BINDING OF OPSONIZED IMMUNE COMPLEXES TO ERYTHROCYTE CR1/CD35 INHIBITS TNF-α PRODUCTION BY RESTRICTING IMMUNE COMPLEX UPTAKE BY MACROPHAGES

BY

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ABSTRACT

Previous studies have shown that children suffering from severe malaria have elevated concentrations of TNF-α, increased levels of circulating immune complexes (ICs) and decreased levels of complement regulatory proteins (mainly CR1/ CD35 and DAF/ CD55) on their erythrocytes. The cross-linking of FcR on the macrophages has been shown to cause activation and subsequently induce release of pro-inflammatory cytokines which could lead to malarial anemia, a major complication of *P. falciparum* and an important cause of child mortality and morbidity. In this study, it was postulated that the erythrocytes of individuals suffering from or at the risk of severe malarial anemia have reduced levels of complement regulatory proteins and this compromises their ability to mop out circulating ICs. As a result, a lot of ICs remain in circulation, engage the macrophages and induce the secretion of TNF-α which is associated with malarial anemia. Using anti-CR1 monoclonal antibody, erythrocyte CR1 copy numbers were determined by flow cytometry and cryopreserved erythrocytes from a cross sectional study in Kombewa were categorized as low, medium and high expressers. 15 individuals from each cohort were selected and IC binding capacity determined by flow cytometry. Using an *in vitro* model system, macrophages were stimulated with a cocktail of erythrocytes and pre-opsonized BSA-anti-BSA ICs, loaded erythrocytes, supernatants and relevant controls. At the end of 8 hour incubation period, the supernatants were harvested and ELISA done to determine the levels of TNF-α present. The data generated in this study indicated that the IC binding capacity was influenced by the CR1 copy number and it was complement dependent. The data did show that the erythrocytes inhibit IC-induced TNF-α production by macrophages and that the buffering capacity was in a manner proportional to the level of CR1. Also, the erythrocytes soaked with ICs stimulated macrophages more than plain erythrocytes though the stimulation was not in a manner proportional to CR1. Based on the findings it was concluded that erythrocyte CR1 may act as a dynamic buffering system which prevents ICs from stimulating macrophages to release TNF-α which is implicated in the pathogenesis of severe malaria. Also, the CR1 enables the erythrocytes to soak in ICs and in the process makes the erythrocytes to become stimulatory leading to secretion of TNF-α by the macrophages.

CHAPTER 1 INTRODUCTION

1.1 General Introduction

Malaria is one of the most striking re-emerging infectious diseases and it is widely spread geographically. The disease is caused by parasites of the genus *Plasmodium*, of which four species infect humans: *P. falciparum, P. vivax, P. ovale* and *P. malariae*. Of the four species, *P. falciparum* continues to represent a tremendous health burden in developing countries resulting in 300-500 million infections with 1.5-2.7 million deaths annually (WHO, 2000). Death is usually due to complications, such as cerebral malaria and severe malarial anemia (Breman *et al.,* 2001). The mortality rates are particularly high for children under the age of five, pregnant women and non-immune persons (Breman *et al.*, 2001). In individuals living in holo-endemic regions, such as Kisumu district in western Kenya, severe malarial anemia is the major complication yet they have low malaria parasitemia (Kitua *et al.,* 1996; Greenwood, 1987). In areas of low malarial endemicity, the level of anemia correlates with high parasitemia (Phillips *et al.*, 1986). It is apparently clear that the relationship between anemia and parasitemia depends on the endemicity of the area.

Despite considerable work spanning many years, the causes of *P. falciparum* induced anemia remain unknown. Increased destruction of infected and uninfected red blood cells and suppression of erythropoiesis seem to be important causative factors of severe malarial anemia (Phillips and Pasvol, 1992). Surprisingly, the most severe cases of anemia (Hb <6 g/dL) typically occur in young children experiencing a low level of

parasitemia, whereas in the less severe anemias, the parasitemia is often much higher (Abdalla *et al.,* 1980). Unlike cerebral malarial (CM) which has been studied extensively as an illness mediated by pro-inflammatory cytokines, the role of pro-inflammatory cytokines in the pathological events leading to severe malarial anemia remains poorly characterized. TNF-α is thought to be involved with both suppression of hemopoiesis in the marrow and erythrophagocytosis of red cells (Clark and Chaudri, 1988; Taverne *et al.,* 1994).

While circumstantial evidence suggests that TNF-α is involved in the cause of malarial anemia, its source has not been clearly defined. *In vitro* studies have shown that the interaction between erythrocytes loaded with ICs and monocytes leads to production of pro-inflammatory cytokines (Chou *et al.,* 1985). One possible mechanism may be that the circulating immune complexes (ICs) formed in response to malaria infection induce the release of cytokines by macrophages within the bone marrow and/or in the blood system (Mibei *et al.*, 2005; Medof and Oger, 1982; Beynon *et al.*, 1994; Nielsen *et al.*, 1994). In this study, the possibility that the binding of ICs by erythrocytes with different CR1 values reduces the availability of ICs for uptake by the phagocytes and thus prevents their IC-induced activation was investigated. This is because gaining an understanding of the mechanisms involved in etiology of severe malarial anemia will be critical in the development of new preventive or curative therapies and/ or vaccines, as well as defining prognostic markers.

1.2 Justification

Malarial anemia in children remains a health burden with the vast morbidity and mortality occurring in the tropical world. Protective malarial immunity is regulated partly by cytokines, yet the mechanisms responsible for the pattern of pro-inflammatory cytokine production that predicts a protective immune response against severe malaria is unknown. Recent observations of severe anemia developing in non-human primates (*Aotus* monkeys), with low-level or microscopically undetectable parasitemia, following vaccination with blood-stage antigens and challenge infection (Egan *et al.,* 2000) suggest that the anemia might have been caused by inhibition of erythropoiesis resulting from haematopoiesis-suppressive cytokine TNF-α and IFN-γ released in the bone marrow of anemic individuals in response to malaria infection (Clark and Chaudhri, 1988). The direct destruction of parasitized erythrocytes cannot account for the anemia experienced by the primates since the parasitemia was microscopically undetectable (Egan *et al*, 2000).

The data from this study reports role of erythrocyte CR1 in the IC-induced production of TNF-α by macrophages. The observations made will contribute to improved understanding of the possible causes of severe malarial anemia and help in the development of safe and effective vaccines.

1.3 Hypotheses

a) The erythrocytes of individuals with low CR1 bind immune complexes (ICs) less effectively hence free ICs interact with macrophages inducing TNF-α production leading to severe anemia.

b) IC-loaded erythrocytes can interact with macrophages and induce TNF-α in a manner proportional to the level of CR1.

1.4 General Objective

To determine the ability of erythrocytes with different levels of CR1 copy numbers to inhibit stimulation of macrophages by ICs to release TNF-α and to stimulate macrophages upon soaking up ICs.

1.5 Specific Objectives

a) To determine the erythrocytes CR1 copy numbers.

b) To determine the ability of erythrocytes with various levels of CR1 copy numbers to bind opsonized ICs.

c) To determine the ability of the supernatants to stimulate TNF-α production from the macrophages.

CHAPTER 2 LITERATURE REVIEW

2.1 Malaria: The Disease

Tremendous efforts have been made in the last decade in understanding the pathological steps that leads to severe malaria. The disease is a complicated syndrome, which is determined by various host and parasite factors. The common clinical presentations of the severe malaria are high fever, progressing anemia, multi-organ dysfunction, and coma, which is a sign of cerebral malaria and one of the causes of the death (Miller *et al.*, 1994). Metabolic acidosis has been recognized as an important pathophysiologic feature in both cerebral malaria and malarial anemia (Marsh *et al.*, 1995). Besides the acidosis leading to respiratory distress, it is a major determinant of survival (Taylor *et al.*, 1993). Children suffering from severe malaria suffer from acidosis which is caused by increased production by parasites, direct stimulation by cytokines, decreased clearance by the liver and most importantly by combined effects of several factors in reducing oxygen delivery to the tissues (English *et al.*, 1997).

Severe malaria occurs after parasite proliferation inside erythrocytes and consequent binding of infected red blood cells to the vascular endothelium (cytoadherance) and to noninfected erythrocytes (rosetting). These sequestrations lead eventually to accumulation of parasitized cells in the local postcapillary microvasculature and block the blood flow thus limiting tissue perfusion. Studies have shown that uninfected erythrocytes of individuals suffering from severe malaria have marked reduction in deformability (Dondorp *et al.*, 2000) and this might play a role in limiting tissue

perfusion. Patients with malaria tend to be dehydrated and hypovolemic (English *et al.*, 1996), potentially worsening the blood flow obstruction caused by sequestration in the microvasculature by reducing perfusion pressure. The erythrocytes destruction is also an inevitable part of malaria, and anemia further compromises oxygen delivery. Pathogenesis of severe malaria is complex and several theories have been proposed to explain it. No single schema can accurately explain all of the epidemiological data.

2.2 Malaria Parasite Life Cycle

The malaria parasite life cycle involves two hosts (see figure 1).

