CYTOKINE AND EFFECTOR MOLECULE REGULATION AS DETERMINANTS OF HEMATOLOGICAL OUTCOMES IN RHESUS MACAQUES DURING *PLASMODIUM COATNEYI*-MALARIA

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Non-human primates are often used in the development and testing of vaccines and therapeutics for malaria. Infection of rhesus macaques (*Macaca mulatta*) with *Plasmodium coatneyi* causes cerebral malaria (CM) at high levels of parasitemia. To prevent mortality, parasitemia is frequently treated prior to this point when cerebral signs are largely absent. The public health significance was to determine the validity of this as a model for studying malarial anemia (MA) by examining hematological profiles, cytokines, and effector molecules shown to be important in childhood MA, including tumor necrosis factor (TNF)-α, interleukin (IL)-10, (IL)-12p40, and interferon (IFN)-γ, and nitric oxide (NO) at eight different time points during the acute infection. Peripheral parasitemia was monitored daily throughout infection. Venepuncture was performed for hematology panels on days 0, 2, 5, 7, 8, (peak parasitemia), 10, 15, 22, 34, 42, 56, and 150 post-infection (PI) for monitoring acute and chronic malaria infection. Complete blood counts (CBC) revealed that monocytes (MO) were highest during the initial rise in parasitemia, neutrophils (NEU) were highest at peak parasitemia, and lymphocytes (LY) remained constant throughout infection, except at peak parasitemia where levels were dramatically reduced. Platelets (PLT) declined shortly after infection and decreased until day 15, where levels sharply increased to higher than baseline values on day 19 PI. Hemoglobin (Hb) was lowest at parasite clearance (day 15 PI). Elevated peripheral blood mononuclear cell

(PBMC) transcripts (determined by real time RT-PCR in circulating blood mononuclear cells) and plasma levels (determined by ELISA) were associated with enhanced disease severity (peak parasitemia, thrombocytopenia and anemia). Plasma cytokine levels were not correlated with PBMC transcript levels, suggesting that the plasma cytokine levels may be from multiple cellular sources in addition to PBMC. Low IL-10 relative to TNF-α (IL-10/TNF-α plasma ratio) coincided with the lowest Hb concentration, a finding we have shown in children with MA. Taken together, these results demonstrate similar patterns of cytokine and effector molecule dysregulation in rhesus macaques infected *P. coatneyi* as that observed in humans with *P. falciparum*-induced malarial anemia.

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DEFINITION OF TERMS

Post-infection (PI)
Hemozoin (Hz)
Interferon (IFN)-γ
Tumor Necrosis Factor (TNF)
Interleukin (IL)
Nitric Oxide (NO)
Hemoglobin (Hb)
Hematocrit (Hct)
World Health Organization (WHO)
Cerebral Malaria (CM)
Malarial Anemia (MA)
Severe Malarial Anemia (SMA)
Monocyte (MO)
Lymphocyte (LYM)
Neutrophil (NEU)
Platelet (PLT)
White Blood Cell (WBC)
Red Blood Cell (RBC)
Mean Cell Volume (MCV)
Mean Corpuscular Hemoglobin (MCH)
Mean Corpuscular Hemoglobin Concentration (MCHC)
Mean Platelet Volume (MPV)
Red Blood Cell Distribution Width (RDW)
Complete Blood Cell Count (CBC)
Peripheral Blood Mononuclear Cells (PBMC)
Thrombospondin Related Adhesive Protein (TRAP)
Parasitized Red Blood Cells (pRBC)
Anticoagulant Citrate Dextrose solution (ACD)
Ethylenediaminetetraacetic acid (EDTA)
Roswell Park Memorial Institute media (RPMI)
Fetal Calf Serum (FCS)
Reactive Nitrogen Intermediates (RNI)
Semel in Die (Latin: Once A Day) (SID)
Per Os (Latin: by mouth, orally) (PO)
Intramuscular (IM)

1.0 INTRODUCTION

1.1 PURPOSE

Malaria is a global health threat with an estimated 300-500 million new infections and 1.5-2.7 million deaths attributed to this disease annually (1, 2). As drug resistance continues to be a problem and with no available vaccination, it is critical to gain a further understanding of the molecular mechanisms and immune regulation of malaria. Developing appropriate model systems is essential for investigating the molecular immunological basis of the disease for use in vaccine and pharmacologic trials. Non-human primates, such as rhesus macaques (*Macaca mulatta)* infected with a species-specific malaria strain *(Plasmodium coatneyi)*, have been shown to be a useful model system for studying CM. (3),(4), and have also been shown as a valid model of malaria in pregnancy (5) Several other model systems exist to study malaria, but the species are not as closely related to humans and differ in response to malaria including murine/*P. berghei* (6) and *Aotus*/*P. falciparum* models (7),(8).

In human infections with malaria, we know that the interactions between pro (Th-1) and anti-inflammatory (Th-2) cytokines play a critical role in the immunopathogenesis of malaria (9), (10). Pro-inflammatory cytokines are released primarily by leukocytes in response to numerous different factors during a malaria infection, and have some similar functions to the cells that produce them. In this project, we are examining the immune modulation from a hematological perspective on the role of cytokine and effector molecules in rhesus macaques during an

infection with *Plasmodium coatneyi* to determine if this could be a suitable model system for examining malarial anemia.

1.2 MALARIA

1.2.1 Global Impact

Malaria exists in 100 countries but is mainly confined to developing tropical areas of Africa, Asia and Latin America. There are over 300 million of people affected by malaria world-wide each year, resulting in more than a million deaths (1). Around 90% of these deaths occur in Africa, with children less than five years of age bearing the largest death burden of malaria, due mostly to their non-immune state (2). *Plasmodium falciparum* (in humans) is the main cause of severe clinical malaria and death. It is estimated that over 50% of the world's population is at risk for malaria, which is a 10% increase in the past ten years (2).

The distribution of malaria is solely dependent upon the occurrence of the *Anopheles* mosquito, as it is the only species capable of serving as the host for the *Plasmodium* parasite (Figure 1). Of the four species of *Plasmodium* that infect humans, *P. falciparum* is most widespread, and causes the most severe malaria. *Falciparum* malaria is also found in areas of Asia and South America, though *P. vivax* malaria is more common. One reason why an overwhelming proportion of the malaria burden exists in Africa is that many countries lack the infrastructure and resources necessary to mount a sustainable campaign against malaria, and as a result, very few African countries have benefited from historical efforts to eradicate malaria. In Africa, malaria accounts for 40% of public health expenditure, 30-50% of inpatient hospital admissions, and up to 50% of outpatient hospital visits in areas with high malaria transmission

(1). Malaria is most prevalent in developing areas of the world (58% of cases). This complicates matters in that these patients have the least access to good health care and have economic struggles that are only exacerbated by the burden of malaria (2).

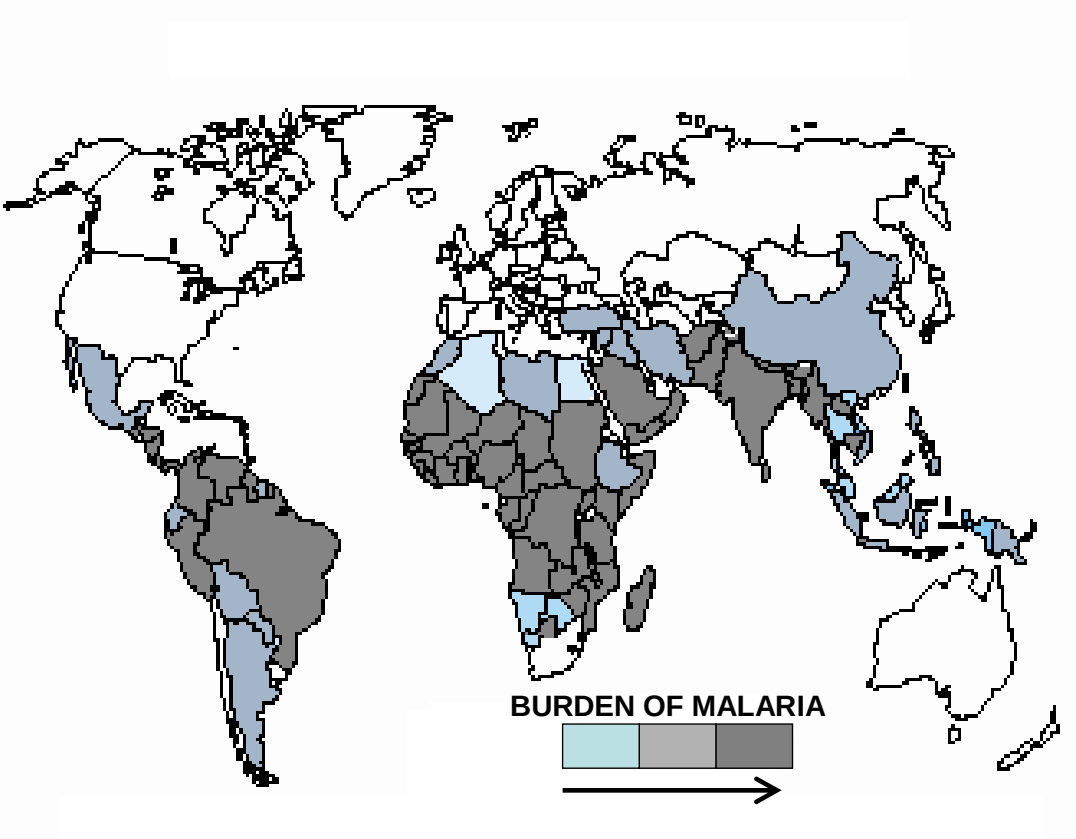


Figure 1: Global Burden of Malaria

1.2.2 *Plasmodium* Life Cycle

Four species (*P. vivax, P. ovale, P. malariae, and P. falciparum*) in the *Plasmodium* genus of protozoa within the subfamily of *Haemosporidia* infect humans. Malaria is a complex disease caused by the single-celled *Plasmodium* blood parasite. The parasite's life cycle is split between the vertebrate host and the insect vector, the *Anopholes* mosquito (Figure 2). The

mosquito becomes infected when it takes a blood meal containing *Plasmodium* gametocytes, which pass into the salivary glands of the mosquito and travel to the gut, where they reproduce sexually and develop into sporozoites. Unlike the human host, the mosquito host does not suffer noticeably from the presence of *Plasmodium* parasites.

