INVESTIGATIONS OF CELLULAR IMMUNE MECHANISMS TO MALARIA DURING PREGNANCY IN A MALARIA HOLOENDEMIC REGION OF WESTERN KENYA

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To James and Emmy Othoro, the best parents anyone could ever have and for whom I have tremendous love and respect.

To Jackie, Anna, Don and Steven, my siblings, for always believing in me particularly when I doubted my capabilities.

To Omondi, my husband, my friend. I thank my God every time I think of you (Phil 1:3).

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ABSTRACT

Women during pregnancy in holoendemic regions of malaria are at an increased risk for peripheral malaria infections with potential for developing placental malaria. The immunological basis of protection and pathogenesis are incompletely understood. This thesis investigates both processes.

Research on maternal placental immune responses necessitates the collection of reliable placental intervillous blood; an appropriate method for placental blood collection was therefore first determined. Five documented methods of collection (perfusion, incision, biopsy, tissue grinding and prick) were compared for foetal blood contamination and mononuclear cell profiles using flow cytometry. Placental blood collection by prick was established as the most appropriate method and was subsequently used for further immunological investigations.

Previous studies conducted in western Kenya have shown that placental IFN-γ confers protection against placental malaria in multigravidae. The nature of this placental response and the peripheral immune response contribution to placental protection were further investigated. Fifty-two HIV negative women, 32 without infection and 20 with placental malaria were enrolled and cell subpopulation numbers for CD4, CD8, CD45RO memory and Natural Killer (NK) cells, as well as IFN-γ production by these cells, in peripheral and placental blood were assessed using flow cytometry. Chemokines IP-10, RANTES, MIP-1α with chemokine receptor CXCR3 and CCR5 were also assessed for their regulatory role of immune responses. Data suggests that protective mechanisms against infection are different in peripheral and placental blood. CD45RO memory T cell responses predominate in peripheral blood and confer protection, while IFN-γ producing NK cells are important in protection at the placental level. Results further showed that IP-10 acting via chemokine receptor CXCR3 are involved in the regulation of the IFN-γ/NK cell mediated protective immune response at the placental level.

Investigations to determine the immune response contribution to maternal anaemia and infant low birth weight, the adverse outcomes of placental malaria, were also carried out in a further group of 48 women (34 without infection, 14 with placental malaria). The results obtained from placental tissue mRNA evaluation using ribonuclease protection assays, provide evidence for a chemokine-chemokine receptor role in the pathogenesis of infection. RANTES, CXCR1, CXCR2 and CCR3 participation in pathogenesis was implied.

CHAPTER ONE

Introduction and Literature Review

1.1 Introduction

Malaria designates the parasitic disease caused by an infection with plasmodial organisms. It is a major public health concern with unbelievable consequences resulting in severe illness and death with particular risk among children and pregnant women. Extensive epidemiological studies have been conducted in pregnant women and although the epidemiology of malaria in pregnancy is well documented, the biological basis of the consequences of disease is incompletely understood. This thesis endeavours to contribute to current knowledge on malaria in pregnancy particularly that which relates to immunological mechanisms of protection. A brief description of malaria as a disease and an overall review of malarial disease during pregnancy is first necessary.

1.2 Malaria parasite species and disease burden

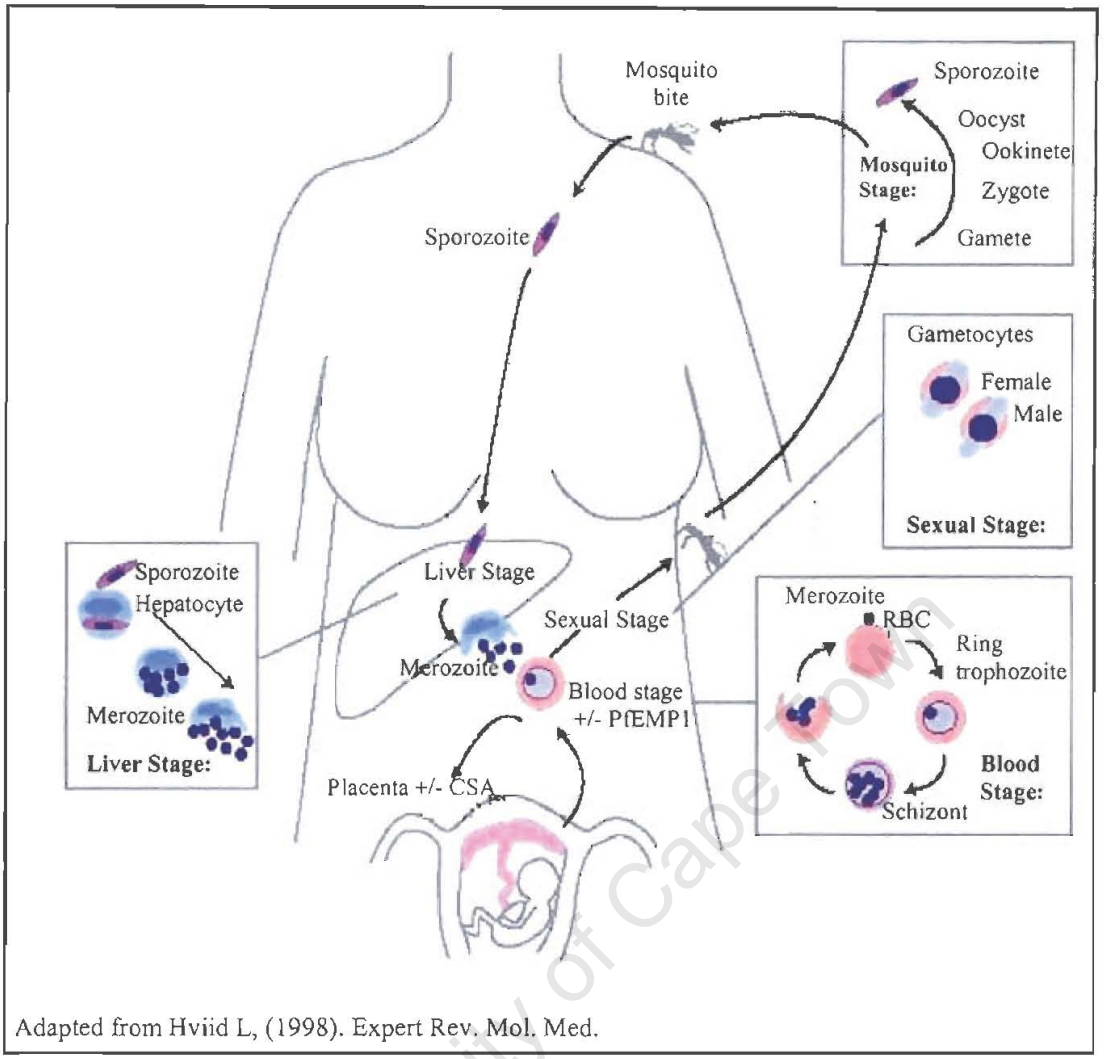
Malaria is caused by parasites of the genus *Plasmodium* with four distinct species infecting man, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium falciparum*. The malarial disease burden is of phenomenal proportions, exacting an enormous toll in terms of morbidity and mortality experienced mostly by children under the age of five and pregnant women. Malaria is present in more than 90 countries with a staggering 400-900 million clinical cases every year and between 1-3 million deaths annually, 90% of which occur in sub-Saharan Africa (Breman, 2001). The World Health Organization estimates that malaria kills one child every 30 seconds resulting in a daily loss of 3000 children per day (WHO, 1998). In pregnant women, where infection has devastating effects on both mother and child, an estimated 3 million newborns suffer each year, from *in utero* related malaria complications, 400,000 of which eventually die (Duffy, 2003). *P.falciparum* is responsible for the tremendous burden of disease experienced in malaria and is the pathogen of interest in this thesis.

1.3 Malaria during pregnancy

Pregnancy is a unique, complex physiological condition where the antigenically distinct foetus develops in an immunologically unfriendly environment. It is associated with changes in the immune system functioning that allows for foetal survival despite foreign genetic material. Paradoxically, these changes or immunomodulatory processes have been associated with an increased risk of acquisition and severity of several diseases during pregnancy (Weinberg, 1984). Malaria is with no exception (Brabin, 1983). Pregnancy provides a highly vascularized organ, the placenta, suitable for uninterrupted malaria parasite life cycle propagation.

1.3.1 *Plasmodium falciparum* life cycle in pregnant women

The plasmodial life cycle is complicated, comprising several developmental stages in a vertebrate host and an insect vector, and passing through three different types of reproduction. Infection of the vertebrate host is initiated when 1-10 plasmodial sporozoites are inoculated by an infected female *Anopheles* mosquito during a blood meal (Figure 1.1). The parasite load which usually does not exceed one hundred sporozoites is injected into subcutaneous tissue, rarely directly into the circulation, and within 30-40 minutes sporozoites that survive host immune defences infect liver hepatocytes via interactions of the circumsporozoite protein (CSP) and receptors on the hepatocyte. In the hepatocyte each sporozoite undergoes asexual division (exo-erythrocytic schizogony) producing hepatic schizonts, which mature, rupture and release thousands of merozoites into the blood. Within 15 to 20 seconds of release, each merozoite attaches to and invades an erythrocyte where they undergo a second phase of asexual reproduction (erythrocytic schizogony) developing first into rings, then trophozoites and finally erythrocytic schizonts. Once mature the schizonts rupture and release more merozoites into the blood, which then immediately invade additional erythrocytes. For each merozoite that infects a red blood cell approximately 10-20 merozoites emerge after schizont rupture. During development in the erythrocyte, parasites insert proteins into the red blood cell membrane one of which is the highly variable *P.falciparum* erythrocyte membrane protein 1(PfEMP1). PfEMP1 mediates adhesion of infected erythrocytes to host tissue resulting



Adapted from Hviid L, (1998). Expert Rev. Mol. Med.

Figure 1.1: The life cycle of *Plasmodium falciparum* in the pregnant woman. Infection is initiated by sporozoite inoculation during a mosquito blood meal. Within 30-40 minutes sporozoites infect liver hepatocytes and undergo exo-erythrocytic schizogony to produce hepatic schizonts. Each schizont matures, ruptures and releases thousands of merozoites into the blood; each invades an erythrocyte, undergoes erythrocytic schizogony and produces more merozoites thus perpetuating the asexual blood stage. During development in the erythrocyte the parasite inserts proteins on the red blood cell membrane, such as PfEMP1 that mediate sequestration to receptors such as placental CSA in the pregnant woman. Some merozoites differentiate into male and female gametocytes and following ingestion by a mosquito and through a number of differentiation stages results in the production of sporozoites ready for infection of the host.

in sequestration, a survival mechanism employed to avoid splenic elimination of infected erythrocytes. In pregnant women the process of sequestration occurs in placental tissue and is responsible for the accumulation of parasites in the placenta, and the consequential phenomenon of placental malaria. The erythrocytic cycle usually continues with parasites proliferating until controlled either by the immune response or chemotherapy, or until the host dies. Some merozoites, by a process of selection not fully understood, develop within erythrocytes into sexual forms (male and female gametocytes). Following ingestion by the female mosquito during a blood meal, these gametocytes mature to male and female gametes. Fertilization occurs and the resultant zygote transforms into an ookinete, which penetrates the mosquito midgut wall and forms an oocyst. Within the oocyst, through several mitotic divisions, sporozoites are formed which eventually burst out and migrate to the salivary glands ready for the next blood meal.

The pathogenecity of *P.falciparum* is exclusively associated with the asexual cycle in the erythrocyte. Sporozoite infection rarely presents with pathology. Pathogenecity arises from the fact that *falciparum* schizonts release a large number of merozoites increasing its capacity for rapid multiplication and consequent massive destruction of erythrocytes. Additionally, merozoites indiscriminately invade erythrocytes regardless of age, thus *P.falciparum* will infect both mature and immature red blood cells resulting in a more rapid rate of increasing parasitemia. Most importantly *P.falciparum* infected erythrocytes become adhesive via parasite derived red cell membrane proteins in tissues concealing the parasite from the immune system.

1.3.2 Malaria epidemiology during pregnancy

Malaria in pregnancy epidemiology is typified by three distinctive features. First, in all malaria endemic regions, pregnant women are more susceptible to malaria infections and suffer more frequent and higher density infections than their non-pregnant counter parts (Brabin, 1983; Menendez, 1995; Nosten *et al.*, 1991). The increased risk of infection is not evenly distributed throughout pregnancy, parasite prevalence tends to be higher in the first half of pregnancy than in the latter months (Brabin, 1991; Nosten *et al.*, 1991).

Secondly, susceptibility to infection and severity of clinical manifestations of infection is determined by pre-pregnancy immunity which is dependent on malaria transmission intensities (Menendez, 1995; Okoko *et al.*, 2003). Malaria parasite transmission intensities may be characterized into a number of endemicities namely holoendemic, mesoendemic and hypoendemic malaria regions. In holoendemic regions, malaria transmission is stable and intense all year round and population immunity against infection is high and efficient. In mesoendemic areas malaria transmission is seasonal and immunity levels are inadequate and therefore does not confer sufficient protection to residents, all age groups therefore suffer from infections. Hypoendemic areas are characterized by very little to no malaria transmission with modest or no immunity to the parasite and are typically prone to malaria epidemics and their devastating effects (Kachur and Bloland, 1998). Women residents of meso- and hypoendemic regions have little pre-pregnancy malaria immunity and are therefore at risk of maternal and foetal morbidity, disease outcomes are often severe (Nosten *et al.*, 1991; Steketee *et al.*, 1996). In contrast, in holoendemic regions of malaria women of childbearing age have relatively high levels of acquired anti-malarial immunity. Disease manifestations during pregnancy are less drastic, infections are mainly asymptomatic, morbidity is low and mortality very rare.

Lastly, in holoendemic regions susceptibility to infections is gravidity dependent. Susceptibility to infection generally decreases with an increase in the number of pregnancies experienced thus primigravidae are more susceptible to malaria infection than multigravidae (McGregor, 1984). In other endemic settings infection is not parity dependent and all pregnancies suffer infections equally (Nosten *et al.*, 1991).

1.3.3 Clinical manifestations of malaria in pregnancy

Clinical manifestations of malaria in pregnancy are numerous and are dependent on many factors with various outcomes for both mother and child. The epidemiological settings discussed above intensely factor on the clinical picture of disease. Consequences of an infection for pregnant women in holoendemic regions are generally uncomplicated. Primigravid women may suffer from fevers and mild symptoms of infection but for the

most part pregnant women will rarely present with clinical signs or symptoms, even in the presence of high parasitemia (Menendez, 1995). Regrettably the adverse effects of a malaria infection in regions of high malaria transmission are manifested in the foetus. Parasite replication in the placenta has the potential to alter and seriously compromise foetal development (Steketee *et al.*, 1996). Maternal malaria causes low birth weight of the infant either through preterm delivery or intrauterine growth retardation (IUGR) with subsequent neonatal and infant complications, sometimes resulting in mortality during the first year of life (Steketee *et al.*, 2001).

In low malaria transmission regions *P. falciparum* infections in pregnancy presents as severe syndromes. Women indiscriminately suffer from cerebral malaria, acute pulmonary oedema, hypoglycaemia and renal failure. In the foetus, maternal malaria infections precipitate abortions or result in still births, premature deliveries and low birth weight infants due to foetal growth retardation (Duffy and Desowitz, 2002; Menendez, 1995).

Notwithstanding malaria endemicity patterns or levels of pre-pregnancy immunity, pregnant women develop severe anaemia as a consequence of infection (Menendez, 1995). Maternal anaemia is a risk factor for perinatal mortality (Zucker *et al.*, 1994) and may influence infant morbidity and mortality through birth weight reduction (Menendez, 1995).

It is unclear precisely how malaria infections contribute to adverse pregnancy outcomes particularly in regions of intense malaria transmission. A hallmark of pregnancy malaria in holoendemic regions is the accumulation of infected erythrocytes in the placenta resulting in placental malaria (Duffy, 2003). The placenta is a flat disc shaped organ that is primarily foetal in origin and consists of numerous intervillous spaces filled with maternal blood and permeated by highly branched syncytiotrophoblast lined villi, housing the foetal circulatory system. Parasites in the placenta accumulate in the large lumen of the intervillous spaces and may be found together with mononuclear cell aggregates and pigment (a parasite by-product of haemoglobin digestion). The events

leading to the adverse consequences of infection are played out here and the outcome of an infection and progression into pathology depends on specific host and parasite properties.

1.3.4 Host-parasite interactions in the placenta

Women in holoendemic regions typically harbour high parasite levels in the placenta, sometimes exceeding 50% of the total placental erythrocyte count (Flick *et al.*, 2001), and frequently with an absence of parasites in peripheral blood (Brabin, 1983). The disparity in placental and peripheral blood parasitemia is thought to be indication of active retention of parasites in the placenta (Andrews and Lanzer, 2002). Histological studies indeed show a concentration of infected erythrocytes in placenta intervillous spaces with some adherent to the syncytiotrophoblast (Bulmer *et al.*, 1993). Investigations in pregnant Malawian and Kenyan women have reported that placental trophozoite infected erythrocytes adhere to placental tissue host receptor cells, specifically to the glycoasminoglycan chondroitin sulphate A (CSA) (Fried and Duffy, 1996; Rogerson *et al.*, 1995) and not to CD36 or intracellular adhesion molecule-1 (ICAM-1) both with previously reported binding properties for non-placental parasite isolates (Beeson *et al.*, 2000; Fried and Duffy, 1996; Ockenhouse *et al.*, 1991). Additionally, isolates from peripheral blood of pregnant women but not isolates from non-pregnant adults and children adhered to CSA (Beeson *et al.*, 1999; Rogerson *et al.*, 1999). A selection process operating in the placenta that leads to the accumulation of CSA binding parasites is suggested (Rogerson and Beeson, 1999) and is supported by investigations further revealing that that these parasite isolates do not occur prior to pregnancy and are in fact a distinct isolate (Beeson *et al.*, 1999). Furthermore, placental parasite strains are recognized by gender specific plasma so that parasites selected for CSA are not recognized by adult male plasma IgG (Ricke *et al.*, 2000).

CSA receptor molecules are not confined to placental tissue and occur in numerous organs of the body. Recently an extra cellular proteoglycan matrix has been described in placenta intervillous spaces with a low sulphated CSA (Achur *et al.*, 2000; Alkhalil *et al.*, 2000) and distinguishes placental CSA from the CSA of other organs. It efficiently binds

infected erythrocytes and may be the likely receptors for the sequestration of parasitized erythrocytes in infected placentas. Two other receptors have been suggested as possible mediators of sequestration, hyaluronic acid (Beeson *et al.*, 2000) and neonatal Fc receptors on the placenta (Flick *et al.*, 2001), both of which need further elucidation due to questionable functional roles in host-parasite interactions. However, it has been suggested that multiple and perhaps synergistic receptor ligand interactions account for parasite sequestration in the placenta and subsequent severity of pathology associated with an infection of the placenta (Andrews and Lanzer, 2002).

Parasite adhesion to CSA is mediated by variant surface antigens (VSA) expressed on the erythrocyte cell membrane and recent studies have shown that the highly variable PfEMP1 protein is the principal VSA in this interaction (Reeder *et al.*, 2000). PfEMP1 is encoded by the diverse *var* gene family containing 50-60 members per haploid parasite genome (Gardner *et al.*, 2002). Different PfEMP1 molecules have different receptor specificities and clonal switching between *var* genes leads to the expression of the various PfEMP1 molecules that allows the parasite to modify its adhesion properties, (Smith *et al.*, 1995) a characteristic thought to be an immune evasion strategy to avoid splenic clearance (Berendt *et al.*, 1990). Several *var* genes encoding PfEMP1 domains with affinity for CSA have been described, with exclusive binding properties for CSA and not CD36 (Buffet *et al.*, 1999; Gamain *et al.*, 2001). Parasites with adhesion specificity for CSA which might be gained as a result of switching between PfEMP1 variant genes are therefore eliminated from non pregnant individuals owing to the lack of suitable host adhesion receptors (Hviid 1998). Likewise in pregnant women where low sulphated CSA is available, parasite variants with adherent properties for CSA enable an infection to take hold and expand unhindered in the relatively protected environment of the placenta.

Besides specific host receptor-parasite VSA interactions, the sequestration of infected erythrocytes in the placenta may also be influenced by placental blood flow rate which is slow compared with that in other vascular beds. Studies have however revealed that CSA supports adhesion at high wall shear stress and may not be an important factor

influencing the adhesion of infected erythrocytes in the placenta over other organs (Rogerson *et al.*, 1997).

1.3.5 Host response to placental *P.falciparum* infections

1.3.5.1 Pathophysiological response to infection

Parasite sequestration in the placenta results in hypoxia, inflammatory reactions and chronic intervillousitis all of which have negative effects on foetal development and growth. *Plasmodium falciparum* infected placentas elicit an inflammatory host response that mediates several events related to pregnancy outcome. Elevated cytokine production due to parasite presence has been proposed as the mediator of trophoblastic damage and the thickening of the basement membrane of the trophoblast cells, causing abnormalities of placental blood flow (Dorman *et al.*, 2002). The trophoblast is the placental parenchyma through which exchanges must occur. The efficiency of the transplacental transfer of nutrients and oxygen to the foetus is therefore diminished (Galbraith *et al.*, 1980) and maybe a likely contributor of IUGR. The pro-inflammatory environment also results in leukocyte infiltration to the placenta. The degree of infiltration maybe intense resulting in massive chronic intervillousitis (Ordi *et al.*, 1998). The enormous numbers of pigment containing mononuclear cells accumulating in intervillous spaces, together with parasite presence and increased fibrinoid deposition also contributes to the obstruction of gas and nutrient exchange (Duffy, 2002).

In holoendemic regions the severity of the host response to infections decreases with an increase in parity. The mechanisms underlying the increased susceptibility in primigravid women and the development of gravidity dependent immune mechanisms are the focus of numerous investigations.

1.3.5.2 Factors responsible for susceptibility to infection

Several hypotheses have been proposed to explain the increased risk of pregnant women to malaria and the high frequency of placental infection. Pregnancy represents a period of immunomodulation to permit foetal survival. The immunomodulatory events have been proposed as shifting the immune response towards a type 2 cytokine environment and

away from a detrimental type 1 response that would compromise the viability of the foeto-placental unit (Wegmann *et al.*, 1993). Malaria as well as other intracellular pathogenic infections require type-1 mediated immune responses for parasite elimination, pregnant women would therefore be susceptible to infection due to the inhibition of type-1 responses (Matteelli *et al.*, 1997).

Pregnancy has been proposed to exacerbate malaria through hormone dependent depression of the immune system (Brabin, 1985). A transient depression of cell-mediated immunity occurs during pregnancy and hormones have been implicated as possible effectors of suppression. Cortisol levels are increased during pregnancy with highest levels reported for primigravid women as compared to multigravid women, and could account for the increased risk of malaria in primigravid women (Menendez, 1995; Roberts *et al.*, 1996). Placental oestrogen levels are associated with lower cell mediated immune responses in the placenta relative to peripheral blood, which might explain parasite accumulation in the placenta. Oestrogen production reduces with parity and may be responsible for the lowered susceptibility in multigravidae as compared to primigravidae (Watkinson *et al.*, 1985).

A third hypothesis for primigravid susceptibility to infection has been suggested by McGregor (1984). McGregor proposed that pregnancy establishes a highly vascular immunologically malaria-naïve placenta, suitable for uninterrupted parasite replication. However the uncontrolled multiplication of parasites is short lived; local immune responses are induced which restrict replication and effect parasite destruction. These responses are least effective in the first pregnancy but are boosted and become more efficient in subsequent pregnancies (McGregor, 1984). Intriguing as it may be, investigations are required to substantiate McGregor's hypothesis.

1.3.5.3 Immunity to infection

In areas of intense malaria transmission the incidence and density of malaria parasitemia in all populations declines with age due to the development of natural anti-malaria immunity (Greenwood and Snow, 1991; Marsh, 1992). Natural acquired immunity to

malaria takes 10-15 years of exposure to develop (Baird, 1998) thus women of reproductive age will have developed sufficient anti *P.falciparum* immunity. In pregnancy, particularly the first and second, susceptibility to infection is reacquired and women suffer from more frequent and higher density infections as well as suffer severe manifestations of infection as delineated above. Intriguingly, multiparous women regain immunity to infection and suffer fewer consequences of a malarial infection during pregnancy. The process of protective immune response acquisition is evidently complex.

Immunity to malaria encompasses several different defence mechanisms both innate and acquired in origin. Innate mechanisms comprise inherent traits as well as first-line cellular and humoral defence events and are largely non-specific in their nature of function, while acquired immunity is as a result of the development of specific immune responses to the presence of parasite. The two immune responses are strongly interactive with innate mechanisms instructing the acquired response to effect protection.

1.3.5.3.1 Innate immune responses in pregnancy

Normal pregnancy reports an increase in monocyte and granulocyte numbers from the first trimester onwards. An increase in monocyte phagosytosis and respiratory burst activity is also reported (Sacks *et al.*, 1999). Many studies have found granulocyte activation in pregnancy as well as changes in plasma levels of soluble innate factors typical of an acute phase response (Shibuya *et al.*, 1987). Natural killer (NK) cell activity, particularly IFN-γ production, is however suppressed and may be specific for particular functions (Colonna *et al.*, 1998). It has been suggested that an increase in innate mechanisms is for the compensation of the decrease in cell mediated immunity that occurs during pregnancy (Sacks *et al.*, 1998).

Numerous studies conducted in children and non-immune adults have shown that innate mechanisms against malarial parasites are important and probably contribute significantly to the initial reduction of parasitemia seen in acute *P.falciparum* infections. Innate immunity against malaria is mediated by the polymorphonuclear and monocyte/macrophage cell lineages, NK cells and γδ T cells. Phagocytosis of free

parasites and parasitized erythrocytes has been reported by neutrophils, monocytes and macrophages and may be antibody mediated (through opsonization) or nonopsonic mechanisms (Smith *et al.*, 2002). NK cell numbers increase in a malarial infection and participate in the elimination of parasites by infected erythrocyte cell lysis (Orago and Facer, 1991) and production of IFN-γ which mediates macrophage parasiticidal activity (Mohan *et al.*, 1997). T cells bearing the γδ T cell receptor are strongly expanded during the early phases of malaria infection (Salerno and Dieli, 1998) and have been shown to inhibit *in vitro* parasite replication (Troye-Blomberg *et al.*, 1999).

Innate mechanisms of protection against malaria during pregnancy have not been explored, however innate immunity against P.*falciparum* is reported to be more important in unexposed individuals in whom severe disease may develop before an acquired immune response is mounted (Smith *et al.*, 2002) and are likely to also be important in the immune response against malaria in pregnancy particularly in the immunologically naïve placenta. Several studies document increased cytokine production in pregnant women infected with malaria (Fievet *et al.*, 2001; Fried *et al.*, 1998a; Moore *et al.*, 1999; Moormann *et al.*, 1999), the source of which may be innate.

1.3.5.3.2 Acquired immune responses in pregnancy

The life cycle of the malaria parasite is complex with distinct phases. Each phase or stage is characterized by the expression of stage specific proteins, and presents a variety of potential targets for the action of protective mechanisms (Li *et al.*, 2001). Acquired immunity results from the generation of specific immune responses to these targets, with different effects resulting for each. Asexual blood forms of parasites are responsible for the disease manifestations of infection thus immune responses to the asexual blood stages are important. A combination of antibody and cellular mediated immune responses are directed against *P. falciparum* asexual blood stages.

1.3.5.3.2.1 Humoral immune responses

Studies conducted in children have shown that the acquisition of specific antibodies directed against malaria parasite proteins is associated with protection (Bull *et al.*, 1998) .

Cytophilic antibody of the IgG1 and IgG3 subclasses are considered to be most important (Aucan *et al.*, 2001). Parasite elimination mechanisms mediated by antibody function by blocking erythrocyte invasion and sequestration, rendering merozoites susceptible to phagocytosis or initiating complement mediated damage are reported (Wipasa *et al.*, 2002).

Numerous studies have examined peripheral blood antibody responses in pregnant women suffering from malaria. Majority of the studies reported no difference in antibody levels on the basis of pregnancy or parity (Duffy, 2002). Work on antibodies against placental parasites has revealed parasite specific antibody responses associated with protection. Variant specific anti-PfEMP1 antibodies have been reported in pregnant women. Multigravid pregnant women have high levels of antibodies to PfEMP1 expressed by CSA-adhering parasites where as non-pregnant women and men have no antibodies to these PfEMP1 parasite variants (Ricke *et al.*, 2000; Staalsoe *et al.*, 2001). The acquisition of CSA-PfEMP1 specific antibodies interferes with parasite adhesion to CSA and confers protection against infection particularly to multigravid women (Fried *et al.*, 1998b).

1.3.5.3.2.2 Cellular immune responses

Documented accounts of cell mediated immune responses involved in protection against infection but also in pathogenesis of the malarial disease in children are numerous. T cells are central to the acquisition and maintenance of protective immunity against the asexual blood stage parasite. CD4 T cells in particular are crucial in orchestrating the events that lead to blood stage parasite elimination and effect their functions through cytokine production. CD4 T cells of the Th1 type activate macrophages and other cell types to produce TNF-α, nitric oxide (NO), reactive oxygen species (ROS) and other mediators through the production of inflammatory cytokines such as IFN-γ. IFN-γ is a potent activator of macrophages that effect parasite phagocytosis and production of parasiticidal factors. CD4 T cells of the Th2 type function as helpers to B cells in humoral immunity through the production of IL-4 and IL-10 (Abbas *et al.*, 1996). During a malaria infection a balance between Th1 and Th2 cytokines is important and

dysregulation of the levels of the CD4 T cell products could result in the persistence of the parasite and lead to pathology.

Pregnancy has a profound effect on CD4 T cells. CD4 T cell percentages decrease in the first and second trimester and their cellular function is downregulated progressively throughout pregnancy, a process that results in the transient depression of cell mediated immunity (Hunt, 1992). As a consequence of non-specific immunosuppression antigen specific cell mediated immunity against several intracellular infections are muted in pregnancy (Guilbert *et al.*, 2002). Peripheral blood mononuclear cells (PBMC) of Gambian pregnant women had lower lymphoproliferative and IFN-γ responses than nulligravidae (Riley *et al.*, 1989). Primigravid Cameroonian women also had decreased proliferative and IL-2 responses than non-pregnant women (Fievet *et al.*, 1995). Placental blood mononuclear cell (PlacBMC) proliferative responses were poor in a group of Gambian women as compared to PBMC responses (Rasheed *et al.*, 1993) and IFN-γ expression by PlacBMCs of Kenyan primigravidae was lower than in multigravidae suggesting parasite specific immune response suppression in first pregnancies (Moore *et al.*, 1999).

Cellular response investigations in relation to pathological events of pregnancy malaria have shown that malaria elicits inflammatory cytokine production. Malaria infections of the placenta alter the local cytokine secretion patterns and may mediate the adverse effects of infection (Fievet *et al.*, 2001). Placental tissue mRNA levels for IL-1β (interleukin-1 beta), TNF-α, IL-8 (interleukin-8) and placental plasma TNF-α levels were upregulated in Malawian women and were associated with poor pregnancy outcomes (Moormann *et al.*, 1999; Rogerson *et al.*, 2003a). Persistently elevated placental blood IL-2 (interleukin –2), TNF-α and IFN-γ even after parasite resolution, were associated with maternal anaemia and LBW in primigravid Kenyan women (Fried *et al.*, 1998a).

1.3.5.3.2.3 Gravidity dependent immunity

Increasing resistance to pregnancy malaria with parity in regions of intense malaria transmission suggests gravidity dependent immunity. McGregor (1984) hypothesized that

placenta specific immunity is acquired and maintained over repeated exposure through successive pregnancies despite placental expulsion at partition much in the same way that children in endemic areas develop resistance through repeated infections over a number of years. Moore *et al.*, 2000b, suggest a lymphocyte recirculation hypothesis to further qualify McGregor's supposition. They propose that during a first malaria exposed pregnancy, the initial exposure to malaria parasites induces the activation of maternal effector cells and generates malaria specific lymphocytes. As part of this response some of these activated effector/memory cells circulate out of the placenta and take up residence in local uterine lymphoid sites, thus when the placenta is expelled at parturition a pool of memory cells is retained. In a subsequent malaria exposed pregnancy, recruitment of memory cells from the lymphoid organs back to the placenta mediates a rapid and focused response. Thus protection is maintained across pregnancies even with new placental tissue, immunologically naïve to malarial parasites.

Recent investigations have provided evidence of gravidity dependent placental mechanisms of protection against infection. Anti adhesion antibodies directed against CSA binding parasites were reported as being highest in protected multigravid women as compared to susceptible primigravid women (Fried *et al.*, 1998b). Placental IFN-γ immune responses were elevated in placenta uninfected multigravid women and lowest in primigravid women and multigravid women with placental malaria (Moore *et al.*, 1999). Both study results suggest malaria parasite specific memory cell production and retention. Definitive characterization of the gravidity dependent immune response may offer some insight into the immune response development against malaria infections in pregnancy.

1.4 Study motivation

Malaria during pregnancy has devastating effects on both mother and child and has therefore recently been considered a priority area for research and control (Menendez, 1995). An interesting feature in endemic regions such as western Kenya is the relative protection against placental malaria over a number of pregnancies. How this protection is acquired and maintained is not understood given that the placenta is expelled at the end

of every pregnancy period. Investigations are evidently required to understand the immunological mechanisms related to protection and pathogenesis. The current study endeavours to characterize immune mechanisms related to protection and pathogenesis in pregnancy-associated malaria. A better understanding of the immunological basis of infection will be useful for the development and design of new interventions for malaria during pregnancy.

1.5 Scope of the thesis

Data collected for this thesis is discussed in four parts: the first part compares different documented methods of placental maternal blood collection for foetal cell contamination and mononuclear cell profiles to determine the best method for placental malaria immunology studies. The second part investigates the source of IFN-γ production and memory cell generation in relation to protection against malaria during pregnancy. The third part investigates IFN-γ producing immune cell trafficking to the placenta for protection while the fourth investigates the association between placental tissue chemokine/chemokine receptor production, upregulated by parasite infection in relation to poor pregnancy outcomes.

This study was initially integrated into a 'Vertical transmission project' that investigated placental malaria infection and perinatal transmission of HIV infection in Kenya and more recently was part of a laboratory research project on ' Immunological mechanisms in protection and pathogenesis in malaria in childhood and during pregnancy in western Kenya'. The study design is outlined below.

1.6 Study Objective

The overall objective of this study was to characterize cellular immune responses in relation to protection and pathogenesis of malaria during pregnancy and consists of four primary objectives as follows:

1. To compare five documented methods of maternal placental blood collection (placenta puncture, perfusion, incision, biopsy and tissue grinding) for suitability for immunology studies.
2. To investigate IFN-γ dependent and immune memory protection from malaria during pregnancy in women resident in an area of high malaria endemicity.
3. To investigate chemokine and chemokine receptor expression in relation to protection from malaria during pregnancy
4. To investigate placental tissue chemokines and chemokine receptors in relation to the poor pregnancy outcomes of malaria during pregnancy.

1.7 Study Design

1.7.1 Study site

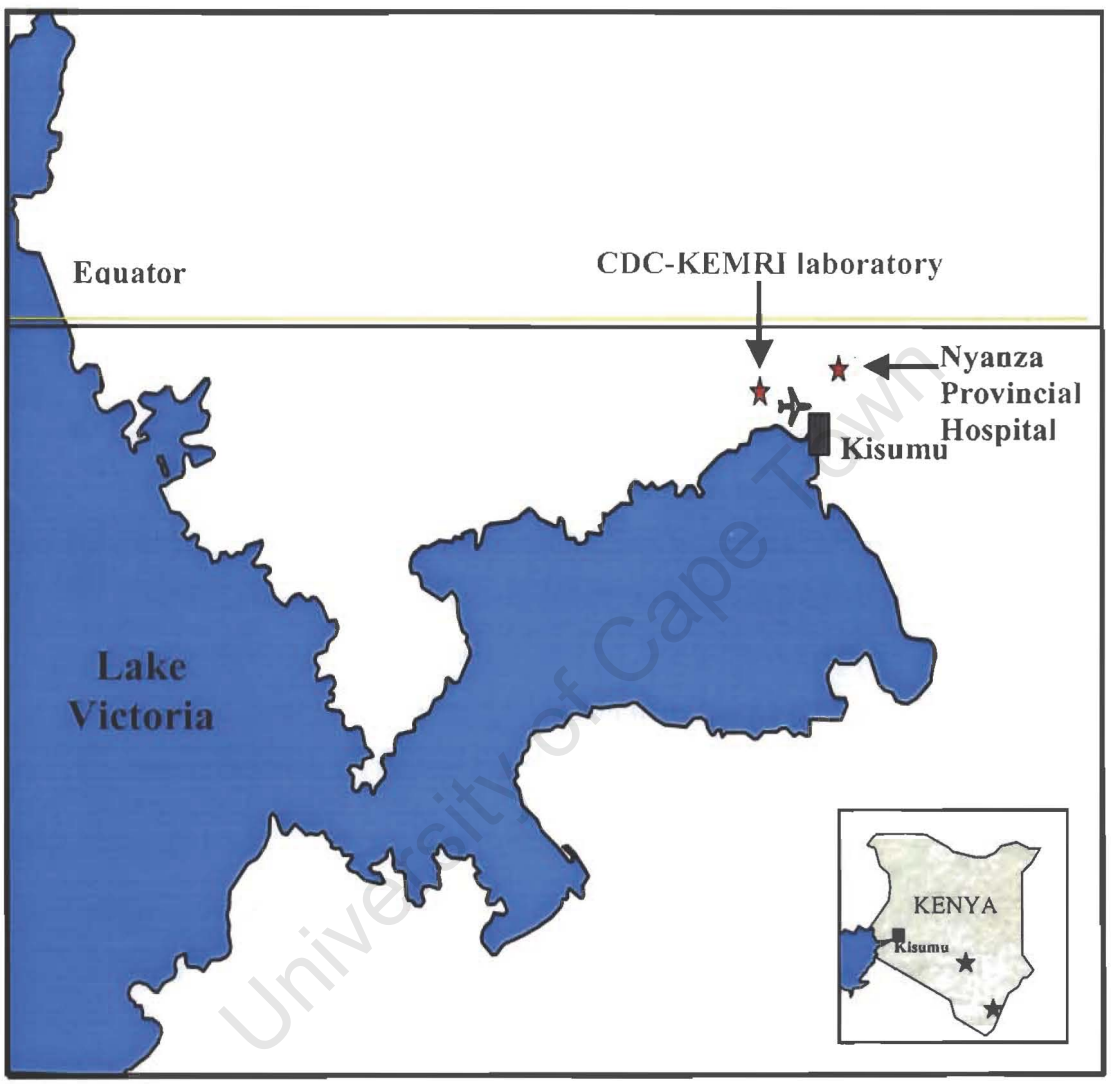
The study was carried out in Kisumu town of western Kenya, a holoendemic region for malaria (Figure 1.2). Kisumu is located on the shores of Lake Victoria with a population of approximately 350,000 inhabitants, the predominant tribal group being the Luo. Malaria is endemic and transmission occurs throughout the year with two intense transmission peaks during and shortly after the rainy seasons of March to May and October to December and with exposure to mosquito bites ranging from 90-400 per year (Githeko *et al.*, 1993). *P.falciparum* is the predominant infecting malaria species accounting for 98% of all malaria cases (Beier *et al.*, 1990).