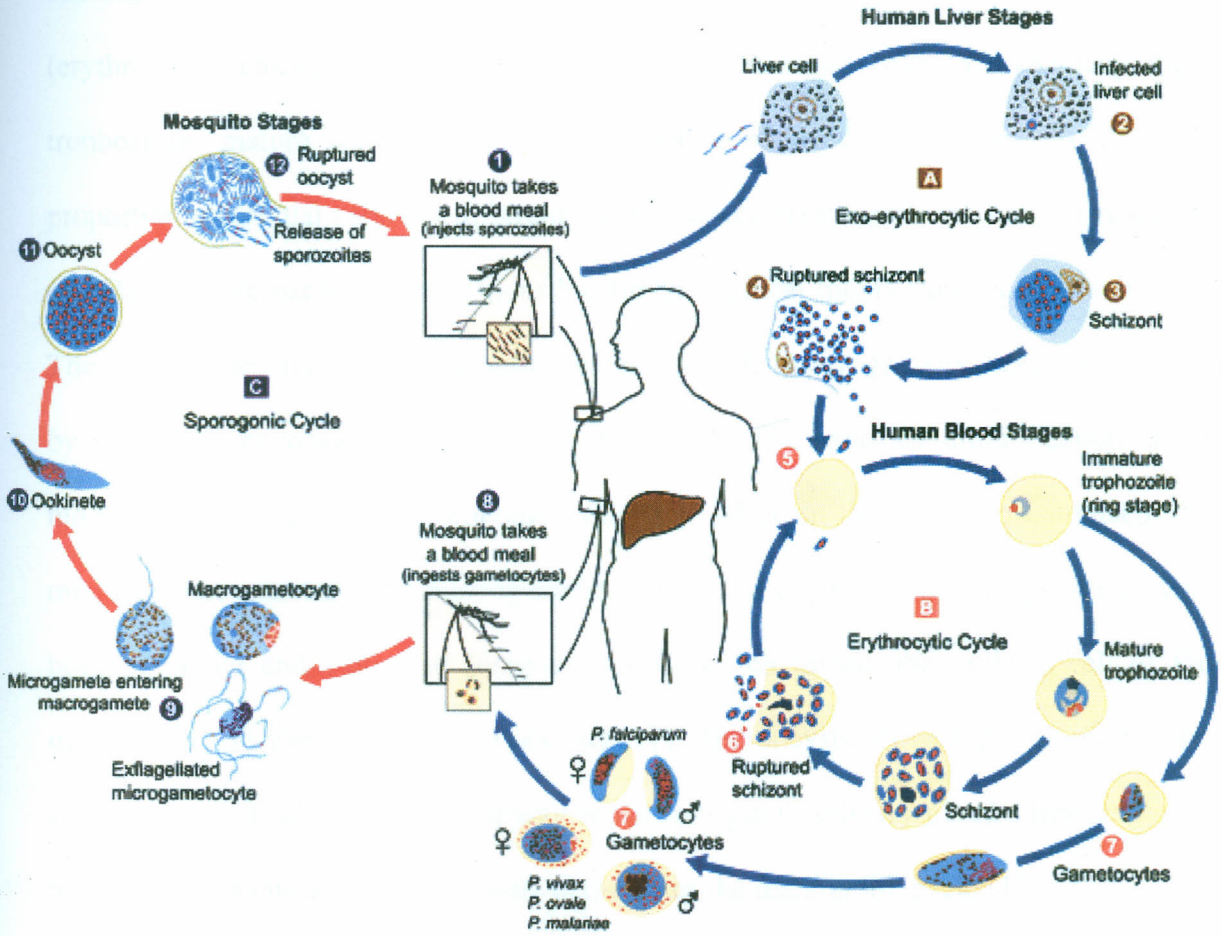

Figure 1 **Life cycle of *Plasmodium falciparum* (Http://www.dpd.cdc.gov/dpdx)**

During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates 10-100 sporozoites into the subcutaneous tissue and less frequently directly into the bloodstream of the human host (1) (Rosenberg *et al.*,1990). Sporozoites infect liver cells-hepatocytes within 30 minutes (2) and mature into schizonts (3), which rupture and release tens of thousands of merozoites (4). In *Plasmodium vivax* and *P. ovale,* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later. After this initial replication in the liver (exo-erythrocytic schizogony [A]), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony [B]). Merozoites infect red blood cells (5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites (6). A small proportion of asexual parasites differentiate into sexual erythrocytic stages (gametocytes) (7). Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal (8). The parasites' multiplication in the mosquito is known as the sporogonic cycle [C]. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes (9). The zygotes in turn become motile and elongated (ookinetes) (10) which invade the midgut wall of the mosquito where they develop into oocysts (11). The oocysts grow, rupture, and release sporozoites (12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (1).

2.3 The Immune System

It is important to consider the complex nature of the host responses to malaria infection. Children born in endemic areas get passive immunity from malaria infection through the transferred maternal antibodies from their immune mothers during the first 6 months of life followed by 1 or 2 years of increased susceptibility before acquisition of clinical immunity (Snow *et al.*, 1998).

2.3.1 Innate immunity

Several studies have indicated the crucial role of innate immune responses in protective immunity to malaria. For instance, in the absence of natural killer (NK) cells, peak parasitemia is higher during acute infection with *P. chabaudi chabaudi AS* and there is marked recurring parasitemia during the chronic phase (Mohan *et al.*, 1997). Early production of interferon gamma (IFN-γ) by NK cells is associated with resolving infection in mice infected with various *Plasmodium* species, whereas absence of early IFN-γ production by NK cells leads to lethal infection (Mohan *et al.*, 1997). Studies have shown that NK cells are frequently the first cells to respond after *in vitro* exposure of human PBMCs to *P. falciparum*-infected erythrocytes (Artavanis-Tsakonas and Riley, 2002). It has been reported that NK cells in malaria-exposed individuals can lyse *P. falciparum*-infected erythrocytes, indicating specific recognition of parasitized cells (Orago and Facer, 1991). γδ T-cell populations are expanded during malaria infection in mice and in their absence parasitemia is prolonged and elimination of parasites is delayed for a couple of days (Langhorne *et al.*, 2002). Polyclonal expansion of γδ T-cell subset has also been reported in acute infection with *P. falciparum* (Langhorne *et al.*, 1994). *P.*

falciparum-activated γδ T-cells produce large amounts of IFN-γ (Hensmann and Kwiatkowski, 2001) and the cytokine is associated with inhibition of parasite growth (Elloso *et al.*, 1994).

Other cells also thought to be playing a role in the innate immunity are the NK-like cells called the NKT cells. The NKT cells in mice carry NK1.1 surface marker and αβ T-cell receptors (TCR). *In vitro* studies have shown that NKT cells are potent inhibitors of liver-stage parasite replication in mouse malaria (Schmieg *et al.*, 2003). Macrophages, which are well known for their antigen presenting role, have been observed to play an important role in innate immunity to malaria due to their ability to phagocytose infected erythrocytes in the absence of cytophilic or opsonizing malaria-specific antibody (Serghides *et al.*, 2003). The opsonin-independent phagocytosis of *P. falciparum*-infected erythrocytes by macrophages from non-immune individuals probably involves the binding of CD36 to PfEMP-1 on the surface of the infected erythrocytes (Serghides *et al.*, 2003). However, macrophages seem to be more important during adaptive immunity as effector cells that can mediate antibody-dependent cellular inhibition or production of anti-parasite molecules after their activation by IFN-γ (Good and Doolan, 1999). By the virtue of their unique ability to sample sites of pathogen entry, dendritic cells (DCs) play an important role in innate and adaptive immune responses. *In vitro* studies have shown that *P. falciparum*-infected erythrocytes bind to CD36 on the surface of human peripheral-blood-derived DCs and inhibit normal lipopolysaccharide (LPS)-induced up regulation of expression of major histocompatability complex (MHC) class II molecules, ICAM-1, CD40, CD80, CD83 and CD86 (Urban *et al.*, 1999). Plasma levels of DC- and

macrophage-derived cytokines are up regulated within hours of the appearance of parasitized erythrocytes in the blood circulation of human (Hermsen *et al.*, 2003) and mice (Stevenson *et al.*, 2001) and the cytokines are required for protection (Stevenson *et al.*, 2001).

2.3.2 Humoral immunity to malaria

Malaria parasites develop within erythrocytes from ring stage to trophozoite and in the process of their growth they express parasite-encoded neoantigens on surface of the erythrocytes which elicits specific homologous responses in the infected host (Marsh and Howard, 1986). These neoantigens are generally referred to as variant surface antigens (VSAs) and the best characterized is PfEMP-1 which is encoded by a multigene family *var* genes with about 60 copies per genome (Su *et al.*, 1995). PfEMP-1 plays an important role in the pathogenesis of malaria by mediating the binding of parasitized erythrocyte to different host tissues via different host receptors, including CD36, ICAM-1, chondroitic sulfate A, and hyaluronic acid (Deitsch and Hviid, 2004). The binding of parasitized erythrocyte to different host tissues results in local pathology (Wahlgren *et al.*, 1992) and enables the parasite to avoid clearance at the spleen (Miller *et al.*, 1994). *In vitro* studies have shown that the sera from individuals in endemic areas are able to disrupt cytoadherent processes (Singh *et al.*, 1988). The clearance of a parasite strain appears to follow the development of a VSA-specific antibody response (Bull *et al.*, 1998). Other multigene families encoding parasite antigens on the erythrocyte surface are the *rif* and *stevor* genes (Cheng *et al.*, 1998). The antigens coded by these two multigene

families are thought to have an accessory role in the binding of uninfected to infected erythrocytes giving rise to rosetting (Cheng *et al.*, 1998).

2.3.3 Antibodies

The humoral response is characterized by enhanced and polyclonal production of IgM and IgG, but also of other immunoglobulin isotypes. In areas that are endemic for malaria, the ratio of IgG1 to IgG3 antibodies appears to be highest in the individuals whose antibodies are most efficient in parasite neutralization (Shi *et al.*, 1999). In some individuals elevated concentration of IgG2 antibodies is associated with decreased risk of *P. falciparum* infection by binding to certain allelic variant of the monocytic Fcγ receptor (Fcγ RIIA) (Aucan *et al.*, 2000). IgE antibodies are implicated in malaria pathogenesis since the elevation of this isotype is higher in patients suffering from severe malaria (Perlmann *et al.*, 1999).