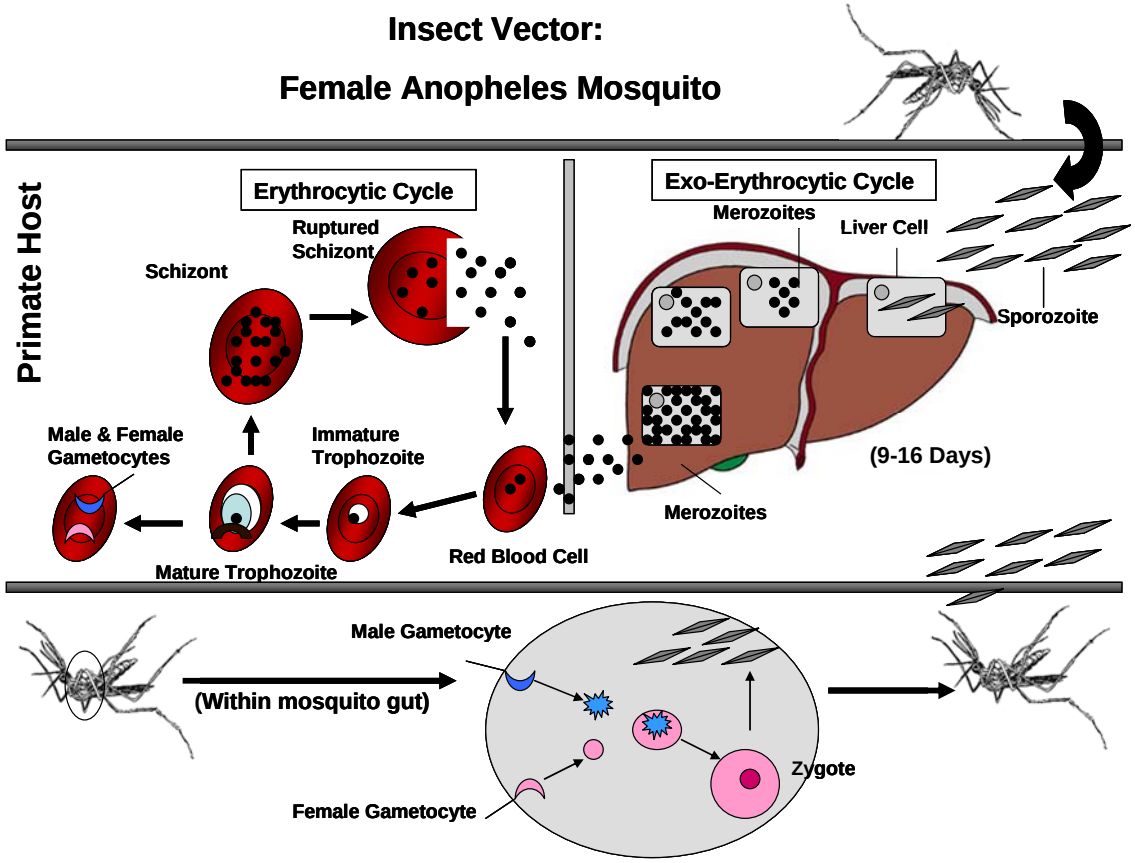


Figure 2: *Plasmodium* Life Cycle

Two stages of the *Plasmodium* life cycle exist in the human host: the exo-erythrocytic and erythrocytic cycle. The exo-erythrocytic cycle occurs when the *Anopholes* mosquito injects its saliva and *Plasmodium* sporozoites into tiny blood vessels of the human host during a blood meal. The sporozoites travel through the bloodstream to the liver, where the parasite enters the liver cells. The co-receptor on sporozoites involves thrombospondin domains on circumsporozoite protein and thrombospondin related adhesive protein (TRAP), which

specifically bind the heparin sulfate proteoglycans on hepatocytes (11). In the liver, the sporozoites divide (tachysporozoites) and mature into thousands of merozoites in period of 9-16 days (12). The distended liver cell bursts open and releases the merozoites into the blood stream. The parasite can only survive if it rapidly attaches to and enters the host RBCs. In order to enter the RBC, the parasite must engage the receptor, undergo apical re-orientation, junction formation and signaling. It is not certain which merozoite surface signal recognizes the RBC surface signal. When the merozoite enters the RBC, the erythrocytic cycle begins. Once inside the RBC, the parasite matures and divides, going from merozoite, to trophozoite and then schizont stage, forming up to 32 daughter merozoites.

Disease begins when the asexual parasite multiplies within the RBC. After expansion of the parasite, the RBC bursts, releasing merozoites to invade other erythrocytes. This exponential growth and destruction of RBCs contributes to the anemia associated with *P. falciparum*-malaria. Within the erythrocytes, merozoites can also differentiate into male and female gametocytes, which are released upon rupture of parasitized RBCs (pRBC). During a blood meal of the infected human host, the mosquito ingests gametes which fuse to form a diploid zygote. The zygote develops into an oocyst, within which sporozoites are produced and then transmitted to the human host during the mosquito's subsequent blood meals (12).

1.2.3 Pathophysiology

The release of merozoites into the bloodstream gives rise to symptoms that include fever, shivering, joint pain, headache, repeated vomiting, generalized convulsion, and coma. According to the World Health Organization (WHO) criteria of malaria, anemia (Hb < 5g/dl), renal failure (serum creatinine >3 mg/dl), pulmonary edema, hyperparasitemia (> 5% of

erythrocytes infected), jaundice, circulatory collapse, and/or shock are all clinical signs of severe malaria (13). The mortality from malaria comes from complications of the infection. Cerebral manifestations can lead to coma and severe and refractory anemia. This can lead to hypoxia and cardiac decompensation. All of the typical clinical and severe disease pathology associated with malaria is caused by the asexual erythrocytic or blood stage parasites.

1.2.3.1 Parasitic Products

Hb is the oxygen-carrying protein in RBCs, made up of globin chains and heme. The *Plasmodium* parasite feeds on Hb, but heme, which is released when the parasite digests Hb, is toxic to and lyses malaria parasites (14), (15), (16). Complete removal of toxic molecules is achieved by the parasite by a process of biocrystallization. In order to avoid the toxic effects of heme, the parasite incorporates the released heme into hemozoin (Hz), malarial pigment, which is an insoluble polymer of ferriprotoporphyrin (FP-IX). (17). When the parasite develops in the erythrocyte, numerous known and unknown waste substances such as Hz and other toxic factors accumulate in the infected RBC. These toxins are emptied into the bloodstream when the infected RBCs lyse and release invasive merozoites.

Monocytes and macrophages phagocytose *P. falciparum*-derived Hz during a malaria infection by either the phagocytosis of pRBCs or Hz released after the RBC bursts (18). The Hz and other toxic factors such as glucose phosphate isomerase (GPI) from merozoite surface proteins (MSP)-1, and -2, stimulate monocytes, macrophages and other cells to produce cytokines and soluble factors which act to produce fever, rigors and influence other severe pathophysiological features associated with malaria. Previous studies indicate that Hz and

synthetic malarial pigment (β-hematin) cause up-regulation of TNF-α from human PBMC (19), (20), (21), (22).

1.2.4 Immune Response

1.2.4.1 Cytokine and Effector Molecule Regulation

The cytokine response that leads to malaria is still not fully explained, nor is the role of cytokines at different stages of infection. The severity of disease may vary depending upon the level and the type of cytokines produced after malaria parasite infection. Cytokines can play both protective and pathogenic roles during malaria infection, and also mediate interactions between humoral and cellular immune responses (23). Cytokine release is triggered by Hz (24), merozoites, rupture of parasitized RBCs, and by parasite antigens, such as the GPI anchor on plasmodial antigen (25).

