravid: r=0.5, p=0.02).

Figure 3.11


PM- multi
PM+ pauci
A.
%CX3CR1 Expression on T Cells
Birth Weight (g)
B.
%CX3CR1 Expression on Monocytes
Birth Weight (g)
C.
%CCR5 Expression on T Cells
Birth Weight (g)
D.
%CCR5 Expression on Monocytes
Birth Weight (g)
E.
%CCR4 Expression on T Cells
Birth Weight (g)
F.
%CCR4 Expression on Monocytes
Birth Weight (g)

CHAPTER 4

CONCLUSIONS

In conclusion, this study contributes to the knowledge of immunological factors and associations between these factors and the pathogenesis of or protection against PM infection. This study describes the associations between pathology (placental parasitemia, malaria pigment deposition, and lower birth weight) of placental malaria infection and the increased expression of MIG, IP-10, MIP-1 alpha, MIP-1 beta and the suppression of RANTES. Also shown are associations of CCR5 expression on maternal immune cell infiltrate with pathogenic factors of PM infection. This study describes possible associations of FKN and cells expressing CX3CR1 with improved pregnancy outcomes and a possible regulatory role for sICAM-1 by preventing maternal immune cell accumulation in PM infection. These findings contribute to the determination of immunological factors and their contribution to the pathogenesis or protection of PM infection.

FUTURE RESEARCH

While this study is representative of a sample of women in western Kenya, it is not representative of pregnancy in other malarious areas. Though there are some reports of similar traits in paucigravid PM+ pregnancies worldwide, such as antibody production to the *var2csa* protein product, there is some possibility for immunological differences between populations. Therefore, it would be beneficial to the full body of placental malaria research to study similar chemokine levels in other populations.

It is also necessary to test maternal leukocytes, isolated from the placenta, for the CXCR3 receptor which is specific for MIG and IP-10 chemokines. For this study, it would be plausible to test CXCR3 expression on maternal leukocytes via immunohistochemistry, because the original samples were not stained for CXCR3 detection via flow cytometry.

Other possible avenues of research in context of the contribution of chemokines to the pathogenesis of PM infection may include isolating maternal placental leukocyte mRNA to identify the source of chemokine secretion.