The study was conducted at the Nyanza Provincial General Hospital (NPGH), a referral hospital for the western Kenya region and at the CDC-KEMRI research facilities. The Centres for Disease Control and Prevention (CDC), research station was established in 1979 as part of the Kenya Medical Research Institute (KEMRI) and has extensive research activities in malaria and HIV.

1.7.2 Study subjects

All gravida pregnant women admitted to the Labour Ward at the NPGH after informed written consent for HIV-1 and malaria parasite testing were enrolled into the study. Recruitment criteria included singleton, uncomplicated, term vaginal deliveries with no

Figure 1.2: CDC-KEMRI placental malaria study region



underlying chronic infection other than malaria. Pre and post HIV-1 test counselling was offered by experienced counsellors and on consent, clinical and reproductive information was obtained. Paired peripheral and placental samples were collected from HIV negative women eligible for this study after delivery. Peripheral blood was collected by finger prick into heparin charged containers, while intervillous blood and cryo-preserved placental tissue were also collected from the placenta.

All samples were transported to the CDC-KEMRI facilities approximately 15kms from the hospital (Figure 1.2) within a maximum time period of 6 hours after collection for further sample processing and immunological investigations (see details in chapters 2, 3, and 4).

1.7.3 Ethical Considerations

All study methods were approved by the Kenya National Ethical Review Committee, Nairobi, Kenya and the Centres for Disease Control and Prevention institutional review board, Atlanta, USA.

1.7.4 Laboratory Procedures

Laboratory procedures used were of two categories, those that determined study subject enrolment and those that investigated maternal immune responses in protection and pathogenesis. Procedures used to determine enrolment are outlined below. Specific immunological procedures employed to investigate maternal immune responses are briefly mentioned here but are outlined in detail within the chapters discussing study results.

1.7.4.1 Malaria Blood Smears

Thick and thin blood smears were prepared from peripheral and placental blood to determine infection. Slides were air-dried, fixed and stained using a 3% Giemsa staining solution and read using a light microscope under oil immersion using a X100 objective. Peripheral malaria infection was defined by the presence of asexual stage parasites in a blood smear. If a smear was positive, infecting parasite species and the number of parasites found were documented. Parasites were counted per 300 white blood cells

(WBC), assuming 8000 WBCs per microlitre of blood, and parasitemia was calculated. A smear was considered negative if at least 200 high power microscopic fields revealed no parasites.

Placental malaria infection was also determined by the presence of asexual stage parasites in placental blood. Infection was also determined by pigment presence in the placental blood smear. Malaria pigment presence was semi quantitatively assessed using a scoring system where PS (pigment score) 0 indicates no detectable pigment, PS1 low pigment presence and PS2 intermediate to high pigment presence (Bulmer *et al.*, 1993).

1.7.4.2 HIV Testing

Mothers' HIV status was determined by two rapid tests: a primary Determine HIV1/2 (Abbott Laboratories, Abbott Park, IL) and a confirmatory Unigold HIV1/2 (Trinity Biotech, Bray, Ireland). A mother was considered negative if results were non-reactive on both rapid tests. Mothers with discordant results were not enrolled.

1.7.4.3 Haemoglobin determination

Haemoglobin was measured using a drop of blood on the Hemocue machine (Mission Viejo, CA).

1.7.4.4 Flow cytometry and RNase protection assays (RPA)

Flow cytometry and RNase protection assays (RPA) were used to investigate maternal immune responses. Three and four-colour analysis was used to characterize intervillous blood. Data acquisition was performed on a FacsCalibur flow cytometer (BD Biosciences, CA) equipped with both an argon ion laser with 530 nm, 585nm and >650nm filter settings and a red iodide laser. Placental tissue messenger ribonucleic acids (mRNA) expression was evaluated using RPAs. Tissue RNA was quantified using phosphorimaging with the Series 400 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). See details in chapters 2, 3 and 4 for flow cytometry procedures and chapter 5 for the RPA technique.

CHAPTER TWO

A comparative analysis of various methods of maternal placental blood collection for immunology studies in placental malaria.

2.1 Introduction

The placenta is a complex, sophisticated organ that is formed 6-7 days after conception and with several important functions to execute throughout gestation. Foetal in origin, it sustains the growth and development of the foetus while acting as a barrier against components of the maternal immune system that may be directed towards paternal-foetal antigens and also against potential infectious organisms. In malaria holoendemic regions malaria parasites typically accumulate in the placenta and although they have rarely been reported to cross into the foetal blood system, infection of the placenta has serious consequences on foetal development. However, not all pregnant women suffer from a malaria infection of the placenta. It has been hypothesized that the placenta possesses immune responses that effect protection against infection. Studies on these protective mechanisms necessitate the collection of placental blood samples using reliable methods. As an initial step for the CDC-KEMRI laboratory research activities on pregnancy associated malaria, this investigation compares five documented methods of intervillous blood (IVB) collection.

2.2 Placenta morphology

The placenta is a flat, round to slightly oval disc shaped organ. At term it weighs about 450g with an average diameter of 18-22cms and an average thickness of 2-2.5cms. It consists of maternal and foetal portions, the maternal portion lying against the uterine wall while the foetal portion is attached to the foetus via the umbilical cord. The maternal portion consists of a basal plate and intervillous spaces lined by a continuous multinucleated syncytiotrophoblast and is supplied by maternal blood through uterine blood vessels. The foetal portion consists of a chorionic plate from which numerous tree-like villus structures emerge, networking towards the maternal portion and terminating in the intervillous spaces as fine projections, bathed in maternal blood (Figure 2.1). Each villus structure contains within it foetal blood vessels. Blood vessels emanate from the

umbilical cord and run along the chorionic plate dispersing throughout the placental disc in a specific pattern and turning away from the surface toward the vascular interior of the placenta through villi. Thus, immediately beneath the chorionic surface villus 'trunks' are sparsely distributed and are separated by intervillous spaces that shine through the glossy epithelium of the chorionic surface as dark purple regions. The maternal surface is subdivided into fleshy lobules or cotyledons beneath which lie densely packed minute villi carrying foetal capillaries.

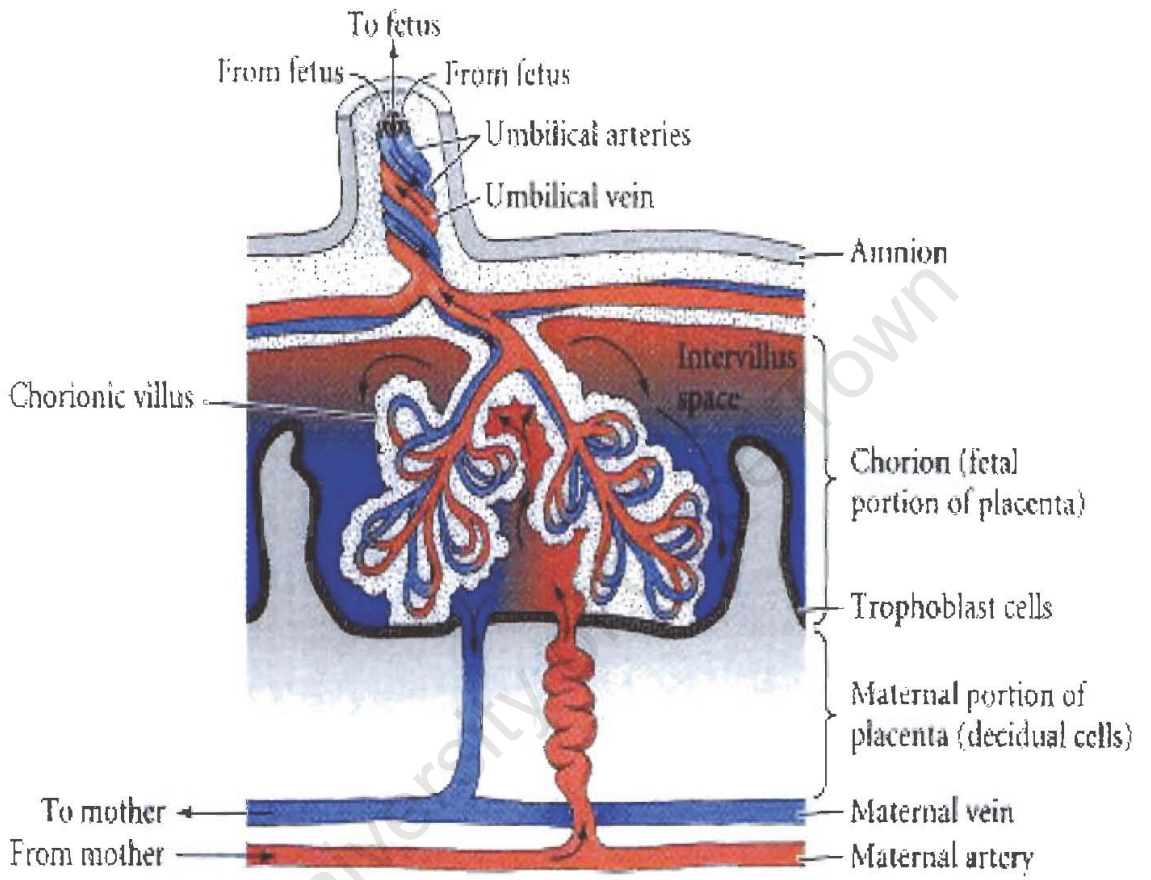
2.3 Placental blood collection methods

Several methods of maternal blood collection have been described. Camelo *et al.* (1995) describe the use of a sharp stylet to puncture (referred to as prick in this thesis) the chorionic plate and the collection of the dripping blood into a collection tube. A perfusion technique has been used to isolate maternal intervillous blood mononuclear cells (Moore *et al.*, 1997), non-invasive in its methodology, perfusion employs a pressure pump to flush phosphate buffered saline (PBS) through fine tubing into intervillous spaces to push out maternal blood. A much simpler technique and widely used is one that involves making shallow cuts, approximately 2mm deep, into the maternal surface of the placenta and collection of the resultant blood from the incision (Bulmer *et al.*, 1993). A mechanical dispersion technique has been reported where biopsied placental tissue was gently teased apart to release blood from within the tissue (Rasheed *et al.*, 1992). Sometimes also referred to as the manual tissue compression method Fried *et al.* (1998a), describe a tissue grinding method of placental tissue to isolate maternal blood. Enzymatic digestion of placental tissue to release decidual immune cells has also been described (Ritson and Bulmer, 1987).

2.4 Study rationale

In selecting the method to be used for placental blood collection for immunology studies, several important factors need to be considered. Foetal cell contamination is probably the most important. Immunological studies on protective immune mechanisms of maternal

Figure 2.1: Schematic of the placenta showing the foetal villous structure and the intervillous spaces carrying maternal blood



H.Gray (1918) Anatomy of the Human Body.

intervillous blood necessitates the collection of uncontaminated maternal blood thus foetal red blood cell levels in maternal blood should be kept as low as possible for results to be reliable. Foetal cells have been reported to modify maternal immune responses particularly in culture (Papadogiannakis *et al.*, 1985; Shohat *et al.*, 1986). Results obtained from maternal blood with considerable foetal contamination would therefore be questionable. Immunological studies also require the collection of blood samples with mononuclear cell profiles that are representative of maternal intervillous blood (IVB). Excessive manipulations of the placenta could potentially alter cell profiles and should therefore be kept at a minimum. Furthermore, unnecessary manipulations of the placenta could artificially induce cell activation making samples inappropriate particularly for RNA or activation assays where minimum manipulation is required (Hartel *et al.*, 2001). The choice of anticoagulant is crucial, different assays require different anticoagulants. Intracellular cytokine staining, for example, requiring calcium, can only be performed with heparin-collected blood. Anticoagulants such as ethylene diamine tetraacetic acid (EDTA) chelate available calcium. Time between placenta collection and placenta blood sampling is vital. Some methods require immediate processing upon expulsion of the placenta before clotting factors take effect. Sample volume issues also require attention, not all collection methods will provide the desired cell volume. And finally the immunological assay requirements in terms of sample type are important, while some assays such as antibody evaluation assays require plasma the technique of choice may significantly dilute plasma thus making results obtained unreliable. In addition to the abovementioned factors that relate directly to the quality of sample, a number of other non-placental related factors need to be considered. For example, essential equipment and special conditions like sterility may need to be contemplated, as should be adaptability to the study set up. Field settings may be limited in the facilities that are available and sophisticated collection methods would therefore be inappropriate. To determine the most appropriate method of placenta blood collection for the purpose of carrying out the investigations that constitute this thesis, five documented placenta collection methods were compared.

2.5 Objectives of the study

2.5.1 General Objective

To compare five documented methods of maternal placental blood collection (placenta puncture, perfusion, incision, biopsy and tissue grinding) for suitability for immunology studies.

2.5.2 Specific Objectives

1. To determine foetal cell contamination in five documented methods of placental blood collection
2. To determine the immune cell profiles for five documented methods of placental blood collection.

2.6 Materials and Methods

2.6.1 Study subjects

Placentas were obtained from mothers who delivered at the Nyanza Provincial Hospital in Kisumu, western Kenya. Mothers were approached and after consenting to the study were screened for HIV and malaria parasites using methods detailed in chapter one. All samples used were from mothers who tested negative for HIV 1/2 antibody and were negative on a giemsa-stained thick blood smear for malaria. Only mothers in their first and second pregnancy were approached.

2.6.2 Sample collection

Placentas were collected immediately upon expulsion into empty sterile containers (without anti-coagulant) and instantly set up for sample processing. Each placenta was carefully examined and only those that were intact and undamaged were processed. Placentas were processed for five methods of blood sample collection, within a maximum period of 45 minutes after expulsion. A peripheral blood sample from the mother was also collected to serve as a reference for the different methods of placental blood collection. Cord blood was collected from the placenta and an HIV negative male venous

blood sample was included with each set of placenta samples collected, for experiment standardization procedures.

2.6.2.1 Placenta processing

The following five methods of collection were compared. All five methods of collection described below were performed on each placenta for consistency of results. Figure 2.2 shows a diagrammatic representation of the series of sampling events from the time of placental expulsion to the end of sample processing.

2.6.2.1.1 Placental prick (Camelo *et al.*, 1995)

A modification of the method described by Camelo *et al.*, (1995) was used and is referred to as the prick method of collection in this thesis. This method employs the insertion of a needle into maternal intervillous spaces through the chorionic plate. On placenta expulsion, the retroplacental clot was removed and a quick examination was performed to determine the placenta's general state. If intact, the placenta was carefully placed on a raised sterile wire mesh, for easy accessibility, with the chorionic plate facing down. The chorionic surface was arbitrarily divided into four quadrants one of which was selected for the prick to be performed. The chorionic surface is characterized by prominent foetal vessels visible just beneath the glossy epithelium and permeating from the point of cord insertion through out the placenta. Beneath the chorionic surface and in between these foetal vessels are intervillous spaces that appear as dark purple regions due to maternal blood shinning through. The area for needle insertion was selected from one of these dark purple intervillous spaces. A large-bore, gauge 14 needle attached to a syringe was carefully inserted from beneath, through the wire mesh, past the chorionic plate and into an intervillous space. Care was taken to make sure that the point of insertion was not in close proximity of any foetal vessels. Approximately 0.5 cm of the needle was inserted and withdrawn soon after. Before withdrawal of the needle, the syringe was gently pulled to create a vacuum that would initiate blood flow out of the IV space. The needle was very carefully withdrawn and blood was allowed to drip freely into eppendorf tubes charged with 25µls of a 1:4 heparin (1000units/ml) in PBS dilution, held beneath the point of insertion.

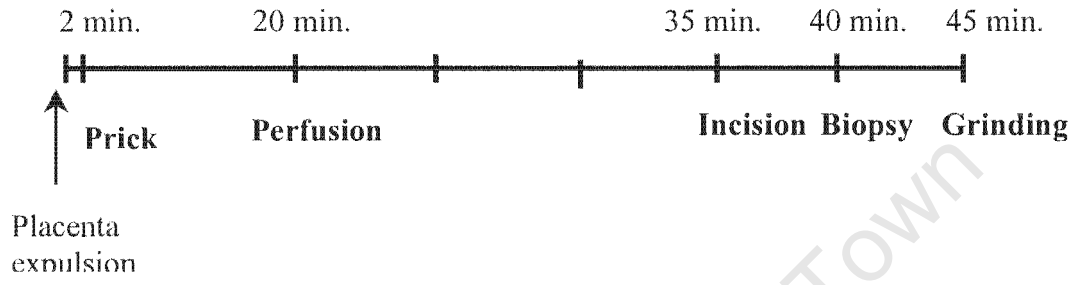


Figure 2.2: Chronology of sampling events. Within two minutes of placenta expulsion the placenta was set up and the prick method of collection performed in a region appropriately chosen for the technique. The perfusion technique was carried out next and perfusate obtained within 30minutes of placental expulsion. Blood collected by incisions was carried out next followed by biopsy within at least five minutes of each other and finally approximately 45 minutes after expulsion; blood by tissue grinding was collected. On average, all techniques were carried out within a maximum period of 45 minutes after placenta expulsion.

2.6.2.1.2 Perfusion (Moore *et al.*, 1997)

On completion of the prick method of collection the placenta was carefully lifted from the wire mesh stand and placed, on a 20° angled slanting surface with the maternal side facing up for perfusion. Perfusion involves the flushing out of maternal blood from intervillous spaces with the aid of a pump. The slanting surface is therefore necessary for efficient drainage of blood from the placenta. Selecting a quadrant different from the one that the prick method was performed, four umbilical vessel catheters (UVC) were inserted into the placental tissue to a depth of about 2-4 mm, 2-4 centimetres apart. Care was taken to ensure that the UVC were not inserted too deeply below the maternal surface where they may damage chorionic villi, consequently causing contamination. Approximately 50mls of a 1:150 heparin (1000units/ml) dilution in PBS at a flow rate of 8mls per minute was delivered into the intervillous spaces by a peristaltic pump (Barnant, Barrington, IL). The resulting diluted blood flushed out by this action was collected using a blunt 20ml syringe and collection continued until the 50mls of PBS pumped in was recovered.

2.6.2.1.3 Incision (Bulmer *et al.*, 1993)

Shallow cuts, approximately 2mm deep, were made into the maternal surface of the placenta's third quadrant. The resultant blood that filled into these incisions was collected using a blunt 1 ml syringe and transferred into heparin charged eppendorf tubes.

2.6.2.1.4 Mechanical dispersion technique (biopsy method) (Rasheed *et al.*, 1992)

Placental biopsies 2cm square and 2mm deep in size were made approximately a quarter of the distance from the centre of the placenta in the last available placental quadrant. Biopsies were transferred to a heparin-PBS (5mls) containing petri dish and using the flat edge of a scalpel the tissue was gently teased apart working from the outer edge inwards to release blood from within the tissue into the PBS. The resulting cell suspension was placed in a 15ml centrifuge tube and the tissue debris allowed to settle. The supernatant was aspirated and transferred to another 15ml centrifuge tube.

2.6.2.1.5 Manual tissue compression technique (tissue grinding) (Fried *et al.*, 1998a)

A pie shaped full thickness block of placental tissue was sliced from any part of the placenta but keeping away from the quadrant that was perfused. Pieces of placental tissue were fed into a meat grinder and blood emerging from the grinder before the tissue squeezed through was collected into heparin charged eppendorf tubes.

Immunological assays were performed within a period of 6 hours after placental sampling.

2.6.2.2 Immunological assays

In comparing the five methods of placental blood collection identified, two issues were considered, foetal blood contamination and maternal mononuclear cell profiles. Foetal blood contamination was determined by measurement of foetal haemoglobin (HbF) levels in maternal placental blood while mononuclear cell profiling was by immune phenotyping.

2.6.2.2.1 Foetal Haemoglobin Determination

The detection of foetal blood contamination was by a single colour flow cytometry. A Fluorescein isothiocyanate (FITC) fluorochrome-labelled monoclonal antibody (mAb) was directed against HbF and used to determine the levels of foetal blood cell contamination. Placental blood samples collected by the five methods outlined were stained for foetal haemoglobin. For each experiment, a negative (0% foetal red blood cells in adult male blood) and a positive (10% foetal RBCs in adult male blood) control were set up alongside the placental samples.

2.6.2.2.1.1 Control preparation

Adult and cord red blood cells were used for preparing the assay controls. HIV negative adult male O-positive blood was obtained from the Ministry of Health Regional Blood Bank while cord blood cells were obtained from placentas of enrolled women. Both adult and cord blood were spun at 1800 revolutions per minute (rpm) for 5 minutes at room temperature (RT) and plasma removed. The red blood cell pellet was washed twice

in Alsever's solution (a red blood cell preservation solution) (Caltag Laboratories, Burlingame, CA.) at 1800rpm for 5 minutes and resuspended in 1ml of the same. RBC counts were determined for both adult and cord blood and using the formula outlined below, the 10% (positive control) cord blood cell suspension in adult blood was prepared. In measuring foetal blood cell contamination, the cut-off for acceptable levels of contaminating HbF in maternal IVB samples was set as 10%. Ten percent foetal RBCs in adult blood was thus prepared as the assay positive control. Adult blood without cord RBC served as the 0% (negative) control.

10% foetal blood in adult blood

Adult RBC count x 0.1= **A**

(**A**/Adult RBC count) x 100= ***X*** µls

X: amount of adult whole blood to remove from a 100µl aliquot of undiluted adult blood suspension.

(**A**/ Cord RBC count) x 100=**Y** µls

Y: amount of cord blood to add back to the adult whole blood to replace the amount X that was discarded.

The amount X was discarded from the adult RBC preparation and Yµls of cord blood was added to the adult whole blood. A new RBC count was performed.

2.6.2.2.1.2 HbF Staining

Foetal haemoglobin determination was by an HbF staining kit (Caltag Laboratories, Burlingame, CA). Blood samples obtained by perfusion and biopsy methods were first pelleted by centrifugation at 2500rpm for 5 minutes and reconstituted with 1ml PBS. RBC counts for all samples including the control preparations were determined and adjustments were made to $2.5x10^9$/ml of red cells in PBS. 10µls of each sample type and control were placed into labelled 3ml polystyrene tubes and cells were fixed by adding 1ml of a 0.05% glutaraldehyde solution (Sigma Chemical Co., St. Louis, MO) for strictly

10 minutes at RT. The cells were washed three times at 2000rpm for 5 minutes each with 2mls of a 1% PBS-Bovine Serum Albumin (BSA) (Sigma Chemical Co., St. Louis, MO) wash buffer solution. Cells were resuspended in 0.5mls of Triton X100 permeabilizing solution (Caltag Laboratories, Burlingame, CA) and incubated for 20 minutes at RT. After incubation cells were washed once and resuspended into 1ml PBS/BSA. Ten microlitres of the resuspended cell suspension was aliquoted into fresh tubes and stained with 5µls of a fluorescein isothiocyanate (FITC) labelled anti-HbF mAb and incubated for 15 minutes at RT in the dark. Two washes were carried out at the end of the incubation period and the resultant pellets were resuspended in 500µls of 1% formaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS/BSA. Acquisition of events was by the FacsCalibur flow cytometer (BD Biosciences, CA, USA).

2.6.2.2.2. Immune Phenotyping

Leucocyte subpopulation phenotypes were determined by using four-colour flow cytometry. Immune phenotyping was performed for the identification of T cells, B cells, NK cells, memory cells and monocytes. T cells were defined as cells expressing the CD3 antigen, B cells the CD19 antigen, NK cells were defined by the presence of CD16 and CD56 and absence of CD3. Monocytes were defined by CD14 and CD45, naive T cells by CD45RA and memory T cells by the absence of CD45RA. Fluorochrome-labelled monoclonal antibody reagents were used for cell surface marker staining using a 'whole blood' staining procedure. The following antibodies were used: Fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies to CD3 (Mouse IgG1), CD4 (Mouse IgG1), CD45 (Mouse IgG1) and CD45RA (Mouse IgG1); phycoerythrin (PE) conjugated antibodies to CD8 (Mouse IgG1), CD14 (Mouse IgG1), CD56 (Mouse IgG1) and CD16 (Mouse IgG1); peridin chlorophyll protein (PerCP) conjugated antibodies to CD3 (Mouse IgG1) and CD45 (Mouse IgG1); allophycocyanin (APC) conjugated antibodies to CD4 (Mouse IgG1) and CD19 (Mouse IgG1). In addition, the following istoype controls were used, mouse IgG1 FITC, mouse IgG1 PE, mouse IgG1 PerCP and mouse IgG1 APC. All were obtained from Becton Dickinson, San Jose, CA.

The antibody-staining panel in Table 2.1 was carried out for every method of sample collection and also for a corresponding peripheral sample of blood from the same mother. A compensation tube was included for every experimental set up and was used for machine parameter settings for each individual. Only peripheral blood was used for this tube. An isotype control tube was included for each type of placental sample and was used to control for non-specific antibody binding.

2.6.2.2.2.1 Immunostaining

Falcon tubes (5ml) (Becton Dickinson, San Jose, CA) were labelled for each type of placenta blood sample as indicated in Table 2.1 and 5µls of appropriate antibodies were added to each tube. Fifty microlitres of each of the placental sample types was subsequently added, gently vortexed and then incubated for 20 minutes in the dark at RT. Three point five millilitres of a 1:10 dilution of room temperature Facs lysing solution (Becton Dickinson, San Jose, CA) in distilled water, was added to each tube for red cell elimination. Samples were then incubated at RT for 10 minutes for effective red cell lysis. Cells were spun at 1500rpm for 7 minutes at RT, the supernatant aspirated and the resultant pellet reconstituted in 2mls wash buffer (1% BSA in PBS) for a further spin. The final cell pellet was fixed in 500µls of 1% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) until analysed on the flow cytometer.

2.6.2.2.2.2. Flow cytometric analysis for HbF and immune phenotyping

Fetal red blood cell and maternal leukocyte subpopulation enumeration was on a FacsCalibur (BD Biosceinces, CA, USA) flow cytometer using the BD Cell Quest Pro software (BD Biosciences, CA, USA). Red blood cells were gated on the FSC-SSC and a total of 50,000 events were acquired. Contamination levels were determined by the subtraction of the negative control value from the sample value. For immunephenotyping, lymphocytes and monocytes were identified and gated on the FSC- SSC and a minimum of 5,000 lymphocytes within the lymphocyte gate were acquired and were used as the guide for total event acquisition. The isotype control tube determined non-specific binding and set markers for distinguishing fluorescence positive and negative populations

	FITC	PE	PerCP	APC
Tube 1 (Compensation)*	CD4	CD8	CD3	CD19
Tube 2 (Isotype control)	IgG1	IgG1	IgG1	IgG1
Tube 3 (T cells)	CD3	CD8	CD45	CD4
Tube 4 (B and NK cells)	CD3	CD56+CD16	CD45	CD19
Tube 5 (naïve and memory T cells)	CD45RA	CD8	CD3	CD4
Tube 6 (monocytes)			CD45	CD14

Table 2.1: Antibody staining panel. The panel above represents the antibody combinations used to stain for various cell subpopulations. Antibody staining was carried out for every method of sample collection and also for a corresponding peripheral sample of blood. A compensation tube was included and was used for machine parameter settings for every individual. *Only peripheral blood was used for this tube. An isotype control tube was included for each type of placental sample.

and also for the subtraction of background. Results for T, B and NK cells were reported as percentages of the lymphocyte gate events while monocytes levels were reported as a percentage of total monocytes gate events.

2.7 Statistical analysis

Data was entered into and analysed using the SPSS statistical software (SPSS 11.0.1, Inc., Chicago, IL) package. Data was analysed for statistical differences between different methods of placenta collection for foetal cell contamination levels and IVB cell subpopulation numbers. Mean values were calculated and were evaluated using the student t test. A two-sided *P* value of less than 0.05 was considered to be statistically significant.

2.8 Results

Results reported here are from 10 placentas that fulfilled the inclusion criteria outlined for placental collection. All 10 placentas were from individuals who were HIV negative, placenta malaria negative (PM-) and were either gravida one (G1) or gravida two (G2). This being the first study comparing different methods of maternal placental blood collection, sample size was determined on the basis of the highly significant differences that were evident from preliminary data collected from only five placentas. Ten placentas were however collected to ensure that the differences reported were indisputable.

2.8.1 Foetal Haemoglobin Contamination Levels

Each method of placental sample collection was assessed for foetal cell contamination. A FITC labelled monoclonal antibody was directed at foetal haemoglobin (HbF), which gave indication of levels of foetal blood cells within maternal IVB. The HbF detection kit used is an extremely sensitive evaluation system, able to detect contamination levels as low as one foetal red cell per 100,000 maternal red cells. A negative and positive control was included. Zero percent foetal red blood cells in adult blood served as the negative control while the 10% preparation was the positive control. Adult male blood was used for the controls to exclude the possibility of high background levels due to pregnancy conditions that may permit foetal cells into maternal peripheral blood during the course

of pregnancy. Figure 2.3 shows an example of the foetal staining plot analysis of the experiment controls used. Gating was done on the FSC-SSC plot for red blood cells (Figure 2.3a) and quadrant markers were set according to 0 and 10% controls. Dots appearing in the upper right quadrant of the 10% plots indicate a positive stain for foetal cells and therefore contamination (Figure 2.3 b and c). The analysis plots for each method of collection for one individual are shown in Figure 2.4 and are representative of the plots obtained for the 10 placentas collected. Lowest levels of positive staining for HbF were for the prick method of collection where as the highest positive staining was from incision. Mean levels of HbF staining from all 10 placentas for each method of collection are shown in Figure 2.5. The lowest levels of foetal RBC contamination was in collection by prick (5.5%) while the highest levels of contamination was with collection by incision (71.6%). The difference of foetal blood contamination between the different methods of maternal IVB collection was significant ($P<0.001$).

2.8.2 Immunephenotyping

The FSC- SSC plot gives an indication of cell size and complexity. Cells with similar size and complexity will be plotted together on the FSC-SSC, thus separating into distinct populations. Gating is the most critical step in the analysis of data generated by flow cytometry. Separation of cells into distinct populations on the FSC-SSC plot is therefore crucial for gating purposes. Cell distributions on FSC-SSC plots were compared for each method of placental sample collection, for cell separation into distinct populations, against a peripheral blood sample from the same individual. Figure 2.6 shows an example of the FSC-SSC analysis plots and is representative of results obtained for each of the 10 placentas. Peripheral blood had excellent separation of cells into three distinct cell subpopulations, lymphocytes monocytes and granulocytes. The prick method of collection had a distribution of cells very similar to peripheral blood with separation into three discrete populations. Incision and tissue grinding had some degree of separation, some cell populations were distinguishable, others were not. In contrast perfusion and biopsy cells did not separate into distinct cell subpopulations making the identification of populations unachievable, particularly for monocytes (Figure 2.7).

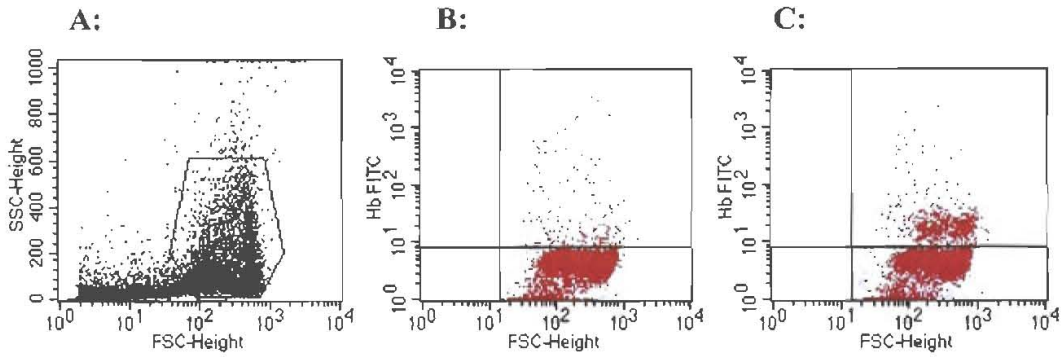


Figure 2.3: Feotal haemoglobin experiment control dot plots for placenta 177. Placenta 177 is representative of results obtained from all 10 placentas. A, indicates the FSC-SSC gating of red blood cells, B and C are the 0 and 10% controls respectively.

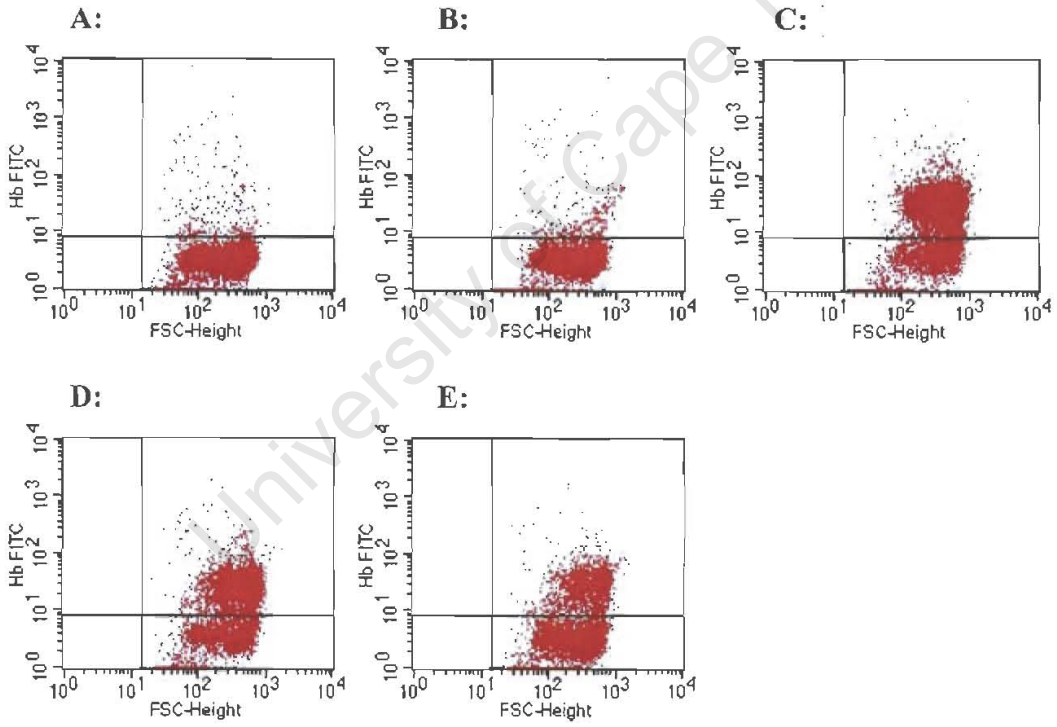


Figure 2.4: Feotal haemoglobin staining dot plots for the five placenta blood collection methods for placenta 177. A- placental prick, B-perfusion, C-incision, D-biopsy and E-tissue grinding. FSC-SSC gating for red blood cells.

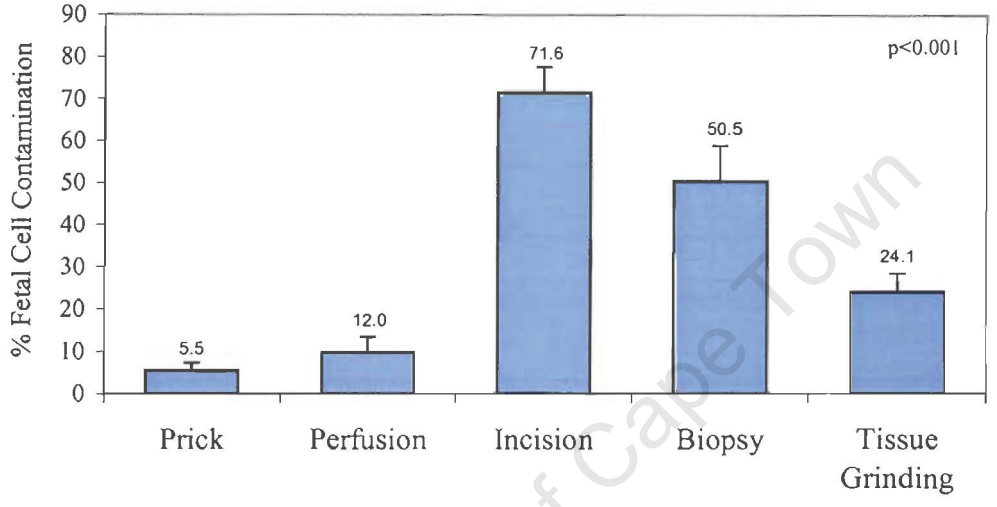


Figure 2.5: Percentage mean fetal cell contamination for the five different methods of collection. Sample collection by prick had the lowest contamination levels (5.5%) while incision had the highest (71.6%). Values obtained by gating for red blood cells on the FSC-SSC analysis plot. Error bars indicate standard error. N=10.