Production of pro-inflammatory cytokines by phagocytic cells is a hallmark of malaria infection (Figure 3). This type 1-biased immune response to *P. falciparum* infection is culminated primarily by the release of TNF-α and IFN-γ which directly or indirectly contribute to parasite killing through activation of phagocytosis or NO dependent pathways. In addition, the pro-inflammatory response to infection is often a precursor to severe clinical sequelae during malaria pathogenesis (26). Protective immunity in malaria is coordinated by the release of pro-inflammatory cytokines such as IL-12, TNF-α, and IFN-γ (27). IL-12, released from primarily monocyte-macrophages, initiates the inflammatory cascade through the activation of cytotoxic and natural killer cells (28). Acute *falciparum*-malaria induces suppression of IL-12 that is associated with leukocyte ingestion of Hz and disequilibrium in the balance of IL-12,

TNF-α, and IL-10 (29), (30). IL-12 induced protection in malaria is related to the ability of IL-12 to promote secretion of IFN-γ from Th1 cells (31), (32), (33). IL-12 has been suggested to stimulate erythropoiesis through the induction of IFN-γ production (34). Elevated IL-12 levels have been shown to be inversely correlated with parasitemia, which may be related to the impaired function of monocytes due to acquisition of Hz. Non-immune African children with severe malaria have been shown to have reduced levels of IL-12 relative to those with mild malaria, and high levels of TNF-α and IL-10 (29).

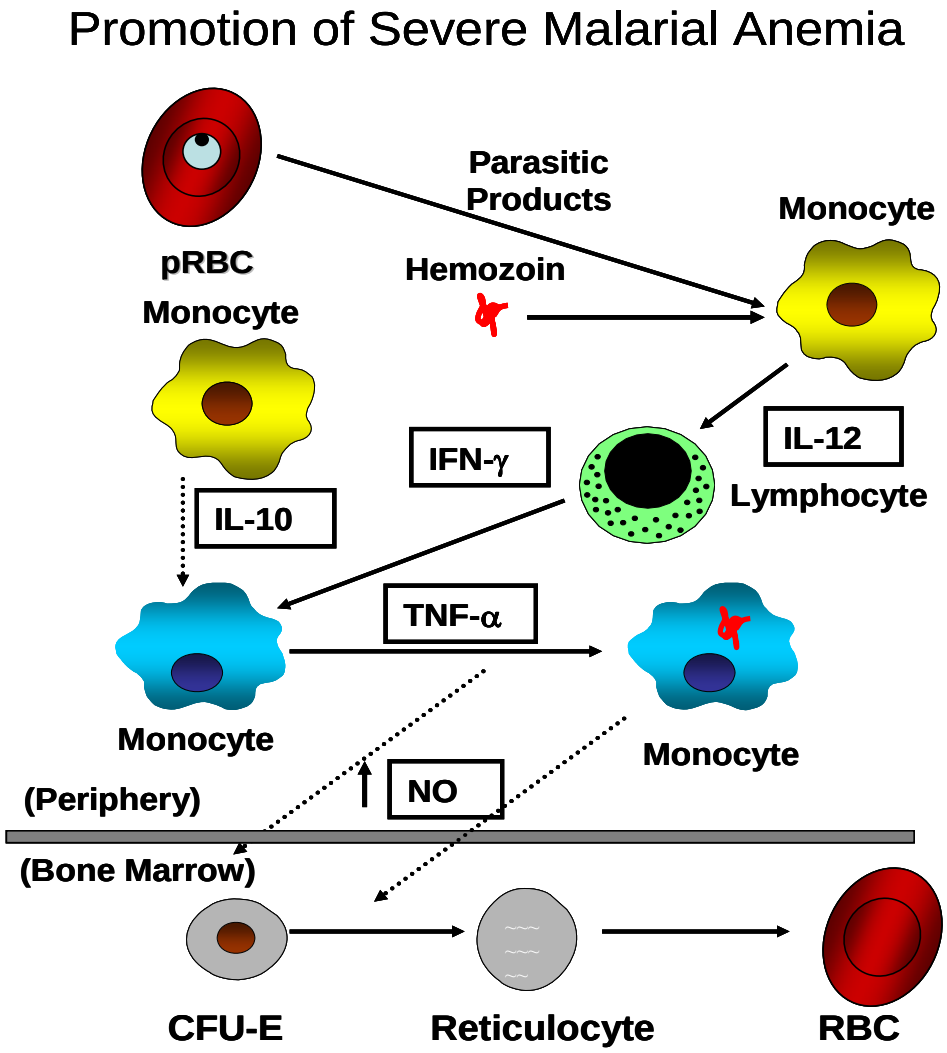


Figure 3: Proposed Model of Cytokine and Effector Molecule Dysregulation during Malarial Anemia

IL-12 stimulates the release of pro-inflammatory IFN-γ from CD4+ T cells. IFN-γ activates macrophages to increase phagocytosis and cytotoxicity, aiding in parasite killing and clearance. High serum levels of IFN-γ have been shown in malaria-naïve adults to correlate with their first clinical episode of acute *P. falciparum* malaria (35) and with level of parasitemia (36). Levels of IFN-γ are lower in humans who live in malaria endemic areas than those who are malaria-naïve and exposed for the first time (37). IFN-γ has been shown to also increase in the 24 hour period before onset of fever and parasitemia (38). It has been suggested that high levels of IFN-γ-inducing macrophage activation may promote anemia (39). IFN-γ upregulates cytoadherence ligand intercellular adhesion molecule (ICAM)-1, which leads to an increase in sequestration of the parasite (40). Children with CM have lower levels of plasma IFN-γ relative to those with uncomplicated malaria, which implicates this cytokine to play a protective role in preventing disease severity and CM (41). While the early acute release of IFN-γ may be protective, the sustained release of IFN-γ may be a factor in severe malarial anemia (SMA) (42), (43).

During malaria, IFN-γ stimulates monocytes-macrophages to release TNF-α. Over-production of TNF-α is associated with promotion of anemia in a variety of chronic and acute inflammatory diseases (44), including anemia associated with rheumatoid arthritis (45). Serum levels of TNF-α have been shown to be correlated with both parasitemia and disease severity and severe anemia (46), (47). However, it is clear that TNF-α is not solely responsible for disease severity as high serum levels have also been found in *P. vivax* infections where severe disease is rare, suggesting that other factors also contribute to pathogenesis (48). TNF-α is a pyrogen, know to cause fever in malaria. Studies have shown that fever induced by TNF-α may substantially reduce the growth of erythrocytic forms of *falciparum* (49). Overall these studies

suggest that low levels of TNF-α may be protective while overproduction of TNF-α is pathogenic during malaria infection.

Anti-inflammatory IL-10 is produced by monocytes and T-cells in response to pro-inflammatory cytokines. B-1 cells require IL-10 for both proliferation and maturation. In a recent study, B cell knock-out mice did not produce IL-10, but only presented a Th1 response, whereas normal mice presented first a Th1 response, then at peak parasitemia, switched to a Th2 response with IL-10 production (50). IL-10 also has been shown to suppress macrophage function (51). IL-10 downregulates pro-inflammatory cytokine production, and has been shown to down-regulate TNF-α over-production from monocytes (52), (53). Induction of IL-10 synthesis by IL-12 serves as a negative feedback loop by which IL-12 limits its own pro-inflammatory effects (54). It has been demonstrated that the serum levels of IL-10 correlate with disease severity in severe malaria, and were up to three times higher than in those with uncomplicated malaria (33). Ratios of IL-10/IL-12 were shown to be higher in children with severe malaria than those with mild malaria (29).

Plasma levels of TNF-α and IL-10 have been shown to be lower in children with SMA relative to those with mild or uncomplicated malaria (29). Children with SMA also show a low IL-10 level relative to TNF-α level (55), (56), (29), (57). IL-10 may prevent development of SMA by controlling excessive levels of TNF-α (58). Increased IL-10 production has been also associated with quicker malarial parasite clearance (59), and is an important regulator in protecting against malarial anemia and the harmful over-production of TNF-α.

Pro-inflammatory cytokines IL-12, IFN-γ and TNF-α increase NO production through the enzyme inducible NO synthase (iNOS), while anti-inflammatory cytokines such as IL-10 decrease NO production (60). NO is produced by monocytes, macrophages, hepatocytes and

other cells and eliminates pathogens through oxidative toxicity (61). Through iNOS, large amounts of NO can be produced for sustained periods of time. There are a number of contradictory results in studies addressing the role of NO in malaria. It has been suggested that the ability to produce more NO is protective against severe malaria (62), (63), (64). NO has also been shown to be protective against blood stage parasites in murine malaria (31). One way NO aids in protection in malaria is thorough destruction of gametocytes (65). NO is a cytotoxic free radical that has been shown to be important in parasite clearance by monocytes, macrophages and polymorphonuclear lymphocytes (66), (67). It has been shown to be protective through the downregulation of ICAM-1, thereby decreasing parasite vascular adherence (68). NO produced from upregulation of iNOS by TNF-α, has been implicated in the pathogenesis of severe malaria such as coma, acidosis, and hypoglycemia, and poor prognosis (69),(70), (71). Patients with mild malaria had significantly higher levels of iNOS expressed by monocytes than those with severe malaria, suggesting it may be an important protective factor in malaria (64),(72). *In vitro,* NO has been shown to suppress the growth of *Plasmodium falciparum* (66).

1.2.5 Cerebral Malaria

Plasmodium falciparum-infected erythrocytes, particularly those with mature trophozoites, can adhere to the vascular endothelium of blood vessel walls instead of freely circulating through the bloodstream. Parasite adhesion is likely dependent both on age and exposure of the host. Vascular adhesion of pRBC is similar to that of leukocytes, undergoing adhesion, rolling, tethering, before firmly attaching. Parasite adhesion involves CD36 and chondrioton sulfate (CSA) receptors on host cells, and both ICAM-1 and vascular cell adhesion molecule (VCAM)-1 (13). Sequestration of infected erythrocytes in the post-capillary venules of

the brain causes CM, which is associated with high mortality. Parasite sequestration may enhance parasite survival by preventing their destruction in the spleen (73).