2.3.4 Cell mediated immunity

Malaria-induced cell-mediated immune responses may be protective against both pre-erythrocytic and erythrocytic parasites. Direct destruction of infected erythrocytes by the CD8+ T cytotoxic cells is MHC restricted and thus does not affect human erythrocytes. *In vitro* studies have shown that CD4+ helper cells can kill malaria parasites (Fell *et al.*, 1994) and that the parasite-specific lymphoproliferative responses of individuals living in malaria endemic areas were predictive of resistance to clinical malaria (Mshana *et al.*, 1993). The studies thus support the important role of CD4+ T cells in the control of the parasite growth. Acute infection also results in a strong and relatively long-lasting

increase in γδ T cells in the circulation. *In vitro* studies have shown that activated γδ T cells from malaria-naïve donors inhibit parasite replication in erythrocytes, supporting their role in parasite clearance in the early phases of infection (Troye-Blomberg *et al.*, 1999).

2.3.5 The cytokines

The main players in protective anti-malarial immunity are lymphocytes and mononuclear phagocytes as reflected in their role in cellular activities such as antibody production, phagocytosis, cellular cytotoxicity and parasite inhibition. Role of both pro-inflammatory and anti-inflammatory cytokines have been studied. It was observed that the elevated IL-6: IL-10 ratios in plasma due to relative IL-10 deficiencies predict a fatal outcome of severe malaria (Day *et al.*, 1999). Othoro and her group showed that children living in endemic area in the Western part of Kenya who were suffering from malarial anemia had lower IL-10: TNF-α ratios than those who had mild disease, suggesting that IL-10 may suppress induction of anemia by TNF-α (Othoro *et al.*, 1999). Recent studies have revealed that IL-10 production induced by malaria antigens may predict resistance to *P. falciparum* infection (Kurtis *et al.*, 1999). Induction of TNF-α in the brain of cerebral malaria patients suggests that local release contributes to pathogenesis of the disease (Brown *et al.*, 1999). Variation in the amounts of TNF-α produced by monocytes/macrophages has a genetic basis, and overproduction of the cytokine is associated with a single nucleotide polymorphism in the TNF-promoter region-308 and with elevated susceptibility to cerebral malaria (Knight *et al.*, 1999). Conversely, children

with low plasma levels of TNF-α due to a single nucleotide polymorphism at the TNF-promoter allele-238A are vulnerable to severe malarial anemia (McGuire *et al.*, 1999).

2.3.6 Nitric oxide

Nitric oxide (NO) can modulate antimicrobial activity, smooth muscle contraction, neurotransmission, cytokine production and adhesion molecules expression on microvascular endothelial cells (MVECs) (MacMicking *et al.*, 1997). Cerebral malaria gives rise to significantly enhanced NO production by inducible NO synthase as has been reported in brain tissue from fatal cases (Maneerat *et al.*, 2000). This seems to suggest that besides the microbial activity, NO is also a pathogenic agent.

2.4 Immunopathogenesis of Anemia in Malaria

Anemia is one of the most common complications in *P. falciparum* malaria and it is the leading cause of severe malarial morbidity in children living in holoendemic regions (Kitua *et al.*, 1996). Hemoglobin of less than 5g/dL is considered to represent severe disease. Anemia is typically normocytic and normochromic, with absence of reticulocytes, although microcytosis and hypochromia may be present due to high prevalence of alpha and beta thalasemia traits and/or iron deficiency in many endemic regions (Newton *et al.*, 1997).

The pathophysiology of severe malarial anemia is complex and has not been extensively studied as cerebral malaria. Hemolysis is considered as the most critical factor responsible for anemia in *P. falciparum* infections. Extravascular hemolysis of both

parasitized and nonparasitized erythrocytes occurs (Looareesuwan *et al.*, 1987[a]; 1987[b]). The destruction of parasitized erythrocytes due to growth and multiplication of intra-erythrocytic parasite population is considered as a major factor responsible for anemia (Greenwood *et al.*, 1991). Some of the parasitized erythrocytes are destroyed by 'pitting' of the parasites from the cells by the macrophages in the spleen (Schnitzer *et al.*, 1972; Schnitzer *et al.*, 1973). After an episode of 'pitting' of the parasites, the erythrocytes become defective nonparasitized cells. As a result their membranes become rheologically altered and are further destroyed in the spleen. The nonparasitized erythrocytes are destroyed by increased activity of the macrophages in the spleen (Mohan *et al.*, 1995).

Blood transfusion of the patients suffering from severe malarial anemia showed a shortened lifespan suggesting that the hemolytic process is not inherent to the patient's erythrocytes and the cause of the hemolysis in malaria infection could be attributable to its prevailing environment (Weathrall, 1988). Intravascular hemolysis also known as 'black water fever' occurs in falciparum malaria and is caused by interactions of three factors. These are the use of antimalarial drugs (especially common with quinine), glucose -6-phosphate dehydrogenase deficiency and malarial fever (Reeve *et al.*, 1992; Sarkar *et al.*, 1993; Hong chau *et al.*, 1996). Anemia in malaria may also be caused by bone marrow suppression. In any hemolytic process, the drastic fall in hemoglobin level usually triggers an increased production of erythropoietin, which results in erythroid hyperplasia of the bone marrow, reflected as reticulocytosis in peripheral blood. Studies have shown that acute malarial infections are accompanied by inappropriate reticulocytosis along with erythroid hypoplasia (Srichaikul *et al.*, 1967; Camacho *et al.*,

1998). Another evidence pointing towards inadequate red blood cell formation was the decreased colony forming unit erythrocytic (CFU-E) and burst forming unit-erythrocytic (BFU-E) in malarial bone marrow mononuclear cells cultured with serum from infected patients (Jootar *et al.*, 1995) along with suppression of the serum erythropoietin response to anemia (Burgmann *et al.*, 1996). Depression of erythropoiesis has been demonstrated in chronic malaria (Philips *et al.*, 1986) and several mechanisms have been implicated. The notable ones being dyserythropoiesis (Philips *et al.*,1986; Abdalla *et al.*,1980) *in vitro* defective heme synthesis and premature death of the normoblasts (Wickramasignh *et al.*, 1982; Srichaikul *et al.*, 1976). Clark and Chaudhri were able to show using mice that TNF-α plays a role in pathogenesis of malarial anemia by inducing dyserythropoiesis and erythrophagocytosis (Clark and Chaudhri, 1988).

2.5 The Complement System

Complement system is part of the host innate immune system and it plays an essential role in the defense against pathogens through the covalent binding of C3 to microorganisms activated by the alternative pathway or by binding of a lectin, mannose-binding protein, to mannose groups on the surface of microorganisms (Dodds and Sim, 1997). To prevent complement mediated autologous attack, host tissues express a number of fluid-phase and membrane-bound inhibitors (Dodds and Sim, 1997). Three of these proteins found on the erythrocyte surface and responsible for regulation of complement system are CD35 (also known as complement receptor 1, CR1), CD55 (also known as decay accelerating factor, DAF) and CD59 (also known as membrane inhibitor of reactive lysis, MIRL). The human C3b/ C4b receptor (CR1) mediates the binding

between particles or immune complexes (ICs) bearing C3b (or C4b) and the cell membrane (Ross and Medof, 1985; Wilson *et al.*, 1987). CR1 have been demonstrated on phagocytes (Ross and Medof, 1985; Wilson *et al.*, 1987; Ehlenberger and Nussenzweig, 1977), glomerular podocytes (Fischer *et al.*, 1986), lymphocytes (Fearson, 1980), erythrocytes in primates (Cornacoff *et al*, 1983) and platelets in nonprimates (Taylor *et al.*, 1985).

Erythrocyte CR1 has been shown to function as a co-factor in the factor I-mediated cleavage of C3b to C3bi and C3dg (Medof *et al.*, 1982) and in the removal of ICs from the circulation by binding ICs containing C3b (Cornacoff *et al.*, 1983). The erythrocyte-bound ICs are removed by macrophages as the erythrocyte transverses the liver and the spleen (Davies *et al.*, 1990) and in the process CR1 molecules are continuously lost but the erythrocytes are recycled. DAF and CD59 are glycosylphosphatidylinositol (GPI)-anchored membrane regulators of complement. DAF inhibits the C3 convertases of both the classical and alternative pathways (Lublin and Atkinson, 1989) whereas CD59 prevents the assembly of the membrane attack complex (MAC) that forms a pore in the cell membrane, leading to lysis by binding to the C5b-8 complex and preventing the polymerization of C9 (Morgan, 1999). Deficiency in CD55 and CD59 results in paroxymal nocturnal haemoglobinuria (PNH) syndrome, a disease characterized by intermittent haemolysis due to increased sensitivity of red blood cells to autologous complement-mediated lysis (Parker, 1996).

2.6 Complement Factors Linked in Severe Malaria

Studies have shown that CR1 might be involved in the pathogenesis of severe malaria (Waitumbi *et al.*, 2000; Stoute *et al.*, 2003; Rowe *et al.*, 1997). However, its exact role is not well understood. CR1 is thought to be the receptor for *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) (Rowe *et al.*, 1997; 2000). The interaction between PfEMP-1 and CR1 may be responsible for rosetting, a phenomenon in which erythrocytes infected with *P. falciparum* late stage parasites bind to uninfected erythrocytes *in vitro* and has been associated with cerebral malaria (Rowe *et al.*, 1995). The greater the number of CR1 molecules per erythrocyte, the greater the tendency to rosetting (Rowe *et al.*, 1997). Rosettes are thought to contribute to the pathogenesis of cerebral malaria by plugging cerebral capillaries and decreasing blood flow (Wahlgren *et al.*, 1992).