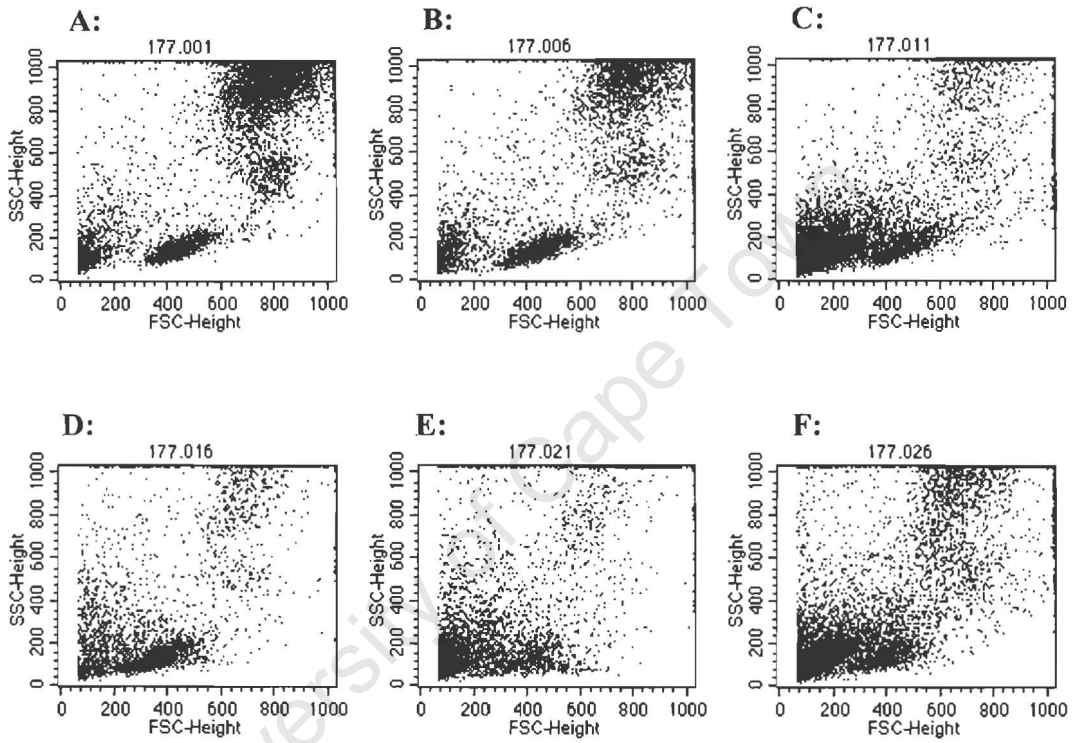


Figure2.6: Study subject number 177 FSC-SSC analysis plots for peripheral blood (A), together with IVB obtained by prick (B), perfusion (C), incision (D), biopsy (E) and tissue grinding (F). Of the five methods of maternal IVB collection, prick has the most distinct separation of cells on the FSC-SSC and closely resembles peripheral blood. Collection by perfusion and biopsy were the least separated.

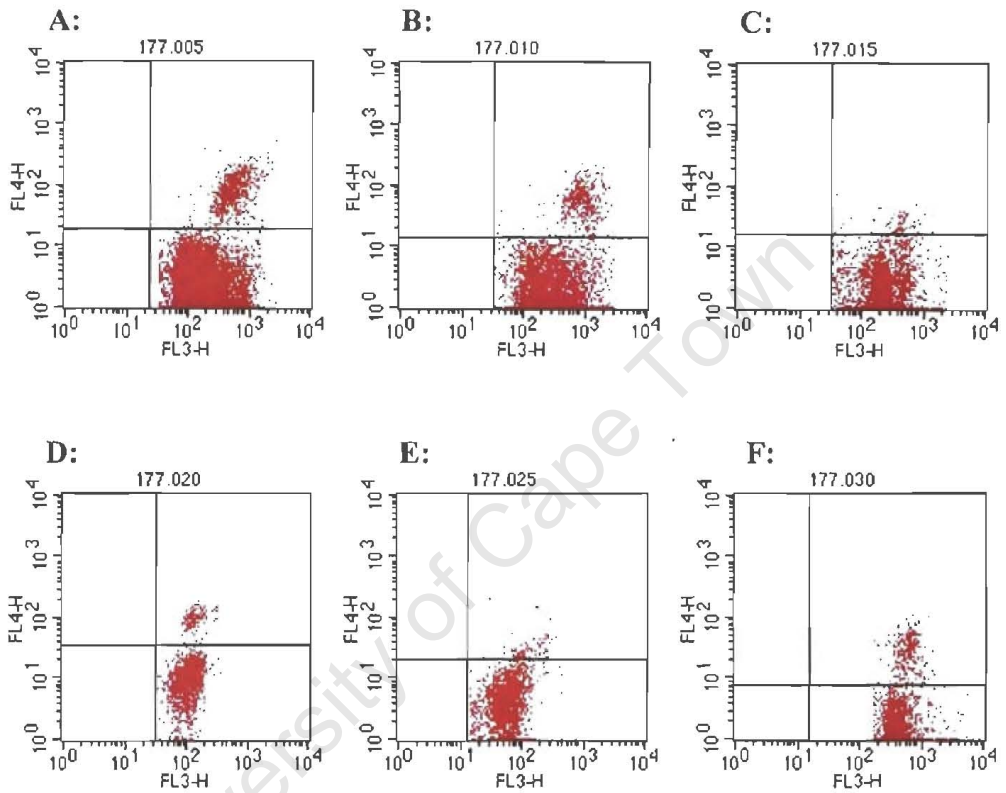


Figure 2.7: Study subject number 177 monocyte analysis plots for peripheral blood (A), prick (B), perfusion (C), incision (D), biopsy (E) and tissue grinding (F) blood. Monocyte population was not clear for some of the methods on the FSC-SSC plots therefore as a further step blood samples were stained with an anti-CD45 PerCP antibody and an anti-CD14 APC antibody for monocyte selection. Prick, incision and tissue grinding monocytes were distinct contrary to perfusion and biopsy monocytes.

2.8.2.1 Cell subpopulation phenotyping

Further analysis of cell populations was carried out using fluorescent antibody staining. Mean levels of the key cell populations (monocytes and lymphocytes) as well as specific cell subpopulations (CD4 and CD8 T cells, B cells, NK cells, memory T cells and monocytes) were obtained and compared. Statistical analysis compared the results obtained from the five methods of maternal IVB collection, peripheral data was not included and therefore the *P* values obtained relate only to the five placental blood sample types. Peripheral blood data was however incorporated in the graphs to highlight the difference between the placental blood samples and peripheral blood.

2.8.2.2 Lymphocyte and monocyte gating

Total lymphocyte and monocyte populations were compared. Percentage mean cell numbers for both cell groups are shown in Figure 2.8. Overall, there was a significant difference in the numbers of cells obtained for the five methods of collection for cell numbers in the lymphocyte and monocyte gates (P=0.01, P=014 respectively). For the lymphocyte gate, the prick method of collection yielded cell numbers similar to the peripheral blood, while perfusion and biopsy had significantly lower numbers of cells than peripheral blood.

2.8.2.3 Lymphocyte gate cell subpopulation numbers

Using quadrant markers and antibody isotype controls, percentage mean levels of specific lymphocyte subpopulations were determined for CD4 and CD8 T cells, B cells, NK cells, and memory T cells. Figure 2.9 shows the staining analysis plots for each of the five methods of maternal IVB collection compared with a peripheral sample from the same individual.

The percentage mean levels of the cell subpopulations within the lymphocyte gate are shown in Figure 2.10, 2.11, 2.12 and 2.13. Total T cell numbers yielded by the five methods of collection were significantly different (P =0.003) and of the five different methods, prick yielded T cell numbers closest to peripheral blood (Fig. 2.10). CD4 and CD8 T cells numbers (Fig. 2.11) were also significantly different between the five

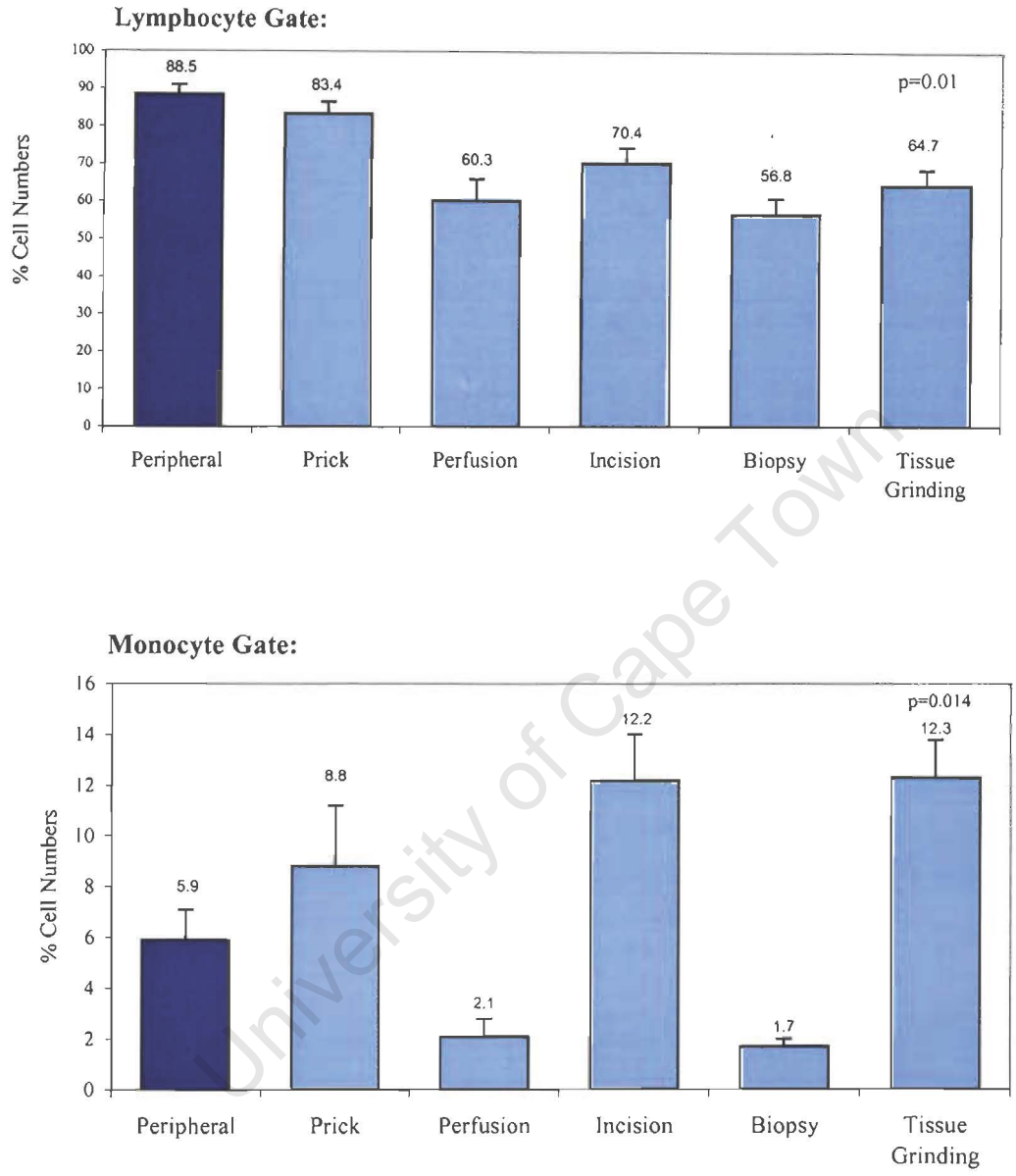


Figure 2.8: Percentage mean cell numbers for lymphocyte and monocyte gating. N=10. The dark bar represents peripheral blood; lighter colored bars represent placental blood. Cell numbers were significantly different for the five methods of collection in the lymphocyte (P=0.01) and monocyte gates (P=0.014). Error bars indicate standard error.

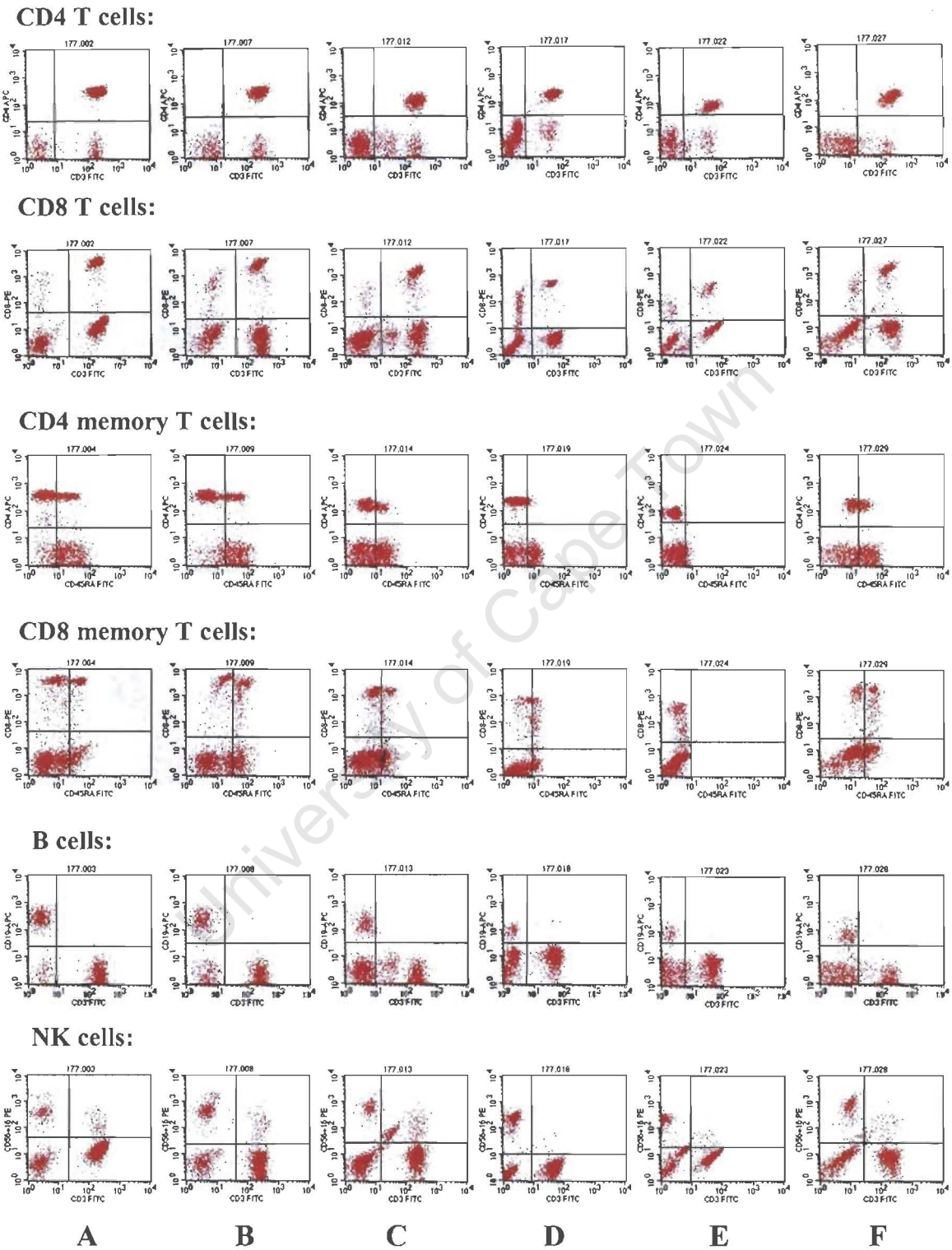


Figure 2.9: Staining dot plots for peripheral blood (A), and IVB obtained by prick (B), perfusion (C), incision (D), biopsy (E) and tissue grinding (F) for CD4 T, CD8 T, CD4 memory, CD8 memory T cells, B cells and NK cells.

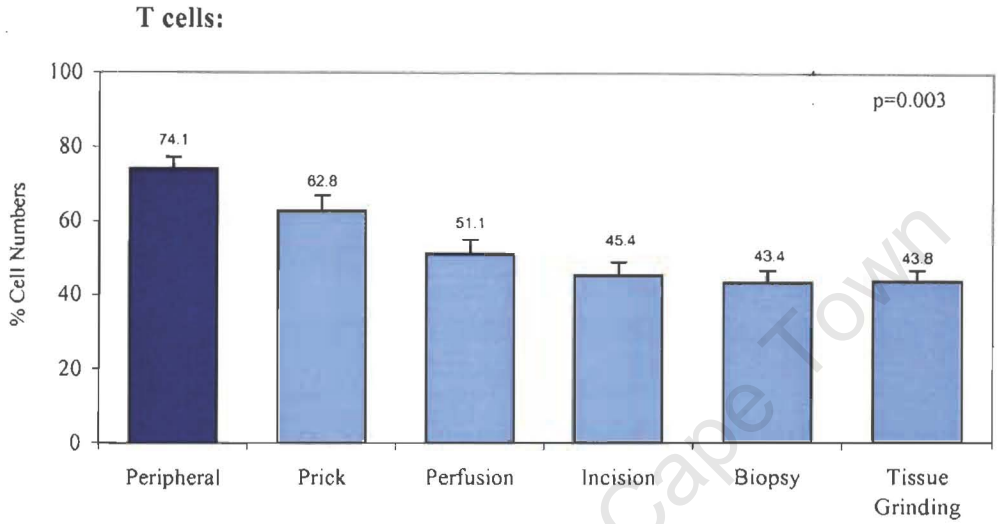


Figure 2.10: Percentage Total T cells numbers yielded by the five methods of maternal IVB collection by lymphocyte gating. Cell numbers were significantly different (p=0.003). Of the five different methods, prick yielded T cell numbers closest to peripheral blood. Error bars indicate standard error.

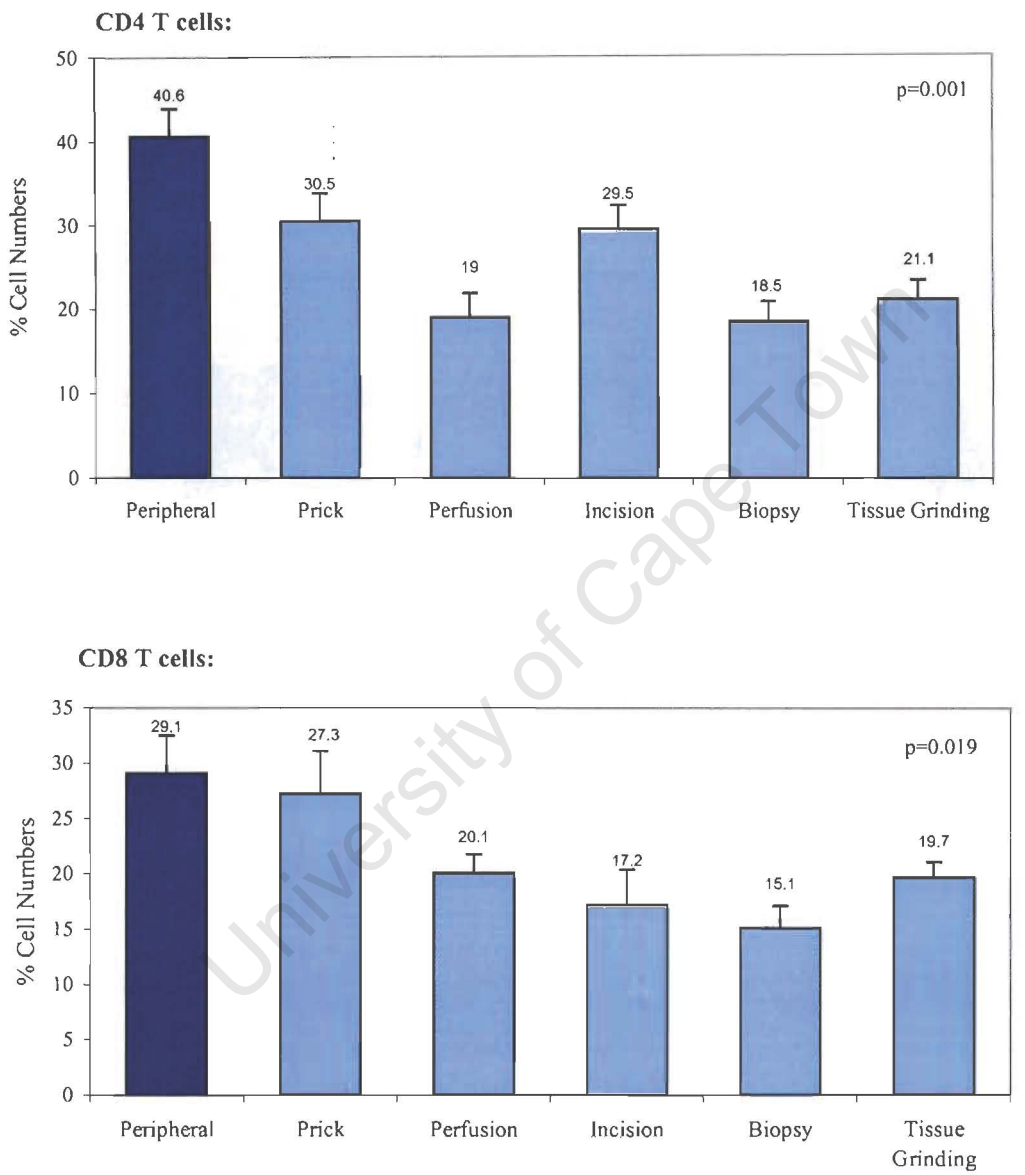


Figure 2.11: Percentage mean cell numbers for CD4 and CD8 cells by lymphocyte gating. CD4 and CD8 T cells numbers were significantly different between the five methods of placental blood collection (P=0.001 and P= 0.019 respectively). Error bars indicate standard error.

methods of placental blood collection and again prick blood had levels most comparable to peripheral blood (P =0.001, P =0.019). When analysed further by CD45RA negative marker distribution for CD45RO indication, CD8 memory T cells (P =0.023) but not CD4 memory (P =0.503) cells showed differences by method of collection (Fig 2.12). Numbers of CD8 memory T cells were surprisingly lower in placental blood than peripheral for all methods of collection, that notwithstanding prick levels were most similar to peripheral blood. Although similar for all methods of collection, CD4 memory cells for all five methods were considerably lower than peripheral blood. B and NK cells numbers were not different between the five methods of collection (P =0.714, P =0.335 respectively) (Fig. 2.13).

2.8.2.4 Percentage similarity of placenta blood cell profiles to peripheral blood

A scoring system was formulated to further evaluate overall results obtained from each method of collection. Taking peripheral blood cell numbers as the reference, the percentage cell yield of each method was calculated against the peripheral blood cell yield for percentage similarity and scored. Scoring results are shown in Table 2.2. Comparisons revealing differences in cell profiles between each method of collection (horizontal comparison) and similarity to peripheral blood composition (vertical comparisons) are summarized. A score of 90-100% indicates high similarity while a score below 50% indicates low similarity.

Horizontal comparisons revealed clear variations in scores obtained for cell subpopulations with significantly different cell numbers by method of collection. Conversely, cell subpopulations that yielded numbers that were not statistically different for the five methods of sample collection were similar in their scores. Prick, incision and tissue-grinding methods scored equally and with high similarity (90-100%) to peripheral blood for monocytes cells, in contrast perfusion and biopsy had less than 50% similarity to peripheral blood. For Total CD3, CD4 and CD8 T cells the prick method of collection scored at least 20% higher than other methods and although CD8 memory cell numbers were fairly similar (80-90%) for four out of the five methods of collection, incision showed low similarity (below 50%) to peripheral blood. Memory CD4 T cells, B cells

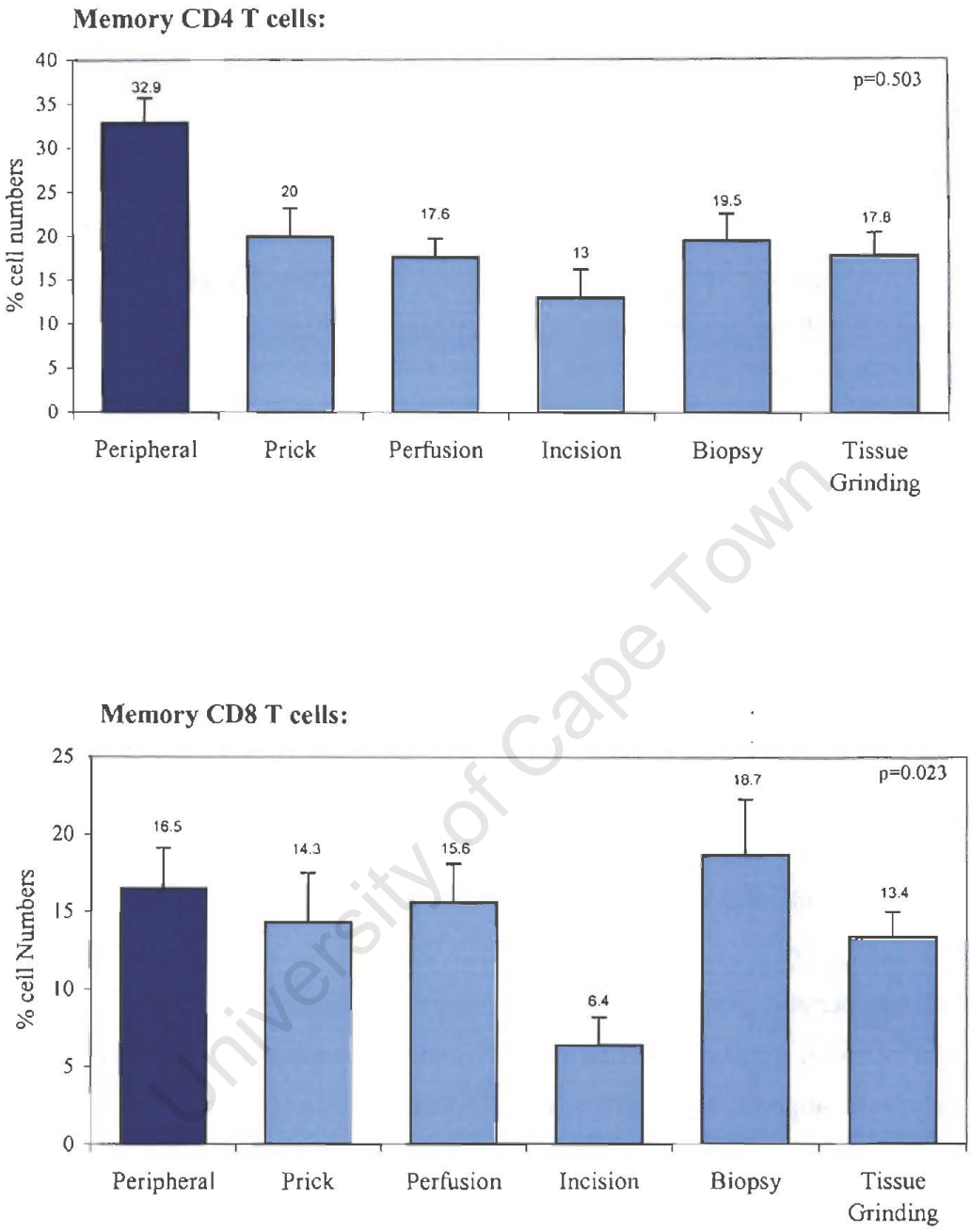


Figure 2.12: Percentage mean cell numbers for CD4 and CD8 T memory cells by lymphocyte gating. CD8 memory T cells (P=0.023) but not CD4 memory (P=0.503) cells showed differences by method of collection. Error bars indicate standard error.

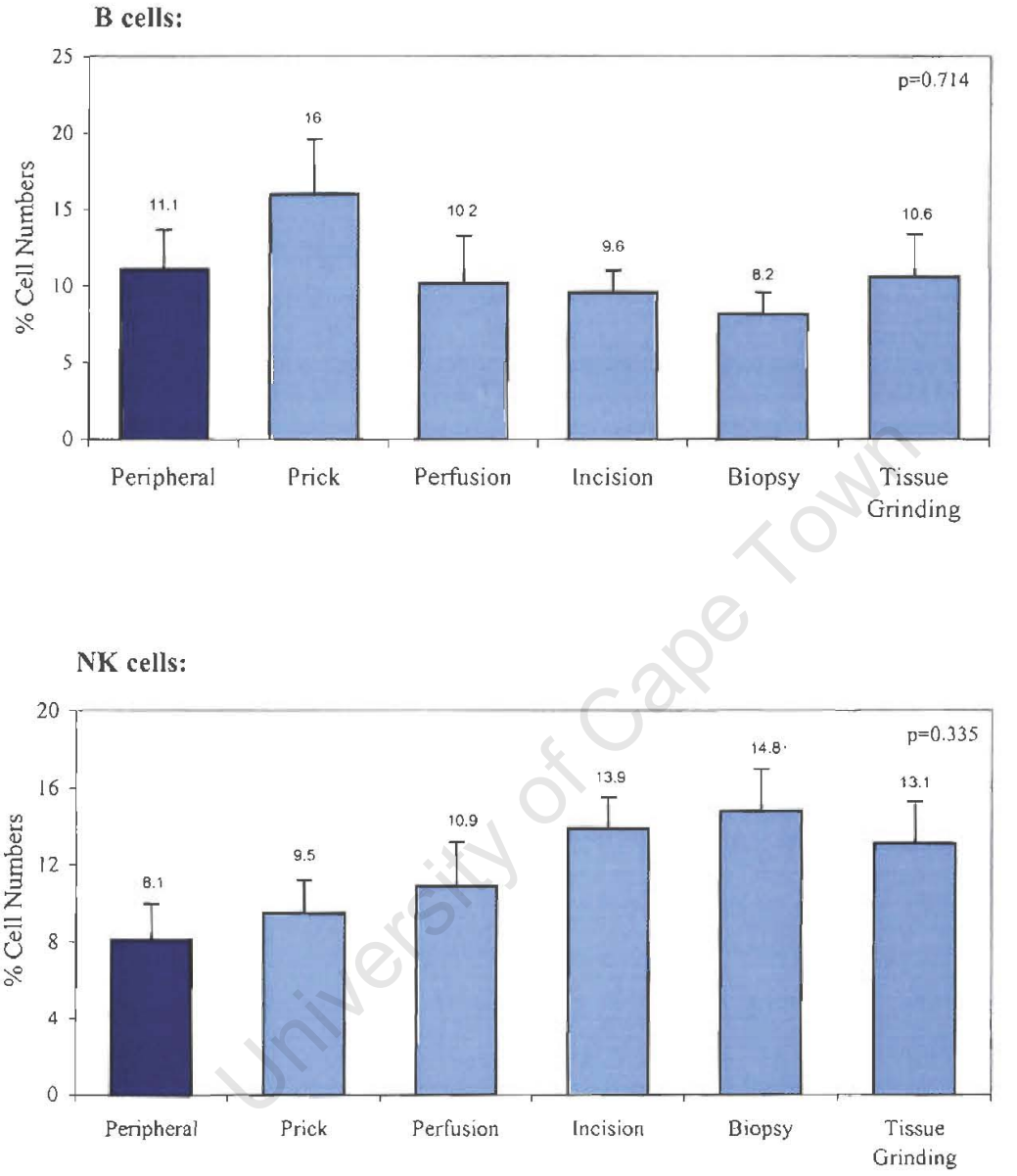


Figure 2.13: Percentage mean B and NK cell numbers by lymphocyte gating. B and NK cells numbers were not different between the five methods of collection (P=0.714, P=0.335 respectively). Error bars indicate standard error.

	Prick	Perfusion	Incision	Biopsy	Tissue Grinding
Monocytes#	******	*	******	*	******
Total CD3 #	*****	***	***	**	**
CD4 T cells#	****	*	****	*	**
CD8 T cells #	******	***	**	**	***
Memory CD4 T cells	*****	**	**	**	**
Memory CD8 T cells#	*****	******	*	******	*****
B cells	******	******	*****	****	******
NK cells	******	******	******	******	******

Table 2.2: Tabulated summary of cell profiles for the five methods of placental blood collection evaluating cell profile divergence from peripheral blood (vertical comparison) and between method cell profile differences (horizontal comparison). ******90-100% ***** 80-90% ****70-80% ***60-70% **50-60 *below 50%
#: $p<0.05$

and NK cell numbers were not significantly different and therefore scored the same for all collection methods. Interestingly, B and NK cells scored high (between 70-100%) for all five methods of placental sample collection.

A vertical assessment of the summarized results (Table 2.2) revealed that collection of maternal IVB by placental prick has highest similarity to peripheral blood for cell number yield (between 70-100%) as compared to the other methods of placental blood sample collection. Incision and biopsy have lowest similarity to peripheral blood.

2.9 Discussion

This thesis focuses on cellular immune response investigations in peripheral and placental intervillous blood in relation to protection from malaria during pregnancy. The collection of placental intervillous blood with minimal foetal cell contamination and reliable maternal mononuclear cell profiles is therefore vital. Several methods of maternal IVB collection have been documented however no comparisons have been made for collection method suitability for immunology studies. Five documented methods of collection were compared to determine the best method of collection for these studies, collection by placental prick, perfusion, incision, biopsy and tissue grinding. Placental prick, like the finger prick technique is a simple method of placental blood collection easy to perform and requiring minimal equipment. If performed correctly and instantly upon placenta expulsion approximately 3-4mls of intervillous blood may be collected. Perfusion requires fairly sophisticated equipment and entails some skill in carrying out the technique. It requires time to perform; of the five methods of collection perfusion took the longest taking on average15 minutes to obtain 50mls of diluted perfusate. Cell volume obtained by perfusion is good; 5mls of packed cells result from 50mls of diluted perfusate. Incision like prick is easy to perform, requiring no special equipment and taking little time for sample collection. Blood volume obtained is low thus several incisions need to be made for 3mls of sample. The biopsy method of collection is easy also requiring little time to perform and with good cell volume yield. Tissue grinding is simple to carry out and also has good cell volume yield; approximately 5-10mls of blood are achieved from a block of placental tissue.

Each of the five methods briefly described above was performed on the same placenta for consistency of results and all were carried out within a maximum period of 45 minutes after placenta expulsion. Technique evaluation was on the basis of foetal cell contamination and maternal mononuclear cell profiling. This report provides information that may be used in the assessment and subsequent selection of appropriate placental blood collection techniques for malaria in pregnancy immunology studies.

2.9.1 Foetal blood contamination of maternal intervillous blood

Foetal blood contamination levels in maternal intervillous blood were determined by foetal haemoglobin detection in the placental blood samples collected. A fluorochrome labelled monoclonal antibody was directed against HbF and is able to detect contamination levels as low as one foetal red cell per 100,000 maternal red cells. Comparisons were made for HbF levels among the five methods of collection. The prick method of collection had the lowest levels of contamination (5.5%). This result is not entirely unexpected. In performing a placental prick, the placenta is raised with the foetal surface facing down, allowing the accumulation of blood towards the chorionic plate and thus demarcating the IVB spaces through the glossy surface of the foetal side. Additionally, entry into IVB spaces without puncturing foetal vessels is better facilitated from the chorionic plate than the basal plate due to the absence of minute foetal vessels in this region. The chorionic plate has prominent vessels that are sparsely distributed and thus easier avoided. Accurate access into an IVB space without foetal vessel perforation is in this way made possible. Thus every time a prick was performed it was carried out with precision; hence the low HbF contamination levels.

Perfusion, like placental prick, directly accesses IVB spaces albeit through the basal plate and could therefore explain the slightly higher levels of foetal blood contamination levels (12%) observed. The UVCs are inserted into the basal plate, which contains a dense lattice of foetal capillaries, and are likely to be broken on insertion of the UVCs. Moreover, the pressure with which PBS is delivered into IVB spaces may damage the fine vessels of the villous trees thus releasing foetal blood.

Sample collection by incision, biopsy and tissue grinding exhibited unacceptable levels of foetal blood contamination. Over 70% of the blood sample obtained by incision was foetal in origin. Referring to the placental architecture, the villous tree that holds the foetal blood circulation starts out as stem villi that emerge from the chorionic plate into the intervillous space. From the stem villi arise smaller intermediate villi, which branch progressively into terminal villi ending close to the maternal surface. It is likely that incisions cut directly into the villous tree and that the blood that wells into the incision is foetal and not maternal blood. The biopsy method cuts into the maternal surface and would be expected to have the same contamination issues. Interestingly, collection by biopsy had lower foetal blood contamination than incision, and even more intriguing are the even lower levels of foetal contamination by the tissue grinding method. It is reported that the total blood volume obtained from a placenta would constitute 30% foetal blood and 70% maternal (Kaufmann, 1981). The foetal haemoglobin results obtained from the tissue grinding technique are consistent with that. A pie shaped piece of placental tissue was processed for the tissue grinding method and the contamination levels obtained were 24%, a figure commensurate with the expected foetal cell presence in maternal blood.

2.9.2 Intervillous blood mononuclear cell profiles

Mononuclear cell profiles were determined for the five methods of placental blood collection. Monocytes, T cells, B cells and NK cell numbers were assessed and yielded interesting results.

Monocyte cell levels are generally increased in the placenta during pregnancy as part of the innate immune response necessary for protection against infection (Ordi *et al.*, 2001). Monocyte numbers may therefore be elevated in placental blood. Three out of the five methods compared reported higher levels of monocyte cells than peripheral blood (prick, incision and tissue grinding) in accordance with the expectation of higher placental blood monocyte levels. The remaining two, perfusion and biopsy, had significantly lower cell numbers than peripheral blood. Conversely, a recent study that employed perfusion to obtain placental blood mononuclear cells, reported mean levels of IVB monocyte cells

49% higher than peripheral blood monocytes (Moore *et al.*, 2003). Monocyte loss in the current investigation using perfusion could be due to cell retention. Monocytes have been reported as having the propensity to aggregate and bind to villous fibrinoid (Walter *et al.*, 1982). Alternatively, the pressure with which PBS is delivered into placental IV spaces may cause monocytes to transform and subsequently adhere to placental tissue thereby causing the apparent monocyte diminution. The use of different anticoagulants in both studies may offer further explanation for the discordance in results. Where as this present study used heparin for placenta sample collection, EDTA was used in the abovementioned study. The precise interaction between the blood sample and anticoagulant is unknown but may involve cell surface marker alteration by anticoagulant. Additionally, the extrication properties of EDTA over heparin may facilitate the dislodging of tissue-attached monocytes/macrophages thereby yielding higher monocyte numbers. Investigations to confirm this will be necessary. In contrast to results obtained in this study, reports from a Gambian study document samples rich in monocytes with the biopsy method of collection (Rasheed *et al.*, 1992). The Gambia is a malaria endemic region characterized by considerable monocyte infiltrations of the placenta, the discrepancy in results obtained between this current study and the Gambian study may be due to this fact.

T lymphocytes are crucial to the immune process and are often the focus of immune response investigations thus the collection of blood samples with accurate lymphocyte numbers is imperative. Significant variation by method in the number of total T cells, CD4 and CD8 T cells was observed, with all five methods yielding lower numbers than peripheral blood. However, for all three lymphocyte subpopulations, sample collection by prick yielded cell numbers most similar to the corresponding peripheral blood. A comparative analysis of IVB and peripheral blood from postpartum women in western Kenya showed no differences in total T cell numbers (Moore *et al.*, 2003). The reduction in T cell numbers observed in this study for all methods of placenta collection implies contamination with foetal blood. Feotal T cell levels are decreased relative to adult blood (Erkeller-Yuksel *et al.*, 1992; Katevas *et al.*, 1999; Milosevits *et al.*, 1995) and the fact that the decreased levels particularly for the incision, biopsy and tissue grinding

methods correspond with the levels of foetal blood contamination for the three techniques, lend support to this. Reduction in T cell numbers in placental blood could also simply be a temporal issue, the numbers of T cells progressively reducing with each successive method employed.