CM is characterized clinically by reduced consciousness and coma, seizures, metabolic acidosis, hypoglycemia and retinal hemorrhaging (74). CM is prevalent in areas of moderate and seasonal transmission, and frequent in children (75). Children who recover from CM typically do so within 48 hours (74). Parasites adhere to the endothelial wall, which hinders the flow of arteriole blood to tissues; the brain particularly is sensitive to this deprivation. About 10% of children who recover from CM will have suffer sustained neurological damage such as blindness, deafness, or cognitive deficits (76), (77). Acidosis can lead to coma in CM, which is associated with poor prognosis (78). Accumulation of infected erythrocytes favors local development of cytokine-mediated inflammatory reactions which can damage endothelial cells and alter nervous system function (41). Cytoadherence of parasitized RBCs is due to interaction of pRBC ligands and receptors on endothelial cells, namely ICAM-1 and (VCAM)-1 (79). Soluble levels of both of these molecules have been positively correlated with disease severity in human malaria (80).

1.2.6 Malarial Anemia

Severe *P. falciparum* malaria in African children is frequently characterized by severe anemia, hypoglycemia, hyperparasitemia, and respiratory distress (81), (82), (83). SMA is responsible for the largest amount of morbidity and mortality associated with *P. falciparum* malaria (84). SMA in Africa is seen most frequently in areas of very high malaria transmission (75). Those most at risk are pregnant women and children under 2 years of age (85), (75), (86).

The WHO defines SMA in humans as having both a parasitemia of greater than 10,000 parasites μL^{-1} and a Hb concentration of less than 5 g/dL or a hematocrit (Hct) of less than 15% (87).

Reference values for Hb and Hct vary in different populations, and are unknown in many tropical areas of the world. Typically, malarial anemia as having a Hb concentration of less than 8 g/dL or less than a 24% Hct (88). Malarial anemia is also defined as Hb concentration two standard deviations below the mean. Anemia associated with malaria is complex, not resulting from a single source, with a wide spectrum of clinical manifestations. Malarial anemia may occur in the absence of overt clinical symptoms and is often disproportionate with parasitemia. Peripheral parasitemia can underestimate parasite burden due to parasite sequestration (89), which could partially explain why the relationship between level of parasitemia and degree of anemia are not always correlated in acute malaria.

Malarial anemia is caused by a number of factors including the excess removal of nonparasitized RBCs, immune destruction of parasitized and non parasitized RBCs, and bone marrow dysfunction (90). There are two main clinical patterns of malarial anemia: chronic and acute (91). Chronic malarial anemia is commonly seen in children that live in malaria-endemic areas and is characterized by a more long-term and controlled parasitemia (43). In chronic malarial anemia, phagocytized Hz is commonly seen in blood films. The clinical presentation appears several weeks prior and includes a low grade fever, general ill health and in some instances, splenomegally (43). The marrow is hyperactive in chronic malaria, and anemia is primarily caused by dyserythropoiesis and no reticulocyte release (92). The mechanism of bone marrow inhibition in the presence of EPO in malaria is not well understood, but may be due to the release of cytokines, such as TNF-α (93), macrophage dysfunction due to ingestion of Hz (94) or effect of parasite products on bone marrow (92).

During acute malarial infections, clinical symptoms may appear prior to the onset of anemia, however the anemia is rapid once it appears (43). Suppression of bone marrow in acute malaria is partly a response to a decrease in erythropoietin (EPO) (90), which is downregulated by pro-inflammatory cytokines. Drop in Hb and Hct can be due to the decline in parasitemia post-treatment, or from the destruction of uninfected RBCs (43).

1.2.6.1 Types of Anemia

There are three main functional classifications of anemia which are based on abnormalities in erythron function which are clinically recognizable (95). They are hypoproliferative, maturation disorder, and hemorrhagic or hemolytic anemia. Hypoproliferative anemia is usually associated with acute or chronic inflammatory disease (95). It can also be due to iron deficiency, a decrease in EPO response or marrow damage (suppression of early stem-cell proliferation) (95). Hypoproliferative anemia is characterized by having normocytic and normochromic erythrocytes, but with a low reticulocyte index (<2.0) relative to the severity of anemia (95). In maturation disorders there is either a micro- or macrocytosis, combined with a low reticulocyte index, abnormal red cell precursors, and a proliferative marrow (95). Maturation disorders are either due to cytoplasmic maturation defects which characterized by a decrease in Hb synthesis resulting in microcytosis, or due to nuclear maturation defects, characterized by a macrocytosis (95). Cytoplasmic maturation defects are caused by iron deficiency, defects in Hb synthesis or defects in mitochondrial function and porphyrin synthesis (sideroblastic anemias) (95). Hemorrhagic/hemolytic anemias also show normocytic and normochromic RBCs (95). Hemolytic anemia is a disorder in which the RBCs are destroyed faster than the bone marrow can produce them (95).

1.2.6.2 Hematological Perspective

In malaria, monocytes phagocytose pRBCs and Hz, and are involved in presentation of antigen and the production of cytokines. Blood monocytes also synergize with antibodies to inhibit parasite growth by antibody-dependent cellular inhibition (96). After ingestion of Hz, the function of monocytes and macrophages is severely altered (94). It has been suggested that macrophage function may also be altered by the binding of infected cells to host cell receptors CD36 or CD51 (97).

Lymphocytes are categorized into two distinct cell lines, which are morphologically identical but distinguishable by cell-surface markers. B cells are the plasma cells and memory cells, and are responsible for humoral immunity. T cells are responsible for both cell-mediated immunity and for activating B cells. Lymphocyte function in malaria includes antibody production and parasite killing. Reactive lymphocytes and plasma cells are increased for a short time during malaria, especially in acute malaria (98), (88). Neutrophils are important in mediating phagocytosis of *Plasmodium falciparum* forms (99). Excess pigment in neutrophils and macrophages and an elevated neutrophil count are indicators of poor prognosis (100).

PLT function in promoting blood clot formation, preventing bleeding, and repairing damaged vessels. PLT, as with RBCs, are produced within the bone marrow and originate from megakaryocytes. Thrombocytopenia is a decreased production of PLT due to suppression of erythropoiesis by pro-inflammatory cytokine over-secretion (101). Thrombocytopenia is thought to be caused by increased sequestration in the spleen, immune-mediated destruction, and decreased PLT survival (102). PLT have been shown to bind parasitized RBC via the PLT surface glycoprotein CD36 (103), and may contribute to the occlusion in vasculature during malaria. Thrombocytopenia and strong PLT stimulation are associated with *falciparum*-malaria

(104). PLT have also been shown to be a factor in pathogenesis of malaria through adhering with the sequestered pRBC, impairing blood flow which results in decreased tissue perfusion (105), (106). Mean platelet volume (MPV) gives information about PLT production in bone marrow. New PLT are larger resulting in an elevated MPV when increased numbers of PLT are being produced (43). MPV has shown to decrease with increasing parasitemia, but after parasite clearance, increased significantly to counterbalance the loss of PLT during parasitemia (107).

Most RBCs are destroyed by the parasite during schizogony, by host immune response through phagocytosis by monocytes and macrophages, by opsonization through immunoglobulins and complement, or less frequently through natural killer cells and antibody-dependent cytotoxicity (108). In addition to removing pRBCs, the spleen is capable of "pitting", which is the extraction of young parasites from the RBC (109). In SMA, destruction of non-parasitized RBCs is what accounts for the majority of the decrease in Hct, whereas in uncomplicated malaria, it accounts for only 10% of the total RBC loss (110). In acute malarial anemia, the destruction of non-parasitized RBCs is up to eight and a half times that of parasitized RBCs (111). In addition to hemolytic anemia, there is also a dyserythropoiesis in malarial anemia. Bone marrow erythroid cells are increased in dyserythropoiesis, but RBC output is decreased, resulting from bone marrow dysfunction (91). Pro-inflammatory cytokines, such as TNF-α have been implicated in bone marrow depression (93). Administration of recombinant TNF-α *in vitro* inhibits the proliferation of hematopoietic stem cells (112) and *in vivo* has been shown to induce early dyserythropoiesis in mice with malaria (93).

The three factors for determining anemia from hematological panels are decreases in the RBC count, Hb and Hct. Hb is necessary for the transport and delivery of oxygen throughout the body. Hct is the percent volume of the blood that is taken up by the RBCs. The Hb count

divided by the Hct gives the mean cell hemoglobin (MCH). A low MCH could indicate that there is a coexistent microcytic anemia (43). Loss of Hb in malarial anemia also occurs before the pRBC is destroyed. Up to 75% of the Hb in the RBC can be digested by parasite (113).