The primate erythrocyte is thought to play a role in the mopping out the circulating ICs (Cornacoff *et al.*, 1983). Opsonized IC, incorporating the C3b, bind CR1 on the erythrocytes (Schifferli *et al.*, 1986; Corncoff *et al.*, 1983) and are then ferried to the reticuloendothelial system (RES) where IC is removed and degraded by the macrophages of the RES (Kimberly *et al.*, 1989). The erythrocytes return to the circulation, apparently able to bind further IC (Corncoff *et al.*, 1983). This mechanism prevents extravascular deposition of IC and subsequent inflammatory tissue damage (Medof *et al.*, 1982). The erythrocytes of children suffering from severe malarial anemia have been shown to be deficient of CR1 and CD55 (Waitumbi *et al.*, 2000) thus increasing their susceptibility to hemolysis through complement attack. The levels of the two molecules increases following treatment (Stoute *et al.*, 2003), thus the condition is acquired and not inherited.

In addition to quantitative changes related to the disease activity, levels of erythrocyte complement-regulatory proteins change with age, being low in young children and increasing into adulthood (Waitumbi *et al.*, 2000). This might partly explain why children are more prone to severe malarial anemia than adults (Luxemburger *et al.*, 1997). The levels of circulating ICs are higher in children with severe malarial anemia than in symptomatic uncomplicated malaria (Stoute *et al.*, 2003; Mibei *et al.*, 2005). This is understandable given that the children's erythrocytes have low levels of complement regulatory proteins (Waitumbi *et al.*, 2000) hence their capacity to mop out circulating ICs is compromised or less efficient.

Immune complexes stimulate macrophages/monocytes to release proinflammatory cytokines (Virella *et al.*, 2002) and induce expression of inducible nitric oxide synthase (iNOS) (Gommez-Guerrero *et al.*, 2002). An interaction between erythrocytes carrying ICs and monocytes resulting in release of pro-inflammatory cytokines has been shown *in vitro* (Chou *et al.*, 1985). Hence, the erythrocytes with high CR1 levels binding more loads of ICs might interact with monocytes and endothelial cells in sequestered brain capillaries to stimulate production of pro-inflammatory cytokines and downstream mediators. The deposition of immunoglobin G on microvascular endothelial cells of cerebral malaria (CM) patients lends further support to a role of ICs in the pathogenesis of the CM (Maeno *et al.*, 2000). In individuals with low levels of erythrocyte CR1, such as young children, IC is bound less effectively, and the free ICs can stimulate monocytes/macrophages to produce systemic levels of pro-inflammatory cytokines that will lead to decreased erythropoiesis (Emlen *et al.*, 1992; Clark and Chaudhri, 1988). The

view is supported by the finding that young children produce more TNF-α than older children do, regardless of the level of parasitemia (Nussenblatt *et al.,* 2001). Several studies have further suggested that the binding of ICs to erythrocytes reduces the availability of ICs for uptake by the blood phagocytes and thus prevents their IC-induced activation (Medof *et al*., 1982; Paccaud *et al*., 1990; Beynon *et al*., 1994; Nielsen *et al*., 1994; Emlen *et al*., 1992).

2.7 Severe Malarial Anemia and Pro-inflammatory Cytokines

It is unclear which factors predispose some children to become markedly anemic than others during malaria infection (Greenwood *et al*., 1991) though it is well known that the more severe the infection, the more profound the anemia (Slutsker *et al.,* 1994). Pathogenic process of malarial anemia is multifactorial and is only partly explained by direct destruction of parasitized erythrocytes due to growth and multiplication of intra-erythrocytic parasite population (Weatherall, 1993). Besides the destruction of parasitized erythrocytes, there is also evidence of accelerated destruction of unparasitized erythrocytes (Hundreiser *et al*., 1988; Phillips *et al.,* 1986; Looareesuwan *et al.,* 1987[b]; 1991) and this has been attributed to the altered membrane properties of the unparasitized erythrocytes and reduced erythrocyte deformability (Naumann *et al.,* 1992). The change of membrane properties has been shown to correlate with the degree of anemia (Dondrop *et al.,* 1999).

Alteration in the level of several cytokines occurs during malaria infection. Inflammatory cytokines such as TNF-α, IL-1, IFN-γ and IL-6 are highly elevated in acute *Plasmodium*

falciparum infections (Grau *et al.*, 1989; Kwiatkowski *et al.*, 1990). TNF-α in particular has been associated with cerebral malaria and death in children (Kwiatkowski *et al.*, 1990; Grau *et al.*, 1989). Subsequently, it was shown that elevation of TNF-α was not exclusively associated with cerebral malaria but also with anemia and high-density *Plasmodium* infections (Shaffer *et al.*, 1991). IFN-γ upregulates the expression of TNF receptors on a variety of cells and its release by activated T helper type-1 (Th1) cells may potentiate the action of TNF in malaria. TNF is a proinflammatory cytokine that plays a role in Th1-like immune responses and its concentration is normally elevated in individuals suffering from severe malaria (Kern *et al.*, 1989). *In vitro*, TNF-α suppresses proliferation of erythroid progenitor cells in human marrow cultures (Roodman *et al.*, 1987). *In vivo*, erythropoiesis is inhibited by chronic exposure to TNF-α, this has been demonstrated by implanting nude mice with TNF-α-secreting Chinese Hamster Ovary (CHO) cells (Johnson *et al.*, 1989). TNF-α has been specifically incriminated as a cause of dyserythropoietic anemia in experimental malaria in mice, which recover after the administration of anti-TNF-α antibodies (Clark and Chaudhri, 1988; Miller *et al.*, 1989).

TNF-α can also promote erythrophagocytosis by activated macrophages as shown by a transgenic murine model in which a human TNF-α gene was constitutively over expressed resulting in development of severe anemia (Taverne *et al.*, 1994). Higher levels of IL-10, an anti-inflammatory cytokine that plays a role in T-helper type-2-like immune responses, over TNF-α may prevent development of malaria anemia by controlling the excessive inflammatory activities of TNF-α (Othoro *et al.*, 1999). Erythropoietin is produced mainly by the kidney in response to tissue hypoxia and is the primary factor

regulating erythrocyte production. When the hemoglobin concentration is low in the body, the serum erythropoietin level is increased. Despite evidence showing that children suffering from severe malarial anemia respond to the low hemoglobin level as reflected by elevated erythropoietin levels (Burchard *et al.*,1995) *in vitro* studies seems to suggest that TNF-α can suppress the production of erythropoietin (Faquin *et al.*, 1992; Jelkmann *et al.*,1992).

CHAPTER 3 MATERIALS AND METHODS

3.1 Study Design

This was a cross-sectional study. The study participants were recruited under human use protocols approved by the Human Subjects Research Board, Office of the Surgeon General, US Army, (WRAIR-1098) and the Ethics Review Committee of the Kenya Medical Research Institute, Nairobi, Kenya (SSC-580). All the procedures were in accordance with Helsinki Declaration (WMA). The study was open to individuals who are well and free of malaria parasites of both sexes of age between 45 years and 0 month who were residents of the study area.

3.2 Study Site

The study samples were collected from Kombewa Division, a malaria holoendemic region of Lake Victoria basin in Western Kenya, where most individuals are of the Luo ethnic group (appendix 1 for the map of the study area). The entomological inoculation rate is estimated to be 50-300.

3.3 Inclusion and Exclusion Criteria

Any persons, resident of the study area, male or female of 45 years and below was included in the study. Subjects were excluded if there was evidence of severe bacterial infection, oral or auxiliary temperature greater than 37.5°C, evidence of severe protein malnutrition, evidence of alcohol abuse and history or presence of malignancy. Blood

was collected only when participant was well and free of malaria as determined by microscopic examination of Giemsa-stained blood smears.

3.4 Approval of the Study

This study was approved by KEMRI and NIH Clinical Research Center.

3.5 Blood Samples and Sample Processing

3.5.1 Blood smears

Triplicate thick and thin blood smears were made from finger prick and stained with Giemsa. A minimum of 50 high power fields was scanned for a positive smear and 200 high power fields for a negative smear.

3.5.2 Blood collection

Two milliliters of whole blood sample was collected from healthy individuals by venipuncture into ethylenediaminetetraacetic acid (EDTA) tubes (BD, cat#367841, New Jersy, USA). Complete blood count was done with a hematology analyzer (Coulter, Hialeah, FL). Blood was centrifuged at 1500 rpm for 5 minutes and plasma removed. The plasma was stored at -70°C. The blood pellet was processed for cryopreservation (step below).

3.5.3 Cryopreservation of erythrocytes

The blood pellet was washed in sterile PBS (see appendix 2 for protocols on buffer preparations) and buffy coat aspirated. The packed cell volume (PCV) was measured and

to 1 part PVC 1.7 parts glycerolizing solution (Baxter cat# 4A7833, USA) was added. The adding of glycerolyzing solution was at 1 drop per second while gently vortexing. The mixture was dispensed in 1 ml volume to NUNC vials (Corning cat#430488, New York, USA) and placed in –70°C REVCO freezer overnight before final transfer to liquid nitrogen storage.

3.5.4 Thawing of cryopreserved erythrocytes

1ml blood contained in the NUNC vial was thawed in water bath set at 37°C for 2 minutes and then placed in 50ml centrifuge tube. 0.2ml of 12% NaCl was added at 1 drop per second with 25 G needle while vortexing. The suspension was let to stand for 5 minutes then 10ml of 1.6% NaCl was added at 1 drop per second with 20 G needle while vortexing. The suspension was centrifuged at 1500 rpm for 5 minutes and supernantant removed. 10 ml of 0.9% NaCl/0.2% dextrose was added at the same rate while vortexing followed with centrifugation. The erythrocyte pellet was washed twice in Alsever's solution before storage at 4°C if not to be used immediately.