The expression of CD45RA molecules characterizes the naïve state of a T lymphocyte while the absence of CD45RA expression characterizes the memory response. CD8 T memory cells but not CD4 T memory cells showed variation in cell numbers by method of sample collection with highest CD8 memory T cell levels yielded by the biopsy method of collection and possibly the reason for the significant variation observed. All other methods of collection yielded numbers lower than peripheral blood. Again, the decreased levels of placental CD8 and CD4 T memory cells are probably a foetal blood contamination issue. Cord blood has been reported to have a greater proportion of naïve cells than memory cells, as high as 91% of cord cells express the CD45RA marker and not the CD45RO memory marker (Hulstaert *et al.*, 1994; Juretic *et al.*, 2000; Rabian-Herzog *et al.*, 1993). Alternatively, the decreased levels of memory cells both for CD8 and CD4 T cells relative to peripheral blood could be due to the fact that the placenta is a new organ and as such would not have appreciable memory responses particularly in the first and second pregnancies (Moore *et al.*, 2000b).

B cell numbers were comparable between all five methods of collection but interestingly numbers by placental prick collection were higher than peripheral blood. In view of the fact that the prick method of collection has minimal foetal blood contamination and the Th2 placental environment that predominates in the placenta for foetal survival, B cell numbers in the placenta may be elevated in comparison to peripheral blood for antibody production. Thus the elevated B cell levels may be indicative of B cell hyper-stimulation for maternal-foetal interactions (Wegmann *et al.*, 1993; Zenclussen *et al.*, 2002) or due to the proliferation of B cells both for transplacental transfer of antibodies and for APC functions.

NK cell numbers showed no difference between each method of collection however all methods reported higher levels as compared to peripheral blood. Studies have reported cord blood NK cell levels two to three-fold higher than adult blood (Beck and Lam-Po-Tang, 1994; Erkeller-Yuksel *et al.*, 1992; Katevas *et al.*, 1999; Rabian-Herzog *et al.*, 1993) and Moore *et al.*, (2003) report a three fold increase in IVB compared to peripheral blood. Contamination of IVB with foetal blood may well be responsible for the higher placental NK cells in this study. Paradoxically, a study conducted by Spanish researchers in Tanzania report results completely contrary to those reported here, showing a total absence of NK cells in the intervillous spaces in all 1179 placentas investigated for cell mediated inflammatory responses regardless of placenta malaria status (Ordi *et al.*, 2001). The basis for the disparity in results may be in the differences of techniques used, where as this study used immunophenotyping by flow cytometry using anti CD56 and CD16 mABs to identify NK cells, the Spanish group used immunohistochemical analysis of formalin fixed paraffin embedded tissue (Ordi *et al.*, 2001). IVB cells are lost in the process of tissue fixation and may therefore not be ideal for the quantitative estimation of cell subpopulations. Intervillous spaces are like large blood vessels, devoid of a matrix within the lumen, which would hinder the retention of intervillous blood cells (Dr. Julie Moore: personal communication). Thus, in processing tissue for immunohistochemical analysis, due to the lack of an interceptive matrix, immune cells are in all probability, washed out.

To evaluate overall results obtained for each of the five methods of placental blood collection a scoring system was formulated summarizing collection method yields in comparison to peripheral blood composition. Ideally, comparisons should have been made against a 'gold standard' of intervillous blood collection for maternal immune cell phenotypes, however in the absence of such a standard which is yet to be identified, peripheral blood was used as a guide to the differences in cell number yields between the five different methods of collection. The scoring system evaluated between method differences and percentage similarity of IVB to peripheral blood. Two important issues were revealed by the scores obtained. If there is no significant difference between methods of collection for a particular cell subpopulation as is evident for NK cells or B

cells, the method used for IVB collection would not have a bearing on results obtained. Results would remain the same regardless of method used. However, if there is a significant difference, and this difference is related to the method of collection, a careful selection of collection methods in relation to the study objective would be necessary.

In conclusion, the results reported here suggest that the placental prick method best represents peripheral blood composition. Prick offers the closest cell profile levels to peripheral blood for each cell population as well as having lowest levels of foetal RBC contamination (5.5%). Furthermore, taking into consideration other factors involved in the decision making process for a particular technique, prick is simple, easy to perform, involves minimal placental manipulation and does not require sophisticated equipment for collection. It presents the most natural method of blood collection much in the same way that finger prick blood is collected. It is easily adaptable for field studies with sterility being relatively high. Plasma is not diluted and can therefore be used for assays requiring plasma. However a draw back to this method of collection is the low volume of sample obtained. Perfusion presents the next best method of choice. Relatively low levels of foetal blood contamination were reported (12%) and with the exception of monocytes, cell profile levels correspond to peripheral blood. However, perfusion employs the use of electric power to artificially flush out maternal cells, placental manipulation is thus high and perfused blood may therefore not be appropriate for assays requiring minimum manipulation such as activation and RNA assays. Perfusion requires special equipment and may therefore not be appropriate for the field set up. Plasma is highly diluted thus antibody and intracellular staining assays requiring autologous plasma cannot be performed using perfused samples. That notwithstanding, perfusion maintains sample sterility during collection and yields high sample volumes. A combination of these two methods of IVB collection performed on the same placenta would be ideal to meet the needs of an immunological study.

Incision, biopsy and tissue grinding methods of collection yield unacceptable levels of foetal blood contamination and display unreliable immune cell profiles thus the three techniques would be inappropriate for immunology studies particularly those that pertain

to malaria in pregnancy. It should be mentioned here that in so far as these three methods present inappropriate methods of collection for immunology studies related to maternal immune mechanisms of protection, they may be suitable for investigations where the study correlate is a disease outcome such as low birth weight (LBW) as opposed to an immune response. Studies investigating the adverse outcomes of a placental infection of malaria using any of the three methods are unlikely to generate unreliable results. Foetal blood contamination of maternal blood, regardless of the level of contamination, will not change associations observed between an immune response and pregnancy outcome.

The placental prick method of collection of placental intervillous blood has been used for the investigations carried out in chapters 3 and 4.

CHAPTER 3

IFN-γ producing NK cells and CD45RO memory cells are associated with protection against malaria during pregnancy

3.1 Introduction

Natural immunity to malaria develops after repeated exposure to the parasite over a period of years. It is not sterile and thus does not prevent infection, rather infected individuals are asymptomatic, harbouring low to moderate parasitemia (Greenwood and Snow, 1991; Moore *et al.*, 2000b). In pregnancy this immunity is altered. Pregnant women suffer from increased prevalence and increased clinical episodes of malaria with higher parasite densities than in the same women before pregnancy and their non-pregnant counter parts (Brabin, 1983; Weinberg, 1984). The susceptibility to infection and the severity of clinical manifestations is determined by the level of pre-pregnancy immunity and, in holoendemic regions, parity (McGregor *et al.*, 1983; Nosten *et al.*, 1991; Steketee *et al.*, 1988). Pregnancy establishes a new rich intrauterine vascular system that provides the parasite a haven for uncontrolled, uninterrupted replication (McGregor, 1987), malaria infections in pregnancy are thus characterized by parasite accumulation in the placenta, sometimes with parasite densities exceeding 50% of the total placental erythrocyte count and occasionally with undetectable parasites in the peripheral blood (Flick *et al.*, 2001). The enhanced susceptibility and the high frequency of placental infection have been attributed to a number of possibilities including immunological factors. One compelling hypothesis suggests a Th1/Th2 paradigm in pregnancy. A Th2 cytokine environment in the placenta is required for the successful maintenance of pregnancy; conversely a Th1 environment is associated with pregnancy failure (Raghupathy, 1997; Smith, 1996). Numerous immunological studies in non-pregnant populations have shown that malaria infection elicits a Th1 inflammatory environment. TNF-α, IFN-γ, IL-1 and IL-12 are produced during a malaria infection and contribute to protection against both pre-erythrocytic and erythrocytic blood stages (Perlmann and Troye-Blomberg, 2002). The inhibition of Th1 responses during pregnancy may therefore confer susceptibility to infections (Matteelli *et al.*, 1997;

Wegmann *et al.*, 1993). Malaria in pregnancy studies have documented increased expressions of type 1 cytokines, however most have been in relation to pregnancy complications due to infection. Excessively elevated placental levels of type 1, TNF-α, IFN-γ and IL-2 cytokines in *Plasmodium falciparum* infected placentas are associated with maternal anaemia and low birth weight babies (Fried *et al.*, 1998a; Moormann *et al.*, 1999). Fewer studies have investigated cytokines in relation to protection; only one study reports elevated levels of cytokines in association with protection. This study, conducted in western Kenya in relation to gravidity specific immunity, showed that IFN-γ levels were significantly elevated in placentas from multigravid women without placenta malaria in comparison to primigravid women with and without placental malaria and placenta malaria positive multigravid women (Moore *et al.*, 1999). Additionally, HIV infected pregnant women from the same region had impaired IFN-γ responses and were more susceptible to malaria (Moore *et al.*, 2000a). IFN-γ seemingly confers protection from parasite infection in pregnant women of western Kenya.

3.2 IFN-γ and protection from malaria

Numerous studies conducted in animal models have shown that resistance to rodent malaria is dependent on IFN-γ (Favre *et al.*, 1997; Waki *et al.*, 1992) and the difference between lethal and non lethal infections depends on the ability to mount an IFN-γ response (Shear *et al.*, 1989). Likewise, studies conducted in children in different malaria endemic settings, have shown that IFN-γ inhibits malaria parasite development (Deloron *et al.*, 1991), significantly delays re-infection (Luty *et al.*, 1999) and is associated with protection from parasitemia, clinical malaria and anaemia (Dodoo *et al.*, 2002).

IFN-γ is a dimeric, type 1 cytokine that is a potent regulator of macrophage function, and elicits the production of reactive oxygen species and nitrogen intermediates (Gyan *et al.*, 1994; Li *et al.*, 2001; Ockenhouse *et al.*, 1984). Produced by T and NK cells, it serves to effect protection through innate and acquired immune mechanisms. Innate mechanisms are non-specific requiring no prior exposure to the parasite and play an important role in

limiting infection; acquired specific immunity requires prolonged exposure to the parasite and is precisely generated, with the development of memory cells central to its execution thus showing capability for future recall. In the innate response IFN-γ is produced by NK cells and γδ T cells and is instrumental in the early control of malaria infections (Li *et al.*, 2001) while in acquired immune mechanisms it is produced by CD4 and CD8 T cells and is crucial in providing direction for cell mediated immune responses and help for antibody mediated mechanisms (Plebanski and Hill, 2000).

Elevated levels of placental IFN-γ have been reported in pregnant Kenyan women protected from placental malaria (Moore *et al.*, 1999). The Kenyan study however did not identify the source or the quality of the protective IFN-γ response. Is the IFN-γ response innate in origin or is it acquired? Additionally, the increased susceptibility during pregnancy of previously protected women implies loss of memory to *P. falciparum*, but even more intriguing is the development of parity specific immunity suggesting the retention of memory with successive pregnancies.

The placenta represents a unique immunological compartment different from the peripheral blood system. It has been shown that the immunological makeup of intervillous blood is distinct from that of peripheral blood (Moore *et al.*, 2000b; Moore *et al.*, 2003), however, unknown are whether immune effector mechanisms operating in each compartment are also distinct particularly in relation to *P.falciparum* infections. Undoubtedly more investigations are required to elucidate further the precise mechanism of the IFN-γ protective response and the course of the development of memory to placental *P.falciparum* infections.

3.3 Study rationale

The biological basis of susceptibility to malaria infections in pregnancy is not well understood. Efforts to understand the principal mechanisms are further complicated by the fact that in holoendemic regions gravidity specific immunity has been observed (McGregor, 1984). Previous studies conducted in Kenya have shown that IFN-γ is associated with protection in multigravid women and the precise mechanism of protection needs to be

further elucidated. IFN-γ is produced by activated T-helper cells, γδ T cells and NK cells and as part of the CDC-KEMRI laboratory research activities on pregnancy associated malaria, this investigation seeks to elucidate the specific source of IFN-γ both in the peripheral blood and at the placental level. This study also investigates memory cells in malaria during pregnancy. The ultimate goal is to determine whether different immune components play different roles in peripheral and placental blood in protection against malaria during pregnancy.

3.4 Objective

To investigate IFN-γ dependent immune protection from malaria during pregnancy in women resident in an area of high malaria endemicity.

3.4.1 Specific Objectives

1. To investigate levels of IFN-γ producing CD3, CD4, CD8 T subpopulations, NK cells and memory cells in peripheral and placental blood.
2. To investigate IFN-γ by CD3, CD4, CD8 T cells, NK cells and memory cells in association with protection from malaria during pregnancy.

3.5 Materials and Methods

3.5.1 Study participants

Mothers were enrolled after delivery and following consent, from the Nyanza Provincial Hospital. Placentas were collected at delivery and immediately processed for prick blood as described in chapter two. A peripheral sample (3mls) was also obtained from the mother. All blood samples were collected into heparin charged eppendorf tubes. HIV testing and parasite determination were carried out. Altogether, 52 women were enrolled 20 with placenta malaria and 32 without placenta malaria. The sample size was calculated at an alpha level of 0.05 and 90% power, to detect at least a 25% difference in immune responses between parasitic and aparasitemic women.

3.5.2 Laboratory methods

3.5.2.1 Cell activation and culture

As a first step in the assessment of peripheral and placental blood related mechanisms of protection, spontaneous as opposed to antigen specific production of IFN-γ was assessed. Spontaneous cytokine production reflects *in vivo* stimulation and thus represents cell activity in the context of the infection (Walker *et al.*, 2002). For spontaneous IFN-γ production, cells were activated using Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St. Louis, MO), which stimulates the release of already synthesized cytokine, and ionomycin (Sigma Chemical Co., St. Louis, MO) in the presence of IL-2 (Genzyme Corporation, Cambridge, MA) with monensin (Sigma Chemical Co., St. Louis, MO) as the transport inhibitor. Blood samples (peripheral and placental) were diluted 1:1 with incomplete RPMI medium supplemented with 2mM L-glutamine (GIBCO, Invitrogen Corporation, UK) and activated using PMA at 15ng/ml, ionomycin at 1μg/ml, IL-2 at 10ng/ml and monensin at a concentration of 1μM/ml. Cells were incubated at 37°C, 5%CO_2 for four hours. In addition, unstimulated aliquots of peripheral and placental blood incubated with monensin, were processed in parallel, to serve as controls. Stimulant concentrations used were predetermined by standardization experiments for optimal IFN-γ production. Staining was carried out in two steps; first, cell surface marker staining for the identification of the following cell subpopulations; total T, CD4 T, CD8 T cells, memory and NK cells and second, intracellular staining for IFN-γ expression by the abovementioned cell subpopulations.

3.5.2.2 Cell surface marker staining

The following antibody reagents were used for cell surface marker staining. Anti-CD3 CyChrome (mouse IgG1), anti-CD4 PE (mouse IgG1), anti-CD8 PE (mouse IgG1), anti-CD45RO PE (mouse IgG1), and anti-CD56 PE (mouse IgG1). In addition an isotype-matched monoclonal antibody (anti-mouse IgG1) conjugated with PE was used as a negative control. All antibodies were obtained from Pharmingen, San Diego, CA.

Five microlitres of each of the above fluorochrome-conjugated antibodies were aliquoted into labelled 5 ml falcon tubes (Becton Dickinson, San Jose, CA) as indicated in Table 3.1.

Table 3.1: Antibody Staining Panel

Tube	FITC	PE	Cy-Chrome
1	IFN		CD3
2	IFN	CD4	CD3
3	IFN	CD56	CD3
4	IFN	CD8	CD3
5	IFN	CD45RO	CD3
6	MOPC21*		CD3
7	MOPC21	IgG1	CD3

The panel above represents the antibody combinations used to stain for IFN-γ production by various cell subpopulations. Tubes 1 to 5 represent the IFN-γ test tubes while 6 and 7 are the isotype controls; tube 6 represents the control for Tube 1 while tube 7 serves as the control for tubes 2-5. In the first step of staining (cell surface marker staining) only PE and CyChrome labeled antibodies were added to tubes. Anti-IFNγ FITC labeled antibody was added in the second step for intracellular staining. *MOPC21, an isotype control antibody, is a non-binding mouse IgG1 antibody clone.

Tube 6 served as the isotype control for tube 1 while tube 7 served as the control for tubes 2 to 5. Only PE and CyChrome labelled antibodies were added in this first staining step. Stimulated and unstimulated whole blood samples were added (100µls) to each tube at the end of the four-hour incubation period; stimulated blood into tubes 2,3,4 and 5 and unstimulated blood into tubes 6 and 7. Cells were then incubated on ice for 30 minutes in the dark after which erythrocytes were eliminated by lysis using a 1:10 dilution of room temperature Facs lysing solution (BD Pharmingen, San Diego, CA) in distilled water (3.5mls per tube) for strictly 10 minutes in the dark. Cells were spun at 1500rpm for 7minutes at 8°C at the end of the incubation period. The supernatant was aspirated and discarded after which the cell pellet was reconstituted in 1ml of ice cold freezing medium. Cells were then frozen at -80°C overnight.

3.5.2.3 Intracellular Cytokine staining

Anti-IFN-γ FITC (mouse IgG1) (Pharmingen, San Diego, CA) at a concentration of 125ng/ml was used for intracellular staining of IFN-γ. FITC labelled mouse IgG1 (Pharmingen, San Diego, CA) at a concentration of 125ng/ml was used as the isotype-matched negative control. Antibody concentrations were predetermined by titration experiments.

Cells were removed from -80°C and quickly thawed in a 37°C water bath while constantly shaking, in preparation for the second staining step. Cells were washed once at 1500rpm for 7 minutes at 8°C and permeabilized using 0.1% saponin for 30 minutes in the dark at 4°C. One hundred microlitres of the anti-IFN-γ FITC antibody was added to tubes 1 to 6 and the FITC labelled negative control into tubes 6 and 7. Cells were further incubated in the dark for 30 minutes on ice or at 4°C after which they were washed once in 2mls of wash buffer by centrifugation at 1500rpm for 7 minutes at 8°C. The supernatant was aspirated and discarded and cells fixed in 500µls of 1% paraformaldehyde per tube. Cells were analysed on a FacsCalibur (BD Biosciences, San Diego CA) calibrated before use with CaliBRITE beads (BD Biosciences, San Diego, CA).

3.5.2.4 Flow Cytometric acquisition and analysis

For data acquisition and analysis the Cell Quest Pro software (BD Biosciences, San Diego, CA.) was used. Ten thousand ungated events were acquired for each antibody combination. Using dot plots the percentage distribution of each cell subpopulation was calculated by an SSC-CD3 gate. The negative controls were used to set quadrant markers for each cell subpopulation allowing a maximum of 2% as background. Cell numbers for CD3, CD4, CD8 and memory T cells were reported as % cell numbers gated in the double positive quadrant with % negative control values subtracted. NK cell percentages were evaluated from the CD56+/CD3- quadrant values after subtracting the % negative control values. % IFN-γ producing cells were calculated from gated cell subpopulation events. The results are reported as proportions (%) of gated leukocyte events for the cell subpopulations of interest and as mean % IFN-γ production by each cell subpopulation.

3.6 Statistical analysis

Data entry and statistical analysis was performed using the SPSS statistical software (SPSS 11.0.1, Inc., Chicago, IL.). Because the data obtained were not normally distributed, natural log transformations were performed for each parameter. Pearson's χ^2 test was used to evaluate gravidity group differences. Differences between peripheral and placental blood as well as between placenta malaria negative and placenta malaria positive individuals were tested using the non-parametric Kruskal-Wallis statistical test. The Spearman's rho was used to test for associations between parasite densities in peripheral and placental blood. Confounding factors were controlled for in the statistical analysis. *P* values (two tailed) less than 0.05 were considered statistically significant while values less than 0.09 but greater than 0.05, were considered marginally significant.

3.7 Results

Fifty-two pregnant women were recruited at the Nyanza Provincial Hospital after delivery. Peripheral and placental blood samples were obtained and immune phenotyping performed for CD3, CD4, CD8 T cells, memory T cells and NK cells and were also assayed for intracellular IFN-γ production, using a three-colour flow cytometry.

3.7.1 Characteristics of study participants

Table 3.2 shows the general characteristics of study participants. Of the 52 women enrolled, 20 (38%) had *Plasmodium falciparum* infections of the placenta (PM+) and 32 (62%) were without placenta malaria (PM-). All 20 PM+ women had both peripheral and placental parasitemia, however of the 32 PM- women, 31 were aparasitemic for both peripheral and placental blood while one had peripheral but not placental parasitemia. Among the 20 PM+ mothers 16 (80%) were primigravidae and secundigravidae while 4 (20%) were multigravidae, while for the PM- mothers 18 (56.3%) were primigravidae and secundigravidae and 14 (43.8%) were mutligravidae (P=0.072). Primigravidae and secundigravidae were grouped together on the basis of the fact that previous work in this region has shown that immunologic characteristics for the two groups are the same (Moore *et al.*, 1999). The mean age between the two groups was not different, PM- mothers had a mean of 24 ± 7.2 years, while PM- mothers were 21± 5.0 years. Mean placental parasite rates for the PM+ mothers were 3326 parasites/µl and 13363 parasites/µl for the peripheral and placental blood respectively, and was not statistically different. Although there was no significant difference in parasitemia density between the two blood compartments parasite levels between peripheral and placental blood were correlated (rho=0.698, P =0.002) (Figure 3.1). Birth weight and haemoglobin levels between the two groups of mothers were comparable (P =0.829 and P =0.266 respectively).

3.7.2 Immune parameter comparisons between peripheral and placental blood

Overall, comparisons of cell subpopulation numbers revealed that CD4/CD3 and CD45RO/CD3 cells were significantly higher in peripheral blood than placental (P=0.024 and 0.038 respectively). All other cell subpopulation numbers were the same for the two blood compartments (Table 3.3).

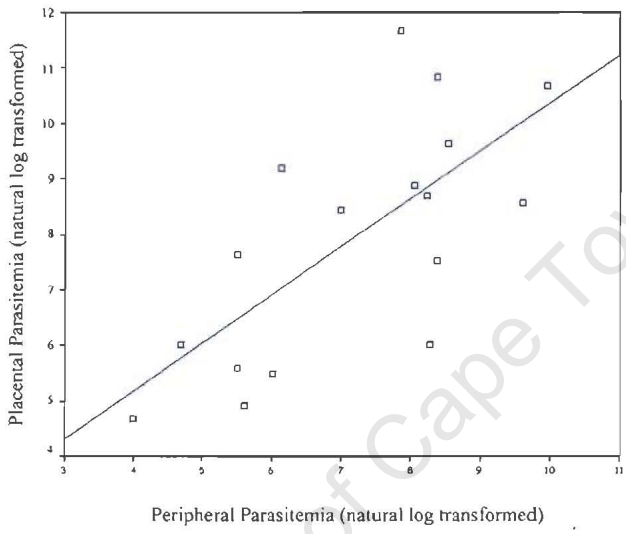
IFN-γ production between peripheral and placental blood for all enrolled mothers, revealed that levels of IFN-γ by CD4+, CD8+ CD45RO+ and CD56+/CD3- were not different (Table 3.3). Table 3.3 also shows that the predominant IFN-γ producing cells appear to be CD8+ cells in both peripheral blood and in the placenta.

Table 3.2: Characteristics of study participants.

	PM-	PM+	P
N	32	20	
Age (yrs)	24+/-7.28	21+/-5.05	**0.124**
Gravidity (n;<=2, >2)	18(56.3%), 14(43.8%)	16(80%),4(20%)	**0.072**
Hb (g/dl)	12.10+/-2.03	11.50+/-1.60	**0.266**
Birth weight (Kg)	3.30+/-0.49	3.33+/-0.47	**0.829**
Peripheral parasitemia (parasites/μl)	243.84+/-243.84*	3326.40+/-1227.52	**0.407**
Placental parasitemia (parasites/μl)	0	13363.65+/-6272.31	

*The placenta malaria (PM) status of enrolled women was determined by a positive placental blood smear. Thirty-two women were classified as being PM-, however one woman had peripheral but no placental parasitemia and was therefore still considered PM-. All characteristics indicated as mean value ± SE.

Figure 3.1: Association between peripheral and placental parasitemia



Peripheral and placental parasitemia levels were highly correlated for the study participants (rho=0.698, p=0.002).

Table 3.3: Comparison of IFN-γ producing cells and memory cells between peripheral and placental blood in relation to protection from malaria in pregnancy

				PM-			PM+		
Parameter	**Peripheral**	**Placental**	***P***	**Peripheral**	**Placental**	***P***	**Peripheral**	**Placental**	***P***
IFN/CD3	19.57	22.1	**0.76**	19.37	21.86	**0.809**	19.9	22.48	**0.705**
CD4/CD3	55.04	50.07	**0.024**	59.64	52.38	**0.023**	47.67	46.38	**0.433**
IFN/CD4	13.93	13.3	**0.838**	13.28	13.53	**0.793**	14.97	12.92	**0.935**
CD8/CD3	41.19	39.58	**0.576**	42.85	42.11	**0.999**	38.54	35.52	**0.441**
IFN/CD8	16.07	16.85	**0.995**	16.96	18.29	**0.83**	14.64	14.54	**0.818**
CD45RO/CD3	36.97	28.81	**0.038**	43.94	31.2	**0.019**	25.81	24.99	**0.705**
IFN/CD45RO	15.11	15.33	**0.938**	16.54	16.2	**0.727**	12.82	13.95	**0.839**
CD56/CD3-	4.56	5.8	**0.114**	4.9	6.34	**0.133**	4.01	4.93	**0.655**
IFN/CD56	7.36	7.09	**0.683**	7.43	7.41	**0.722**	7.24	6.55	**0.255**

Values indicated are mean % cell numbers obtained from flow cytometry. Comparisons were done using the Kruskal-Wallis statistical non-parametric statistical test. P (two sided) <0.05 considered as significantly different.

When stratified by presence of parasites in the placenta, PM- mothers had significantly higher levels of CD4+ (P =0.023) and CD45RO+ (P = 0.019) cells in the periphery than in the placenta (Table 3.3). No significant differences were seen in the PM+ group. All IFN-γ responses measured were also not different between PM- and PM+ mothers (Table 3.3).

3.7.3 Immune parameter comparisons between PM+ and PM- mothers

Taking peripheral immune responses alone, PM- mothers had significantly higher numbers of CD4+ and CD45RO+ cells than the PM+ mothers (P =0.001, P =0.002). IFN-γ production by the different cell subpopulations were not statistically different between the two groups of women (Table 3.4A).

At the placental level, comparisons between PM- and PM+ parameters revealed significantly higher levels of CD4+ (P =0.033) and CD8+ cells (P =0.039) (Table 3.3) in the PM- mothers. CD56+/CD3- cell numbers showed a trend towards higher levels in PM- individuals than in PM+ mothers (P =0.09). IFN-γ expression by CD56+/CD3- cells in the placenta was significantly higher in PM- individuals than in PM+ (P =0.008). IFN-γ production by other cell subpopulations showed no difference in expression between PM+ and PM- mothers (Table 3.4B).

3.7.4 Immune parameters and gravidity

To further investigate whether or not the above potential protective parameters were related with gravidity, mothers were divided into four groups based on gravidity and presence of parasites. The four groups obtained were as follows: PM-, gravidity less than or equal to 2; PM-, gravidity more than 2; PM+, gravidity less than or equal to 2; and PM+, gravidity more than 2. As mentioned earlier, primigravidae and secundigravidae were grouped together on the basis of similar immunological characteristics (Moore *et al.*, 1999).

Immune responses that were either significant or showed borderline significance (CD4+, CD45RO+ in peripheral blood and CD4+, CD8+, CD56+/CD3-, IFN-γ/CD56+ in the

Table 3.4: Determination of IFN-γ by CD4, CD8, CD45RO and CD56/CD3- responses between PM- and PM+ women in peripheral (A) and placental (B) blood.

A:

	Peripheral		
Parameter	**PM-**	**PM+**	***P***
IFN/CD3	19.37	19.9	**0.693**
CD4/CD3	59.64	47.67	**0.001**
IFN/CD4	13.28	14.97	**0.605**
CD8/CD3	42.85	38.54	**0.24**
IFN/CD8	16.96	14.64	**0.573**
CD45RO/CD3	43.94	25.81	**0.002**
IFN/CD45RO	16.54	12.82	**0.118**
CD56/CD3-	4.9	4.01	**0.236**
IFN/CD56	7.43	7.24	**0.375**

B:

	Placental		
Parameter	**PM-**	**PM+**	***P***
IFN/CD3	21.86	22.48	**0.97**
CD4/CD3	52.38	46.38	**0.033**
IFN/CD4	13.53	12.92	**0.851**
CD8/CD3	42.11	35.52	**0.039**
IFN/CD8	18.29	14.54	**0.397**
CD45RO/CD3	31.2	24.99	**0.301**
IFN/CD45RO	16.2	13.95	**0.56**
CD56/CD3-	6.34	4.93	**0.09**
IFN/CD56	7.41	6.55	**0.008**

Values indicated are mean % cell numbers obtained form flow cytometry. Comparisons were done using the Kruskal-Wallis statistical non-parametric statistical test. P (two sided) <0.05 considered as significantly different.

placenta) were compared. No significant difference was seen for CD4+, CD8+ and CD56+/CD3- in placental blood. However, levels were significantly different for peripheral CD4 + and CD45RO+ cells (P =0.005, P =0.008; P value calculated for presence/absence of parasitemia stratified by gravidity) with highest levels found in the PM- multigravid group. Interestingly, levels of IFN-γ/CD56+ in the placenta showed a trend towards significance (P =0.067) with higher levels in the PM- groups and lower levels in the PM+ groups regardless of gravidity (Figure 3.2A, B and 3.3). Further analysis of IFN-γ/CD56+ levels between primigravid and multigravid women, in PM- and PM+ women taken separately, revealed no difference in IFN-γ/CD56+ levels (P =0.805 and P =0.549 respectively) suggesting that gravidity may not have bearing on IFN-γ/ NK cell protection from infection.

Taken together, results here suggest that CD4 and CD45RO memory cells maybe important in peripheral protection against infection while IFN-γ/CD56+ cells are probably essential for placental protection.

3.8 Discussion

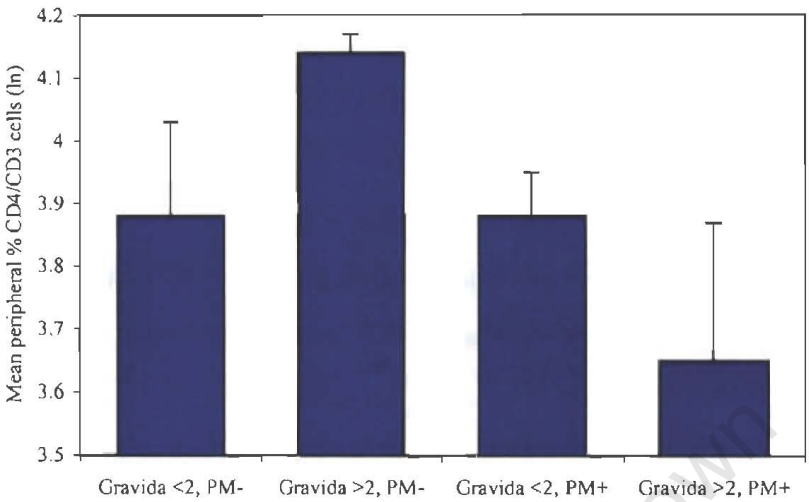
Malaria infections commonly elicit inflammatory cytokines and IFN-γ has long been established as an important factor in protection against both rodent and human malaria. IFN-γ's protective functions have also been recently extended to malaria in pregnancy where elevated levels of IFN-γ are associated with protection from placental infection (Moore *et al.*, 1999). This current study investigated further the nature of the IFN-γ protective response in a group of women, resident in a holoendemic region for malaria. Results here suggest that different immune components may play different roles in protection from malaria during pregnancy, with CD45RO cells critical in peripheral blood and IFN-γ producing NK cells important in the placenta.

3.8.1 Peripheral and placental immune component differences

The previous study in Kenyan women did not address the source of the IFN-γ response although results obtained in that study alluded to the fact that the response was a

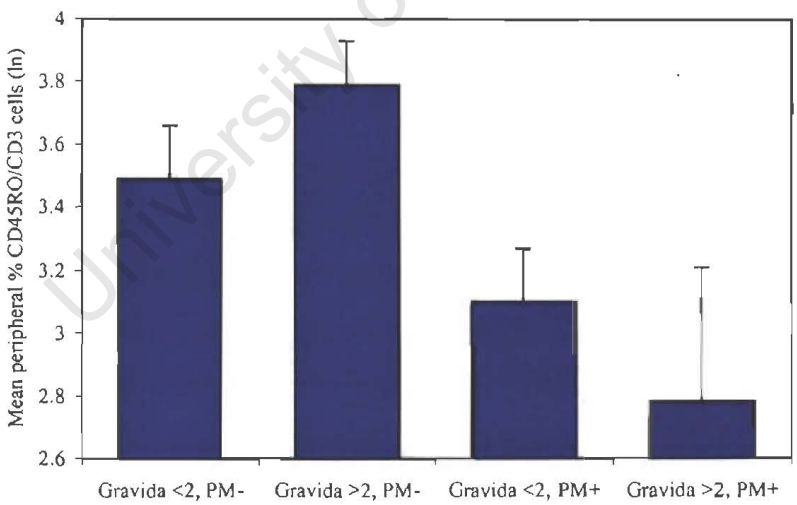
Figure 3.2: Comparisons of peripheral CD4 and CD45RO T cells between four groups of women categorized by placenta malaria status and gravidity

A:



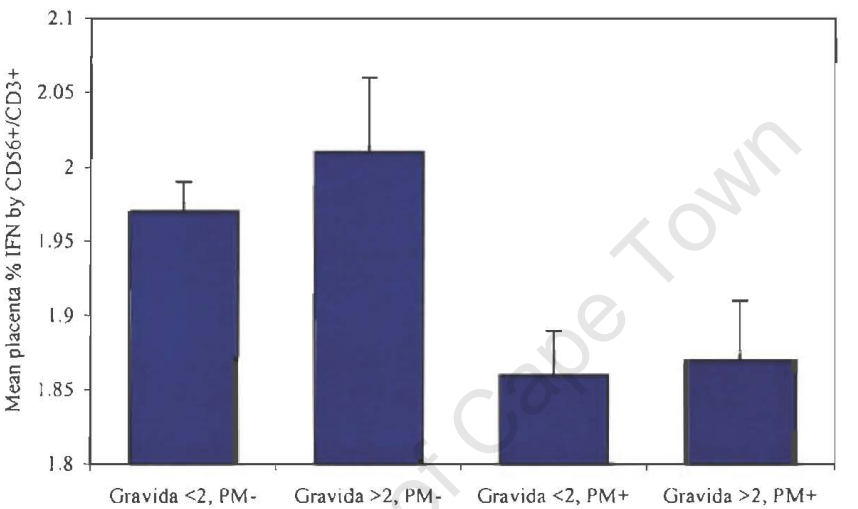
Mean % cell numbers for peripheral CD4 cells. Peripheral levels of CD4 were significantly different between all four groups (P=0.005). Note: P value calculated for all four groups. N: Gravida ≤2,PM-18; Gravida>2,PM-14; Gravida ≤2,PM+ 16; Gravida>2,PM+4.

B:



Mean % cell numbers for peripheral CD45RO cells. Peripheral levels of CD45RO were significantly different between all four groups (P=0.008). Note: P value calculated for all four groups. N: Gravida ≤2,PM-18; Gravida>2,PM-14; Gravida ≤2,PM+ 16; Gravida>2,PM+4.

Figure 3.3: Comparisons of placental immune IFN-γ by CD56/CD3- between four groups of women categorized by placenta malaria status and gravidity



Mean % IFN-γ producing CD56/CD3- placental cell numbers. Levels were marginally different between all four groups (p=0.067). Note: p value calculated for all four groups. Further statistical analysis between primigravid and multigravid women in PM- and PM+ women taken separately revealed no difference in IFN-γ/CD56 levels (p=0.805 and p=0.549). N: Gravida ≤2,PM-18; Gravida>2,PM-14; Gravida ≤2,PM+ 16; Gravida>2,PM+4.

component of adaptive immune mechanisms probably involving memory. Whether or not the protective immune response was the same in peripheral and placental blood was also not addressed. Given the fact that the placenta is increasingly being recognized as an immunological entity (Moore *et al.*, 2003) and the fact that the parasite invading the placenta has been suggested as being of a unique phenotype (Fried and Duffy, 1996), this study investigated differences in immune responses between the two blood compartments in relation to protection against malaria during pregnancy. Peripheral and placental immune responses were therefore assessed to address these issues.

CD4, CD8, CD45RO memory cell as well CD56+/CD3- NK cell numbers were investigated to determine if there was an increase in cell numbers in association with protection. Only CD4 and CD45RO cells revealed differences when comparing peripheral and placental immune parameters. Overall, CD4 and CD45RO levels were elevated in peripheral blood as compared to placental blood. In comparing immune responses between PM+ and PM- mothers, peripheral levels of CD4 and CD45RO memory cells were raised in PM- mothers as compared to PM+ mothers, while placental levels of CD4 and CD45RO were not different. Seemingly in peripheral blood, CD4 T and CD45RO memory cells were associated with protection from infection. Previous studies conducted in The Gambia show that lymphoproliferative responses of placental cells to malarial antigen were poor compared with cells of peripheral blood and the reduced proliferative capacity of placental cells was suggested as a contributing factor to placental parasitization (Rasheed *et al.*, 1993). Results from the current study showing association between CD4 T cells and memory cells with protection against infection in peripheral blood and not placental blood, lends support to the previous Gambian findings.

3.8.2 CD4 and CD45RO cells in relation to protection against peripheral malaria

Pregnancy has been described as a state of immune suppression for foetal survival. The observation that both CD4 T cells and CD45RO memory cells are generally higher in peripheral blood than placental and higher in PM- than in PM+ in peripheral blood but not placental, raises the possibility that immune responses in the peripheral system are not entirely suppressed and that they still function, albeit limited, to protect against

infection. Luppi *et al.* (2002) in studies conducted to investigate immune leukocyte changes throughout gestation suggest that the immunosuppressive condition associated with pregnancy does not exist throughout the entire body. Results reported here are in agreement with this suggestion. Women in holoendemic regions having established a robust pre-pregnancy anti-malaria protective response probably retain segments of its activity in peripheral blood during pregnancy, perhaps through CD4 T and CD45RO cells. The elevated levels of peripheral CD4 T and CD45RO memory cells in PM- women may contribute to the elimination of parasites in peripheral blood before they anchor in the placenta. Indeed, early pregnancy infections and subsequent clearance of parasites during gestation has been suggested in studies from Cameroon where primiparous women, with no indication of a placental infection at delivery, proliferated to a placenta parasite line indicating previous exposure to the parasite (Fievet *et al.*, 2002).