The mean corpuscular volume (MCV) provides a measurement of the average size of the red blood cell, which is useful in determining the factors of anemia. An elevated MCV indicates macrocytic RBCs, while a decreased MCV is indicative of microcytic RBCs, such as seen in anemia associated with iron deficiency (95). Mean corpuscular hemoglobin concentration (MCHC) is a calculation of the percentage of Hb in the RBC. Hypochromia is an anemic condition in which the percentage of Hb in RBCs is abnormally low (decreased MCHC), resulting in decreased oxygen carrying capacity (95). Red cell distribution width (RDW) is a calculation of the variation in the size of RBCs. Increased red cell population dispersions or red cell distribution width (RDW) has been observed in malaria, and has been attributed to the red cell response to malarial parasite, and correlated with the degree of macrocytosis (114). Low RDW signifies a normal, homogeneous population of cells, where as an increased RDW signifies heterogeneity in cell size as a result of active hemtaopoiesis due to large immature red cells (reticulocytes) (95). RDW can also be used as a marker of release of early erythrocytes and reticulocytes (95). A study in Ghanaians with *falciparum*-malaria showed that high RDW correlates with the initial levels of EPO, suggesting that there is a quick, reversible suppression of the bone marrow response to EPO during malaria (115).

1.2.6.3 Hematological Profile during Acute Malaria

Leukopenia during acute *falciparum*-malaria is common in non-immune adults (43). Changes in total WBC in malaria are generally reflective of neutrophil counts, hence

neutropenia, due to increased neutrophil margination, is common in acute malarial infections (43). Pro-inflammatory cytokine release, such as IL-12 induces neutrophil release from the marginating pools. Attraction of neutrophils to the site of inflammation is caused by chemotactic factors including complement (51). Lymphopenia is common in acute malaria of non-immune adults, and appears to resolve within 72 hours of anti-malarial treatment (43). But in some cases, more common in children with acute malaria, there is a mild lymphocytosis rebound (43). Monocytosis is common in children and non-immune adults with acute malaria (116). Acute infections in non-immune people present in mild to moderate thrombocytopenia, which is less frequent in immune and semi-immune populations (117). Adults with acute uncomplicated malaria have >90% RBC loss accounted for by the destruction of nonparasitized RBCs (118), therefore anti-malarial treatment will only influence one in ten RBCs and the recovery from anemia will occur rapidly. As well, non-immune individuals have suppression of erythropoiesis in acute malaria (119), due, in part, to the overproduction of TNF-α (93). There is a decrease in Hb after treatment of acute malaria, which has been shown to correlate with the level of parasitemia when the patient was admitted to the hospital (91).

1.2.6.4 Hematological Profile During Chronic Malaria

Leukocytes play an important role in malaria through phagocytosis, cytotoxicity, and cytokine production. Leukocytosis is common in severe and complicated malaria, and has been associated with severe disease (120). Neutrophils function in phagocytosis, respiratory burst, and release of defensins during malaria. Neutrophilia is seen in patients with severe malaria and has been associated with poor prognosis (116). Monocytosis is less common in children with chronic malaria. MPV has been shown to be increased in children with malaria (121), which

may be due to compensation for peripheral PLT destruction through the early release of PLT from the bone marrow. In chronic malaria, dyserythropoiesis and functionally ineffective erythropoiesis are the primary factors in anemia (88), (92). Chronic malaria is characterized by a low grade parasitemia, slow recovery and long-lasting anemia (91).

1.2.6.5 Impact of Cytokines and Effector Molecules on Hematological Outcomes

Monocytes and macrophages function in phagocytosis, release of cytokines, reactive nitrogen intermediates, lysozymes and other cytotoxic molecules (51). Pro-inflammatory cytokines IFN-γ and TNF-α activate monocytes to induce phagocytosis and release of other cytokines and effector molecules, whereas anti-inflammatory cytokines, such as IL-10, prevent the overproduction of pro-inflammatory cytokines through feedback inhibition (122), (123), (60). A high pro- to anti-inflammatory cytokine ratio is associated with higher Hb concentrations (124), whereas overproduction of TNF-α and decreased IL-10 levels are associated with low Hb concentrations (125). A balance between Th-1 and Th-2 responses is critical in the outcome of the pathogenesis of malaria. Pathogenesis in malaria is also dependent upon the order of cytokine exposure, which determines the function of macrophages and other immune cells (126).

The overproduction of TNF-α during malaria has been shown to impact RBC production through both ineffective erythropoiesis and suppression of erythropoiesis. (127). Erythropoiesis is controlled by EPO, secreted by the kidneys. High levels of EPO stimulation cause a shift of marrow reticulocytes into the blood circulation. Chronic and acute inflammation influence iron-deficient erythropoiesis since inflammatory cytokines suppress EPO and stem-cell proliferation. Key cytokines involved in the suppression of EPO are TNF-α, IL-1, and IFN-γ, and effector molecules such as NO (127). In children with malarial anemia, NO is elevated and has been

inversely correlated with levels of Hb (128). Increases in plasma TNF-α during acute inflammatory infections directly suppresses EPO, and blocks the release of iron from reticuloendothelial stores (129). In chronic infections, IFN-γ has been shown to suppress erythroid precursor growth. Inflammatory cytokines impact RBC survival, production, and interfere with the delivery of iron to the erythroid marrow, resulting in a hypochromic anemia (95).

1.2.7 Animal Model Systems for Malarial Research

Malaria is a global health threat with an expanding number of cases each year. The situation is complicated by the increase of anti-malarial drug resistance and the lack of an effective vaccine. Understanding the immunologic basis of protective immunity and identification of novel therapeutic targets are therefore increasingly important. A model system is required for investigation of molecular- and mechanistic-based immunologic studies for use in vaccine and pharmacologic trials. There are virtually no longitudinal cohort studies looking at cytokines and hematology during *P. falciparum* infection in children. This is complicated due to many factors including the limited amount of blood that can be taken from children, the actual time of primary infection, duration of primary infection, and occurrence of subsequent malarial infections. Therefore animal models are able to provide an ideal and controlled model to examine the pathogenesis of malaria over time.

1.2.7.1 *Plasmodium falciparum* and *Plasmodium coatneyi*

An effective disease model should closely mimic the pathogenesis of malaria seen in humans. Non-human primates are and ideal candidate because they are susceptible to many species of *Plasmodium* and have humoral and cellular responses similar to those in humans (130). Macaques used in malaria research include pigtailed (*M. nemenstrina)*, cynos (*M. fascicularis)*, and rhesus *(M. mulatta).* Of these three species, rhesus are the best characterized and the most widely available. *Plasmodium coatneyi* is a simian malarial parasite that shares many clinical and pathological similarities to severe *falciparum* malaria in the human host (130). Rhesus infected with *P. coatneyi* have been shown to be a valid model system for CM (131). Rhesus with *P. coatneyi*-malaria share many pathologic characteristics are similar to humans with CM, such as presence of knobs, binding pRBC to endothelial cells, and the pattern of sequestration in the brain, evident from synchronized appearance of ring stage parasites and absence of late trophozoite/schizonts in the peripheral blood (4), (132).

Although the natural host for *P. coatneyi* is *M. fascicularis,* the best experimental vertebrate host of *P. coatneyi* has been the rhesus monkey. Blood induced infection in rhesus can be explosive with 33% mortalities in intact monkeys and up to 100% mortality in splenectomized monkeys. (130). *P. coatneyi* is a tertian malaria, completing one asexual life cycle every 48 hours. The species was discovered by Eyles in the *Anopholes hackeri* mosquito (130). One year later in 1962, *coatneyi* was isolated from *M. fasicularis,* it's natural host, in which it produces a mild persistent infection (130). *P. coatneyi* in the rhesus monkey results in a rapid developing, high parasitemia (300,000-500,000 parasites/mm^3), which is often fatal when left untreated (130). Cytokine profiles in rhesus with *coatneyi*-malaria resemble human cytokine profiles in *falciparum*-malaria (133), (132). However, if animals are treated with anti-malarials

at a 5% parasitemia, the infection remains sub-clinical for CM. Experimental infections of rhesus with *P. coatneyi* may be characterized by jaundice, anorexia, listlessness, fever, anemia, and splenomegally in spleen intact animals (130).

Sequestration of mature schizonts in *coatneyi*-malaria requires that blood films be performed at 24-hour intervals during the course of infection to monitor parasitemia. The prepatent period (before clinical symptoms of disease) ranges from 10-14 days, and peak parasitemia occurs between the 7-9 days PI (130). The parasite sequestration rate in the microvasculature in *P. coatneyi* infected rhesus macaques is approximately 80%, which is comparable to that of cerebral *falciparum*-malaria in humans (134). *P. coatneyi* exhibits many clinical manifestations similar to *P. falciparum* and is a valid model system for CM. Thus, it may represent a valid model system for examining malarial anemia associated with *P. falciparum.*

2.0 SPECIFIC AIMS AND HYPOTHESES

The goal in this study is to examine the immunopathogenesis of malaria in a potential model system for malarial anemia: rhesus macaques (*Macaca mulatta*) infected with *Plasmodium coatneyi*.

2.1 SPECIFIC AIM 1

Characterize the hematological profile during *P. coatneyi*-malaria infection, and determine if there is an association between hematological profiles and disease manifestations.

2.1.1 Hypothesis

Parameters of disease severity, such as a decrease in Hb and Hct or change in platelet levels are correlated with changes in parasitemia, and necessary factors to characterize anemia associated with malaria.