3.6 Measurement of Erythrocyte CR1

Upon thawing, a 10µl aliquot of erythrocyte pellet was washed twice in Alserver's solution and finally placed in 1ml of the same medium for storage at 4°C until fluorescent staining was performed, usually within 48 hours. 100µl of the reconstituted erythrocyte was placed in each of the four wells in a U-bottom 96-well plate and the supernatant was aspirated after centrifugation at 1300 rpm for 5 minutes. The pellet at the bottom of the well was further washed twice with PBS containing 1% BSA and 0.1% NaN_3 (see

appendix 2). Indirect fluorescent staining was done using monoclonal antibody against erythrocyte CR1 (Clone E11, Pharmigen, San Diego, CA, USA) and a secondary goat anti-mouse fluorescein isothiocynate (FITC)-labeled polyclonal antibody (Pharmigen, San Diego, CA, USA). Irrelevant monoclonal antibody of the same isotype was used as negative control. The primary antibody was used at a dilution of 1:20 while secondary antibody was used at 1:50. The antibody diluent was PBS containing 1% BSA. All incubations were done in the dark and at room temperature for 30 minutes followed by washing with 1% BSA-PBS. After final incubation, the samples were resuspended in PBS containing 1% paraformaldehyde and then stored in the dark at 4 °C until acquisition was performed. Cytoflourometry was performed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). One drop of Full Spectrum Beads was added to 500µl of PBS and it was used to adjust the FL1 PMT to the standard value. The values were set prior to each acquisition to insure day-to-day comparability of assays. The value was arrived at in advance after preliminary experiments had determined the optimal initial parameters for acquisition. Acquisition and analysis were performed using WinFCM and EXPO software packages (Applied Cytometry Systems, Sheffield, UK). The individuals were then grouped in three categories depending on the expression of CR1 on the surface of the erythrocytes as low, medium and high expressers.

3.7 Immune Complex Preparation

Rabbit IgG fraction to bovine serum and endotoxin free bovine serum albumin was obtained from Sigma Chemical Company (St. Louis, MO). The equivalence point for the

antigen-antibody reactions was determined using turbidometric assay previously described (Miletic and Rodic, 1984). In brief, antigen concentration was held at 1µg/ml in sterile PBS while increasing amounts of antibody was added to 0.6ml reaction mixtures. The reactions were mixed, incubated at 37°C for 1 hour and then optical densities obtained using a Perkin-Elmer Bioassay spectrophotometer HTS 7000 (Perkin Elmer, USA). The equivalence point was designated as that antigen:antibody ratio at which the highest turbidometric reading was obtained.

The IC was formed at 2x antigen excess. This ensured that large aggregates were obtained enough to cross-link the Fc receptors. After the 1 hr incubation at 37°C, the IC was incubated further at 4°C overnight. After which the formed IC was centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant discarded. The IC was washed three times by resuspending in sterile PBS. The protein concentration was determined by use of UV spectrophotometry using a known protein standard. The IC was prepared in large batch and aliquoted in volumes of 100µl in eppendorf tubes for storage at -70°C.

3.8 Measurement of RBC IC Binding Capacity

The IC was prepared as described above except the BSA used in this case was conjugated to FITC. The anti-BSA: BSA-FITC IC was incubated with AB+ serum for 30 minutes at 37°C for opsonization. IC preparation to be used as unopsonized IC had 100 mM EDTA included in the cocktail. Opsonized and unopsonized ICs were added to wells containing 1x 10^7 erythrocytes. The plate was covered with aluminium foil and incubated at 37°C for 30 minutes. The erythrocytes were washed with ice cold plain RPMI 1640 (see appendix

2). After aspiration of the supernatant, the erythrocytes were resuspended in 1% paraformaldehyde in PBS (see appendix 2) and stored at 4°C in the dark until acquisition. Acquisition and analysis were performed using WinFCM and EXPO software packages (Applied Cytometry Systems, Sheffield, UK).

3.9 Preparation of Human Peripheral Blood Mononuclear Cells (PBMCs)

The PBMCs were obtained under endotoxin-free conditions from fresh whole blood of a healthy adult volunteer after informed consent was obtained. The venous blood was drawn in heparinized vacutainers. The whole blood was diluted 1:1 with sterile PBS, layered on a histopaque cushion (Sigma cat#116K6070, USA) and centrifuged at 400g for 30 minutes at room temperature. The interphase between plasma and histopaque, corresponding to PBMCs fraction, was collected and then washed 4 times with ice-cold PBS. The cells were suspended in plain RPMI 1640 media and counting done using hemocytometer (Cambridge Instruments Inc., Buffalo, New York, USA). Viability of the cells was >95% as determined by the trypan blue exclusion test. The cells were plated at 1.5×10^6 /well in 96-well flat-bottom culture plate. The plate was covered with a lid and incubated at 37°C in a CO_2 incubator for 1 hour to allow the macrophages to adhere at the bottom of the plate because of their adherent property. The plate was washed three times with sterile PBS to remove nonadherent cells within the PBMCs population that was plated. 75µl of plain RPMI containing polymyxin B (PMXB) at 10µg/ml was dispensed into each well that had the adherent macrophages before addition of the stimulants into the appropriate wells. PMXB helped to keep the level of endotoxin low.

3.10 Stimulation of Macrophages

The endotoxin free IC was pre-opsonized using AB+ serum at 37 °C for 30 minutes. The thawed erythrocytes were counted using hemocytometer and pelleted in sterile eppendorf tubes at 1x 10^8 cells per tube. Each sample had six tubes of which four had opsonized ICs added while two tubes had only media and serum added. The tubes were incubated at 37°C for 30 minutes to allow the erythrocytes bind the ICs. At the end of the incubation period, the contents of the four tubes- two tubes with IC loaded erythrocytes and two tubes with unloaded erythrocytes- were introduced into the culture plate containing adherent macrophages. The remaining two tubes containing loaded erythrocytes were span down and the supernatants introduced to the culture plate. The erythrocytes that pelleted down were washed with plain RPMI and also introduced in their respective wells in the plate. Duplicate wells representing stimulation by ICs alone without erythrocytes were included as the positive control. The final volume in each well was 150µl. The adherent macrophages were stimulated for 8 hours at 37°C in a CO_2 incubator. The IC concentration per well was 35µg/ml as this had been shown in a number of experiments to induce maximal release of cytokines (Debets *et al.*, 1988). Other control stimuli included 7µg/ml LPS (from *Escherichia coli* 055:B5, Sigma, USA), and IgG anti-BSA at 35 µg/ml. The concentration of anti-BSA was chosen to match that of the I C. The immune complexes and other proteins used in the stimulation assays were screened for endotoxin contamination by the *Limulus* amebocyte lysate assay (Bio Whittaker, USA) and found to contain less than 0.01 EU/ml of protein. The supernatants harvested at the end of the designated incubation time were stored at -70°C before ELISA assays were done.

3.11 TNF-α Specific ELISA

TNF-α concentration of cell-free supernatants of the cell cultures was determined by a TNF-α specific sandwich E LISA as previously described (Abrams, 1995). The high binding immunoassay plates (96-well plates; Dynex Immulon 2H) were coated overnight with an anti-TNF-α monoclonal antibody (Endogen M-303-E, Pierce, MA, USA) at a concentration of 6µg/ml. The plates were then blocked by adding to each well 200µl of 0.5% (w/v) boiled casein for two hours. The supernatants from the *in vitro*-stimulated macrophages were diluted in a sample diluent in the ratio of 1:1 before being added to the plate at a volume of 100µl per well. The plates were incubated for 2 hours at room temperature. A standard titration curve was obtained by making serial dilutions of a known sample of human recombinant TNF-α (Endogen R-TNFA-50, Pierce, MA, USA). The plates were washed with 0.05% Tween-PBS wash buffer and then incubated for 1 hour with 100µl/well of biotinylated rabbit anti-TNF-α secondary antibody (Endogen M-302-B, Pierce, MA, USA) that had been diluted in sample diluent at a ratio of 1:400. At the end of the incubation period, the plates were washed and 100µl /well of peroxidase-conjugated streptavidin (Kirkegard and Perry Laboratories, Maryland, USA) at a dilution of 1:5000 added. The plates were incubated at room temperature for 30 minutes, after which they were washed and 100µl/well of substrate containing 1mg/ml 2,2`-azinobis (ethylbenzthiazolinesulfonic acid) (Kirkegard and Perry Laboratories, Maryland USA), 0.0003% hydrogen peroxide in Na_2HPO_4, and 0.05M citric acid was added. After adding the substrate, the plates were incubated in the dark at room temperature for 30 minutes after which the reaction was stopped with 50µl/well of 1% sodium dodecyl sulphate (SDS). The light absorption was measured with a Perkin-Elmer Bioassay HTS 7000 plate

reader using a 415nm filter. The software used in reading the plate was HTSoft version 1.0.

3.12 Sample Size Determination

A total of 344 individuals were enrolled in the study. Based on CR1 copy numbers, 45 individuals were selected for the stimulation assays. The selection was based on CR1 copy numbers: 15 individuals representing low cohort, 15 individuals representing medium cohort and 15 individuals representing high cohort. Calculation by software PS (version 2.1.31) showed that with sample size of 45, the power was 0.98 at p=0.05 (fig 2).