The elevated levels of peripheral CD4 T and memory cells numbers reported for PM- as compared to PM+ mothers may be for help with anti-parasite antibody production. CD4 T cells are required for help with antibody production by B cells. Anti-placental parasite specific antibody activity has been reported in placental malaria infections and are associated with greatly reduced prevalence and density of parasites (Fried and Duffy, 2002). Moreover, data from a series of related studies in Cameroon, using a CSA adhering parasite line RP5, have demonstrated the acquisition of parasite specific RP5 cellular immune responses and of RP5 specific antibody production (Fievet *et al.*, 2002; Maubert *et al.*, 1999; Staalsoe *et al.*, 2001) supporting the possibility that the elevated CD4 T cell numbers reported here, maybe for the regulation of placental parasite specific antibody production. Studies to further evaluate the association between placenta parasite specific CD4 T cell responses and antibody production would be necessary.

In view of the gravidity dependent IFN-γ protective immune response reported previously in this study region, CD4 T and CD45RO memory cells, were assessed to determine their contribution to this immunological phenomenon that has been suggested as requiring both repeated exposure during successive pregnancies, and the development of memory (McGregor, 1984; Moore *et al.*, 2000b). In this study, levels of CD4 T and CD45RO

memory cell subpopulations were highest in multigravid aparasitemic women and lowest in parasitemic multigravid women suggesting their involvement in gravidity dependent mechanisms of protection. These results are in agreement with the abovementioned Cameroonian study that also reported evidence of parity related peripheral blood cellular responses to malarial antigen (Fievet *et al.*, 2002). It may be therefore postulated that in the gravidity dependent protective mechanisms, peripheral blood via memory cells, dictates the course of protection from infection in the placenta. Thus epidemiological data documented on the phenomenon of gravidity dependent protection in malaria during pregnancy may well be related with peripheral immune responses rather than placental. This presumption deserves further investigations.

3.8.3 IFN-γ producing NK cells and association with protection against malaria in the placenta

In the event that the peripheral blood compartment is unable to control an infection, placental parasitization is likely to occur. Investigations of placental immune responses in this study revealed that IFN-γ producing NK cells and not memory cells were associated with protection in the placenta. Numerous immunological studies have shown that IFN-γ exhibits both innate and acquired response activity. It is expressed by NK cells and γδ T cells (characteristic innate components) and also by activated CD4 and CD8 T cells (adoptive immune cells). An increasing body of evidence from both murine models and human studies indicates that innate IFN-γ production contributes significantly to the elimination of malaria infections. The resolution of the non-lethal *Plasmodium yoelii* infection is dependent on early IFN-γ production by NK cells among other immune components (De Souza *et al.*, 1997) and *Plasmodium chabaudi* AS infections were resolved by early expression of IFN-γ by NK cells (Mohan *et al.*, 1997). In adult *P. falciparum* infections NK cells have been shown to be major contributors to the early IFN-γ response against infection (Artavanis-Tsakonas and Riley, 2002). Thus the results obtained in this study are in agreement with those from mouse models and human infection studies and suggest that protection from infection at the placental level is mediated by the innate immune response. The data here provides the first demonstration

of a role for innate immune mechanisms in protection against placental *P.falciparum* infection.

In reviewing immunity to asexual blood stages of *Plasmodia,* Fell and Smith (1998) have proposed an "innate to Th2" model of malaria immunity. They suggest that Th1 type responses may not be important in controlling malaria infection in the blood where parasites are shielded in erythrocytes from direct attack due to the lack of MHC molecules, rather innate immune responses are more important in the control of early parasitemia and once infection is reduced, parasitemia is eliminated by the activation of T cells that drive the production of antibodies (Fell and Smith, 1998). Placental malaria investigations have documented the acquisition of antibodies specific to placental binding parasites (Fried *et al.*, 1998b; Fried and Duffy, 2002) and taken together with the IFN-γ producing NK cell activity along with the absence of placental memory cell function reported here, the current data suggests the possibility of an innate to Th2 mechanism of protection against placenta malaria. The speculation for a Th2 response development in the later stages of infections is further supported by the finding that placental CD4 T cell levels were elevated in PM- women as compared to PM+ women, perhaps for antibody production.

An assessment of IFN-γ producing NK cell association with gravidity, showed that placenta IFN-γ levels by NK cells were high in PM- women and low in PM+ women regardless of gravidity, suggesting that gravidity may not be associated with this placenta protection mechanism. In the context of the investigations carried out here, these findings seemingly further support the suggestion that memory cells may be more critical to the development of gravidity related protection in peripheral blood. However a lymphocyte recirculation hypothesis proposes the local development and maintenance in local lymphoid tissues, of placental parasite specific memory responses, as being important in gravidity-dependent immunity (Moore *et al.*, 2000b). The absence of gravidity associated protective mechanisms in the placenta reported here and the suggestion that memory cells are more instrumental in peripheral protection than placental is not necessarily a contradiction of the recirculation hypothesis. While the current study showed that

memory cells in the placenta were not associated with protection, it did not investigate specific memory cell subset responses. Memory cells mediate two functions, each carried out by distinct cell types. Protective memory is mediated by effector memory T cells (T_{EM}) that home to inflamed peripheral tissues and display immediate effector function, while reactive memory is mediated by a distinct subset of central memory T cells (T_{CM}) that retain lymph node homing receptors and high proliferative capacity in response to antigenic challenge (Sallusto *et al.*, 1999). Further studies to elucidate particularly the role of central memory T cell subset responses in relation to protection from placental malaria would be important. Nevertheless, in the context of the recirculation hypothesis and the findings reported here, it would be interesting to determine the biological basis of NK cell recruitment to the placenta in relation to the protection that NK cells afford the placenta against a P.*falciparum* infection (see chapter four). Mononuclear cell infiltration in the placenta is a histopathological hallmark of malaria during pregnancy (Brabin *et al.*, 1993). NK cells may constitute a proportion of this placental infiltrate although investigations have documented otherwise (Ordi *et al.*, 2001).

In conclusion, the results presented here indicate that different immune mechanisms operate within different compartments to effect protection from malaria during pregnancy. CD4 T cells together with CD45RO memory cells are associated with protection in peripheral blood while IFN-γ producing NK cells maybe responsible for initiating a protective response in the placenta. The immunological basis of gravidity dependent protection in malaria during pregnancy in holoendemic areas is still open to speculation.

CHAPTER FOUR

The balance of IP-10 production between peripheral and placental blood is associated with the regulation of IFN-γ/NK cell mediated protection against placental malaria

4.1 Introduction

In recent years much progress has been made in understanding effector immunity to *P.falciparum* and NK cells are increasingly being recognized as important effectors of the protective response. Studies of malaria infections in rodents have provided evidence for NK cell mediated protection. Enhanced NK cell activity in mice infected with irradiated *Plasmodium berghei* sporozoites has been demonstrated (Ojo-Amaize *et al.*, 1984) and *Plasmodium yoelii* sporozoite infections have been shown to induce a rapid protective inflammatory response characterized by NK cells, macrophages, T cell infiltration and IFN-γ production (Pasquetto *et al.*, 2000). In addition, *Plasmodium chabaudi* infections in NK cell depleted mice results in a more severe course of infection with higher parasitemia and increased mortality (Mohan *et al.*, 1997). Regarding human malaria investigations, studies in Nigeria observed raised NK cell levels in malaria infected children, with a positive correlation between parasitemia and lytic activity (Ojo-Amaize *et al.*, 1981) suggesting an NK cell role in protection. *In vitro* studies of NK cell activity reports the lysis of schizont infected erythrocytes by CD56+/CD3-NK cells (Orago and Facer, 1991). More recently, results from flow cytometric studies report NK cells as a significant source of IFN-γ in the crucial first few hours of a human malaria infection in relation to parasite control (Artavanis-Tsakonas and Riley, 2002). Data obtained in chapter three further reports IFN-γ producing NK cells activity in malaria during pregnancy; levels were related to protection from *P. falciparum* infections of the placenta regardless of gravidity.

In keeping a close surveillance for potential pathogen invasion, cellular components of the immune system circulate in the blood and are recruited to sites of infection where they execute their function. Cell recruitment is a well-orchestrated event that is an essential component of the immune response and is largely under the control of the chemokine-chemokine receptor system. NK cell interaction with chemokine-chemokine

receptors would be important in the development of the IFN-γ producing NK cell protection reported in chapter 3.

4.2 Chemokines and chemokine receptors in leukocyte trafficking

A group of specialized cytokines responsible for the migration of cells within the body has been described. Referred to as chemokines or chemotactic cytokines, these small 8-14kD proteins, numbering more than 50 in total (Bacon *et al.*, 2002) perform multiple biological activities including the recruitment and activation of various cell types (Matsukawa *et al.*, 2000). Chemokines may be divided into 4 subfamilies on the basis of the position of a pair of highly conserved cysteines (C) in the amino acid sequence (α [CXC], β [CC], δ [CX_3C] and γ [XC] chemokines) (von Andrian and Mackay, 2000) however, a more recent classification system suggests the division of chemokines into two broad categories based on site of production and function. Thus chemokines are either inducible or homeostatic (Sallusto *et al.*, 1998). Inducible chemokines also referred to as inflammatory chemokines were the first to be discovered and are expressed in inflamed tissues on stimulation by pro-inflammatory cytokines or during contact with pathogenic agents. They are specialized for the recruitment of effector cells and primarily attract granulocytes, monocytes, NK cells and effector T cells. Homeostatic or lymphoid chemokines are produced in discrete microenvironments and are involved in maintaining physiological processes and immune surveillance and act predominantly on monocytes, mast cells and eosinophils. Members of both families attract lymphocytes and NK cells.

Chemokines do not act alone in cell recruitment, they mediate their functions through 7-transmembrane (TM) G–protein coupled receptors expressed on leukocytes and other cells types (Murdoch and Finn, 2000). These chemokine receptors are part of a larger super family of G-protein coupled receptors that include receptors for hormones, neurotransmitters and enzymes but are relatively small compared with other TM receptors (339-373 amino acids) (Balkwill, 1998). Eighteen human chemokine receptors have been identified to date (Moser and Loetscher, 2001) and are divided into subfamilies that correspond to the chemokine groups, CXCR receptors bind α-chemokines, CCR receptors bind β-chemokines, and CX_3CR1 and XCR1 receptors bind δ and

γ chemokines respectively (von Andrian and Mackay, 2000). The expression of chemokine receptors varies greatly among different leukocyte subsets and the number of receptor molecules per cell dictates cellular migratory patterns, generally however, chemokine receptors will cause the movement of target cells against a chemoattractant gradient.

In addition to chemoattractant properties, chemokines are also potent activators of the innate immune response to infectious diseases including parasitic infections.

4.3 Chemokines and parasitic infections

Parasitic infections elicit the production of chemokines. Certain chemokines enhance phagocytic killing activity towards parasites via the production of nitric oxide (NO), in *Leishmania major* infections NO aided leishmanicidal activity was exhibited by eosinophils and IL-8 was implicated (Oliveira *et al.*, 1998). MIP-1α (macrophage inflammatory protein-1 alpha), MIP-1β (macrophage inflammatory protein-1 beta) and RANTES (Regulated on Activation Normal T-cell Expressed and Secreted) enhance the intracellular destruction of *Trypanosoma cruzi* by macrophages (Aliberti *et al.*, 1999; Villalta *et al.*, 1998) and the elimination of *Wuchereria bancrofti* parasites has been associated with an increase in plasma levels of RANTES (Gopinath *et al.*, 2000).

4.3.1 Chemokines and malaria in pregnancy

Chemokine-chemokine receptor biology in relation to malaria infections is a relatively new field with few studies documented on chemokine role in protection. Of the few documented studies, data obtained from adult Thai populations report IL-8 (interleukin-8) levels correlated with parasitemia and severity of disease, and elevated serum levels of MIP-1α with maximal concentrations coinciding with parasite clearance (Burgmann *et al.*, 1995; Friedland *et al.*, 1993). In malaria during pregnancy in which most chemokine studies have focused perhaps because the placenta is a new organ requiring immune cell recruitment for protection against infection, chemokine expression has been documented particularly in the context of monocyte infiltrations to the placenta. Placental MIP-1α, MCP-1 (monocyte chemotactic protein-1), I 309 (Inducible 309) and IL-8 mRNA

expression was increased in pregnant Malawian women during a malaria episode and was suggested as being the trigger for monocyte accumulation in the placenta (Abrams *et al.*, 2003). High IP-10 (interferon-γ inducible protein-10) levels were observed in Kenyan women without a placental infection of malaria as compared to women with placental malaria (Chaisavaneeyakorn *et al.*, 2002) and in a separate study in the same region levels of MIP-1β were increased in malaria-infected placentas of Kenyan women. The increased chemokine levels were hypothesized as being important in the mobilization of protective Th1 lymphocyte populations (Chaisavaneeyakorn *et al.*, 2003b).

Evidently information on the role of chemokines and chemokine receptors in relation to protection against malaria infection is limited. Clearly more investigations are required.

4.4 Study rationale

Results from chapter three have shown that perhaps different immune components possibly work to effect protection in different blood compartments. IFN-γ producing NK cells are associated with protection at the placental level, while CD45RO memory cells were associated with protection in peripheral blood. However the underlying mechanisms of IFN-γ producing NK cell related protection against infection in the placenta is unclear. The requirement for two different mechanisms of protection acting in different compartments particularly in view of the fact that the peripheral and placental blood system are arguably one continuum, is intriguing. NK cells mediate their cytolytic and cytokine secreting functions in intimate proximity to infected sites (Robertson, 2002). It is therefore conceivable that NK cells are recruited into the placenta in response to parasite presence. The chemokine/chemokine receptor system is hypothesized as being important in the regulation of NK cell trafficking and activation. NK cells express CXCR1, CXCR2, CXCR3, CXCR4 and CX3CR1 of the CXC receptors and CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8 and CCR9 of the CC receptors. These receptors are selectively expressed on resting and activated NK cells (Robertson, 2002). NK cells migrate in response to MIG (monokine induced by interferon-γ), IP-10, I-TAC (interferon inducible T-cell α chemoattractant) and SDF-1 (stromal cell -derived factor 1) CXC chemokines and to I 309, MCP-1, MIP-1α, MIP-1β, RANTES and MCP-3

(monocyte chemotactic protein-3) of the CC chemokines, among others (Robertson, 2002). Investigations of chemokine and chemokine expression in relation to the NK cell mediated protective mechanism reported in chapter three may offer a better understanding of the mechanisms of protection against placenta malaria.

4.5 Objective

To investigate chemokine and chemokine receptor expression in relation to protection from malaria during pregnancy.

4.5.1 Specific Objectives

1. To determine levels of chemokines IP-10, RANTES and MIP-1α along with corresponding receptors CXCR3 (for IP-10) and CCR5 (for RANTES and MIP-1α), in peripheral and placental blood.
2. To compare levels of IP-10, RANTES and MIP-1α between placenta malaria positive and placenta malaria negative mothers.
3. To investigate IFN-γ producing NK Cells in relation to chemokines and chemokine receptors in association with protection from malaria infections of the placenta.

The combination of chemokines and chemokine receptors was selected based on their NK cell recruitment and activation ability. All three chemokines are expressed by a variety of cell types however investigations were carried out only for IP-10 expression by monocytes (CD14+ cells), RANTES by T lymphocytes (CD3+ cells) and monocytes and MIP-1α expression by monocytes since these cell types are found in peripheral and placental blood as compared to others that reside mainly in tissues.

4.6 Materials and Methods

4.6.1 Study participants

Mothers were enrolled after delivery following consent from the Nyanza Provincial Hospital. Placentas were collected at delivery and immediately processed for prick blood as described in chapter two. A peripheral sample (3mls) was also obtained from the mother.

All blood samples were collected into heparin charged eppendorf tubes. HIV testing and parasite determination were carried out. Altogether, 52 women were enrolled, 20 with placenta malaria and 32 without placenta malaria. The sample size was calculated at an alpha level of 0.05 and 90% power to detect at least a 25% difference in immune responses between parasitic and aparasitemic women.

4.6.2 Laboratory methods

Blood samples were divided into two for chemokine receptor staining and chemokine intracellular staining purposes. Cells for chemokine receptor staining did not require any activation or incubation and were processed immediately upon collection while intracellular chemokine production required incubation and stimulation.

4.6.2.1 Cell activation and culture

Cells samples for intracellular chemokine production were activated using lipopolysaccaride (LPS) (Sigma Chemical Co., St. Louis, MO), in the presence of monensin (Sigma Chemical Co., St. Louis, MO) as the transport inhibitor to determine spontaneous chemokine production. Blood samples (peripheral and placental) were diluted into an equal volume of incomplete RPMI medium supplemented with 2mM L-glutamine (GIBCO, Invitrogen Corporation, UK) and activated using LPS at 10ng/ml and monensin at a concentration of 1μM/ml for IP-10 and MIP-1α expression. RANTES expression did not require stimulation (spontaneous production of RANTES occurs from cell culturing without stimulation). Cells were incubated at 37°C, 5%CO_2 for 24 hours. In addition unstimulated aliquots of peripheral and placental blood, incubated with monensin, were processed in parallel, to serve as controls. Stimulant concentrations used were on the basis of previous standardization experiments carried out to determine concentrations for optimal chemokine production.

Staining was carried out in two steps. First, cell surface marker staining for the identification of monocytes and T cells and second, intracellular staining for chemokine expression by monocytes and T cells.

4.6.2.2 Cell surface marker staining

Cell surface marker staining was carried out to evaluate chemokine receptor (CXCR3 and CCR5) expression by both monocytes and T cells and to pre-select monocytes and T cells for further intracellular chemokine staining. The following antibody reagents were used for cell surface marker staining. Anti-CD3 CyChrome (Mouse IgG1), anti-CD14 FITC (Mouse IgG2a), anti-CXCR3 PE (Mouse IgG1) and anti-CCR5 PE (Mouse IgG2a). In addition isotype-matched monoclonal antibodies, anti human mouse IgG1 PE and anti-human mouse IgG2a PE were used as negative controls for chemokine receptors CXCR3 and CCR5. All antibodies were obtained from Pharmingen, San Diego, CA.

4.6.2.2.1 Chemokine receptors evaluation

Five microlitres of fluorochrome-conjugated antibodies were aliquoted into labelled 5ml falcon tubes (Becton Dickinson, San Jose, CA) as indicated in Table 4.1. Tubes 1,2, 6 and 7 represent the chemokine receptor antibody staining combinations. Tubes 6 and 7 served as the isotype controls for tubes 1and 2. Fifty microlitres of unstimulated, unactivated blood was aliquoted into each of the four tubes and processed using the protocol outlined below for surface marker staining.

4.6.2.2.2 CD14 and CD3 cells selection

Tubes 3,4,5,8 and 9 represent the antibody combinations for intracellular staining. Tube 8 served as the isotype control for tube 3 while 9 served as the control for 4 and 5. Stimulated and unstimulated whole blood samples were added (100µls) to tubes at the end of the 24-hour incubation period; stimulated blood into tubes 4 and 5 and unstimulated blood into tubes 3, 8 and 9.

4.6.2.2.3 Staining procedure

Cells were incubated on ice for 30 minutes in the dark after which erythrocytes were eliminated by lysis using a 1:10 dilution of room temperature Facs lysing solution (BD Pharmingen, San Diego, CA) in distilled water (3.5mls per tube) for strictly 10 minutes in the dark. Cells were spun at 1500rpm for 7minutes at 8°C at the end of the incubation period. The supernatant was aspirated and discarded after which the cell

Table 4.1: Antibody staining panel

Tube	FITC	PE	Cy-Chrome
1	CD14	CXCR3	CD3
2	CD14	CCR5	CD3
3	CD14	RANTES	CD3
4	CD14	MIP-1α	CD3
5	CD14	IP-10	CD3
6	CD14	IgG1	CD3
7	CD14	IgG2a	CD3
8	CD14	IgG1	CD3
9	CD14	IgG2a	CD3

The panel above represents the antibody combinations used to stain for chemokine/chemokine receptor expression monocytes and T cells. Tubes 1 to 5 represent the chemokine receptor and chemokine test tubes while tubes 6 to 9 are the isotype controls; tube 6 and 7 represent the controls for Tube 1 and 2, tube 8 represents the control for Tube 3 while tube 9 served as the control for tubes 4 and 5. In the first step of cell surface marker staining only PE and CyChrome labeled antibodies were added for tubes 3, 4,5, 8 and 9 while tubes 1,2, 6 and 7 had all three antibody sets added at this stage. PE conjugated antibodies for tubes 3,4, 5, 8 and 9 were added in the second step for intracellular chemokine staining.

pellet was reconstituted in 1ml of ice cold freezing medium. Cells were then frozen at -80°C overnight.

4.6.2.3 Intracellular Cytokine staining

Anti human RANTES PE (mouse IgG1, 30ng/ml), anti-human MIP-1α PE (mouse IgG2a, 30ng/ml) and anti-human IP-10 PE (mouse IgG2a, 30ng/ml) were used for intracellular chemokine staining with PE labelled mouse IgG1 (30ng/ml) and mouse IgG2a (30ng/ml) as the isotype-matched negative control. Antibody concentrations were predetermined by titration experiments.

Cells were removed from -80°C and quickly thawed in a 37°C water bath while constantly shaking, in preparation for the second staining step. Cells were washed once at 1500rpm for 7 minutes at 8°C and permeabilized using 0.1% saponin for 30 minutes in the dark at 4°C. One hundred microlitres of the appropriate flourochrome labelled antibodies were added and cells were further incubated in the dark for 30 minutes on ice or at 4°C. Cells were washed once in 2mls of wash buffer by centrifugation at 1500rpm for 7 minutes at 8°C. The supernatant was aspirated and discarded and cells fixed in 500µls of 1% paraformaldehyde per tube. Cells were analysed on a FacsCalibur (BD Biosciences, San Diego,CA).

4.6.2.4 Flow Cytometric acquisition and analysis

For data acquisition and analysis the Cell Quest Pro software (BD Biosciences, San Diego, CA.) was used. Ten thousand ungated events were acquired. Using dot plots the analysis for cell subpopulations was by SSC-CD3 and SSC-CD14 gating. The negative controls were used to set quadrant markers for each cell subpopulation allowing a maximum of 2% as background. Percentage chemokine and chemokine receptor expressing cells were calculated from CD3 and CD14 gated cell subpopulation events. Results are reported as % mean chemokine/chemokine receptor production by each cell subpopulation.

4.7 Statistical analysis

Data entry and statistical analysis was performed using the SPSS statistical software (SPSS 11.0.1, Inc., Chicago, IL). Because the data obtained were not normally distributed, natural log transformations were performed for each parameter. Pearson's χ^2 test was used to evaluate group differences. Differences between peripheral and placental blood as well as between placenta malaria negative and placenta malaria positive individuals were tested using the non-parametric Kruskal-Wallis statistical test. The Spearman's rho was used to test for associations. Confounding factors were controlled for in the statistical analysis. *P* values (two tailed) less than 0.05 were considered statistically significant while values less than 0.09 but greater than 0.05 were considered marginally significant.

4.8 Results

The results reported here were obtained from intracellular chemokine staining of peripheral and placental intervillous blood. Staining was carried out for IP-10, RANTES and MIP-1α. Cell surface marker staining for the corresponding chemokine receptors CXCR3 (for IP-10) and CCR5 (for RANTES and MIP-1α) was also carried out.

4.8.1 Characteristics of study participants

Study subject characteristics are shown in Table 3.2 of Chapter Three.

4.8.2 Comparisons of peripheral and placental chemokine-chemokine receptor levels in PM- and PM+ women

Peripheral and placental chemokine and chemokine receptor expression levels were compared and Table 4.2 shows the comparison results. There was no significant difference between peripheral and placental blood for all chemokines and chemokine receptors and no difference in placental levels of the same parameters between PM+ and PM- women.

Table 4.2: Peripheral and placental chemokine-chemokine receptor levels in PM- and PM+ women

				Placental		
Parameter	**Peripheral**	**Placental**	***P* value**	**PM-**	**PM+**	***P* value**
IP-10/CD14	2.15	2.29	**0.482**	2.24	2.35	0.429
RANTES/CD3	2.5	2.43	**0.355**	2.43	2.43	0.611
RANTES/CD14	1.98	2.05	**0.729**	2.19	1.86	0.222
MIP-1α/CD14	2.09	2.05	**0.882**	2.02	2.08	0.499
CCR5/CD3	3.75	3.54	**0.301**	3.5	3.62	0.110
CCR5/CD14	0.86	0.85	**0.625**	0.89	0.8	0.300
CXCR3/CD3	4.04	4.02	**0.932**	4.1	3.89	0.205
CXCR3/CD3-	7.07	7.21	**0.917**	7.51	6.71	0.739

Values indicated are mean % cell numbers obtained from flow cytometry. Comparisons were done using the Kruskal-Wallis statistical non-parametric statistical test. P (two sided) <0.05 considered as significantly different.

4.8.3 Ratios of peripheral and placental chemokine expression

To investigate the balance between peripheral and placental chemokines in association with cell migrations, IP-10, RANTES and MIP-1α were assessed at the individual level. Ratios of peripheral over placental chemokine levels were calculated for each study participant on the premise that chemokine responses would be different in placental and peripheral blood for cell migration to occur in either direction. A ratio value of one would indicate equal levels of chemokine production in the placenta and peripheral blood and hypothetically no cellular migratory movements between the two compartments. In contrast, a ratio less than one would point to movement towards the placenta due to higher chemokine production within the placental environment than in peripheral blood, similarly a ratio more than one would indicate cellular movement away from the placenta in response to higher chemokine expression by peripheral blood. Study participants were grouped according to cell migration towards or away from the placenta, cellular migration towards the placenta would indicate protection while migration away from the placenta would signify susceptibility. A cut-off of 0.9, was chosen for grouping into protected and susceptible women based on the above-described potential two directional cellular movement of cells as well as for a balanced distribution of study participants. The ratio values of chemokines were evaluated for association with clinical parameters as shown in Table 4.3. An IP-10 ratio less than 0.9 had borderline association with a low frequency of mothers with parasitemia (33.3%) while a ratio value greater than 0.9 showed marginal association with a higher frequency of mothers with parasitemia (72.7%) (P=0.055). There were no associations between IP-10 ratio values and gravidity, haemoglobin and anaemia. There were also no differences in MIP-1α and RANTES ratios for all the clinical parameters including parasite rate. These results suggest that the IP-10 ratio maybe a potential marker for the clinical outcome of placenta malaria infections; a low IP-10 ratio (less than 0.9) would be an indicator of protection while a high IP-10 ratio value (greater than 0.9) would imply susceptibility.

Table 4.3: Association of chemokine ratios with study participant clinical parameters

A:

	Ratio IP-10/CD14		
Characteristic	<=0.9	>0.9	*P* value
Gravidity (<=2, <2)*	66.7%(10),33.3%(5)	72.7%(8),27.3%(3)	**0.543**
Haemoglobin#	11.15(15)	12.03(11)	**0.236**
Anaemia*	40.0%(15)	27.3%(11)	**0.402**
Malaria infection rate: placenta#	33.3%(15)	72.7%(11)	**0.055**
Parasite density: placenta*	7.93(5)	7.94(8)	**0.993**

B:

	Ratio RANTES/CD3			Ratio RANTES/CD14		
Characteristic	<=0.9	>0.9	*P* value	<=0.9	>0.9	*P* value
Gravidity (<=2, <2)	58.3%(7),41.7%(5)	67.6%(25),32.4%(12)	**0.401**	55.6%(5),44.4%(4)	70%(14),30%(6)	**0.364**
Haemoglobin	11.23(12)	12.19(35)	**0.125**	11.90(9)	11.59(19)	**0.681**
Anaemia	33.3%(12)	28.6%(35)	**0.511**	22.2%(9)	36.8%(19)	**0.374**
Malaria infection rate: placenta	58.3%(12)	59.5%(37)	**0.602**	88.9%(9)	75.0%(20)	**0.375**
Parasite density: placenta	7.21(5)	7.86(15)	**0.568**	8.13(3)	7.83(9)	**0.82**

C:

	Ratio MIP-1a/CD14		
Characteristic	<=0.9	>0.9	*P* value
Gravidity (<=2, <2)	71.4%(5),28.6%(2)	68.8%(11),31.3%(5)	**0.649**
Haemoglobin	11.57(7)	12.04(16)	**0.566**
Anaemia	28.6%(7)	25.0%(16)	**0.618**
Malaria infection rate: placenta	85.7%(7)	75.0%(16)	**0.508**
Parasite density: placenta	7.76(4)	7.40(7)	**0.16**

Chemokine ratios for IP-10 (A), RANTES (B) and MIP-1α (C). *Calculation by student t test, #calculation by Pearson's chi sq test

4.8.4 Relationship of IP-10 ratio with immune cell numbers and IFN-γ producing NK cells and CXCR3

Since IP-10 ratio values less than 0.9 are associated with protection and given that IFN-γ producing NK cells were associated with protection in the previous chapter, the association between IP-10 ratios, placental immune cell numbers and IFN-γ production were assessed to investigate possible cell migration in relation to protection (Table 4.4). The group defined as protected had marginally higher CD45RO numbers as compared to the susceptible group (P=0.092) and IFN-γ producing NK cell levels were significantly higher in the former group than the latter (P =0.046). A further evaluation of IP-10 ratio values and IFN-γ producing NK cells stratified by parasitemic and aparasitemic groups revealed that aparasitemic women had a strong negative correlation between the two parameters (rho= -0.488, P =0.013) while there was no relationship for parasitemic women between IP-10 ratios and IFN-γ producing NK cells (Figure 4.1). These results suggest that parasite infection possibly alters the relationship between IFN-γ producing NK cells and the IP-10 ratio.

Since CXCR3 is the IP-10 ligand that enables cell recruitment and trafficking, a further evaluation of CXCR3 expression by placental CD3 negative cells (representing NK cells) was performed. Figure 4.2 illustrates that placental levels of CXCR3 expression on CD3 negative cells were highly correlated with IFN-γ producing NK cells (P =0.014). Taken together this suggests that IP-10 trafficking of IFN-γ producing NK cells is mediated by CXCR3 and confers protection.

4.9 Discussion

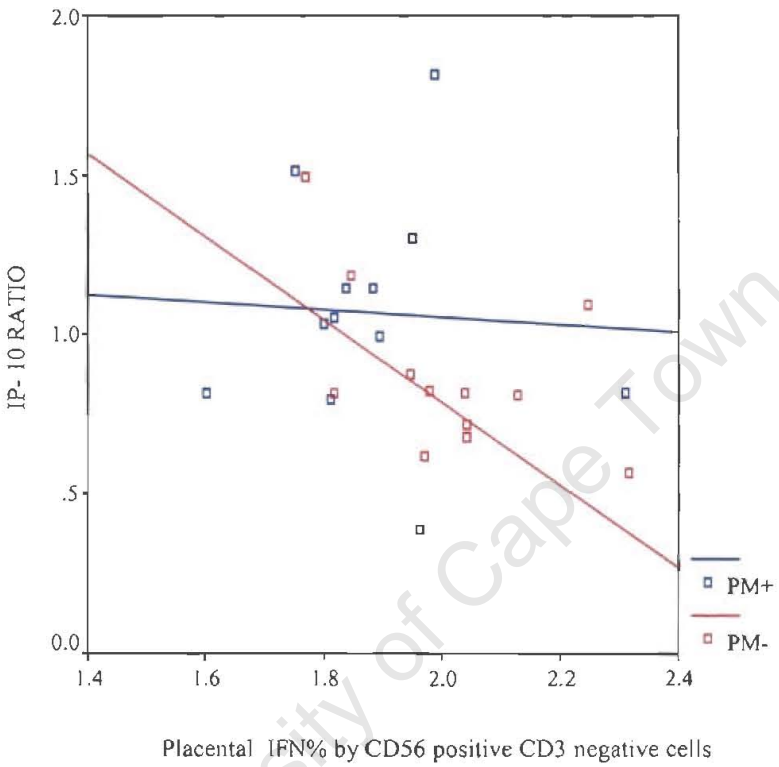
Malaria in pregnancy results in parasite infections of the placenta and has deleterious effects on the foetus due to the accumulation of parasites (Brabin *et al.*, 1993). Previous studies in western Kenya have shown that IFN-γ production in the placenta is associated with protection against placental infections (Moore *et al.*, 1999). The results from the previous chapter have further shown that IFN-γ producing NK cells in the placenta are associated with protection, suggesting that non-specific innate IFN-γ expression maybe critical to protection. However the regulation of this NK cell-IFN-γ protection is

Table 4.4: Relationship of IP-10 ratio with immune cell subpopulations and IFN-γ producing cells.

	Ratio IP-10/CD14		
Parameter	**<=0.9(15)**	**>0.9(11)**	***P* value**
IFN/CD3	2.58	2.7	**0.815**
CD4/CD3	4.01	3.8	**0.337**
IFN/CD4	2.35	2.44	**0.897**
CD8/CD3	3.69	3.47	**0.139**
IFN/CD8	2.48	2.42	**0.775**
CD45RO/CD3	3.17	2.73	**0.092**
IFN/CD45RO	2.63	2.28	**0.243**
CD56/CD3-	3.07	2.87	**0.529**
IFN/CD56	2	1.89	**0.046**

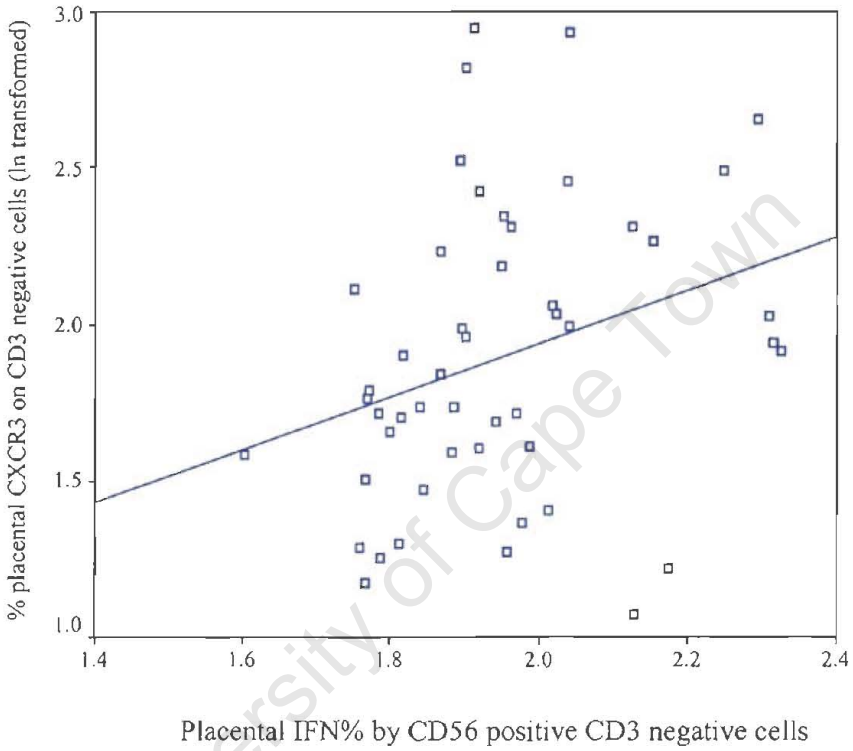
Values indicated are ratios of % cell numbers of chemokine producers obtained from flow cytometry. Comparisons were done using the Kruskal-Wallis statistical non-parametric statistical test. P (two sided) <0.05 considered as significantly different.

Figure 4.1: IP-10 ratios and IFN-γ producing NK cells in PM+ and PM- women



IP-10 ratio values and IFN-γ producing NK cells stratified by placental parasitemia. PM- women had a strong positive correlation between the two parameters (rho= -0.488, p=0.013).There was no relationship for PM+ women between IP-10 ratios and IFN-γ producing NK cells.

Figure 4.2: CXCR3 expression by placental CD3 negative cells in association with with IFN-γ producing NK cells



Placental levels of CXCR3 expression on CD3 negative cells were highly correlated with IFN-γ producing NK cells (p=0.014).

unknown. Chemokines are proteins belonging to the large super family of chemotactic cytokines. In conjunction with their receptors they are responsible for immunoregulatory events that effect protection, mainly by the recruitment of cells to sites of injury or pathogen entry, and by cell activation. This chapter assesses chemokine and chemokine receptor expression in relation to IFN-γ producing NK cells and the potential role that they play in protection against infection.

4.9.1 Chemokine and Chemokine receptor expression in peripheral and placental blood in association with protection

In this study, an assessment of levels of IP-10, RANTES and MIP-1α together with CXCR3 and CCR5 in peripheral and placental blood between aparasitemic and parasitemic individuals revealed no differences for each of the parameters assessed. Contrasting results have been reported for the same chemokine measurements by other studies. In a previous study conducted within the same region, Chaisavaneeyakorn *et al.*, (2002) reported different IP-10 results from those reported here. Intervillous blood mononuclear cell (IVBMC) IP-10 expression levels to PHA were higher in aparasitemic than in parasitemic Kenyan women. These two studies used different experimental approaches and the discrepancy in results may be attributed to the fact that one study quantified IP-10 protein expression levels by IVBMC while the current study assessed single cell intracellular chemokine expression in CD14 cells. Thus a careful interpretation of results from the two studies would be required. A further study by the same group also reported unelevated MIP-1α levels in the presence of infection and is in agreement with reports documented here (Chaisavaneeyakorn *et al.*, 2003a). A more recent study in Malawian pregnant women measuring chemokine mRNA expression of placental tissue reported unelevated placental IP-10 levels in malaria infected women. However, they reported elevated levels of MIP-1α in the same women by mRNA expression and plasma chemokine protein levels (Abrams *et al.*, 2003).

Despite the fact that chemokine/chemokine receptor expression levels were not different between parasitemic and aparasitemic women, IP-10 but not RANTES or MIP-1α ratios showed marginal association with placental malaria. An IP-10 ratio less than 0.9 had a

low frequency of mothers with placental malaria while a ratio value greater than 0.9 had a high frequency of mothers with placental malaria. The finding that IP-10 ratios were indicative of susceptibility or protection together with the reported absence of elevated IP-10 levels in parasitemic women in this study alludes to the possibility that the IP-10 expression balance between peripheral and placental blood may play more of a regulatory role than a parasite elimination role. From these observations it may be even further suggested that peripheral to placental chemokine ratios may be better protection indicators than placental chemokines expression levels taken exclusively.