2.1.2 Rationale and Interpretation

To test this hypothesis, four adult, malaria-naïve rhesus monkeys were infected with *P. coatneyi* (1 x 10^6 pRBC from a donor monkey). On days 0, 2, 5, 7, 8 (peak parasitemia), 10, 15

(7 days post-anti-malarial treatment) and 22 (14 days post-treatment) 34, 42, and 56, and 150 PI, venous blood (0.5 mL) was collected in EDTA-containing vacutainer tubes for hematological analyses using a Coulter Counter. A CBC and differential was performed at each time point. Pre-infection samples from day 0 were used as baseline values for each of the four animals.

The hematological panels examined were: white blood cell (WBC) count, LY, MO, MPV and PLT counts. Red cell indices included RBC count, Hb, Hct, RDW, MCV, MCH, and MCHC. These values were analyzed relative to baseline using the Mann-Whitney U for statistical significance. Hematological indices were also compared to the parasite burned at different time points to examine the hematological regulation during malaria in rhesus macaques. Likewise, the infection was sectioned into an acute phase, which was from day 0 (initial infection) through day 15 PI (primary parasite clearance) and a chronic phase of infection, which was from day 22 [re-emergence of parasitemia (secondary)] through day 150 PI (complete parasite clearance). Regulation of hematological parameters was compared to disease progression during both the acute and chronic phases of malaria. Significance was tested by calculating levels of significance using randomization/permutation tests to obtain p-values for Pearson correlations.

2.2 SPECIFIC AIM 2

Determine the profile of cytokine and effector molecule production associated with *P. coatneyi*-malaria in rhesus macaques, and to determine if changes in soluble inflammatory mediators is regulated at the transcriptional level in circulating blood mononuclear cells.

2.2.1 Hypothesis

There is a unique profile of soluble cytokine and effector molecules regulated by transcriptional changes in circulating blood mononuclear cells associated with the pathogenesis of blood-stage malaria that can be used to predict disease severity and clinical outcomes.

2.2.2 Rationale and Interpretation

To test this hypothesis, four malaria-naïve adult rhesus macaques, *Macaca mulatta* were infected intravenously on day 0 with 1 x 10^6 *Plasmodium coatneyi*-infected erythrocytes. Parasitemia was monitored daily throughout the course of the infection by peripheral blood smears from a tail-prick. Venous blood (8 mL) was collected in ACD vacutainers on days 0, 2, 5, 7, 8 (peak parasitemia), 10, 15 (7 days post-treatment) and 22 PI (14 days post-treatment). Baseline values were established from the pre-infection samples on day 0 for the four animals. PBMCs were isolated from whole blood using a Lymphocyte Separation Media gradient via centrifugation. Cells were washed and re-suspended in complete DMEM. Plasma was aliquotted, SNAP frozen, and stored at -80°C for use in soluble cytokine quantification using ELISA. PBMCs were centrifuged, SNAP frozen, and stored at -80°C for RNA isolation. Plasma was examined for levels of soluble cytokines (IL-10, IL-12 IFN-γ and TNF-α) by ELISA and effector molecule (NO) using the Greiss reaction for total NO. Quantitative sandwich enzyme immunoassay technique was used with commercially available reagents. Each sample was assayed in duplicate, and the mean of the two values were used in all analyses.

RNA was isolated from PBMC pellets and by the Guanidinium Thiocyanate (GITC) method. Total RNA (1μg) was reverse transcribed into cDNA using an ABI GeneAmp 9700.

Gene expression of IL-10, TNF-α, IL-12, and IFN-γ were analyzed in triplicate using real time RT-PCR on an ABI Prism 7700 Sequence Detection System, using gene-specific primers and probes. Primers for iNOS were also tested in rhesus cDNA, but the levels could not be detected. For each reaction plate, non-template controls (NTC) were added in quadruplicate. β-actin was used as an endogenous control gene.

Due to the lack of information available for macaque mRNA sequences, Taqman primer and probe sets specific for human cDNA were tested for cross-reactivity with rhesus cDNA. The cDNA was amplified for 40 cycles. Data was analyzed using the –ΔΔCT method where the endogenous control gene cycle threshold (CT) value was subtracted from the experimental gene CT for each sample. The ΔCT for each experimental sample was then subtracted from the ΔCT of the baseline control sample. Fold change was expressed as $2^{-\Delta\Delta}$CT relative to baseline conditions.

Plasma and transcript cytokine levels from *ex vivo* PBMCs were expressed as mean ± standard error of the mean (SEM). Regression analysis was used to determine the correlation between variables. The plasma level of cytokines was graphed against transcript levels to determine if plasma levels are regulated at the gene expression level in PBMC. To examine the patterns of immune regulation, levels of pro-inflammatory cytokines were compared to levels of anti-inflammatory cytokines.

2.3 SPECIFIC AIM 3

Determine if there is a correlation between the hematological profiles and soluble inflammatory mediators (cytokine and effector molecule dysregulation).

2.3.1 Hypothesis

Dysregulation of cytokine and effector molecule production promotes changes in the hematological profile and clinical outcomes of *P. coatneyi* infection in rhesus macaques.

2.3.2 Rationale and Interpretation

Plasma and transcript levels of cytokines (IL-12p40, IFN-γ, IL-10 and TNF-α) and effector molecules (NO) were compared with parasitemia, Hb, Hct, and PLT levels over the course of infection (days 0-22 PI). Cytokine production was examined relative to MO, NEU, LY, WBC and RBCs. Ratios of anti-/pro-inflammatory cytokines were examined relative to markers of disease severity (Hb, Hct, PLT and parasitemia). Statistical analyses were performed to determine if the levels of cytokines and effector molecules over the course of the infection significantly correlated with the hematological indices and clinical parameters of disease severity using and Mann-Whitney U and randomization/permutation analysis for determining p-values for Pearson-correlations.

3.0 MATERIALS AND METHODS

3.1 RHESUS/COATNEYI-MALARIAL INFECTION

3.1.1 Experimental Animals

One adult male malaria-naïve rhesus macaque (14.3 kg) (*M. mulatta)* of Indian origin was used for the initial trial infection to provide sufficient quantities of inoculum for the study. Four adult female rhesus macaques (4.8-7.0 kg) of Indian origin were used in the study. Animals were housed according to American Association of Laboratory Animal Care Standards in a (AALAC-accredited BSL2+) primate facility at the University of Pittsburgh (Pittsburgh, PA) under the direction of a qualified veterinarian. All monkeys were obtained commercially and free of natural malaria infections. The study was performed in accordance with the animal use protocols approved by the Institutional Animal and Use Committee (IACUC) at the University of Pittsburgh, School of Medicine.

3.1.2 Malarial Infection

3.1.2.1 Initial Infection of M11700

One vial of 0.5mL *P. coatneyi*-infected erythrocytes (Parasite Stock 8-38, 1.Sept, 1990) was generously donated from the laboratories of Dr. William Collins at the Centers for Disease

Control and Prevention, Department of Parasitic Infectious Diseases, Atlanta GA. *P. coatneyi* cannot be cultured *in vitro*, therefore one rhesus macaque was used to grow a supply of parasites for use in the subsequent malaria infection of four rhesus macaques. Frozen parasite stock was thawed in a 37°C waterbath for 1-2 minutes and transferred to a sterile 50 mL centrifuge tube. To prevent lysis, the parasites were treated with a series of decreasing salt concentrations. A 12% NaCl solution (0.2 mL/mL of thawed parasite stock) was added at a rate of 1-2 drops/second. The tube was vortexed, allowed to stand for three minutes and 1.6% NaCl solution (10 mL/mL of thawed parasite stock) was added at a rate of 1-2 drops/second. The solution was vortexed, centrifuged at 400 x g for 10 minutes and supernatant was decanted. The final gradient of 0.9% NaCl/0.2% dextrose (10 mL/mL of thawed parasite stock) was added dropwise in same fashion as described above. The solution was vortexed, centrifuged at 400 x g for 10 minutes and supernatant was decanted. Parasites were re-suspended in 2 mL sterile USP-grade 0.9% NaCl, drawn up in a syringe and used for inoculation of the monkey.

For the initial infection, M11700 was sedated with 10mg/kg of Ketamine IM, and infected with 1 x 10^6 *P. coatneyi*-infected erythrocytes, IV in the saphenous vein using a 23g butterfly catheter. The line was flushed with 3 mL of USP-grade 0.9% NaCl solution. Parasitemia was monitored daily with thin films of peripheral blood from a tail-prick using a disposable lancet. Slides were fixed with methanol, stained with Giemsa (pH 7.25) for 45 minutes, and parasites were enumerated microscopically under oil immersion. Parasitemia was quantified by counting the number of pRBCs relative to 10,000 total RBCs, yielding a percent parasitemia. Parasitemia was also quantified as number of parasites/mm^3 of blood by taking the number of infected RBCs multiplied by the total number of RBCs divided by 1,000.

Venepuncture (9.5 mL ACD, 0.5 mL EDTA blood) was performed on days 0, 2, 5, 7, 9, and 19 PI for hematology and cytokine profiles. Parasitemia peaked on day 12 (7.0%). At peak parasitemia, the animal was sedated and 40 mL of venous blood was drawn in ACD for use in making new parasite stock solutions. Additionally, 9.5 mL (ACD) and 0.5 mL (EDTA) of venous blood was drawn for cytokine and hematological profiles, respectively. At peak parasitemia on day 12 PI, M11700 was treated with 150 mg chloroquine and 150 mg quinine PO at 0 and 48 hours. The infection was completely cleared by day 16 PI and remained clear for the next 40 days of monitoring by thin film blood smears.