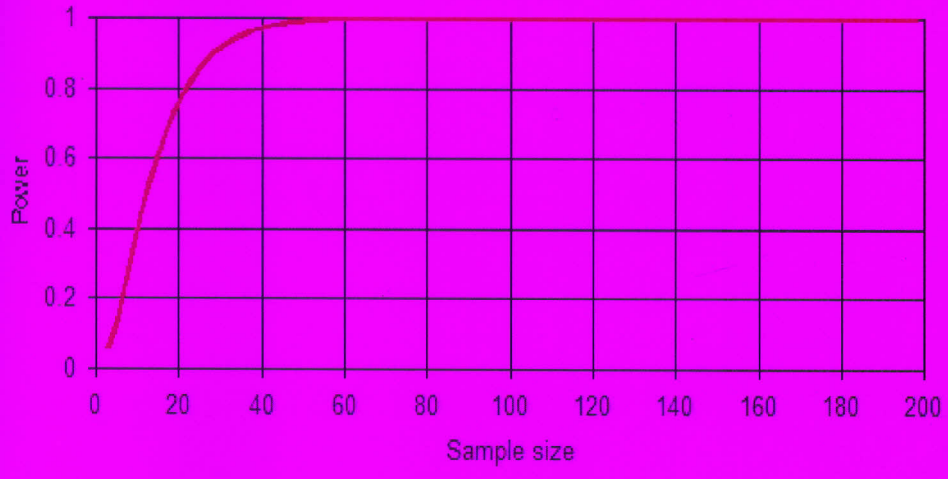

Figure 2 Power Graph for Paired T-test

3.13 Data Analysis

The SPSS software package (version 11.5; SPSS) was used for data analysis. The statistical comparison of CR1 levels and immune complex (IC) binding capacity was done using ANOVA supplemented with Bonferroni's post-hoc test. The statistical comparison of cytokine release induced by IC stimulus was done using ANOVA supplemented with Bonferroni's post-hoc test and paired-sample t test procedure. In the ANOVA analysis the sample were matched using the assay dates and cohorts to take care of day to day variations in the PBMCs stimulations. Differences were considered statistically significant if $p<0.05$.

CHAPTER 4 RESULTS

4.1 Erythrocyte CR1/CD35 Levels

Individuals selected to represent each category were clearly distinct in terms of the CR1 copy numbers. The low CR1 expressers had individuals with median fluorescence intensity (MFI) range of 5-7 (200-300 CR1 copy numbers), for the medium expressers the MFI range was 11-14 (400-500 CR1 copy numbers) while for the high expressers the range was 11-22 (700-1000 CR1 copy numbers). The differences between the categories (fig 3) were highly significant (p=0.0005 using ANOVA).

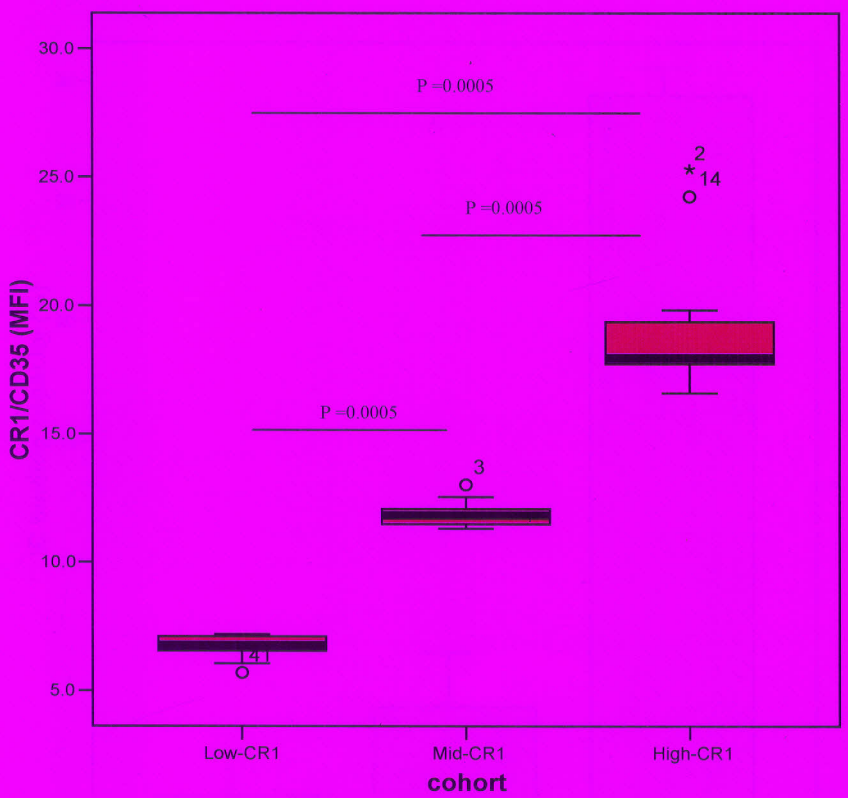

Figure 3 CR1/CD35 levels on the Erythrocytes

4.2 Immune Complex Binding

In the diagram below (Fig.4), the first bar represents the untreated erythrocytes from the 45 individuals who were selected. It serves as the background since the fluorescence registered is due to autofluorescence. The second bar represents binding of ICs to the erythrocytes through complement independent pathway. The erythrocytes were treated with ICs incubated in AB+ serum plus 10mM EDTA. The third bar represents binding of ICs through complement dependent pathway. The erythrocytes were treated with pre-opsonized ICs.

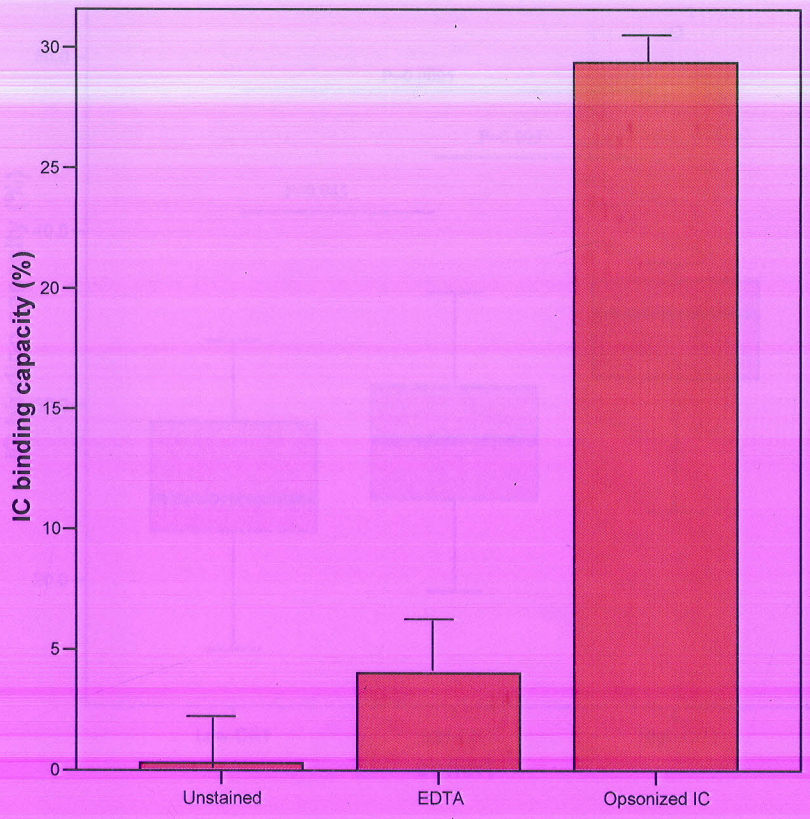

Figure 4 IC-binding to the erythrocytes

To study the effect of variation in CR1 numbers of the three cohorts on the binding of ICs to the erythrocytes, the immune complex binding capacity values of the erythrocytes in groupings based on the CR1 was determined. The results showed that the IC binding was influenced by CR1 numbers (fig 5). The difference was highly significant between the low and high expressers (P=0.0005 using ANOVA). The difference between medium and high expressers was significant (p=0.003 using ANOVA). There was no the significant difference between low and medium expressers (p=0.945).

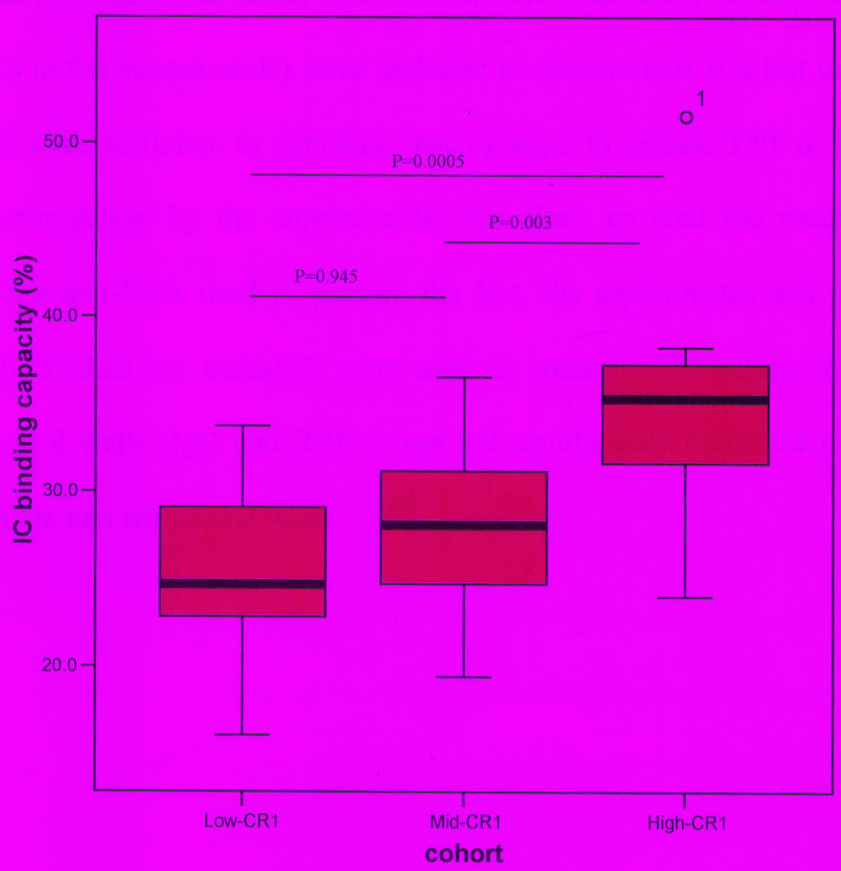

Figure 5, Influence of CR1 on IC Binding Capacity of the Erythrocytes

4.3 Insoluble ICs induce TNF-α secretion by Macrophages

To determine whether erythrocytes could inhibit the stimulation of the macrophages by opsonized ICs, the TNF levels in the supernatants of macrophages incubated with a cocktail of RBCs with ICs, and ICs alone were measured. As can be seen in figure 6, the erythrocytes were able to inhibit the IC-induced TNF production by the macrophages ($p<0.0005$ using paired t test). Loaded RBCs (RBCs incubated with opsonized ICs and washed to remove unbound ICs) were included in separate wells to determine if the erythrocytes upon soaking the ICs become stimulatory. There was significant difference in TNF secretion between loaded RBCs and RBCs only ($p<0.0005$ using paired t test). The unbound ICs (incubated RBCs with ICs and then span down the cells to remain with unbound ICs in the supernatants) were included to determine if ICs left unbound in the supernatants were sufficient to stimulate macrophages to release TNF-α. There was no significant stimulation by the supernatants. ICs alone as well the medium, the IgG fraction of the anti-BSA used to prepare the ICs, the erythrocytes and a well known stimulator LPS (data not included) were used as controls. The release of TNF in the negative control wells (IgG and RBCs) was not significantly increased over the levels measured in the non stimulated wells.