4.9.2 Regulatory role of IP-10 in relation to IFN-γ producing NK cells and protection

In this study peripheral over placental chemokine ratios were further evaluated to assess cell migration and activation in association with protection from placental infection. IP-10 ratios were assessed in relation to immune cell levels; women defined as protected had marginally higher frequencies of placental CD45RO than susceptible women suggesting a role for memory cells in IP-10 activity. In the mouse model of *Leishmania donovani* T cells are implicated in the regulation of IP-10. T cell presence in this mouse model was necessary for the maintenance of maximal tissue IP-10 mRNA expression (Cotterell *et al.*, 1999). It is postulated that the increased levels of memory cells in protected women may be for the enhancement and sustenance of IP-10 production enabling the recruitment of additional NK cells. These results suggest that protection in uninfected women may be partially memory cell driven. Further investigations are required to establish the nature of this memory cell role in chemokine-mediated protection.

IP-10 ratios were associated with IFN-γ producing NK cells with higher levels in protected women as compared to susceptible women. Further assessment stratified by parasitemia revealed a strong negative relationship between placenta IP-10 ratios and IFN-γ producing NK cells in aparasitemic women. Parasitemic women had no associations between IP-10 ratios and IFN-γ producing NK cells, suggesting that parasite infection of the placenta may alter the regulatory role of IP-10. Additionally, the correlation of CXCR3 expression by CD3 negative cells with placental IFN-γ producing

NK cells provides further evidence that suggests cell recruitment to the placenta for protection mediated by IP-10 in conjunction with CXCR3.

Chemokine functions are manifold and more recently the function of cell activation has been added to the list of chemokine activities (Matsukawa *et al.*, 2000). Numerous immunological studies have shown that IP-10 has activation mediated functions (Robertson, 2002). The strong correlation of IP-10 ratios with IFN-γ producing NK cells not only suggests a role for cell recruitment but also suggests a role for cell activation. *Leishmania major* susceptible mice recruit NK cells to the site of infection but appear to have defective NK cell activation. IP-10 has been found to be expressed in higher levels in resistant mice as compared to the susceptible in the early phases of infection. Likewise, in this study IP-10 expression may facilitate NK cell activation for IFN-γ production or for cytolysis of infected erythrocytes and subsequent parasite elimination. Indeed recombinant IP-10 enhances the cytolytic activity of human NK cells *in vitro* (Maghazachi *et al.*, 1997; Taub *et al.*, 1995). Investigations are required to further assess NK cell cytolytic activity in placental infections of malaria to determine possible associations with IP-10 production. If established, one might therefore postulate that in placenta malaria susceptible women, IP-10 levels were insufficient to elicit protection despite sufficient NK cell recruitment to the placenta.

4.9.3 A proposed mechanism of protection from placental malaria

The results obtained from this study suggest that the mechanism for the IP-10/IFN-γ producing NK cell protection is complex and involves numerous parameters. Based on results reported in previous studies by others and results from the current study, the following model of protection from placenta malaria is proposed. Upon infection of the placenta by *P. falciparum,* parasite presence elicits a pro-inflammatory environment with IFN-γ production via IL-12 ensuing. IP-10 is subsequently upregulated which initiates the recruitment of NK cells to the placenta through interaction with CXCR3, while augmenting NK cell IFN-γ production and cytotoxicity. These events lead to parasite elimination. Memory T cells of the CD4 type ensure that IP-10 levels are maintained at a maximum resulting in more recruitment of NK cells and further parasite elimination. This

innate non-specific immune response probably continues until infection is cleared or the adaptive immune response is developed as discussed in chapter three. Total elimination of parasites from the placenta is probably dependent on the production and peripheral to placental balance of IP-10 early in infection for rapid efficient NK cell recruitment and innate IFN-γ production. However in a delayed IP-10 response situation or an imbalance of IP-10 production between peripheral and placental blood, parasitemia is not controlled and individuals experience placental malaria (Figure 4.3). In view of this proposed IP-10 dependent mechanism of protection, it would be interesting to investigate further the timing of IP-10 production in relation to the mother's prior malaria experience during pregnancy and whether with each pregnancy one's ability to rapidly express IP-10 becomes more efficient. If found to be so, this could offer a possible explanation to the phenomenon of gravidity-dependent protection from placenta malaria in pregnant women resident in malaria holoendemic regions.

In conclusion the present study describes for the first time chemokine dependent regulation of protective mechanisms against placental malaria. The results obtained here provide evidence for a possible IP-10 role and expression balance, acting via CXCR3, in the recruitment and potential activation of NK cells in defence of the placenta from *P. falciparum* infection. The results reported here together with results obtained in the previous chapter signify the complexity of the IFN-γ /NK cell protection against placental infection. It is likely that the NK cell dependent protective mechanisms involve more parameters than those reported here. Recently, Pearson (2001) suggested a possible role for prolactin dependent NK cell cytolytic activity against malaria parasites. Investigations are clearly required to delineate further NK cell dependent mechanisms of protection against placental malaria.

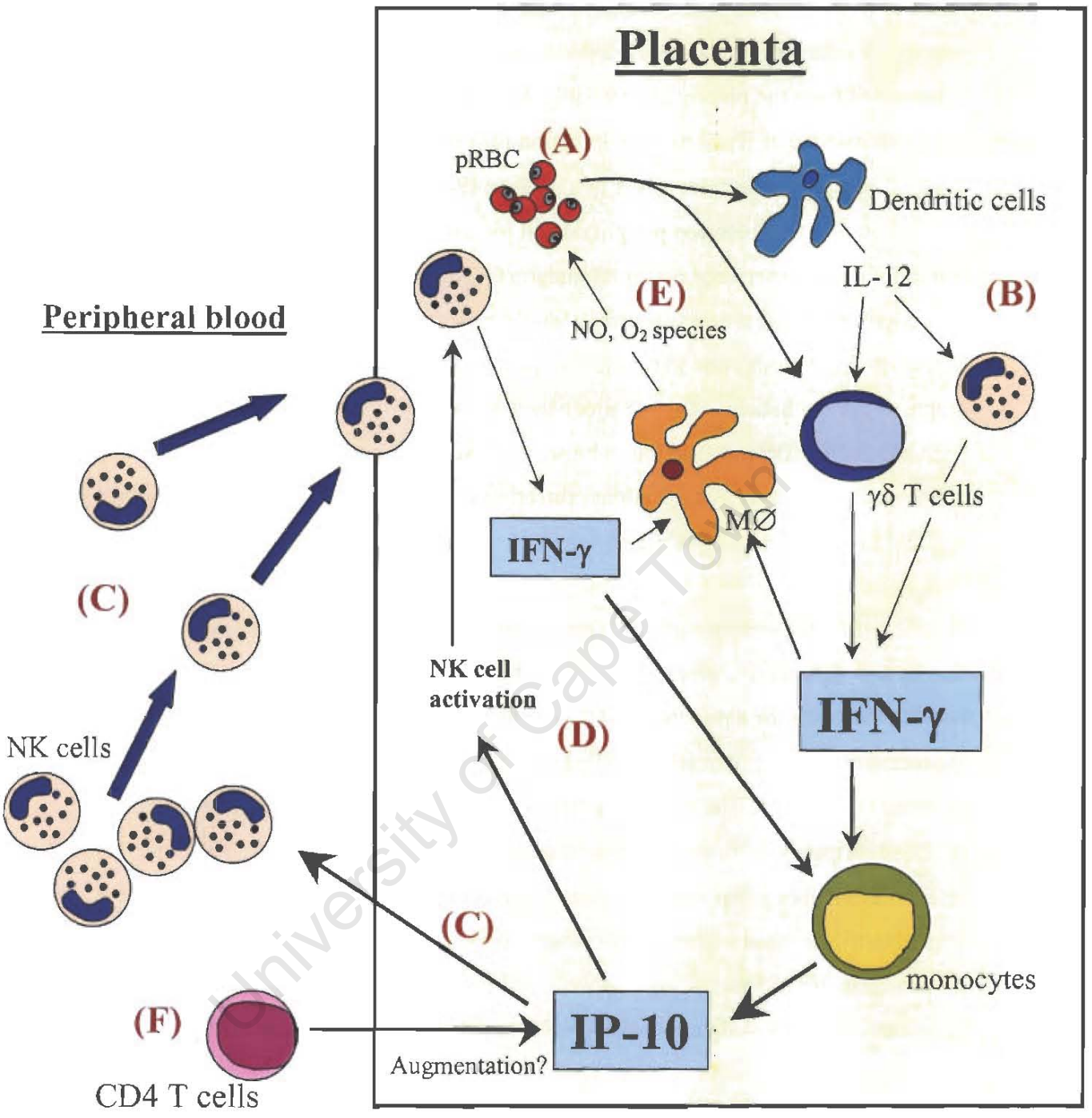


Figure 4.3: Proposed IP-10 regulatory mechanism of IFN-γ producing NK cells in protection againt Placental malaria. Parasitized red blood cells (pRBC) (**A**) elicit IL-12 production by dendritic cells, which causes IFN-γ production by γδ T cells, and NK cells (**B**). Consequently IP-10 is upregulated and initiates the recruitment of NK cells to the placenta through interaction with CXCR3 (**C**). IP-10 production also mediates recruited NK cell activation for IFN-γ production and cytotoxicity against the pRBCs (**D**). IFN-γ elicits the production of NO and reactive O_2 species by macrophages that effect parasite elimination (**E**). CD4 T cells ensure that IP-10 levels are maintained at a maximum resulting in more recruitment of NK cells and further parasite elimination (**F**).

CHAPTER FIVE

Chemokine and chemokine receptor expression in placenta malaria

5.1 Introduction

Malaria during pregnancy in holoendemic regions has adverse consequences. It carries a high-risk of developing severe pathology: anaemia in the mother and low birth weight (LBW) in the infant (Steketee *et al.*, 2001). The consequences of infection cannot be overemphasized, severe maternal anaemia has been reported as the main cause of 8-20% of maternal deaths in sub-Saharan African hospitals (Shulman, 1999), while an estimated 3 million newborns per year, suffer complications arising from a *P. falciparum* infection that include LBW (Phillips, 2001). LBW is the single most important risk factor for infant mortality during the first year of life with mortality rates for LBW babies four times more than mortality for normal birth weight babies (Bloland *et al.*, 1996; McCormick, 1985). LBW associated complications kill an estimated 62,000-363,000 infants in sub-Saharan Africa each year (Murphy and Breman, 2001).

5.2 Pathogenesis of adverse outcomes during pregnancy

The severe consequences of a malaria infection during pregnancy in holoendemic regions are confined mainly to first but sometimes also to second pregnancies, i.e. women with a higher risk of parasite accumulation in the placenta. Parasite accumulation in the placenta initiates a series of events that lead to pathology and subsequent adverse outcomes of infection. Pregnancy is characterized by a type 2 cytokine profile for the foetus's continued existence (Wegmann *et al.*, 1993), *P. falciparum* adhesion to CSA in the intervillous spaces of the placenta however has been shown to direct the immune environment towards a detrimental type 1 response and may be responsible for pathology observed. Type 1 cytokines IFN-γ, TNF-α and IL-2 have been reported in a large proportion of placentas from a holoendemic region (Fievet *et al.*, 2001; Fried *et al.*, 1998a), with placental TNF-α levels associated with anaemia particularly in primigravid women (Fried *et al.*, 1998a). In addition to anaemia being a risk factor for maternal mortality, low haemoglobin levels have also been suggested as a cause for LBW (Shulman, 1999). Severe maternal anaemia may cause placental and foetal tissue hypoxia and subsequent foetal intrauterine growth retardation (Brabin *et al.*, 2003). A further

consequence of parasite presence is the phenomenon of leukocyte infiltration-a histopthological hallmark of placental malaria (Bulmer *et al.*, 1993; Walter *et al.*, 1982). Leukocyte infiltrates are predominantly monocytic in composition, may contain pigment and may be responsible for the pro-inflammatory milieu elicited by infection (Moormann *et al.*, 1999). Monocyte infiltration of the placenta has been suggested as being a crucial mediator of LBW; monocytic infiltrates were associated with reduced birth weight in a Tanzanian study (Ordi *et al.*, 1998). Placental TNF-α levels in a Malawian study were higher in women with placental infections than in women without infection and were associated with monocyte infiltration (Rogerson *et al.*, 2003a). Parasite presence in the placenta also leads to haemozoin (malaria pigment) accumulation within the placenta. Malaria pigment is a by-product of parasite digestion of haemoglobin and has been used to indicate evidence of active or past infections. Pigment has been suggested as contributing to the pathogenesis of malaria; histological examinations of parasitized placenta have revealed associations with anaemia and LBW (Rogerson *et al.*, 2003b).

5.3 Chemokines and chemokine receptors in malaria pathogenesis in pregnancy

Chemokines and their receptors in the recent years have been the focus of many immunological functions. Initially investigated for their role in immune cell migrations, chemokines are known to participate in many physiological conditions but have also been implicated in pathological processes. In pregnancy, chemokines are involved in different physiological processes including embryonic implantation and parturition. They have also been observed in pathological conditions such as preterm deliveries- one of the causal factors of LBW (Simon *et al.*, 1998). IL-8 concentrations in amniotic fluid were significantly higher in women with preterm labour than in controls (Romero *et al.*, 1994). Predictably, investigations of pathology associated with malaria infections during pregnancy have documented chemokine production. Levels of MIP-1β were elevated in Kenyan women with placental malaria infections and were associated with both anaemia and pigment load (Chaisavaneeyakorn *et al.*, 2003a). Studies in Malawi showed increased gene expression levels of MIP-1α, MCP-1, IL-8 and I 309 associated with placenta malaria (Abrams *et al.*, 2003). All four chemokines correlated with monocyte densities however only IL-8 levels correlated with infant birth weight. MIP-1α, MIP-1β and MCP-

1 are potent chemoattractants of monocytes while IL-8 attracts neutrophils, and in each of the studies outlined above the expression of these chemokines were implicated as being responsible for monocyte infiltration to the placenta in malaria during pregnancy. The precise function and mechanism of chemokines in relation to maternal anaemia and LBW pathogenesis is still not clear. However, chemokines mediate their biological responses by binding to chemokine receptors and therefore by inference chemokine receptors may be predicted as being important in the process. Moreover several lines of evidence indicate that chemokine receptor expression is as important as chemokine production in the regulation of the immune response (Patel *et al.*, 2001). Chemokine receptor role in the aetiology of malaria pathology in pregnancy has not been explored therefore the nature of chemokine/chemokine receptor expression patterns in relation to pregnancy-associated malaria requires further investigation.

5.4 Study Rationale

Immune mechanisms against malaria may be beneficial when produced in appropriate quantities but can be harmful when produced in excessive amounts and could lead to pathology. Therein lies a paradox; factors important in protection against malaria may also be damaging. Malaria during pregnancy is characterized by monocyte infiltration of the placenta, an event that has been associated with LBW and anaemia. Immune mechanisms otherwise considered protective may be involved in the pathogenesis of both conditions. Placental chemokine and chemokine receptor expression has been considered to be primarily beneficial in protection, as reported by others and also by the data reported in the preceding chapters of this thesis. However, associations have recently been made between chemokines and adverse pregnancy outcomes particularly in relation to monocyte infiltration. None of the chemokines so far investigated have been studied with corresponding chemokine receptors. Studies are therefore required to further investigate chemokines together with parallel chemokine receptors in relation to maternal haemoglobin levels and birth weight of the infant. Also, malaria pigment influence on chemokine and chemokine receptor expression is not well defined and requires more investigation. The current investigations were carried out to address these issues in an effort to assess malaria infection outcomes during pregnancy.

5.5 Objective

To investigate placental tissue chemokines and chemokine receptors in relation to malaria in pregnancy.

5.5.1 Specific Objective

1. To determine placental tissue chemokine and chemokine receptor gene expression patterns in relation to placental parasitemia.
2. To determine the influence of pigment on the gene expression levels of placental tissue chemokine and chemokine receptors.
3. To associate tissue chemokine and chemokine receptor gene expression with maternal haemoglobin and birth weight of the infant.

5.6 Materials and methods

5.6.1 Study participants

Mothers were enrolled after delivery and their placenta collected for tissue sampling. A placental tissue biopsy (2cm square by 2cm) was collected in an area approximately 1 inch from the insertion of the cord as described by Bulmer *et al.*, (1993). Samples were rinsed in PBS, placed in cyrovials containing 1ml RNA extraction fluid (Biotecx Laboratories, Houston, TX) and immediately frozen in liquid nitrogen.

5.6.2 RNA extraction

Total RNA was extracted from the placental tissue samples using the method of Chomczynski and Sacchi (1987) with modifications as follows:

Samples were homogenized in 1ml of Solution D (4M guanidine thiocyanate; 25mM sodium citrate, pH 7.0; 0.5% sarcosyl; Sigma Chemical Co., St. Louis, MO) with 0.1M β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) using loose fitting Wheaton dounces (0.0035-0.0055 inches clearance). Homogenates were transferred to eppendorf tubes, vortexed and then given a quick spin (14,000rpm for 30seconds). RNA was extracted by a series of steps carried out sequentially as follows; 60µls of 2M sodium acetate (Sigma Chemical Co., St. Louis, MO), followed by 600µls of H_2O-saturated

phenol (Sigma Chemical Co., St. Louis, MO) were added to each tube and mixed by inversion after which 120µls of chloroform:isoamyl alcohol (50:1) (Sigma Chemical Co., St. Louis, MO) was added and vortexed until an emulsion formed. Tubes were then incubated for 15minutes at 4°C and then spun for 20 minutes at 12,000rpm at 4°C. The aqueous phase containing RNA was extracted carefully and transferred to clean labelled tubes. RNA was precipitated by adding 600µls of isopropanol (Sigma Chemical Co., St. Louis, MO) and incubated for 1 hour at –20°C. Further purification was performed by resuspending the RNA in 50µls of solution D and β-mercaptoethanol and was followed by an ethanol precipitation step (125µls of 100% ethanol incubated for 1hour at –20°C). Sample tubes were centrifuged at 14,000rpm for 20 minutes at 4°C, the supernatant removed and 15µls of ice-cold 80% ethanol added and centrifuged again at 14,000 for 10 minutes at 4°C as a final wash. Pellets were air dried and dissolved in HPLC-grade, RNase-free water (Fisher Scientific, Pittsburgh, PA). The absorbance of the RNA concentration at 260 nm was measured by spectrophotometry. Extracted RNA was stored in aliquots at –80°C until use.

5.6.3 Ribonuclease Protection Assay (RPA)

Specific mRNA for chemokines and chemokine receptors were detected by hCK-5 hCR-5 and hCR-6 multiprobe template sets (RiboQuant, Pharmingen, San Diego, CA) containing riboprobes for specific human chemokines/chemokine receptors and rpL32. The hCK-5 detected mRNA for RANTES, IP-10, MIP-1α, MIP-1β, MCP-1 and IL-8, the hCR-5 detected CCR1, CCR2, CCR3, CCR5 and CCR8 while the hCR-6 detected CXCR1, CXCR2 and CXCR3.

5.6.4 Probe synthesis and prurification

For probe synthesis the following were added to a an eppendorf tube, 1µl RNasin Rnase inhibitor, 1µl GACU pool (10uls of 10mM GTP, ATP, CTP, UTP), 2µls DTT, 2µl 5x transcription buffer, 1µl multiprobe template set, 10µl [α-^{32}P] 1µl T7 RNA polymerase, gently mixed by a quick spin (14,000rpm for 30seconds) and then incubated at 37°C for 1 hour. The reaction was terminated by adding 2µl of DNase and mixed by a quick spin in

a micro-centrifuge and incubated for a further 30 minutes at 37°C. 26µls of 20mM EDTA, 25µls of Tris-saturated phenol, 25µl chloroform:isoamyl alcohol (50:1) and 2µl of yeast tRNA were added sequentially and vortexed into an emulsion then spun for 5 minutes at RT. The top aqueous phase was transferred into a new eppendorf tube and 50µl chloroform:isoamyl alcohol (50:1) was added and mixed by vortexing and then spinning for 2minutes at RT. The upper aqueous phase was transferred to a fresh eppendorf tube and 50µl of 4M ammonium acetate and 250µls ice-cold 100% ethanol were added. The tubes were inverted to mix and then incubated for 30 minutes at –70°C. After incubation tubes were spun in a micro-centrifuge for 20-30 minutes at 4°C. The supernatant was carefully removed and 100µls of ice-cold 90% ethanol was added to the pellet and spun for 10-15 minutes at 4°C. The supernatant was removed and the pellet allowed to air dry for 10 minutes. Fifty microlitres of hybridization buffer was added to the dried pellet to solubilize it and the suspension was vortexed and then given a quick spin. One microlitre of the probe suspension was quantified in duplicate on a scintillation counter and the yield noted.

5.6.5 RNA hybridization

Fifteen microlitres of extracted placental RNA or appropriate volumes as determined by the yield of RNA in each sample were aliquoted into eppendorf tubes along with yeast tRNA as a background control. All samples were vacuum dried including the negative control in a vacuum evaporator centrifuge with no heat applied. 8µls of hybridization buffer was added to each sample and first vortexed and then given a quick spin. The previously prepared probe was diluted in hybridization buffer and 2µl added to each RNA sample. A drop of mineral oil was added to each tube and given a quick spin then samples were placed in a pre-warmed 90°C heat block for 3 minutes after which the temperature was set to 56°C for an overnight incubation.

5.6.6 RNase treatment

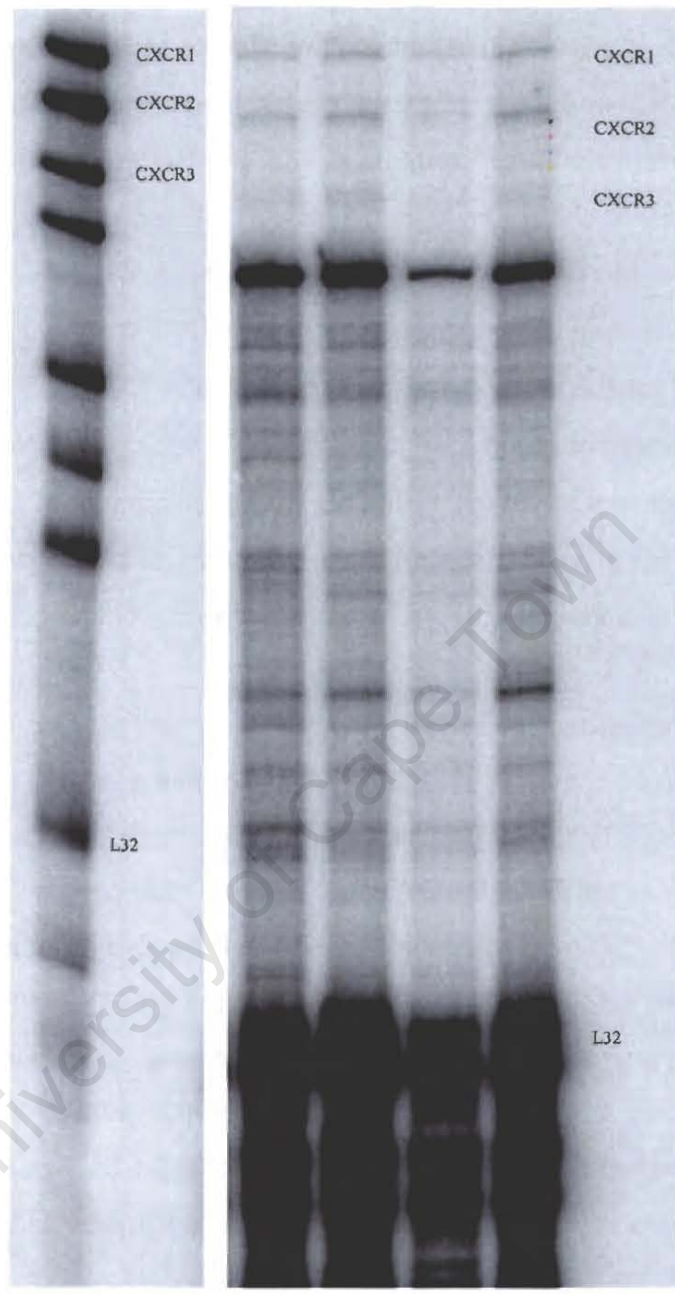
Samples were removed from the heat block 15 minutes prior to RNase treatment and left at RT to allow temperatures to equilibrate slowly. An RNase cocktail (2.5ml RNase buffer, 6µls RNase A plus T1 mix) was pipetted underneath the oil layer into the sample

and spun for 10 seconds on the micro-centrifuge. Samples were incubated for 45minutes at 30°C. 18µls of Proteinase K cocktail (390µls proteinase K buffer, 30µls Proteinase K, 30µls yeast tRNA) was aliquoted into empty labelled eppendorf tubes and the corresponding RNase digests from each sample tube were added. After a quick spin samples were incubated for 15 minutes at 37°C. Sixty-five microlitres of Tris-saturated phenol and 65µls of chloroform:isoamyl alcohol (50:1) was added to each tube and spun for 5 minutes at RT. The aqueous layer was extracted and transferred to labelled tubes. 120µls of 4M ammonium acetate and 650µls ice cold 100% ethanol were added. Tubes were mixed by inversion and incubated for 30 minutes at –70°C. On completion of incubation, tubes were spun for 30 minutes at 4°C. Supernatants were removed, 100µls of ice-cold 90% ethanol added and spun for a further 15 minutes at 4°C. Supernatants were air-dried and 5µls of the loading buffer was added to each dried pellet and vortexed for 2-3 minutes and then given a quick spin ready for loading onto a gel.

5.6.7 Polyacrylamide gel analysis

A 4.75% acrylamide gel was prepared and poured into a gel cast making sure that all bubbles were expelled. After polymerisation (1 hour) gels were inserted into a gel rig with TBE running buffer in the reservoirs. Samples and controls were heated for 3minutes at 90°C before loading and were carefully loaded into the gel. Gels were run at 55watts until the gel loading dye had reached 30cms from the bottom of the wells. Once removed the gels were layered between filter papers and dried in a gel dryer under vacuum for 1 hour at 80°C. Dried gels were exposed to film at –70°C for 3 hours and probe bands were visualized by autoradiography (XAR film, Kodak, Rochester, NY). Probe quantification was with the Series 400 PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Rectangular objects were used to generate PhosphorImager (PI) counts for each RNA volume. Each probe was normalized to account for varying numbers of incorporated [α-^{32}P] UTPs and to account for lane-to-lane and assay-to-assay variability, and are presented as a percentage of the internal housekeeping signal (rpL32) in each lane (Figure 5.1).

Figure 5.1: Autoradiogram of a protection assay utilizing hCR6 multiprobe template set with RNA from placental tissue



The figure above is an example of an autoradiograph for detection of placental tissue chemokine receptor mRNA using the hCR6 template. The hCR6 multi- template kit detects CXCR1, CXCR2 and CXCR3 among other chemokine receptors. Lane 1 shows the mRNA of the RNase unprotected probe while lanes 2,3 and 4 show mRNA of protected probes of placental tissue.

5.7 Statistical analysis

Data entry and statistical analysis was performed using the SPSS statistical software (SPSS 11.0.1, Inc., Chicago, IL.). Natural log transformations for each chemokine/chemokine receptor mRNA data, was performed before analysis. Study participant characteristic differences were calculated using the student t test while Pearson's χ^2 test was used to evaluate gravidity and delivery period group differences. Differences in chemokine/chemokine receptor mRNA expression between placenta infected and placenta uninfected women, pigment presence and absence, were tested using the non-parametric Kruskal-Wallis statistical test. The Spearman's rho was used to test for associations between chemokine/chemokine receptor mRNA expression levels and parasite density, pigment, maternal haemoglobin levels and infant birth weight. *P* values (two tailed) less than 0.05 were considered statistically significant while values less than 0.09 were considered marginally significant.

5.8 Results

5.8.1 Study subject characteristics

Results are reported from 48 women enrolled after delivery, at the Nyanza Provincial Hospital (Table 5.1). Of the 48 women, 34 had negative placental thick blood smears while 14 were positive for *P. falciparum* parasites in the placenta with a mean parasite rate of 12,556.93 parasites/μl. Of these 14 placental malaria positive mothers 11 had (78.5%) haemozoin. All 34 mothers without placental parasitemia did not have detectable levels of haemozoin. Due to the prohibitive small sample size of this study, statistical analysis stratified by gravidity was not performed, however 19 (55.9%) of the placenta malaria negative women were either a primigravida or a secundigravida as compared to 12 (85.9%) of the placenta malaria positive women while 15 (44.1%) of the uninfected women were multigravid as compared to 2 (14.3%) of the infected women. Age did not differ between the two groups. Both haemoglobin levels and birth weight were comparable between PM- and PM+ groups. There were no significant differences in the in the frequency of deliveries between the high and low malaria transmission seasons.

Table 5.1: Characteristics of study participants

	PM-	PM+	P
N	33	14	
Age (yrs)*	23.2+/-1.12	21.2+/-1.45	**0.306**
Gravidity <=2,>2 (%) #	19(55.9%), 15(44.1%)	12(85.9%), 2(14.3%)	**0.048**
Parasitemia/ul	0	12556.93 +/- 8558.43	
Pigment presence	0	78.50%	
Hb (g/dl)*	12.1+/- 0.37	11.3+/-0.48	**0.225**
Baby Weight (kg)*	3.20+/-0.10	3.29+/-0.12	**0.65**
Season born (high, low%)#	58.3%, 75.0%	41.7%, 25.0%	**0.228**

*Calculated by student t test. Mean +/- S.E
Calculated by Pearson's chi sq

5.8.2 Comparison of tissue chemokine-chemokine receptor mRNA levels between uninfected and infected women.

Tissue chemokine and chemokine receptor mRNA levels were compared between placenta uninfected and infected mothers for three CXC, five CC receptors and six chemokines, to determine gene expression levels in relation to placental infection (Table 5.2). Detection was carried out using the Ribonuclease (RNase) protection assay (RPA) technique. Of the eight receptors and the six chemokines assayed MIP-1β and IL-8 showed significantly different levels of mRNA between uninfected and infected mothers, with higher levels of expression for both chemokines by PM+ women (P=0.018 and P =0.001 respectively) (Table 5.3). mRNA expression levels were marginally higher in PM+ women than in PM- women for CXCR1, CXCR2 and RANTES (P =0.051, P =0.069 and P =0.066).

5.8.3 Comparison between women with and without pigment.

Study subjects were divided into three groups on the basis of placental pigment load as determined by a pigment score (Table 5.4). Pigment scoring was as follows; PS0 (no detectable pigment), PS1 (low pigment score) and PS2 (intermediate and high pigment score) (Bulmer *et al.*, 1993). MIP-1β and IL-8 levels were significantly different for the three groups, with highest levels mRNA expression by the PS1 group for MIP-1α and the PS2 group for IL-8 (P =0.049 and P =0.007 respectively); CCR3 levels showed marginal differences between the three groups with highest levels in the PS0 group P =0.056). All other chemokines and chemokine receptors mRNA levels were comparable between the three groups.

5.8.4 Association between chemokine- chemokine receptor mRNA levels and parasite density, haemoglobin and birth weight

Taking only immune parameters with a significant or marginal difference between placental infected and uninfected mothers or in pigment score (CXCR1, CXCR2, CCR3,

Table 5.2: Chemokine-chemokine receptors investigated for MRNA gene expression

CHEMOKINES	Target cells	Receptors bound
CC chemokines		
MCP-1	M, T, NK, DC, N, BA	CCR2, 10, 11
MIP-1α	M, T, NK, E, DC, BA	CCR1, 5
MIP-1β	M, T, NK, DC	CCR5, 8
RANTES	T, E, NK, BA, NK	CCR1, 3, 5,11
CXC chemokines		
IL-8	N, MC, E, NK	CXCR1, 2
IP-10	T, NK, EC	CXCR3

CHEMOKINE RECEPTORS	Cellular Distribution	Ligand
CC receptors		
CCR1	N, M, T, NK, B, MC	RANTES, MIP-1α
CCR2	M, T, B, BA	MCP-1
CCR3	E, BA, T	RANTES
CCR5	T, M, MC, DC	RANTES, MIP-1α, MIP-1β
CCR8	M	MIP-1β
CXC receptors		
CXCR1	N, M, T, NK, BA, MA,	IL-8
CXCR2	N, M, T, NK,	IL-8
CXCR3	T, NK	IP-10

Abbreviations: B, Bcell; BA, basophil; DC, dendritic cell; E, eosinophil; M, Monocyte/macrophage; MC, mast cell; N, neutrophil; T, T cell.

Table 5.3: Comparison of chemokine and chemokine receptors between placenta malaria infected and uninfected mothers.

	PM-	PM+	*P*
N	33	14	
CXCR1	1.37	1.46	**0.051**
CXCR2	1.49	1.63	**0.069**
CXCR3	1.5	1.57	**0.18**
CXCR4	6.42	7.19	**0.143**
CCR1	6.35	7.57	**0.376**
CCR2	3.04	3.25	**0.414**
CCR3	5.04	1.06	**0.212**
CCR4	3.59	2.97	**0.421**
CCR5	3.05	2.77	**0.642**
CCR7	2.06	2	**0.364**
CCR8	1.23	1.23	**0.819**
RANTES	3.64	4.69	**0.066**
IP-10	2.87	4.27	**0.129**
MIP-1α	5.42	8.77	**0.143**
MIP-1β	4.04	8.21	**0.018**
MCP-1	3.64	4.91	**0.734**
IL-8	1.23	1.31	**0.001**

Table values are mean mRNA levels f chemokine and chemokine receptors for the 33 aparasitemic and 14 parasitemic enrolled women. Each values obtained from each enrolled individual is a ratio of the volume of chemokine/chemokine receptor signal to that of rpL32, a housekeeping gene on the same gel. PM+, placenta malaria infected; PM-, placenta malaria uninfected.

Table 5.4: Comparison of chemokines and chemokine receptors between groups of mothers categorized by pigment scores

	PSO	PS1	PS2	*P*
N	37	9	2	
CXCR1	1.38	1.46	1.59	**0.100**
CXCR2	1.49	1.62	1.79	**0.120**
CXCR3	1.5	1.56	1.72	**0.306**
CXCR4	6.49	7.07	7.57	**0.428**
CCR1	6.63	7.86	2.85	**0.131**
CCR2	3.06	3.35	2.71	**0.857**
CCR3	5.13	3.86	1.81	**0.056**
CCR4	3.59	2.98	1.96	**0.254**
CCR5	3.07	2.78	1.95	**0.399**
CCR7	2.03	2.19	1.6	**0.199**
CCR8	1.23	1.23	1.23	**0.675**
RANTES	3.67	5.01	4.1	**0.221**
IP-10	2.89	4.77	3.83	**0.405**
MIP-1α	5.38	8.67	15.04	**0.277**
MIP-1β	4.07	10.1	5.09	**0.049**
MCP-1	3.56	4.99	7.94	**0.349**
IL-8	1.23	1.29	1.44	**0.007**

Pigment scoring was as defined by Bulmer *et al.*, 1993; Chaisavaneeyakorn *et al.*, 2003a. PS0 (no detectable pigment), PS1 (low pigment score) and PS2 (intermediate and high pigment score). *P* (two tailed) <0.05 considered statistically significant, *P*(two tailed)<0.09 considered marginally significant.

RANTES, MIP-1β and IL-8) further investigations for associations between these chemokine/chemokine receptor mRNA levels and parasite density/haemoglobin levels/birth weight, were performed. Table 5.5A shows marginal correlation of RANTES with parasitemia (rho=0.464, P =0.095) when stratified by parasite presence in the placenta (Figure 5.2). All other parameters were not associated with parasite density (Table 5.5).

Investigations were carried out to determine the association between haemoglobin concentrations and the immune parameters even though initial comparisons showed that haemoglobin levels were not different between PM+ and PM- mothers. Categorization into PM+ and PM- revealed associations for PM+ but not for PM- for some of the parameters (Table 5.5B). CXCR1 mRNA levels had a strong negative correlation with haemoglobin levels (rho= -0.596, P =0.025) (Figure 5.3) while CCR3 mRNA levels had a strong positive association with haemoglobin levels (rho=0.599, P =0.024) (Figure 5.4). CXCR2 showed a marginal negative association with maternal haemoglobin levels (rho= -0.493, P = 0.073) (Figure 5.3). Infection seemingly alters the relationship between haemoglobin and chemokine/chemokine receptor levels.

Associations between all immune parameters and birth weight were also investigated despite the absence of a difference in birth weight between PM- and PM+ women. Investigations of associations stratified by placental parasitemia revealed strong negative associations for CXCR1 and CXCR2 but not other parameters with birth weight in PM+ women (rho=0.639, P =0.014; rho=0.730, P =0.003). PM- women had no associations between CXCR1, CXCR2, CCR3, RANTES, MIP-1β and IL-8 and birth weight of the infant. These results suggest that infection alters the relationship between chemokines and chemokine receptors and birth weight (Table 5.5C; Figure 5.5).