3.1.2.2 Parasite Preparation

Parasites from M11700 were prepared for freezing using 40 mL whole blood from peak parasitemia, which was centrifuged at 400 x g for 10 minutes. Plasma was separated, aliquotted and frozen at -80°C. Blood was washed twice in Roswell Park Memorial Institute media (RPMI) 1640, (Invitrogen, Carlsbad, CA, US) and centrifuged at 400 x g for 10 minutes. Supernatant was carefully decanted without removing the brown layer on top of the RBCs. The volume of packed cells was estimated at 18 mL. To prevent the parasites from lysing during the freezing process, increasing salt concentrations were gradually added. An equal amount of glycerolyte (1/3 the volume of the packed cells) was added at a rate of 1-2 drops/second. The tube was vortexed and allowed to stand for five minutes. The second glycerolyte gradient was added (amount equal to 4/3 the original volume of the packed cells), at a rate of 1-2 drops/second. The solution was vortexed and aliquotted at 1.0 mL/cryovial. The vials were placed in a slow freezing apparatus (at a rate of -1°C/minute), and placed in a -80°C freezer. After 24 hours, the cryovials were transferred to liquid nitrogen storage.

3.1.2.3 Experimental Infection

Four rhesus macaques were infected intravenously with a fresh inoculum of 1 x 10^6 viable *Plasmodium coatneyi*-infected erythrocytes, which had been frozen from the previous infection of the donor monkey infected with a thawed cryostabilate of *P. coatneyi*. From infection onward, parasitemia was monitored daily by thin blood smears from a tail-prick. Thin blood films were prepared by the method of Earle and Perez (135), fixed with methanol, stained with Giemsa, examined microscopically under oil immersion and parasitemia was enumerated. Malarial infections were recorded as the percentage of infected RBCs per 10,000 RBCs counted, with a minimum of twenty fields in each thin film counted, and also as parasites/mm^3 of blood (as described above).

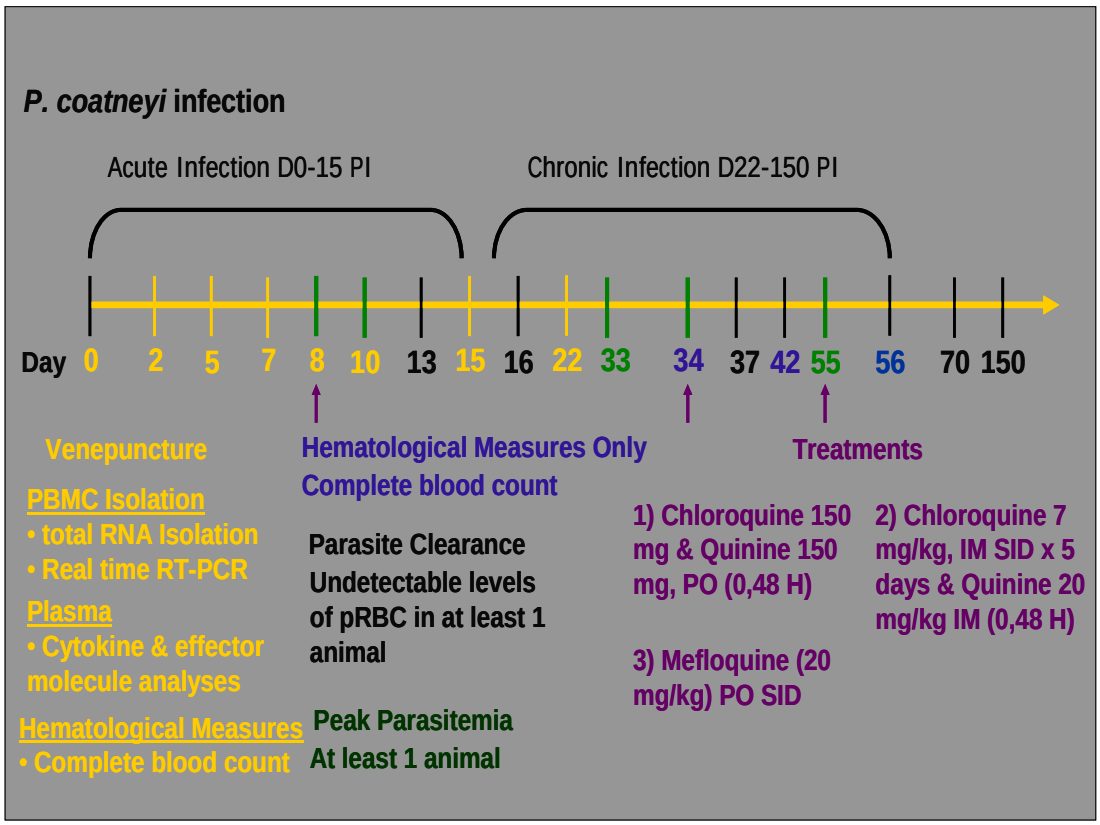


Figure 4: Experimental Design

Acute infection was defined as the primary infection from days 0-15 PI, which was two weeks after the initial treatment and after the first parasite clearance (Figure 4). Chronic infection was defined as the duration after the primary acute infection which includes the re-emergence of parasites after initial clearance (day 22-day 150 PI), followed by two subsequent treatment regimens. Animals were sedated for venepuncture with 10 mg/kg Ketamine. Venous blood was collected in ACD (8mL) and EDTA (0.5mL for hematology panels) vacutainers prior to challenge (day 0) and on days 2, 5, 7, 8 (peak parasitemia), 10, 15 (7 days post-treatment) and 22 PI (14 days post-treatment) during infection. Venous blood was also collected for hematology panels only (0.5mL in EDTA) in the same manner on days 34, 42, 56, and 150 PI during the chronic infection, since there is a limited amount of blood that can be withdrawn from the animals during a defined period. At each time point the animals were sedated, both weight, and rectal body temperature were recorded. Hematological parameters were measured using a Coulter Counter (Beckman Coulter, Miami, US). Both complete blood counts (CBC) and differentials were performed. Baseline values were established using the pre-challenge samples from day 0 for the malaria-infected animals. Reference values were also taken from Buchl and Howard's (1997) hematological values in clinically normal captive bred rhesus macaques (136) (Table 1).

Animals were treated when parasitemia reached 5% or greater with chloroquine (150mg PO) and quinine (150 mg PO) on days 8 and 10 PI. This treatment did not effectively clear the malaria infection. Parasites were undetectable by days 15-16 PI, but all animals relapsed with parasites re-emerging by day 20 PI. This enabled for the comparison between the acute primary infection and the chronic secondary infections. Three animals (M2198, M10800, and M11799) had an increase in parasitemia of 5% or greater by day 33 PI, while M11600 had a controlled

parasitemia of 0.25%. Animals were monitored and treated again on day 34 PI with combination therapy of quinine (20 mg/kg IM SID at 0 and 48 hours) and chloroquine (7mg/kg, IM SID x 5 days). This treatment regimen was not effective at completely clearing the malarial infection. By day 54 PI, parasitemia ranged from 1-3.5% in all four monkeys. Mefloquine is used to treat *coatneyi*-malaria if an isolate is suspected to be chloroquine-resistant. On day 55 PI, animals were treated with a single dose of mefloquine (20 mg/kg SID PO). Parasitemia cleared by day 70 PI, and remained clear through day 150 PI.

Table 1: Normal Hematological Values in Healthy Adult Rhesus Macaques

Leukocyte Indices				**Red Blood Cell Indices**	
WBC	**(x10^6/mL)**			**RBC**	**(x10^6/mL)**
	4.2-8.1				**5.1-5.6**
LY	**(%)**	**LY****	**(x10^3/mL)**	**Hb**	**(g/dL)**
	39-72		**3.1-4.1**		**12.0-13.1**
MO	**(%)**	**MO****	**(x10^3/mL)**	**Hct**	**(%)**
	1-4		**0.2-0.6**		**37-40**
NEU	**(%)**	**NEU****	**(x10^3/mL)**	**MCV**	**(fL)**
	26-52		**2.1-3.3**		**71-75**
Platlet Indices				**MCH**	**(pg)**
Plt	**(x10^3/mL)**				**22.8-24.5**
	260-361			**MCHC**	**(g/dL)**
MPV**	**(fL)**				**31.0-33.4**
	5-15			**RDW****	**(%)**
TEMP	**(^{o}C)**				**12.4-13.9**
	37.2-39.2				

** calculated from the mean +/- standard deviation of the animals preinfection

All other values from Buchl SJ, Howard B. Hematologic and serum biochemical and electrolyte values in clinically normal domestically bred rhesus monkeys (*Macaca mulatta*) according to age, sex, and gravidity. *Lab Anim Sci.* 1997 Oct;47(5):528-33.

Malarial anemia is defined in humans as Hb concentration of less than 8 g/dL or less than a 24% Hct, and severe malaria anemia as Hb concentration of less than 5 g/L or a Hct of less than 15% (87). Since reference values for Hb and Hct vary, and are not known for many tropical

areas of the world, anemia can be defined as Hb concentration two standard deviations below the mean based on age, sex, and pregnancy state. There are no reference values for malarial anemia in rhesus macaques, therefore two standard deviations from both the mean Hb and Hct levels were calculated from the animals in this study (n=4) (Table 2).