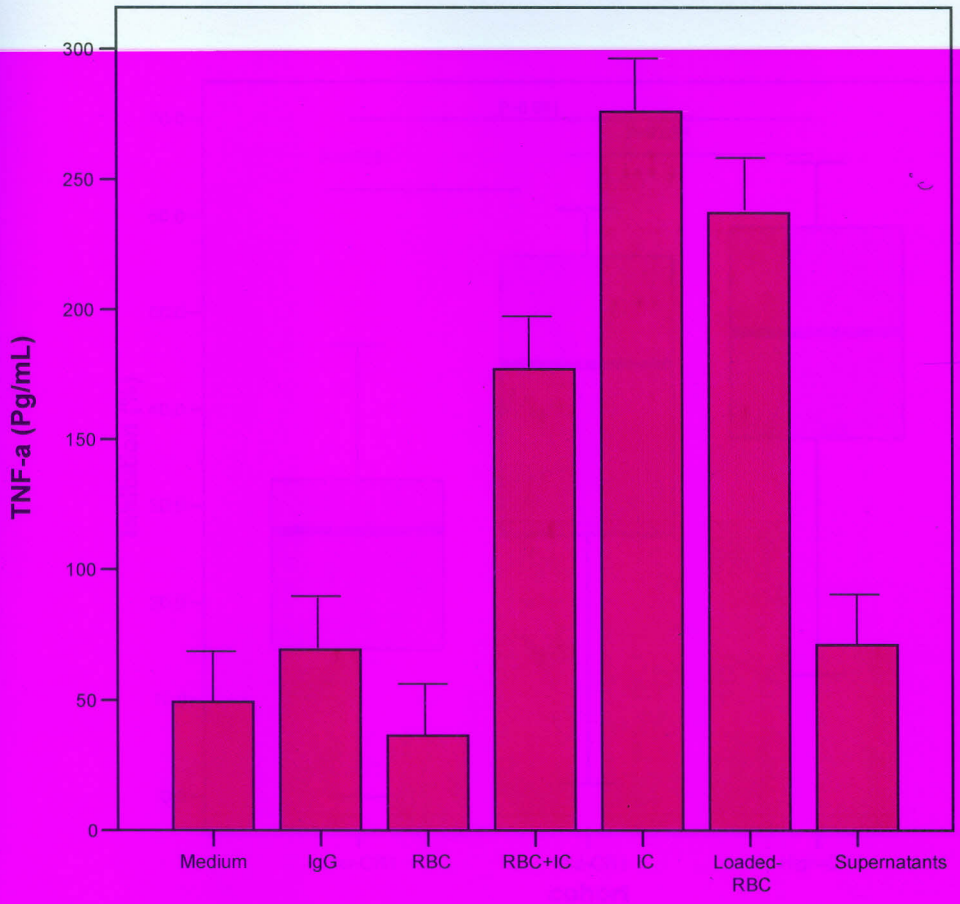

Figure 6. TNF secretion by macrophages when stimulated with ICs

To determine if the erythrocytes inhibitory effect was influenced by the surface CR1 copy numbers, the TNF-α produced when the macrophages were incubated with ICs in the presence erythrocytes from the three categories-low, medium and high expressers were compared (fig 7). The results obtained showed significant difference between low expressers and medium expressers ($p=0.025$ using ANOVA). The difference between low expressers and high expressers was highly significant ($p=0.001$ using ANOVA). However the difference between medium and high expressers was not significant ($p=0.639$).

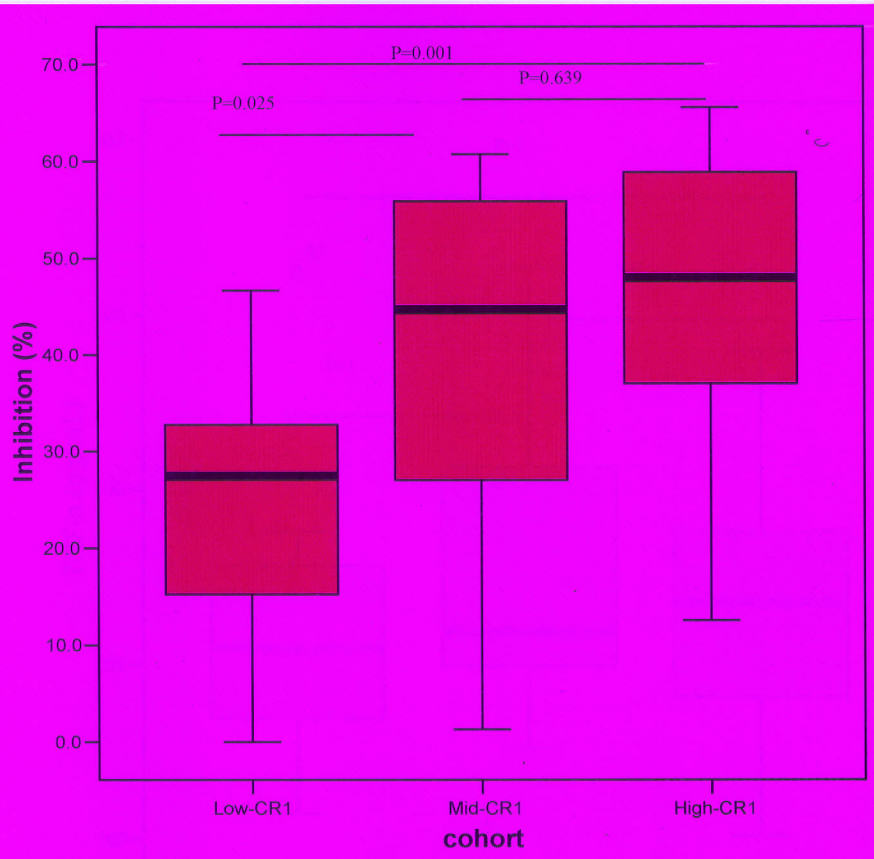

Figure 7 **CR1 influence on inhibitory effect of Erythrocytes on the TNF-α secretion**

The erythrocytes with varying CR1 copy numbers and soaked with ICs were compared to determine if the level of stimulation of the macrophages was influenced by the CR1 on the surface of the erythrocytes. There were no significant differences in TNF-α production between either of the CR1 level cohorts (fig 8).

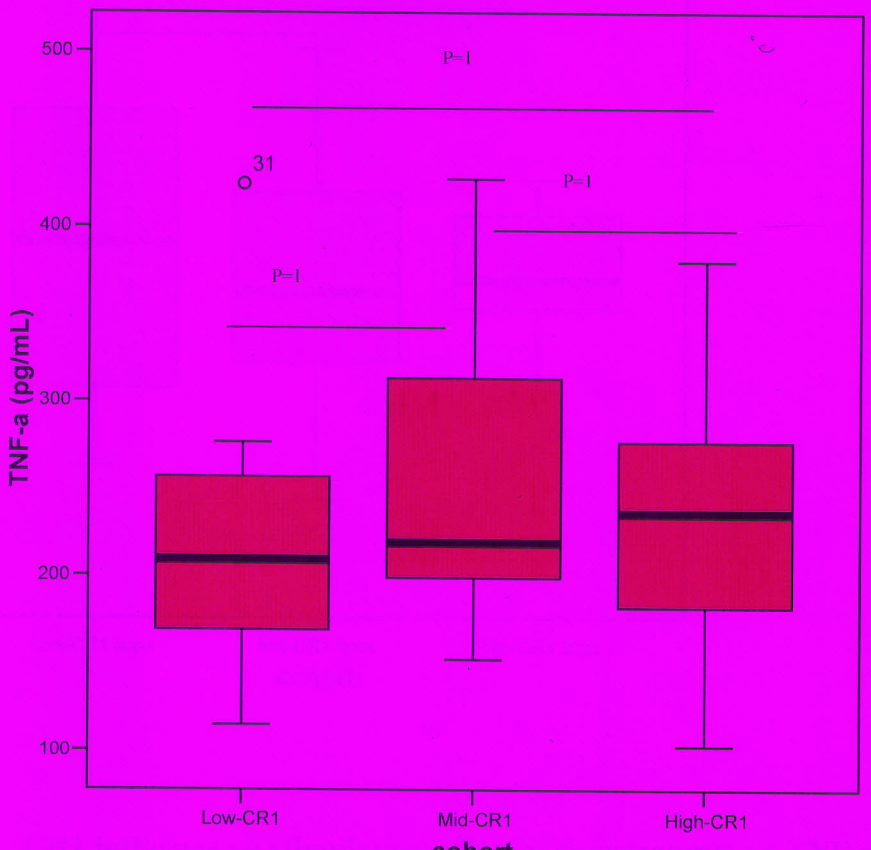

Figure 8 **CR1 influence on the stimulatory effect of loaded Erythrocytes on the TNF-α secretion**

The supernatants were obtained after the erythrocytes with varying CR1 copy numbers were incubated with ICs then span down. The supernatants were compared to determine if level of unbound ICs in the supernatants were influenced by the CR1 copy numbers as would be reflected by varying levels of TNF secreted. There were no differences among the 3 groups: low, medium and high expressers (fig 9).