5.9 Discussion

Chemokines are considered to be primarily beneficial in host defence mechanisms against invading pathogens. However the responses induced by chemokines may be excessive resulting in a detrimental outcome. Malaria pathology has been documented as

Table 5.5: Association between chemokine-chemokine receptor mRNA levels and **A**: parasite density, **B**: haemoglobin and **C**: birth weight

A: Parasitemia

	PM+	
	Corr. Coeff.	P
CXCR1	-0.22	0.94
CXCR2	-0.187	0.523
CCR3	0.191	0.513
RANTES	0.464	0.095
MIP-1β	0.411	0.144
IL-8	-0.247	0.394

B: Haemoglobin

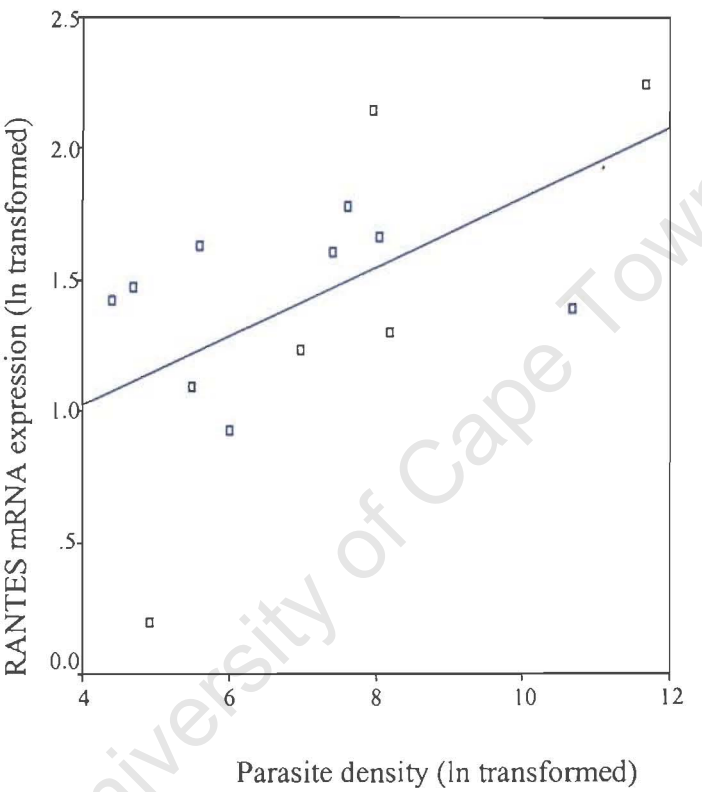
	PM-		PM+	
	Corr. Coeff.	**P**	**Corr. Coeff.**	**P**
CXCR1	0.011	0.953	-0.596	0.025
CXCR2	0.09	0.618	-0.493	0.073
CCR3	0.061	0.738	0.599	0.024
RANTES	-0.059	0.743	-0.392	0.166
MIP-1β	-0.153	0.394	-0.262	0.365
IL-8	-0.108	0.548	-0.021	0.943

C: Birth Weight

	PM-		PM+	
	Corr. Coeff.	**P**	**Corr. Coeff.**	**P**
CXCR1	0.261	0.136	-0.639	0.014
CXCR2	0.125	0.481	-0.730	0.003
CCR3	0.212	0.229	0.284	0.324
RANTES	0.003	0.986	-0.265	0.361
MIP-1β	0.155	0.381	-0.055	0.852
IL-8	0.024	0.895	-0.210	0.471

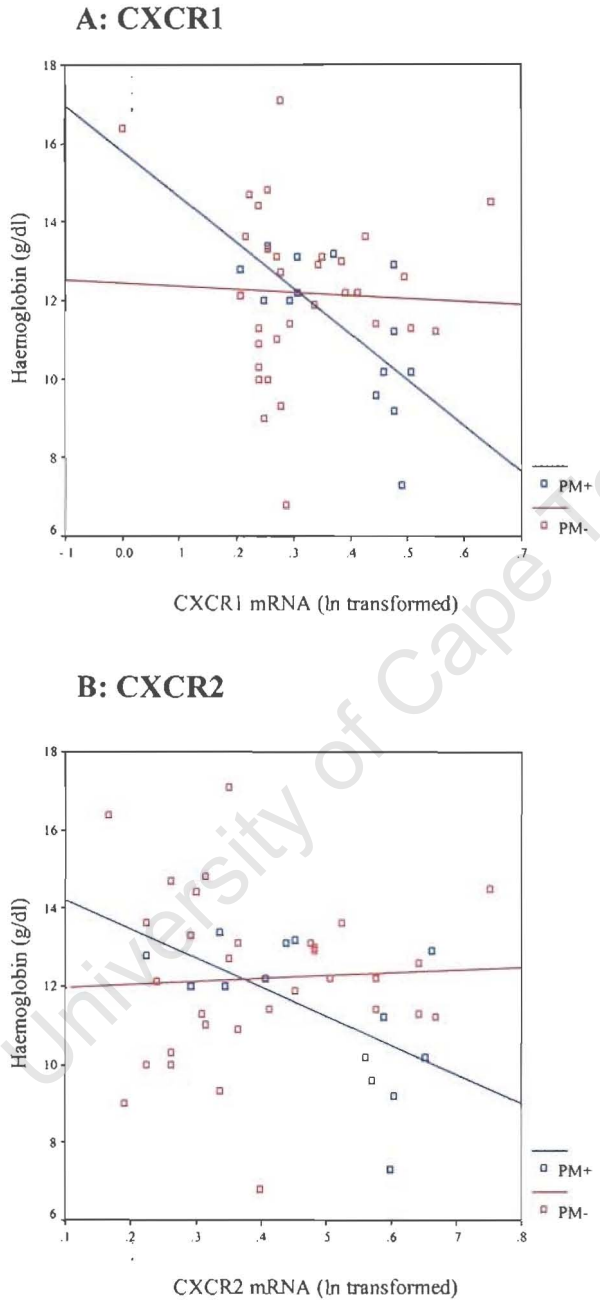
Corr. Coeff, correlation coefficient by Pearson's chi Sq. P(two tailed)<0.05 considered statistically significant.

Figure 5.2: Association between parasite density and RANTES mRNA expression in placenta-infected women



RANTES mRNA expression marginally correlated with parasite density in placenta malaria infected women (rho=0.464, p=0.095).

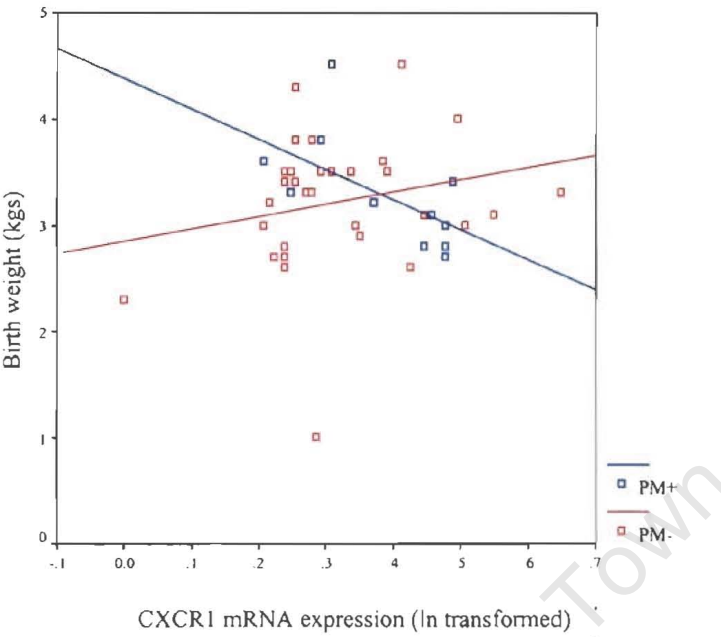
Figure 5.3: Associations between haemoglobin levels and chemokine receptor expression



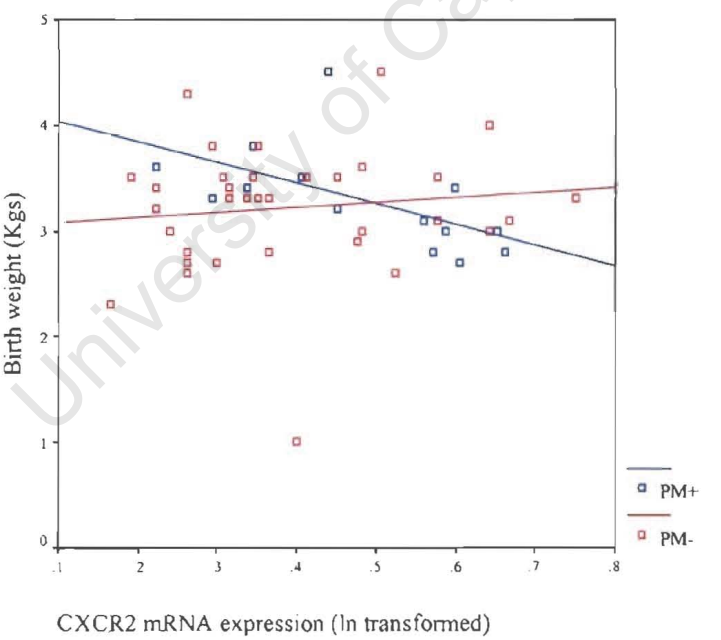
Correlation of placental tissue CXCR1 and CXCR2 mRNA expression with haemoglobin in malaria infected (n=14) and uninfected (n=34) women. CXCR1 and CXCR2 values are % L32 expression. CXCR1 was negatively correlated to haemoglobin levels; women with high CXCR1 levels tended to have lower haemoglobin levels (rho=0.596, p=0.025). CXCR2 showed the same trend as CXCR1, however the relationship was not as strong (rho=0.493, 0.073). PM- mothers did not have associations with haemoglobin.

Figure 5.4: Associations between birth weight levels and chemokine receptor expression

A: CXCR1

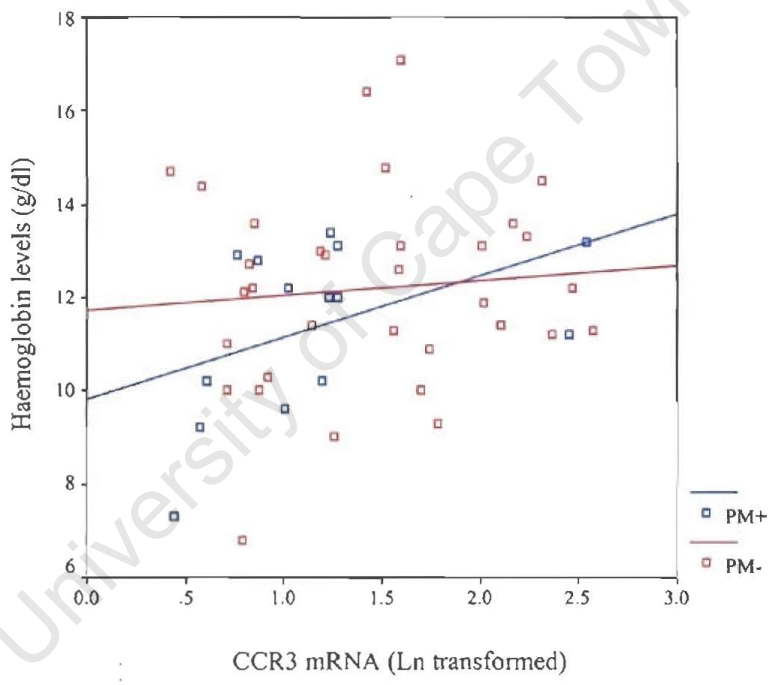


B: CXCR2



CXCR1 and CXCR2 showed a strong negative association with birth weight in PM+ mothers (rho=0.639,p=0.014; rho=0.730,p=003 respectively)

Figure 5.5: Associations between maternal haemoglobin levels and chemokine receptor expression



CCR3 mRNA levels had a strong positive association with haemoglobin levels (rho=0.599, p=0.024).

a consequence of excessive host responses. For instance, the cytokine TNF-α was first described as a mediator of protection from malaria, however exceedingly elevated TNF-α levels have been implicated in the pathogenesis of cerebral malaria and severe malarial anaemia (Clark and Chaudhri, 1988; Shaffer *et al.*, 1991). Chemokines in malaria and pregnancy have likewise been implicated in the pathogenesis of the adverse effects of infection. This study investigated the expression of chemokines and chemokine receptors in relation to malaria in pregnancy and the pathogenesis of maternal anaemia and LBW. Since immune responses associated with pregnancy outcome are likely to be localized in the placenta in which chemokine responses are predominantly elicited to achieve a local as opposed to a systemic effect, placental tissue chemokine and chemokine receptor expression were measured to investigate the local immune response contribution to maternal haemoglobin levels and birth weight of the infant. A total of 14 chemokines and chemokine receptors were measured for placental tissue mRNA expression using the ribonuclease protection assay (RPA) that permits the simultaneous measurements of multiple chemokine and chemokine receptors. Chemokines and chemokine receptors were randomly selected to include a broad variety of both CXC and CC chemokines and their corresponding receptors, essentially to investigate the overall mRNA expression picture in relation to LBW and maternal anaemia. Investigations assessing chemokines with corresponding receptors in relation to the pathogenesis of maternal malaria are currently absent.

Results obtained from this study confirm data from other studies on the expression of placental RANTES, IP-10, MIP-1α, MIP-1β, MCP-1 and IL-8 (Douglas *et al.*, 2001; Simon *et al.*, 1998) and provide evidence for the expression of CXCR1, CXCR2, CXCR3, CCR1, CCR2, CCR3, CCR5 and CCR8 receptors by placental tissue. These data are in agreement with previous investigations that showed placental tissue as being capable of expressing chemokine receptors (Douglas *et al.*, 2001).

Chemokine and chemokine receptor expression was compared in placentas from malaria infected and uninfected women. Taking into consideration chemokine expression as determined on the basis of mRNA levels, MIP-1β and IL-8 were significantly elevated in

infected placentas as compared to the uninfected suggesting upregulation in response to parasite presence. It should be noted that although the statistical difference reported here for IL-8 mRNA expression by parasitemic and aparasitemic mothers is significant, the actual difference is small (1.23 vs. 1.31 respectively) and therefore may not be biologically significant. RANTES levels were marginally different between the two groups. MIP-1α and IL-8 upregulation due to infection have been previously reported (Abrams *et al.*, 2003; Chaisavaneeyakorn *et al.*, 2003a; Moormann *et al.*, 1999) , however this report is the first for placental tissue RANTES elevation in relation to malaria infection. RANTES levels unlike MIP-1β and IL-8 correlated with parasite density but not with maternal haemoglobin levels and infant birth weight, indicating a parasite dose dependent relationship. RANTES is a β-chemokine with chemoattractant properties for T, NK cells basophils and eosinophils (Matsukawa *et al.*, 2000). It is not entirely known for what purpose RANTES levels were elevated, however it may be postulated that expression could be in response to the inflammatory environment and subsequent recruitment of immune cells or for regulatory purposes of the immune response.

An assessment of chemokine receptor expression levels revealed significantly higher CXCR1 levels in PM+ women as compared to PM- women. Marginally higher levels of CXCR2 levels in PM+ women were also reported. No other chemokine receptor levels showed differences in mRNA expression between PM+ and PM- women. So far only one study on placental chemokine receptor expression levels in relation to malaria is documented. This study, conducted in Malawi reported increased expression of CCR5 in malaria infected compared to uninfected placentas but not associated with maternal haemoglobin levels or infant birth weight (Tkachuk *et al.*, 2001). They also report malaria related elevated levels of CCR3. Results reported from the Malawi study differ from those reported here for CCR5. HIV infection in study participants for the Malawi study was not ruled out and may explain the difference in results reported. HIV may modulate chemokine receptor expression particularly since CCR5 is a co-receptor for HIV infectivity (Shearer *et al.*, 1997).

5.9.1 Chemokine-chemokine receptors and pigment

Pigment is a final degradation product of haemoglobin by the malarial parasite (Walter *et al.*, 1982). Pigment contribution to the pathogenesis of malaria remains unclear and contradictory results have been documented with reference to pigment role in anaemia and LBW. A recent study in Malawi reports the presence of malaria pigment containing placental monocytes as a predictive factor for anaemia and LBW (Rogerson *et al.*, 2003b) while an earlier Thai study reports the absence of an association with anaemia and LBW with pigment (McGready *et al.*, 2002). Nevertheless, immune cell responses to pigment have been documented and may have downstream effects on pregnancy outcome. TNF-α and IL-8 were expressed only in pigment-laden placental macrophages (Moormann *et al.*, 1999) and IFN-γ levels were highest in placentas containing pigment (Fievet *et al.*, 2001). Chemokine expression in response to pigment has also been investigated and MIP-1α and MIP-1β levels were related to malaria pigment load (Chaisavaneeyakorn *et al.*, 2003a; Sherry *et al.*, 1995). This current study reports significantly elevated levels of MIP-1β and IL-8 in relation to pigment load, in agreement with the above previous studies. The association of the chemokines with pigment may be a factor of parasite density and subsequent upregulated chemokine expression more than it is a direct result of pigment induced immune activation. This is in agreement with studies by Chaisavaneeyakorn *et al.*, 2003 that also suggest elevated chemokine levels due to parasite density and not pigment presence.

Of the eight chemokine receptors measured, only CCR3 levels were elevated in relation to pigment load with highest levels expressed by women with no detectable levels of pigment suggesting a role for CCR3 in pigment clearance. CCR3 is distributed on eosinophils, basophils and T cells. It would be interesting to investigate the relationship between these cells and pigment laden cell elimination. Evidence for pigment participation in the pathology of malaria is modest. Pigment influence on the pathogenesis of anaemia and LBW clearly warrants further investigations.

5.9.2 Chemokine-chemokine receptors and haemoglobin levels

Maternal anaemia is the most common consequence of malaria during pregnancy for women residents of holoendemic regions. Anaemia places the mother at risk for mortality and her foetus for poor pregnancy outcome (Brabin *et al.*, 1990; Meuris *et al.*, 1993). Chemokine and chemokine receptor expression levels were investigated in relation to maternal haemoglobin levels to determine their role in anaemia pathogenesis. Haemoglobin levels were not statistically different when comparing parasitemic and aparasitemic women although parasitemic women reported lower mean haemoglobin levels than aparasitemic women. Despite this fact a couple of associations were evident. A strong negative correlation was observed between haemoglobin and mRNA levels for CXCR1 and CXCR2 in the parasitemic women. An increase in chemokine receptor mRNA expression seemingly results in lowered haemoglobin levels thus suggesting a possible role in the pathogenesis of anaemia. Other chemokine and chemokine receptors measured in this study were not associated with haemoglobin levels.

CXCR1 and CXCR2 are receptors for IL-8 and are predominantly expressed by neutrophils (Godaly *et al.*, 2000). IL-8 mRNA levels were significantly higher in parasitemic as compared to aparasitemic women, however IL-8 mRNA levels were not correlated with maternal haemoglobin levels and from results reported here it is therefore unlikely that IL-8 is directly involved in the progression of malaria-associated anaemia in pregnant women. Alternatively IL-8 mRNA expression levels which were considerably lower than other chemokines assayed in this study may have been undetectable by the measurement system used here thus correlations were not revealed, also the possibility that the reported statistical IL-8 difference between aparasitemic and parasitemic women as not being biologically viable could further explain the lack of haemoglobin correlation with IL-8. That notwithstanding, overall IL-8 levels were highly correlated with parasitemia, an observation in agreement with reports by other groups (Burgmann *et al.*, 1995; Friedland *et al.*, 1993), this presents the possibility that CXCR1 and CXCR2 receptor expression is increased by infection and may be critical in the migration of neutrophils to the placenta. Anaemia related to malaria in pregnancy is usually haemolytic (Brabin, 1991; Gilles *et al.*, 1969). Neutrophil related responses have been

implicated in the development of haemolytic anaemia (Graca-Souza *et al.*, 2002) and the results here point to possible neutrophil recruitment as a contributing factor to anaemia in pregnancy via proinflammatory response development. Again this presents an interesting situation that requires further investigation.

CCR3 levels were positively associated with maternal haemoglobin levels in PM+ women and may also be involved in the immunological events leading to anaemia particularly those that relate to pigment.

5.9.3 Chemokine-chemokine receptors and birth weight

Birth weights were similar for all infants of mothers enrolled in this study, no differences were observed between infants born to mother with placental infection and mothers without placental infection. However an association analysis of birth weight with chemokines revealed a negative correlation with CXCR1 and CXCR2 in parasitemic women. Higher levels of CXCR1 and CXCR2 result in lower birth weights of the infant. Although CXCR1 and CXCR2 levels showed associations with birth weight, no relationship was observed between IL-8 (CXCR1 And CXCR2 ligand) and infant birth weight in this study possibly due to insufficiently sensitive methods of mRNA quantitation and the prohibitive small sample size as mentioned above. Neutrophil recruitment to the placenta is however implied and may be important in the aetiology of LBW probably through the amplification of pro-inflammatory events (Graca-Souza *et al.*, 2002) that result in reduced birth weight. Elevated placental tissue mRNA and plasma IL-8 levels in placenta parasitemic women have been reported elsewhere as having strong positive correlation with birth weight (Abrams *et al.*, 2003; Moormann *et al.*, 1999).

This study investigated chemokine and chemokine receptor expression in relation to malaria infections of the placenta and in the context of maternal haemoglobin levels and infant birth weight. Among all women enrolled for this study a marked absence of malaria pathology defined as maternal anaemia and LBW was observed. Ayisi *et al.*, (2003) document a 6.7% LBW rate among 2466 singleton deliveries in a hospital-based study of western Kenya attributed to both HIV and malaria infections. The small sample

size of the present study is therefore a major limitation; a larger number of enrolled women would be necessary to detect maternal anaemia and LBW. In addition, the Kenyan Ministry of Health has in place an intervention program for malaria in pregnancy that distributes prophylaxis to pregnant women attending antenatal clinics. The probability of a persistent placental malaria infection that results in pathology is therefore low. However trends in immune responses reported in this study may offer leads to future investigations.

In conclusion, this study presents descriptive data of chemokine and chemokine receptor expression patterns in relation to placenta malaria and is the first to do so for chemokines matched with their corresponding receptors. Results obtained from this study provide new evidence for 1. RANTES association with parasite density 2. CXCR1 and CXCR2 expression negatively correlated with maternal haemoglobin levels and infant birth weight and 3. CCR3 association with haemoglobin levels and pigment presence. The nature of mechanisms involved in LBW and maternal anaemia progression are complex, however this study is important as a first step in the elucidation of the role of chemokines and chemokine receptors in the pathological process.

CHAPTER SIX

General discussion of overall results and suggestions for future investigations

Pregnant women living in high malaria endemic areas are at an increased risk for malaria infection compared to non-pregnant women, and the prevalence and density of parasitemia is higher in pregnant women than their non-pregnant counterparts (Brabin, 1983). The placenta is especially susceptible to infection and may result in placental malaria, characterized by the accumulation of parasites within intervillous spaces, with severe adverse effects on both mother and child. Interestingly, women residents of malaria holoendemic regions are most susceptible to infection in their first and second pregnancies. Protection is seemingly gravidity dependent. Several immunological hypotheses have been proposed to explain the enhanced susceptibility to malaria that is evident in first and second pregnancies and the gravidity dependent immunity exhibited by multigravid women (Brabin, 1985; Smith, 1996; Weinberg, 1984). However, the acquisition of protective immunity is not fully understood and the role of the immune system in the pathogenesis of placental malaria remains unclear.

Previous studies conducted in western Kenya reported an association between placental IFN-γ levels and protection. Multigravid women had higher IFN-γ levels than primigravid women and multigravid malaria infected women (Moore *et al.*, 1999). These findings form the basis of the work carried out for this thesis. The previous study did not address the source of the IFN-γ response. Also not addressed was whether or not protective immune responses were the same in peripheral blood and in the placenta. Investigations related to this thesis were carried out to elucidate further the IFN-γ protective immune mechanisms and also to evaluate the immune responses in relation to the pathogenic process of malaria during pregnancy.

A comprehensive literature review of work on maternal placental immune responses revealed numerous methods of placental blood collection. Placenta related maternal immune response investigations necessitate the collection of maternal intervillous blood

without contamination of foetal blood and with an accurate mononuclear cell profile. Therefore as a first step in these investigations five documented methods of placental blood collection were compared to establish the most appropriate method of collection for immunology studies. Perfusion, incision, biopsy, placental tissue grinding and placental prick methods were assessed by flow cytometry for foetal red blood cell contamination and monocyte, T, B and NK cell levels. Results are reported from ten HIV and malaria negative placentas from primigravid and secundigravid women. Foetal cell contamination was significantly lower for the prick and perfusion methods (5.5%, 12% respectively) than for incision (71%), biopsy (51%) and tissue grinding (24%). There was significant variation by method in the number of monocytes, total T cells, CD4 and CD8 T cells for all five methods, however collection by prick yielded results most similar to corresponding peripheral blood for the four cell subpopulations. No significant differences were observed for B cell and NK cell numbers by method. The prick method of collection was therefore established as the most appropriate method for intervillous blood collection particularly for immunological studies and was the method used for placental IVB sample collection in this thesis. It would be interesting to carry out additional comparisons particularly for immune cell profiles using malaria infected placentas between the five different methods to determine the extent of cell profile alterations and subsequent functional differences, in response to infection and the influence of infection on the choice of method for immunological studies. Also important would be a comparison of parasite densities across the five methods of collection to determine the best method particularly for placental parasite studies. Further investigations would be necessary to address these issues.

IFN-γ exhibits innate and acquired response activity. If expressed by NK cells and γδ T cells IFN-γ is considered innate in origin while if expressed by activated CD4 and CD8 T cells it is considered an acquired immune response. The previous study in western Kenyan women documented placental IFN-γ production as a protective mechanism against placental malaria. The source of the IFN-γ was not investigated, neither were peripheral protective responses simultaneously assessed to determine peripheral immune system contribution to protection in the placenta. Using flow cytometry, cell

subpopulation numbers for CD4, CD8, CD45RO memory and CD56+/CD3- Natural Killer (NK) cells, as well as IFN-γ production by these cells, in maternal peripheral and placental intervillous blood were investigated in association with malaria parasitemia. Fifty-two HIV negative pregnant women, 20 with placental malaria and 32 without placental infections, were enrolled for the study. Results obtained reported two important findings. First, the IFN-γ protective response in the placenta is mediated by NK cells and is therefore innate in origin. Secondly, protective immune responses in the peripheral blood were different from those in the placenta. Memory cells and not IFN-γ dependent mechanisms of protection were reported in peripheral blood. In addition, the peripheral memory cell response was postulated as contributing to the placental immune response against infection and possibly even the gravidity dependent IFN-γ mechanisms of protection previously reported. In the context of the results reported here it would be intriguing to further elucidate the influence of the peripheral system on the innate placental immune response and the precise nature of gravidity dependent immunity in malaria during pregnancy. Investigations of antigen specific IFN-γ responses would also be interesting.

NK cells are potent mediators of the innate immune response and are increasingly recognized as important effectors of early host resistance to infection (Artavanis-Tsakonas and Riley, 2002). IFN-γ producing NK cells in the current study have been shown to be important in protection against infection of the placenta, however the regulatory basis of this innate response is not known. NK cell participation in parasite elimination necessitates the migration, accumulation and subsequent cell activation at the site of infection (Matsukawa *et al.*, 2000; Robertson, 2002). The presumption therefore was that NK cell interaction with chemokine-chemokine receptors responsible for leukocyte trafficking and immune activation would be important in the development of the IFN-γ producing NK cell protection. NK cells were assessed in relation to three chemokines reported with activity for NK cells, IP-10, RANTES and MIP-1α, together with the parallel chemokine receptors, CXCR3 and CCR5 (Robertson, 2002). Results showed that a low peripheral to placental IP-10 but not RANTES or MIP-1α ratio is associated with protection against placental malaria in relation to elevated IFN-γ

producing NK cell levels, suggesting that the IP-10 balance between peripheral and placental blood is important in protection. Results further suggested that IP-10 regulation of NK cells was mediated via CXCR3 and possibly augmented by CD4 memory T cells for maximum recruitment of NK cells. Chemokines are potent activators of leucocytes (Robertson, 2002) and the strong association of IP-10 peripheral to placenta ratios with IFN-γ producing NK cells suggests a cell activation role for IP-10. Activated NK cells produce cytokines but may also initiate a cascade of events that result in cytolysis of pathogen-infected cells. On this basis, NK cells have been suggested as belonging to one of two subsets (Cooper *et al.*, 2001) and it is therefore conceivable that both NK cell groups are recruited in response to IP-10 expression. Investigations are required to assess NK cell cytolytic activity in placental infections of malaria to determine possible associations with IP-10 production particularly in relation to chemokine expression levels. IP-10 production may achieve NK cell recruitment to the placenta however a threshold of IP-10 expression may also be required to effect NK cell activation. One might therefore postulate that in placenta malaria susceptible women, IP-10 levels were insufficient to elicit protection despite sufficient NK cell recruitment to the placenta. Alternatively early IP-10 responses to infection may determine the elimination of infection and a delayed IP-10 response situation may result in infection. It would be interesting to investigate further the timing of IP-10 production in relation to gravidity dependent immune mechanisms of protection.

Numerous studies have documented excessive immune response mediators as being detrimental to the host and resulting in pathology. Malaria during pregnancy in holoendemic regions may result in maternal anaemia and infant low birth weight, consequences of infection that characterize the burden of maternal malaria in holoendemic regions. Chemokines have been implicated in the pathogenesis of the adverse outcomes of placental infection. MIP-1α, MCP-1, I 309 and IL-8 have been previously reported to be associated with placental monocyte recruitment and subsequent low birth weight of the infant (Abrams *et al.*, 2003; Rogerson *et al.*, 2003b). However chemokine receptors have not been simultaneously investigated with parallel ligands. Thus six chemokines, MIP-1α, MIP-1β, RANTES, MCP-1, IP-10 and IL-8 together with

corresponding chemokine receptors (CXCR1, CXCR2, CXCR3, CCR1, CCR2, CCR3, CCR5 and CCR8) were assessed in placental tissue in relation to maternal haemoglobin levels and infant low birth weight. This investigation is one of few documented studies assessing chemokines in relation to placental malaria pathogenesis and only the second reporting results for chemokine receptor expression in relation to placental infection. Results obtained from this study provide new evidence for RANTES association with parasite density. Results also report CXCR1 and CXCR2 expression inversely correlated with maternal haemoglobin levels and infant birth weight suggesting neutrophil participation in pathogenesis for which further investigations are required. CCR3 association with haemoglobin levels and pigment presence was also reported and may contribute to pregnancy complications. These results are important as a first step in the elucidation of the role of matched chemokines and chemokine receptors in the pathological process of a placental malaria infection. More in depth investigations to further determine mechanisms of the pathologic process are warranted.

REFERENCES

Abbas, A.K., Murphy, K.M. and Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature* **383**(6603):787-793.

Abrams, E.T., Brown, H., Chensue, S.W., Turner, G.D., Tadesse, E., Lema, V.M., Molyneux, M.E., Rochford, R., Meshnick, S.R. and Rogerson, S.J. (2003) Host response to malaria during pregnancy: placental monocyte recruitment is associated with elevated beta chemokine expression. *J Immunol* **170**(5):2759-2764.

Achur, R.N., Valiyaveettil, M., Alkhalil, A., Ockenhouse, C.F. and Gowda, D.C. (2000) Characterization of proteoglycans of human placenta and identification of unique chondroitin sulfate proteoglycans of the intervillous spaces that mediate the adherence of Plasmodium falciparum-infected erythrocytes to the placenta. *J Biol Chem* **275**(51):40344-40356.

Aliberti, J.C., Machado, F.S., Souto, J.T., Campanelli, A.P., Teixeira, M.M., Gazzinelli, R.T. and Silva, J.S. (1999) beta-Chemokines enhance parasite uptake and promote nitric oxide-dependent microbiostatic activity in murine inflammatory macrophages infected with Trypanosoma cruzi. *Infect Immun* **67**(9):4819-4826.

Alkhalil, A., Achur, R.N., Valiyaveettil, M., Ockenhouse, C.F. and Gowda, D.C. (2000) Structural requirements for the adherence of Plasmodium falciparum-infected erythrocytes to chondroitin sulfate proteoglycans of human placenta. *J Biol Chem* **275**(51):40357-40364.

Andrews, K.T. and Lanzer, M. (2002) Maternal malaria: Plasmodium falciparum sequestration in the placenta. *Parasitol Res* **88**(8):715-723.

Artavanis-Tsakonas, K. and Riley, E.M. (2002) Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live Plasmodium falciparum-infected erythrocytes. *J Immunol* **169**(6):2956-2963.

Aucan, C., Traore, Y., Fumoux, F. and Rihet, P. (2001) Familial correlation of immunoglobulin G subclass responses to Plasmodium falciparum antigens in Burkina Faso. *Infect Immun* **69**(2):996-1001.

Ayisi, J.G., van Eijk, A.M., ter Kuile, F.O., Kolczak, M.S., Otieno, J.A., Misore, A.O., Kager, P.A., Steketee, R.W. and Nahlen, B.L. (2003) The effect of dual infection with HIV and malaria on pregnancy outcome in western Kenya. *Aids* **17**(4):585-594.

Bacon, K., Baggiolini, M., Broxmeyer, H., Horuk, R., Lindley, I., Mantovani, A., Maysushima, K., Murphy, P., Nomiyama, H., Oppenheim, J., Rot, A., Schall, T., Tsang, M., Thorpe, R., Van Damme, J., Wadhwa, M., Yoshie, O., Zlotnik, A. and Zoon, K. (2002) Chemokine/chemokine receptor nomenclature. *J Interferon Cytokine Res* **22**(10):1067-1068.

Baird, J.K. (1998) Age-dependent characteristics of protection v. susceptibility to Plasmodium falciparum. *Ann Trop Med Parasitol* **92**(4):367-390.

Balkwill, F. (1998) The molecular and cellular biology of the chemokines. *J Viral Hepat* **5**(1):1-14.

Beck, R. and Lam-Po-Tang, P.R. (1994) Comparison of cord blood and adult blood lymphocyte normal ranges: a possible explanation for decreased severity of graft versus host disease after cord blood transplantation. *Immunol Cell Biol* **72**(5):440-444.

Beeson, J.G., Brown, G.V., Molyneux, M.E., Mhango, C., Dzinjalamala, F. and Rogerson, S.J. (1999) Plasmodium falciparum isolates from infected pregnant women

and children are associated with distinct adhesive and antigenic properties. *J Infect Dis* **180**(2):464-472.

Beeson, J.G., Rogerson, S.J., Cooke, B.M., Reeder, J.C., Chai, W., Lawson, A.M., Molyneux, M.E. and Brown, G.V. (2000) Adhesion of Plasmodium falciparum-infected erythrocytes to hyaluronic acid in placental malaria. *Nat Med* **6**(1):86-90.

Beier, J.C., Perkins, P.V., Onyango, F.K., Gargan, T.P., Oster, C.N., Whitmire, R.E., Koech, D.K. and Roberts, C.R. (1990) Characterization of malaria transmission by Anopheles (Diptera: Culicidae) in western Kenya in preparation for malaria vaccine trials. *J Med Entomol* **27**(4):570-577.

Berendt, A., R, Ferguson, D.J. and Newbold, C. (1990) sequestration of Plasmodium falciparum: sticky cells and sticky problems. *Parasitology Today* **6**:247-254.

Bloland, P., Slutsker, L., Steketee, R.W., Wirima, J.J., Heymann, D.L. and Breman, J.G. (1996) Rates and risk factors for mortality during the first two years of life in rural Malawi. *Am J Trop Med Hyg* **55**(1 Suppl):82-86.

Brabin, B. (1991) The risks and severity of malaria in pregnant women. Applied field research report No. 1. Geneva: UNDP/World Bank/WHO-TDR, pp. 1-52.

Brabin, B., Maxwell, S., Chimsuku, L., Verhoeff, F., van der Kaay, H.J., Broadhead, R., Kazembe, P. and Thomas, A. (1993) A study of the consequences of malarial infection in pregnant women and their infants. *Parassitologia* **35 Suppl**:9-11.

Brabin, B.J. (1983) An analysis of malaria in pregnancy in Africa. *Bull World Health Organ* **61**(6):1005-1016.

Brabin, B.J. (1985) Epidemiology of infection in pregnancy. *Rev Infect Dis* **7**(5):579-603.

Brabin, B.J., Ginny, M., Sapau, J., Galme, K. and Paino, J. (1990) Consequences of maternal anaemia on outcome of pregnancy in a malaria endemic area in Papua New Guinea. *Ann Trop Med Parasitol* **84**(1):11-24.

Brabin, B.J., Fletcher, K.A. and Brown, N. (2003) Do disturbances within the folate pathway contribute to low birth weight in malaria? *Trends Parasitol* **19**(1):39-43.

Breman, J.G. (2001) The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am J Trop Med Hyg* **64**(1-2 Suppl):1-11.

Buffet, P.A., Gamain, B., Scheidig, C., Baruch, D., Smith, J.D., Hernandez-Rivas, R., Pouvelle, B., Oishi, S., Fujii, N., Fusai, T., Parzy, D., Miller, L.H., Gysin, J. and Scherf, A. (1999) Plasmodium falciparum domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. *Proc Natl Acad Sci U S A* **96**(22):12743-12748.

Bull, P.C., Lowe, B.S., Kortok, M., Molyneux, C.S., Newbold, C.I. and Marsh, K. (1998) Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nat Med* **4**(3):358-360.

Bulmer, J.N., Rasheed, F.N., Francis, N., Morrison, L. and Greenwood, B.M. (1993) Placental malaria. I. Pathological classification. *Histopathology* **22**(3):211-218.

Burgmann, H., Hollenstein, U., Wenisch, C., Thalhammer, F., Looareesuwan, S. and Graninger, W. (1995) Serum concentrations of MIP-1 alpha and interleukin-8 in patients suffering from acute Plasmodium falciparum malaria. *Clin Immunol Immunopathol* **76**(1 Pt 1):32-36.

Chaisavaneeyakorn, S., Moore, J.M., Otieno, J., Chaiyaroj, S.C., Perkins, D.J., Shi, Y.P., Nahlen, B.L., Lal, A.A. and Udhayakumar, V. (2002) Immunity to placental malaria. III. Impairment of interleukin(IL)-12, not IL-18, and interferon-inducible protein-10

responses in the placental intervillous blood of human immunodeficiency virus/malaria-coinfected women. *J Infect Dis* **185**(1):127-131.

Chaisavaneeyakorn, S., Moore, J.M., Mirel, L., Othoro, C., Otieno, J., Chaiyaroj, S.C., Shi, Y.P., Nahlen, B.L., Lal, A.A. and Udhayakumar, V. (2003a) Levels of Macrophage Inflammatory Protein 1alpha (MIP-1alpha) and MIP-1beta in Intervillous Blood Plasma Samples from Women with Placental Malaria and Human Immunodeficiency Virus Infection. *Clin Diagn Lab Immunol* **10**(4):631-636.

Chaisavaneeyakorn, S., Othoro, C., Shi, Y.P., Otieno, J., Chaiyaroj, S.C., Lal, A.A. and Udhayakumar, V. (2003b) Relationship between plasma Interleukin-12 (IL-12) and IL-18 levels and severe malarial anemia in an area of holoendemicity in western Kenya. *Clin Diagn Lab Immunol* **10**(3):362-366.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**(1):156-159.

Clark, I.A. and Chaudhri, G. (1988) Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoiesis and erythrophagocytosis. *Br J Haematol* **70**(1):99-103.

Colonna, M., Samaridis, J., Cella, M., Angman, L., Allen, R.L., O'Callaghan, C.A., Dunbar, R., Ogg, G.S., Cerundolo, V. and Rolink, A. (1998) Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J Immunol* **160**(7):3096-3100.

Cooper, M.A., Fehniger, T.A. and Caligiuri, M.A. (2001) The biology of human natural killer-cell subsets. *Trends Immunol* **22**(11):633-640.

Cotterell, S.E., Engwerda, C.R. and Kaye, P.M. (1999) Leishmania donovani infection initiates T cell-independent chemokine responses, which are subsequently amplified in a T cell-dependent manner. *Eur J Immunol* **29**(1):203-214.

De Souza, J.B., Williamson, K.H., Otani, T. and Playfair, J.H. (1997) Early gamma interferon responses in lethal and nonlethal murine blood-stage malaria. *Infect Immun* **65**(5):1593-1598.