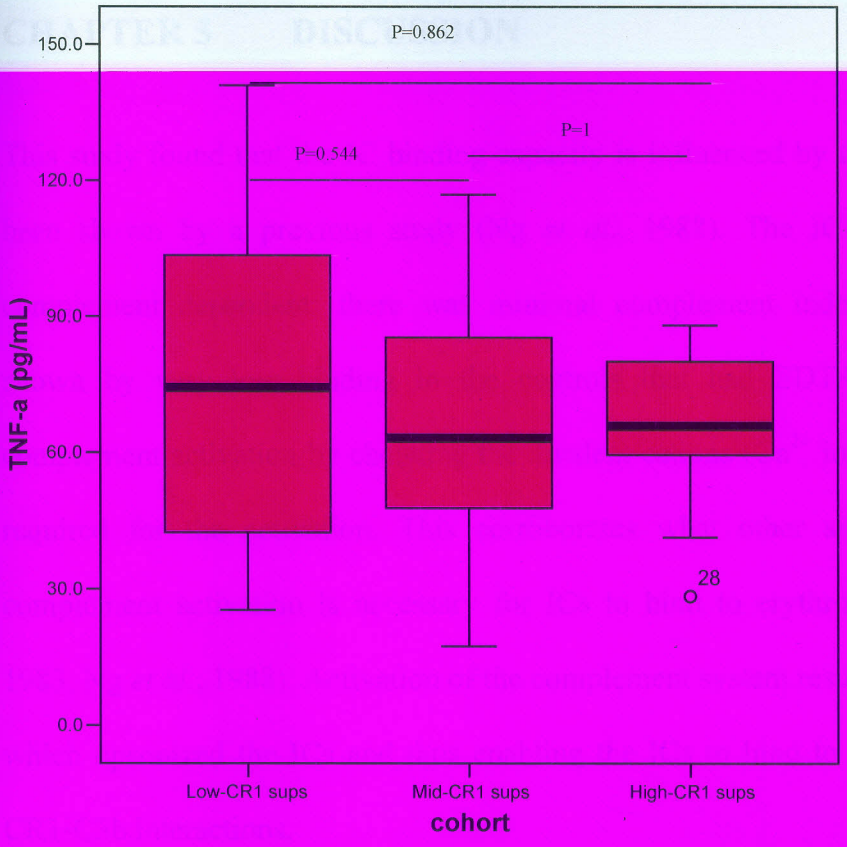

Figure 9 **CR1 influence on stimulatory effect of supernatants on TNF-α secretion**

CHAPTER 5 DISCUSSION

This study found that the IC binding capacity is influenced by the levels of CR1 as has been shown by a previous study (Ng *et al.*, 1988). The IC binding was primarily complement dependent; there was minimal complement independent IC binding as shown by very low binding in the controls that had EDTA. EDTA inhibited the complement activation by chelating the divalent cations (Ca^{2+} ions, Mg^{2+} ions) that were required for the activation. This corroborates what other studies have shown that complement activation is necessary for ICs to bind to erythrocytes (Cornacoff *et al.*, 1983, Ng *et al.*, 1988). Activation of the complement system resulted in formation of C3b which opsonized the ICs and thus enabling the ICs to bind to the erythrocytes via the CR1-C3b interactions.

This study also set out to determine if CD35/CR1 present on the surface of the erythrocytes played a crucial role in the development of severe malaria by inhibiting the IC-induced production of TNF-α by the macrophages. The data reported in this study showed clearly that erythrocyte CR1 inhibited the TNF-α production by macrophages ($p<0.0005$ using paired t test). Studies have shown that individuals with low levels of erythrocyte CR1 bound IC less effectively (Ng *et al.*, 1988), and it was thought that the free ICs could stimulate macrophages to produce high systemic levels of TNF-α that lead to bone marrow dysfunction and decreased erythropoiesis (Akanmori *et al.*, 2000) or increased erythrophagocytosis in the spleen (Clark and Chaudhri, 1988). This view was supported by the finding that young children produce more TNF-α than older children do, regardless of the level of parasitemia (Nussenblatt *et al.*, 2001). It should be noted that

the erythrocyte CR1 also varies with age, with level being low in childhood and high in adulthood (Waitumbi *et al.*, 2004).

The observations in this study supported the hypothesis that erythrocytes in primates may function as a buffering system for complement-bearing ICs in the circulation, preventing their interaction with leukocytes bearing complement and Fc receptors and the potential activation of these cells (Beynon *et al.*, 1994). The significant finding in this study was that it demonstrated clearly that level of CR1 on erythrocytes was critical in stimulation of the macrophages by the ICs. The erythrocytes with low CR1 were able to inhibit less TNF-α production compared to the erythrocytes from high expressers CR1 ($p=0.001$ using ANOVA) and from medium expressers ($p=0.025$ using ANOVA). There was no significant difference in the inhibition of TNF-α production between medium and high expressers ($p=0.639$ using ANOVA). This observation seems to suggest that there is a threshold level of CR1 that is necessary for inhibition and this was achieved at medium level onwards.

Previous studies have suggested that CR1 might be important in the pathogenesis of severe malaria (Rowe *et al.*, 1997). Since CR1 has the ability to bind opsonized ICs, it was possible that individuals with erythrocytes carrying high levels of CR1 might be likely to form rosettes (Rowe *et al.*, 1997) and at the same time bind more ICs (Ng *et al.*, 1988). When these erythrocytes are sequestered in the microvasculature with IC load, they interact with the macrophages (Virella *et al.*, 2002), leading to production of high levels of TNF-α which is localized and thus causes cerebral malaria. This study also

showed that erythrocytes, besides inhibiting the TNF-α production, can become stimulatory upon soaking ICs on their surfaces (P=0.0005 using paired t-test). This supports the theory that loaded erythrocytes sequestered in the microvasculature stimulated macrophages to release TNF-α hence contributing to pathogenesis in cerebral malaria.

This study did not show that bound ICs stimulated macrophages in a manner proportional to the CR1 copy numbers on the surface of the erythrocytes. Probably the model used in thesis research had some inherent artifacts that could have contributed to the lack of significant differences in macrophages stimulation by loaded erythrocytes from the 3 groups. The IC concentration of 35μg/ml could have been insufficient hence the erythrocytes were not saturated enough especially the erythrocytes with high CR1. The TNF production increased from loaded erythrocytes with low CR1 but then went down when the erythrocytes with high CR1 were used. Apparently the level of saturation was minimal in erythrocytes with medium and high CR1 due to insufficient IC available. This argument was further corroborated by no difference in the stimulation of macrophages to release TNF-α by the supernatants obtained after the ICs were incubated with erythrocytes from the three categories with varying levels of CR1. The erythrocytes with low CR1 were supposed to bind less ICs hence more ICs should have remained unbound in the supernatants and resulted in relatively high stimulation of the macrophages. The supernatant obtained from the erythrocytes with high CR1 should have stimulated less since most of the opsonized ICs were bound and very little remained unbound to interact with the macrophages.

The data from this thesis research seems to support the suggestion that the type of severe malaria to which an individual is susceptible can be influenced by changes in the level of CR1 that occur with age. Thus, young children could be at risk of suffering from severe malarial anemia because they have low baseline levels of erythrocyte CR1, hence predisposing their erythrocytes to complement-mediated damage (Waitumbi *et al.*, 2004) and low IC binding capacity of their erythrocytes exposes them to the likelihood of having high systemic levels of TNF-α leading to anemia. Ironically, the low CR1 should offer protection to the young children from developing cerebral malaria due to reduced ability to rosette (Rowe *et al.*, 1997) and low IC binding capacity on the surface of erythrocytes. For the same reason, older children or individuals with high level of CR1 could be at risk of suffering from cerebral malaria but are protected from severe malarial anemia.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, this study has demonstrated that erythrocytes from different individuals are able to bind immune complexes in a manner proportional to the level of CR1 present on their surfaces and that the binding is complement dependent. It was observed that opsonized ICs induced macrophages to release TNF-α, and that the erythrocytes buffer macrophage activation by inhibiting the IC-induced TNF-α production. The erythrocytes inhibition ability or buffering capacity was in a manner proportional to CR1 levels on the surface of the erythrocytes. It was also noted that the erythrocytes upon soaking the ICs activated the macrophages to release TNF-α. However, this study was unable to clearly show that the loaded erythrocytes stimulated macrophages in a manner proportional to the CR1 levels probably due to the insufficient amount of ICs used. However, the data supports suggestions that the levels of CR1 maybe critical in determining whether an individual is predisposed to severe malarial anemia or cerebral malaria and is involved in the pathogenesis of severe malaria.

6.2 Recommendations and Suggestions for Future Research

a) There is need to design experiments with sufficient ICs to saturate the erythrocytes to determine more clearly if loaded erythrocytes are able to stimulate TNF-α production in a manner proportional to the CR1 level on the surface. The results might partly help in explaining the cerebral malaria pathogenesis.

b) A study designed to address the questions that arise concerning the exact receptors on the macrophages that are involved in the activation is necessary. Use of antibodies to block Fc receptors and/or CR1 will go a long way in answering such questions.

c) The erythrocytes used in the study were from healthy (and malaria free) individuals, red cells from individuals suffering from cerebral malaria and malarial anemia should be used to repeat the study and check if the outcome corroborates the findings in this study.

d) The CR1 on the erythrocytes seems to be involved in the pathogenesis of severe malaria and intervention strategies such as the use of vaccines requires a clear understanding of the mechanisms that come into play during the disease state.

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