Deloron, P., Chougnet, C., Lepers, J.P., Tallet, S. and Coulanges, P. (1991) Protective value of elevated levels of gamma interferon in serum against exoerythrocytic stages of Plasmodium falciparum. *J Clin Microbiol* **29**(9):1757-1760.

Dodoo, D., Omer, F.M., Todd, J., Akanmori, B.D., Koram, K.A. and Riley, E.M. (2002) Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to Plasmodium falciparum malaria. *J Infect Dis* **185**(7):971-979.

Dorman, E.K., Shulman, C.E., Kingdom, J., Bulmer, J.N., Mwendwa, J., Peshu, N. and Marsh, K. (2002) Impaired uteroplacental blood flow in pregnancies complicated by falciparum malaria. *Ultrasound Obstet Gynecol* **19**(2):165-170.

Douglas, G.C., Thirkill, T.L., Sideris, V., Rabieh, M., Trollinger, D. and Nuccitelli, R. (2001) Chemokine receptor expression by human syncytiotrophoblast. *J Reprod Immunol* **49**(2):97-114.

Duffy, P.E. (2002) Immunity to Malaria During Pregnancy: Different Host, Different Parasite. In *Malaria in Pregnancy. Deadly Parasite, Susceptible host*. Duffy, P.E. and Fried, M. (eds). NY: Taylor and Francis, pp. 71-126.

Duffy, P.E. and Desowitz, R.S. (2002) Pregnancy malaria throughtout history: Dangerous labours. In *Malaria in pregnancy: Deadly parasite, Susceptible host.* Duffy, P.E. and Fried, M. (eds). New York: Taylor and Francis, pp. 1-25.

Duffy, P.E. (2003) Maternal immunization and malaria in pregnancy. *Vaccine* **21**(24):3358-3361.

Erkeller-Yuksel, F.M., Deneys, V., Yuksel, B., Hannet, I., Hulstaert, F., Hamilton, C., Mackinnon, H., Stokes, L.T., Munhyeshuli, V., Vanlangendonck, F. and et al. (1992) Age-related changes in human blood lymphocyte subpopulations. *J Pediatr* **120**(2 Pt 1):216-222.

Favre, N., Ryffel, B., Bordmann, G. and Rudin, W. (1997) The course of Plasmodium chabaudi chabaudi infections in interferon-gamma receptor deficient mice. *Parasite Immunol* **19**(8):375-383.

Fell, A.H. and Smith, N.C. (1998) Immunity to asexual blood stages of Plasmodium: is resistance to acute malaria adaptive or innate ? *Parasitol Today* **14**:364-369.

Fievet, N., Cot, M., Chougnet, C., Maubert, B., Bickii, J., Dubois, B., Le Hesran, J.Y., Frobert, Y., Migot, F., Romain, F. and et al. (1995) Malaria and pregnancy in Cameroonian primigravidae: humoral and cellular immune responses to Plasmodium falciparum blood-stage antigens. *Am J Trop Med Hyg* **53**(6):612-617.

Fievet, N., Moussa, M., Tami, G., Maubert, B., Cot, M., Deloron, P. and Chaouat, G. (2001) Plasmodium falciparum induces a Th1/Th2 disequilibrium, favoring the Th1-type pathway, in the human placenta. *J Infect Dis* **183**(10):1530-1534.

Fievet, N., Tami, G., Maubert, B., Moussa, M., Shaw, I.K., Cot, M., Holder, A.A., Chaouat, G. and Deloron, P. (2002) Cellular immune response to Plasmodium falciparum after pregnancy is related to previous placental infection and parity. *Malar J* **1**(1):16.

Flick, K., Scholander, C., Chen, Q., Fernandez, V., Pouvelle, B., Gysin, J. and Wahlgren, M. (2001) Role of nonimmune IgG bound to PfEMP1 in placental malaria. *Science* **293**(5537):2098-2100.

Fried, M. and Duffy, P.E. (1996) Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. *Science* **272**(5267):1502-1504.

Fried, M., Muga, R.O., Misore, A.O. and Duffy, P.E. (1998a) Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes. *J Immunol* **160**(5):2523-2530.

Fried, M., Nosten, F., Brockman, A., Brabin, B.J. and Duffy, P.E. (1998b) Maternal antibodies block malaria. *Nature* **395**(6705):851-852.

Fried, M. and Duffy, P.E. (2002) Analysis of CSA-binding parasites and antiadhesion antibodies. *Methods Mol Med* **72**:555-560.

Friedland, J.S., Ho, M., Remick, D.G., Bunnag, D., White, N.J. and Griffin, G.E. (1993) Interleukin-8 and Plasmodium falciparum malaria in Thailand. *Trans R Soc Trop Med Hyg* **87**(1):54-55.

Galbraith, R.M., Fox, H., Hsi, B., Galbraith, G.M., Bray, R.S. and Faulk, W.P. (1980) The human materno-foetal relationship in malaria. II. Histological, ultrastructural and immunopathological studies of the placenta. *Trans R Soc Trop Med Hyg* **74**(1):61-72.

Gamain, B., Smith, J.D., Miller, L.H. and Baruch, D.I. (2001) Modifications in the CD36 binding domain of the Plasmodium falciparum variant antigen are responsible for the inability of chondroitin sulfate A adherent parasites to bind CD36. *Blood* **97**(10):3268-3274.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Craig, A., Kyes, S., Chan, M.S., Nene, V., Shallom, S.J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M.W., Vaidya, A.B., Martin, D.M., Fairlamb, A.H., Fraunholz, M.J., Roos, D.S., Ralph, S.A., McFadden, G.I., Cummings, L.M., Subramanian, G.M., Mungall, C., Venter, J.C., Carucci, D.J., Hoffman, S.L., Newbold, C., Davis, R.W., Fraser, C.M. and Barrell, B. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* **419**(6906):498-511.

Gilles, H.M., Lawson, J.B., Sibelas, M., Voller, A. and Allan, N. (1969) Malaria, anaemia and pregnancy. *Ann Trop Med Parasitol* **63**(2):245-263.

Githeko, A.K., Service, M.W., Mbogo, C.M., Atieli, F.K. and Juma, F.O. (1993) Plasmodium falciparum sporozoite and entomological inoculation rates at the Ahero rice irrigation scheme and the Miwani sugar-belt in western Kenya. *Ann Trop Med Parasitol* **87**(4):379-391.

Godaly, G., Hang, L., Frendeus, B. and Svanborg, C. (2000) Transepithelial neutrophil migration is CXCR1 dependent in vitro and is defective in IL-8 receptor knockout mice. *J Immunol* **165**(9):5287-5294.

Gopinath, R., Hanna, L.E., Kumaraswami, V., Perumal, V., Kavitha, V., Vijayasekaran, V. and Nutman, T.B. (2000) Perturbations in eosinophil homeostasis following treatment of lymphatic filariasis. *Infect Immun* **68**(1):93-99.

Graca-Souza, A.V., Arruda, M.A., de Freitas, M.S., Barja-Fidalgo, C. and Oliveira, P.L. (2002) Neutrophil activation by heme: implications for inflammatory processes. *Blood* **99**(11):4160-4165.

Greenwood, B. and Snow, R.W. (1991) Why do some African children develop severe malaria? *Parasitol Today* **7**:277-281.

Guilbert, L., Abbasi, M. and Mosmann, T.R. (2002) The immunology of pregnancy: Maternal defenses against infectious diseases. In *Malaria in Pregnancy. Deadly Parasite, Susceptible host*. Duffy, P.E. and Fried, M. (eds). New York: Taylor and Francis, pp. 53-70.

Gyan, B., Troye-Blomberg, M., Perlmann, P. and Bjorkman, A. (1994) Human monocytes cultured with and without interferon-gamma inhibit Plasmodium falciparum parasite growth in vitro via secretion of reactive nitrogen intermediates. *Parasite Immunol* **16**(7):371-375.

Hartel, C., Bein, G., Muller-Steinhardt, M. and Kluter, H. (2001) Ex vivo induction of cytokine mRNA expression in human blood samples. *J Immunol Methods* **249**(1-2):63-71.

Hulstaert, F., Hannet, I., Deneys, V., Munhyeshuli, V., Reichert, T., De Bruyere, M. and Strauss, K. (1994) Age-related changes in human blood lymphocyte subpopulations. II. Varying kinetics of percentage and absolute count measurements. *Clin Immunol Immunopathol* **70**(2):152-158.

Hunt, J.S. (1992) Immunobiology of pregnancy. *Curr Opin Immunol* **4**(5):591-596.

Hviid , L. (1998) Clinical disease, immunity and protection against Plasmodium falciparum malaria in populations living in endemic areas. *Exp. Rev. Mol. Med*:http://www-ermm.cbcu.cam.ac.uk/lhc/txt001lhc.htm.

Juretic, E., Uzarevic, B., Petrovecki, M. and Juretic, A. (2000) Two-color flow cytometric analysis of preterm and term newborn lymphocytes. *Immunobiology* **202**(5):421-428.

Kachur, P. and Bloland, P. (1998) Diseases Transmitted Primarily by Arthropod Vectors: Malaria. In *Maxcy-Rosenau-Last Public Health and Preventive Medicine*. Wallace, R.B. (ed). Iowa City: Appleton & Lange, pp. 313-326.

Katevas, P., Lembesopoulos, C., Kamitaki, C., Sakellariou, D. and Douna, V. (1999) Establishment of normal ranges of lymphocyte subpopulations in cord blood and in peripheral blood in infants and in children. *HAEMA* **2**(3):145-151.

Kaufmann, P. (1981) Entwicklung der Plazenta. In *Die plazenta des Menschen*. Becker, V., Schiebler, T.H. and Kublis, F. (eds). Stuttgart: Thieme Verlag.

Li, C., Seixas, E. and Langhorne, J. (2001) Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite. *Med Microbiol Immunol (Berl)* **189**(3):115-126.

Luppi, P., Haluszczak, C., Betters, D., Richard, C.A., Trucco, M. and DeLoia, J.A. (2002) Monocytes are progressively activated in the circulation of pregnant women. *J Leukoc Biol* **72**(5):874-884.

Luty, A.J., Lell, B., Schmidt-Ott, R., Lehman, L.G., Luckner, D., Greve, B., Matousek, P., Herbich, K., Schmid, D., Migot-Nabias, F., Deloron, P., Nussenzweig, R.S. and Kremsner, P.G. (1999) Interferon-gamma responses are associated with resistance to

reinfection with Plasmodium falciparum in young African children. *J Infect Dis* **179**(4):980-988.

Maghazachi, A.A., Skalhegg, B.S., Rolstad, B. and Al-Aoukaty, A. (1997) Interferon-inducible protein-10 and lymphotactin induce the chemotaxis and mobilization of intracellular calcium in natural killer cells through pertussis toxin-sensitive and -insensitive heterotrimeric G-proteins. *Faseb J* **11**(10):765-774.

Marsh, K. (1992) Malaria--a neglected disease? *Parasitology* **104 Suppl**:S53-69.

Matsukawa, A., Hogaboam, C.M., Lukacs, N.W. and Kunkel, S.L. (2000) Chemokines and innate immunity. *Rev Immunogenet* **2**(3):339-358.

Matteelli, A., Caligaris, S., Castelli, F. and Carosi, G. (1997) The placenta and malaria. *Ann Trop Med Parasitol* **91**(7):803-810.

Maubert, B., Fievet, N., Tami, G., Cot, M., Boudin, C. and Deloron, P. (1999) Development of antibodies against chondroitin sulfate A-adherent Plasmodium falciparum in pregnant women. *Infect Immun* **67**(10):5367-5371.

McCormick, M.C. (1985) The contribution of low birth weight to infant mortality and childhood morbidity. *N Engl J Med* **312**(2):82-90.

McGready, R., Brockman, A., Cho, T., Levesque, M.A., Tkachuk, A.N., Meshnick, S.R. and Nosten, F. (2002) Haemozoin as a marker of placental parasitization. *Trans R Soc Trop Med Hyg* **96**(6):644-646.

McGregor, I.A., Wilson, M.E. and Billewicz, W.Z. (1983) Malaria infection of the placenta in The Gambia, West Africa; its incidence and relationship to stillbirth, birthweight and placental weight. *Trans R Soc Trop Med Hyg* **77**(2):232-244.

McGregor, I.A. (1984) Epidemiology, malaria and pregnancy. *Am J Trop Med Hyg* **33**(4):517-525.

McGregor, I.A. (1987) Thoughts on malaria in pregnancy with consideration of some factors which influence remedial strategies. *Parassitologia* **29**(2-3):153-163.

Menendez, C. (1995) Malaria during pregnancy: a priority area of malaria research and control. *Parasitol Today* **11**:178-183.

Meuris, S., Piko, B.B., Eerens, P., Vanbellinghen, A.M., Dramaix, M. and Hennart, P. (1993) Gestational malaria: assessment of its consequences on fetal growth. *Am J Trop Med Hyg* **48**(5):603-609.

Milosevits, J., Pocsik, E., Schmidt, B., Remenyi, P., Intodi, Z.S., Reti, M., Batai, A., Illes, P., Mihalik, R., Petranyi, G.G. and et al. (1995) Immunophenotypic and functional characteristics of haemopoietic cells from human cord blood. *Scand J Immunol* **42**(4):493-500.

Mohan, K., Moulin, P. and Stevenson, M.M. (1997) Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage Plasmodium chabaudi AS infection. *J Immunol* **159**(10):4990-4998.

Moore, J.M., Nahlen, B., Ofulla, A.V., Caba, J., Ayisi, J., Oloo, A., Misore, A., Nahmias, A.J., Lal, A.A. and Udhayakumar, V. (1997) A simple perfusion technique for isolation of maternal intervillous blood mononuclear cells from human placentae. *J Immunol Methods* **209**(1):93-104.

Moore, J.M., Nahlen, B.L., Misore, A., Lal, A.A. and Udhayakumar, V. (1999) Immunity to placental malaria. I. Elevated production of interferon-gamma by placental blood

mononuclear cells is associated with protection in an area with high transmission of malaria. *J Infect Dis* **179**(5):1218-1225.

Moore, J.M., Ayisi, J., Nahlen, B.L., Misore, A., Lal, A.A. and Udhayakumar, V. (2000a) Immunity to placental malaria. II. Placental antigen-specific cytokine responses are impaired in human immunodeficiency virus-infected women. *J Infect Dis* **182**(3):960-964.

Moore, J.M., Nahlen, B.L., Lal, A.A. and Udhayakumar, V. (2000b) Immunologic memory in the placenta: a lymphocyte recirculation hypothesis. *Med Hypotheses* **54**(3):505-510.

Moore, J.M., Shi, Y.P., Othoro, C., Nahlen, B.L., Lal, A.A. and Udhayakumar, V. (2003) Comparative flow cytometric analysis of term placental intervillous and peripheral blood from immediate postpartum women in Western kenya. *Placenta* **24**(7):779-785.

Moormann, A.M., Sullivan, A.D., Rochford, R.A., Chensue, S.W., Bock, P.J., Nyirenda, T. and Meshnick, S.R. (1999) Malaria and pregnancy: placental cytokine expression and its relationship to intrauterine growth retardation. *J Infect Dis* **180**(6):1987-1993.

Moser, B. and Loetscher, P. (2001) Lymphocyte traffic control by chemokines. *Nat Immunol* **2**(2):123-128.

Murdoch, C. and Finn, A. (2000) Chemokine receptors and their role in inflammation and infectious diseases. *Blood* **95**(10):3032-3043.

Murphy, S.C. and Breman, J.G. (2001) Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. *Am J Trop Med Hyg* **64**(1-2 Suppl):57-67.

Nosten, F., ter Kuile, F., Maelankirri, L., Decludt, B. and White, N.J. (1991) Malaria during pregnancy in an area of unstable endemicity. *Trans R Soc Trop Med Hyg* **85**(4):424-429.

Ockenhouse, C.F., Schulman, S. and Shear, H.L. (1984) Induction of crisis forms in the human malaria parasite Plasmodium falciparum by gamma-interferon-activated, monocyte-derived macrophages. *J Immunol* **133**(3):1601-1608.

Ockenhouse, C.F., Ho, M., Tandon, N.N., Van Seventer, G.A., Shaw, S., White, N.J., Jamieson, G.A., Chulay, J.D. and Webster, H.K. (1991) Molecular basis of sequestration in severe and uncomplicated Plasmodium falciparum malaria: differential adhesion of infected erythrocytes to CD36 and ICAM-1. *J Infect Dis* **164**(1):163-169.

Ojo-Amaize, E.A., Salimonu, L.S., Williams, A.I., Akinwolere, O.A., Shabo, R., Alm, G.V. and Wigzell, H. (1981) Positive correlation between degree of parasitemia, interferon titers, and natural killer cell activity in Plasmodium falciparum-infected children. *J Immunol* **127**(6):2296-2300.

Ojo-Amaize, E.A., Vilcek, J., Cochrane, A.H. and Nussenzweig, R.S. (1984) Plasmodium berghei sporozoites are mitogenic for murine T cells, induce interferon, and activate natural killer cells. *J Immunol* **133**(2):1005-1009.

Okoko, B.J., Enwere, G. and Ota, M.O. (2003) The epidemiology and consequences of maternal malaria: a review of immunological basis. *Acta Trop* **87**(2):193-205.

Oliveira, S.H., Fonseca, S.G., Romao, P.R., Figueiredo, F., Ferreira, S.H. and Cunha, F.Q. (1998) Microbicidal activity of eosinophils is associated with activation of the arginine-NO pathway. *Parasite Immunol* **20**(9):405-412.

Orago, A.S. and Facer, C.A. (1991) Cytotoxicity of human natural killer (NK) cell subsets for Plasmodium falciparum erythrocytic schizonts: stimulation by cytokines and inhibition by neomycin. *Clin Exp Immunol* **86**(1):22-29.

Ordi, J., Ismail, M.R., Ventura, P.J., Kahigwa, E., Hirt, R., Cardesa, A., Alonso, P.L. and Menendez, C. (1998) Massive chronic intervillositis of the placenta associated with malaria infection. *Am J Surg Pathol* **22**(8):1006-1011.

Ordi, J., Menendez, C., Ismail, M.R., Ventura, P.J., Palacin, A., Kahigwa, E., Ferrer, B., Cardesa, A. and Alonso, P.L. (2001) Placental malaria is associated with cell-mediated inflammatory responses with selective absence of natural killer cells. *J Infect Dis* **183**(7):1100-1107.

Papadogiannakis, N., Johnsen, S.A. and Olding, L.B. (1985) Human fetal/neonatal suppressor activity: relation between OKT phenotypes and sensitivity to prostaglandin E2 in maternal and neonatal lymphocytes. *Am J Reprod Immunol Microbiol* **9**(4):105-110.

Pasquetto, V., Guidotti, L.G., Kakimi, K., Tsuji, M. and Chisari, F.V. (2000) Host-virus interactions during malaria infection in hepatitis B virus transgenic mice. *J Exp Med* **192**(4):529-536.

Patel, L., Charlton, S.J., Chambers, J.K. and Macphee, C.H. (2001) Expression and functional analysis of chemokine receptors in human peripheral blood leukocyte populations. *Cytokine* **14**(1):27-36.

Pearson, R.D. (2001) Prolactin and NK cells in maternal malaria. *J Infect Dis* **184**(5):662.

Perlmann, P. and Troye-Blomberg, M. (2002) Malaria and the Immune System in Humans. *Chem Immunol* **80**:229-242.

Phillips, R.S. (2001) Current status of malaria and potential for control. *Clin Microbiol Rev* **14**(1):208-226.

Plebanski, M. and Hill, A.V. (2000) The immunology of malaria infection. *Curr Opin Immunol* **12**(4):437-441.

Rabian-Herzog, C., Lesage, S., Gluckman, E. and Charron, D. (1993) Characterization of lymphocyte subpopulations in cord blood. *J Hematother* **2**(2):255-257.

Raghupathy, R. (1997) Th1-type immunity is incompatible with successful pregnancy. *Immunol Today* **18**(10):478-482.

Rasheed, F.N., Bulmer, J.N., Morrison, L., Jawla, M.F., Hassan-King, M., Riley, E.M. and Greenwood, B.M. (1992) Isolation of maternal mononuclear cells from placentas for use in in vitro functional assays. *J Immunol Methods* **146**(2):185-193.

Rasheed, F.N., Bulmer, J.N., Dunn, D.T., Menendez, C., Jawla, M.F., Jepson, A., Jakobsen, P.H. and Greenwood, B.M. (1993) Suppressed peripheral and placental blood lymphoproliferative responses in first pregnancies: relevance to malaria. *Am J Trop Med Hyg* **48**(2):154-160.

Reeder, J.C., Hodder, A.N., Beeson, J.G. and Brown, G.V. (2000) Identification of glycosaminoglycan binding domains in Plasmodium falciparum erythrocyte membrane protein 1 of a chondroitin sulfate A-adherent parasite. *Infect Immun* **68**(7):3923-3926.

Ricke, C.H., Staalsoe, T., Koram, K., Akanmori, B.D., Riley, E.M., Theander, T.G. and Hviid, L. (2000) Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on Plasmodium falciparum-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. *J Immunol* **165**(6):3309-3316.

Riley, E.M., Schneider, G., Sambou, I. and Greenwood, B.M. (1989) Suppression of cell-mediated immune responses to malaria antigens in pregnant Gambian women. *Am J Trop Med Hyg* **40**(2):141-144.

Ritson, A. and Bulmer, J.N. (1987) Extraction of leucocytes from human decidua. A comparison of dispersal techniques. *J Immunol Methods* **104**(1-2):231-236.

Roberts, C., Satoskar, A. and Alexander, J. (1996) Sex steroids, pregnancy associated hormones and immunity to parasitic infections. *Parasitol Today* **12**:382-388.

Robertson, M.J. (2002) Role of chemokines in the biology of natural killer cells. *J Leukoc Biol* **71**(2):173-183.

Rogerson, S.J., Chaiyaroj, S.C., Ng, K., Reeder, J.C. and Brown, G.V. (1995) Chondroitin sulfate A is a cell surface receptor for Plasmodium falciparum-infected erythrocytes. *J Exp Med* **182**(1):15-20.

Rogerson, S.J., Novakovic, S., Cooke, B.M. and Brown, G.V. (1997) Plasmodium falciparum-infected erythrocytes adhere to the proteoglycan thrombomodulin in static and flow-based systems. *Exp Parasitol* **86**(1):8-18.

Rogerson, S.J. and Beeson, J.G. (1999) The placenta in malaria: mechanisms of infection, disease and foetal morbidity. *Ann Trop Med Parasitol* **93 Suppl 1**:S35-42.

Rogerson, S.J., Tembenu, R., Dobano, C., Plitt, S., Taylor, T.E. and Molyneux, M.E. (1999) Cytoadherence characteristics of Plasmodium falciparum-infected erythrocytes from Malawian children with severe and uncomplicated malaria. *Am J Trop Med Hyg* **61**(3):467-472.

Rogerson, S.J., Brown, H.C., Pollina, E., Abrams, E.T., Tadesse, E., Lema, V.M. and Molyneux, M.E. (2003a) Placental tumor necrosis factor alpha but not gamma interferon is associated with placental malaria and low birth weight in Malawian women. *Infect Immun* **71**(1):267-270.

Rogerson, S.J., Pollina, E., Getachew, A., Tadesse, E., Lema, V.M. and Molyneux, M.E. (2003b) Placental monocyte infiltrates in response to Plasmodium falciparum malaria infection and their association with adverse pregnancy outcomes. *Am J Trop Med Hyg* **68**(1):115-119.

Romero, R., Gomez, R., Bauman, P., Mazor, M. and Cotton, D. (1994) The role of infection and cytokines in preterm parturition. In *Basic Mechanisms Controlling Term and Preterm Birth*. Chwalisz, K. and Garfiels, R. (eds). Berlin: Springer-Verlag, pp. 197-240.

Sacks, G., Sargent, I. and Redman, C. (1999) An innate view of human pregnancy. *Immunol Today* **20**(3):114-118.

Sacks, G.P., Studena, K., Sargent, K. and Redman, C.W. (1998) Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *Am J Obstet Gynecol* **179**(1):80-86.

Salerno, A. and Dieli, F. (1998) Role of gamma delta T lymphocytes in immune response in humans and mice. *Crit Rev Immunol* **18**(4):327-357.

Sallusto, F., Lanzavecchia, A. and Mackay, C.R. (1998) Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunol Today* **19**(12):568-574.

Sallusto, F., Lenig, D., Forster, R., Lipp, M. and Lanzavecchia, A. (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**(6754):708-712.

Shaffer, N., Grau, G.E., Hedberg, K., Davachi, F., Lyamba, B., Hightower, A.W., Breman, J.G. and Phuc, N.D. (1991) Tumor necrosis factor and severe malaria. *J Infect Dis* **163**(1):96-101.

Shear, H.L., Srinivasan, R., Nolan, T. and Ng, C. (1989) Role of IFN-gamma in lethal and nonlethal malaria in susceptible and resistant murine hosts. *J Immunol* **143**(6):2038-2044.

Shearer, W.T., Reuben, J., Lee, B.N., Popek, E.J., Lewis, D.E., Hammill, H.H., Hanson, I.C., Kline, M.W. and Langston, C. (1997) Role of placental cytokines and inflammation in vertical transmission of HIV infection. *Acta Paediatr Suppl* **421**:33-38.

Sherry, B.A., Alava, G., Tracey, K.J., Martiney, J., Cerami, A. and Slater, A.F. (1995) Malaria-specific metabolite hemozoin mediates the release of several potent endogenous pyrogens (TNF, MIP-1 alpha, and MIP-1 beta) in vitro, and altered thermoregulation in vivo. *J Inflamm* **45**(2):85-96.

Shibuya, T., Izuchi, K., Kuroiwa, A., Okabe, N. and Shirakawa, K. (1987) Study on nonspecific immunity in pregnant women: increased chemiluminescence response of peripheral blood phagocytes. *Am J Reprod Immunol Microbiol* **15**(1):19-23.

Shohat, B., Hirsch, M., Jardena, O., Henry, J., Levy, I. and Trainin, N. (1986) Cellular immune aspects of the human fetal-maternal relationship. *Am J Reprod Immunol Microbiol* **11**(4):125-129.

Shulman, C.E. (1999) Malaria in pregnancy: its relevance to safe-motherhood programmes. *Ann Trop Med Parasitol* **93 Suppl 1**:S59-66.

Simon, C., Caballero-Campo, P., Garcia-Velasco, J.A. and Pellicer, A. (1998) Potential implications of chemokines in reproductive function: an attractive idea. *J Reprod Immunol* **38**(2):169-193.

Smith, J.D., Chitnis, C.E., Craig, A.G., Roberts, D.J., Hudson-Taylor, D.E., Peterson, D.S., Pinches, R., Newbold, C.I. and Miller, L.H. (1995) Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* **82**(1):101-110.

Smith, N.C. (1996) An immunological hypothesis to explain enhanced susceptibility to malaria during pregnancy. *Parasitol. Today* **12**:4-6.

Smith, T.G., Ayi, K., Serghides, L., McAllister, C.D. and Kain, K.C. (2002) Innate immunity to malaria caused by Plasmodium falciparum. *Clin Invest Med* **25**(6):262-272.

Staalsoe, T., Megnekou, R., Fievet, N., Ricke, C.H., Zornig, H.D., Leke, R., Taylor, D.W., Deloron, P. and Hviid, L. (2001) Acquisition and decay of antibodies to pregnancy-associated variant antigens on the surface of Plasmodium falciparum-infected erythrocytes that protect against placental parasitemia. *J Infect Dis* **184**(5):618-626.

Steketee, R.W., Breman, J.G., Paluku, K.M., Moore, M., Roy, J. and Ma-Disu, M. (1988) Malaria infection in pregnant women in Zaire: the effects and the potential for intervention. *Ann Trop Med Parasitol* **82**(2):113-120.

Steketee, R.W., Wirima, J.J., Slutsker, L., Heymann, D.L. and Breman, J.G. (1996) The problem of malaria and malaria control in pregnancy in sub-Saharan Africa. *Am J Trop Med Hyg* **55**(1 Suppl):2-7.

Steketee, R.W., Nahlen, B.L., Parise, M.E. and Menendez, C. (2001) The burden of malaria in pregnancy in malaria-endemic areas. *Am J Trop Med Hyg* **64**(1-2 Suppl):28-35.

Taub, D.D., Sayers, T.J., Carter, C.R. and Ortaldo, J.R. (1995) Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis. *J Immunol* **155**(8):3877-3888.

Tkachuk, A.N., Moormann, A.M., Poore, J.A., Rochford, R.A., Chensue, S.W., Mwapasa, V. and Meshnick, S.R. (2001) Malaria enhances expression of CC chemokine receptor 5 on placental macrophages. *J Infect Dis* **183**(6):967-972.

Troye-Blomberg, M., Worku, S., Tangteerawatana, P., Jamshaid, R., Soderstrom, K., Elghazali, G., Moretta, L., Hammarstrom, M. and Mincheva-Nilsson, L. (1999) Human gamma delta T cells that inhibit the in vitro growth of the asexual blood stages of the Plasmodium falciparum parasite express cytolytic and proinflammatory molecules. *Scand J Immunol* **50**(6):642-650.

Villalta, F., Zhang, Y., Bibb, K.E., Kappes, J.C. and Lima, M.F. (1998) The cysteine-cysteine family of chemokines RANTES, MIP-1alpha, and MIP-1beta induce trypanocidal activity in human macrophages via nitric oxide. *Infect Immun* **66**(10):4690-4695.

von Andrian, U.H. and Mackay, C.R. (2000) T-cell function and migration. Two sides of the same coin. *N Engl J Med* **343**(14):1020-1034.

Waki, S., Uehara, S., Kanbe, K., Ono, K., Suzuki, M. and Nariuchi, H. (1992) The role of T cells in pathogenesis and protective immunity to murine malaria. *Immunology* **75**(4):646-651.

Walker, D., Jason, J., Wallace, K., Slaughter, J., Whatley, V., Han, A., Nwanyanwu, O.C., Kazembe, P.N., Dobbie, H., Archibald, L. and Jarvis, W.R. (2002) Spontaneous cytokine production and its effect on induced production. *Clin Diagn Lab Immunol* **9**(5):1049-1056.

Walter, P.R., Garin, Y. and Blot, P. (1982) Placental pathologic changes in malaria. A histologic and ultrastructural study. *Am J Pathol* **109**(3):330-342.

Watkinson, M., Rushton, D.I. and Lunn, P.G. (1985) Placental malaria and foetoplacental function: low plasma oestradiols associated with malarial pigmentation of the placenta. *Trans R Soc Trop Med Hyg* **79**(4):448-450.

Wegmann, T.G., Lin, H., Guilbert, L. and Mosmann, T.R. (1993) Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* **14**(7):353-356.

Weinberg, E.D. (1984) Pregnancy-associated depression of cell-mediated immunity. *Rev Infect Dis* **6**(6):814-831.

WHO (1998) Fact Sheet No. 94. Geneva: World Health Organization.

Wipasa, J., Elliott, S., Xu, H. and Good, M.F. (2002) Immunity to asexual blood stage malaria and vaccine approaches. *Immunol Cell Biol* **80**(5):401-414.

Zenclussen, A.C., Fest, S., Busse, P., Joachim, R., Klapp, B.F. and Arck, P.C. (2002) Questioning the Th1/Th2 paradigm in reproduction: peripheral levels of IL-12 are down-regulated in miscarriage patients. *Am J Reprod Immunol* **48**(4):245-251.

Zucker, J.R., Lackritz, E.M., Ruebush, T.K., Hightower, A.W., Adungosi, J.E., Were, J.B. and Campbell, C.C. (1994) Anaemia, blood transfusion practices, HIV and mortality

among women of reproductive age in western Kenya. *Trans R Soc Trop Med Hyg* **88**(2):173-176.

APPENDIX A:

Solutions and reagents

A1 Fetal haemoglobin Staining

0.1% wash buffer
100ml DH2O
0.1g BSA, dissolve.

Triton-X 100 diluent
0.1g NaN_3
100ml, 0.1% wash buffer

0.1% TritonX 100 working solution
1µL TritonX 100
100ml TritonX 100 diluent

A2 Cell surface marker and intracellular cytokine/chemokine staining

Phosphate buffered saline (PBS)
0.23g Na_2HPO_4 (anhydrous)
1.15g Na_2HPO_4 (anhydrous)
9.00g NaCl
dH_2O to 1 litre

1:10 Lysis Buffer
9 parts DH_2O
1 part BD FACS lysing solution

1% wash buffer
500ml PBS
5g BSA
0.5g NaN_3

Freezing medium
9 parts of 1% BSA in PBS
1 part Dimethyl Sulfoxide (DMSO)

0.1% staining buffer
100ml PBS
0.1g saponin

1:10 Perm Wash buffer
9 parts DH_2O
1 part BD Perm Wash buffer

1% Paraformaldehyde
97.5 ml dH2O
2.5ml 40% formaldehyde

A3 Ribonuclease Protection assay

TBE buffer (X10)
108g Trizma base
55g boric acid
40ml EDTA
dH_2O to 1 litre

4.75% Gel
44.7g
9.3mls TBE (X10)
38.1ml dH_2O, mix
11.6ml 40% acrylamide solution
563 µls ammonium persulphate
75 µls TEMED

APPENDIX B:

Abbreviations

PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PlacBMC	Placental blood mononuclear cell
APC	Allophycocyanin
BSA	Bovine Serum Albumin
CDC	Centres for Disease Control and Prevention
CSA	Chondroitin Sulphate A
CSP	Circumsporozoite protein
EDTA	Ethylene diamine tetraacetic acid
FITC	Fluorescein isothiocyanate
FSC	Forward scatter light
HbF	Foetal haemoglobin
HIV	Human immunodeficiency virus
I309	Inducible 309
ICAM-1	Intracellular adhesion molecule –1
IFN-γ	Interferon-gamma
IL-10	Interleukin-10
IL-1β	Interleukin 1beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-8	Interleukin-8
IP-10	Interferon–γ inducible protein-10
I-TAC	Interferon inducible T-cell α chemoattractant
IUGR	Intrauterine Growth Retardation
IVB	Intervillous blood (IVB)
IVBMC	Intervillous blood mononuclear cells
KEMRI	Kenya Medical Research Institute
LBW	Low birth weight
MCP-1	Monocyte chemotactic protein-

MCP-3	Monocyte chemotactic protein-3
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon -γ
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
NK cell	Natural killer cell
NO	Nitric oxide
NPGH	Nyanza Provincial General Hospital
PBS	Phosphate buffered saline
PerCP	Peridin chlorophyll protein
PfEMP1	*P.falciparum* erythrocyte membrane protein 1
PI	Phosphoimager units
PM-	Placenta malaria negative
PM+	Placenta malaria positive
PS	Pigment score
RANTES	Regulated on activation normal T-cell expressed and secreted
RBC	Red blood cells
RNA	Ribonucleic acids
ROS	reactive oxygen species
RPA	RNase protection assays
rpm	Revolutions per minute
RT	Room temperature
SDF-1	Stromal cell-derived factor 1
SSC	Side scatter light
TNF-α	Tumour necrosis factor alpha
UVC	Umbilical vessel catheters
VSA	Variant surface antigens)
WBC	White blood cells
WHO	World Health Organization

APPENDIX C

Abstracts from thesis data submitted for conference presentations

A comparative analysis of various methods of maternal placental blood collection for immunology studies in placental malaria.

Submitted and accepted for the 52nd Annual Meeting of the American Society Of Tropical Medicine Hygiene And Medicine, Philadelphia, USA. 3-7th December 2003

The collection of maternal intervillous blood without contamination of fetal blood and with an accurate mononuclear cell profile is essential for immunologic studies on placental malaria. We used flow cytometry to compare five documented methods of collection: perfusion, incision, biopsy, placental tissue grinding and placental puncture or prick. Ten placentas were obtained from HIV and *P. falciparum* negative primigravid and secundigravid women residing in Kisumu city of western Kenya. All five were performed on the same placenta and for each method we assessed fetal cell contamination and mononuclear cell profiles. We found that fetal cell contamination was significantly lower for the prick and perfusion methods (5%, 12% respectively) than for incision (71%), biopsy (51%) and tissue grinding (24%). There was significant variation by method in the number of monocytes, total T cells, CD4+ and CD8+ T cells. The prick method of collection yielded cell profiles and numbers most similar to corresponding peripheral blood. No significant differences were observed for B cell and NK cell numbers by method. In addition to low levels of fetal blood contamination, other advantages of the prick method of collection include simple to perform, minimal manipulation, no sophisticated equipment needed, easily adaptable for field studies, high sample sterility and intact plasma. However the limitation of this method is low volume of the sample. On the other hand, perfusion, despite low levels of fetal blood cell contamination and high volume yield and sterility, involves considerable placental manipulation, it requires special equipment, and results in diluted plasma. In conclusion, prick and perfusion are both appropriate methods of maternal intervillous blood collection for placenta malaria studies depending on the study objectives and the field setting.

IFN-γ producing NK cells and CD45RO+ memory cells are associated with protection against malaria during pregnancy

Submitted and accepted for the 52nd Annual Meeting of the American Society Of Tropical Medicine Hygiene And Medicine, Philadelphia, USA. 3-7th December 2003

Previous immunologic studies conducted in western Kenya have shown that IFN-γ production in the placenta is associated with protection against placental malaria in multigravidae. It remains unknown which IFN-γ producing cell subpopulations and T memory cells are involved in this protection, and whether the immune components for protection are the same in the peripheral system and the placenta. Using flow cytometry we investigated cell subpopulation numbers for CD4, CD8, CD45RO memory and CD56+/CD3- Natural Killer (NK) cells, as well as IFN-γ production by these cells, in maternal peripheral and placental intervillous blood in association with malaria parasitemia. Of 51 HIV negative pregnant women resident in Kisumu, western Kenya, 20 had both peripheral and placental parasitemia (N=16≤ gravida 2, N=4> gravida 2) and 31 had no parasitemia (N=17 ≤ gravida 2, N=14> gravida 2). Levels of CD45RO+ memory cell were significantly higher in peripheral than in placental blood in aparasitemic women, but not in the parasitemic group. A further analysis of only peripheral immune responses between parasitemic and aparasitemic women showed that levels of CD45RO+ cells were higher in the aparasitemic than the parasitemic group, with aparasitemic multigravid women having highest levels of CD45RO+ cells. At the placental level, IFN-γ production by CD56+/CD3-cell was significantly higher in aparasitemic women compared to the parasitemic group, regardless of gravidity. These results suggest that different immune components in the peripheral system and placenta may be involved in the control of malaria infections during pregnancy: peripheral CD45RO+ memory cells are associated with protection against peripheral malaria infection, while IFN-γ production by non-specific CD56+/CD3- NK cells appears to play a role in protection from placental malaria.