Table 2: Classification of Anemia in Rhesus Macaques Infected with *P. coatneyi*

Characterization of Anemia based on normal values				
HEMOGLOBIN		**HB**	**HEMOGLOBIN**	
AVG (n=4)	10.7		**25% Reduction**	**50% Reduction**
STDEV	*0.4113*		10.675	10.675
2STDEV	*0.8226*	**9.9**	*2.66875*	*5.3375*
3STDEV	*1.2339*	**9.4**	**8.0**	**5.3**
4STDEV	*1.6452*	**9.0**		
HEMATOCRIT		**HCT**	**HEMATOCRIT**	
AVG (n=4)	33.2		**25% Reduction**	**50% Reduction**
STDEV	*1.3401*		33.225	33.225
2STDEV	*2.6802*	**30.5**	*8.30625*	*16.6125*
3STDEV	*4.0203*	**29.2**	**24.9**	**16.6**
4STDEV	*5.3603*	**27.9**		

3.1.3 Isolation of Peripheral Blood Mononuclear Cells

Plasma was separated, SNAP frozen and cryopreserved at -80°C. Blood cells were re-suspended in media, layered over Lymphocyte Separation Medium (Mediatech/Cellgro, Herndon, VA, US) and centrifuged at 2000 rpm for 30 minutes. Cells in the interface were collected and washed twice with RPMI 1640 supplemented with 10% Fetal Calf Serum (FCS) (Hyclone, Logan, UT, US). Cells were cryopreserved in individual pellets and stored at -80°C.

3.1.4 Plasma Cytokine Determination

Venous blood was drawn in ACD and EDTA containing tubes just prior to challenge (day 0) and on days 2, 5, 7, 8, 10, 15, and 22 PI. Freshly thawed plasma samples were assayed for levels of soluble cytokines [IL-10 (BioSource ELISA sets, Camarillo, CA, US) , IL-12 (BioSource ELISA set Camarillo, CA, US), IFN-γ (Opt-EIA ELISA Sets, BD PharMingen, San Jose, CA, US), and TNF-α (Opt-EIA ELISA Sets, BD PharMingen, San Jose, CA, US) using quantitative sandwich enzyme immunoassay technique with commercially available reagents. Each sample was measured in duplicate, and the mean of the two values were used in all analyses. Flat-bottomed Maxisorb (Nalge Nunc, Rochester, NY, US) plates were coated overnight with 100 μL of the capture antibody coating buffer (diluted to optimal concentration with phosphate buffered solution (PBS). Plates were washed with wash buffer and blocked with 200 μL blocking solution for the optimal time for each assay at 37°C. Plasma (100 μL) was added to each well and allowed to incubate for 1 hour (in the case of IL-10 and IL-12, the incubation time was 2 hours, and biotinylated capture antibody was added prior to the incubation). After several washes, biotinylated capture antibody and avidin-labelled horseradish peroxidase were added to each well and allowed to incubate for 30 minutes. Plates were developed for 30 minutes using TMB substrate reagent set (BD PharMingen, San Jose, CA, US) in the dark. The reaction was stopped using 2M phosphoric acid solution. The absorbance was read on a spectrophotometer at a 450 nm wavelength. Monoclonal antibodies against human IL-12 are specific for the IL-12p40 subunit and react with both human and rhesus IL-12, and thus detect both human and rhesus IL-12 levels.

3.1.5 Total NO Determination

NO is an evanescent molecule, with a half-life of 4 seconds (137) and can not be accurately measured *in vivo.* Plasma levels of stable metabolites of NO, nitrate (NO_2^-) and nitrate (NO_3^-) collectively termed reactive nitrogen intermediates (RNI) are measured. As the relative proportion of NO_2^- and NO_3^- is variable, the sum of the RNI is the best index of total NO. Plasma levels of total NO were determined using the Greiss reaction (138), (139). Samples were assayed in duplicate. NO_3^- is converted into NO_2^- by utilizing nitrate reductase. To each well, 50 μL of sample, 15 μL of de-ionized water, 10 μL of 1M TRIS (pH 7.5), 1 μL of 0.2 mM nicotinamide adenine dinucleotide phosphate (reduced form, (NADPH) (Sigma, St. Louis MO, US), 20 μL of 5 mM glucose-6-phosphate (G6P), 3 μL of 10.0 U/mL glucose-6-phosphate dehydrogenase (G6PDH) and 1 μL of 1.0 U/mL nitrate reductase were added on ice. After incubating at ambient temperature for 2 hours, the volume in each well is divided between two plates and 50 μL of each Greiss Reagent I (1% sulfanilamide in 2.5% phosphoric acid) and II (0.1% naphthyethylenediamine in 2.5% phosphoric acid) were added to each well. After incubating for 10 minutes at room temperature, the absorbance was read on a plate reader at 550 nm.

3.1.6 RNA Extraction

RNA was extracted from individual PBMC pellets and by using the guanidinium isothiocyanate (GITC) method (140). This extraction was performed by adding a solution comprised of filter-sterilized water, 25mM sodium citrate (pH 7.0), 10% Sarkosyl (MP Biomedicals, Irvine, CA, US), 4M of GITC (Gibco/Invitrogen, Carlsbad, CA, US) and β-

mercaptoethanol (MP Biomedicals, Irvine, CA, US) to cells. After incubating for 5 minutes, 2M sodium acetate, phenol, and chloroform/isoamyl alcohol (MP Biomedicals, Irvine, CA, US) were each added, and contents mixed thoroughly. Samples were incubated on ice for 30 minutes, which permitted complete dissociation of nucleoprotein complexes. Samples were centrifuged at 3750 rpm for 20 minutes at 4^{o}C. The upper three quarters of the aqueous phase was transferred and an equal volume of cold isopropanol (MP Biomedicals, Irvine, CA, US) was added to the extracted RNA. The solution was allowed to precipitate at -20^{o}C for two days. The precipitated RNA was pelleted by centrifugation at 10,000 rpm for 30 minutes at 4^{o}C. Isopropanol was decanted and evaporated. RNA was dissolved on ice with DEPC-treated water, held on ice for 15 minutes, and heated at 65^{o}C for 10 minutes to solubilize RNA.

The RNA was quantified on a spectrophotometer (Amersham Bioscience, Piscataway, NJ, US). Total RNA (1μg) was reverse transcribed using random hexamers and murine leukemia virus (MuLV) (Applied Biosystems, Foster City, CA, US) into cDNA on a ABI GeneAmp 9700 (Applied Biosystems, Foster City, CA, US).

3.1.7 Quantitative Real Time RT-PCR

Gene expression of IL-10 (Accession Number NM_000572, Applied Biosystems, Foster City, CA), TNF-α, (Accession Number NM_000594, Applied Biosystems, Foster City, CA), IL-12 (Accession Number NM_002187, Applied Biosystems, Foster City, CA), and IFN-γ were analyzed using real time RT-PCR on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) utilizing each gene-specific primers and probes. For IFN-γ, primers were designed using IDT Oligonucleotide design software (Integrated DNA Technologies, Inc., Coralville, IA) by the following sequence: RhIFN-γ AS Taq primer (5'-ATC

TGG ATC ACC TGC ATT AAA ATA TTT-3'), RhIFN-γ S Taq primer (5'-CAG CTC TGC ATT GTT TTG G-3') Probes were designed by the Primer Express software (PE Applied Biosystems, Inc. Foster City, CA) for the following sequence: RhIFN-γ TaqProbe (5'-FAM-CTT GGC TGT TAC TGC CAG GAC CCA TAT GTA A-TAMRA-3'). iNOS (Accession Number NM_153292.1 Applied Biosystems, Foster City, CA) was assayed several times in these samples, but the levels were undetectable.

For each reaction plate, non-template controls (NTC) were added in quadruplicate. β-actin (Applied Biosystems, Foster City, CA) was used as an endogenous control gene. The human-specific β-actin primer and probe set, which is a good housekeeping gene for human PBMC, also recognized macaque cDNA from four independent samples. Due to the lack of information available for macaque mRNA sequences, Taqman® primer and probe sets specific for human cDNA were tested for cross-reactivity with rhesus cDNA. The cDNA was amplified for 40 cycles. Data was analyzed using the $\Delta\Delta C_T$ method where the endogenous control gene cycle threshold (CT) value was subtracted from the experimental gene CT for each sample. The ΔC_T for each experimental sample was then subtracted from the ΔC_T of the unstimulated control sample to calculate the $\Delta\Delta C_T$. Fold change was expressed as $2^{-\Delta\Delta C_T}$ relative to unstimulated conditions.

3.1.8 Statistical Analyses

Plasma cytokine levels and cytokine transcript levels from non-cultured PBMC were measured in duplicate. Data were expressed as mean ± standard error of the mean (SEM). Comparisons between groups were made using the Mann-Whitney U tests with statistical

significance set at $p < 0.05$. Statistical significance was set at $P < 0.05$. Randomization-based p-values for Pearson correlations, with Bon Feroni corrections were calculated for all timepoints in the four animals for each hematological parameter against cytokine levels, parasitemia, anemia (Hb), and thrombocytopenia (PLT).

4.0 RESULTS

4.1 CLINICAL AND HEMATOLOGICAL PROFILES: ACUTE RELATIVE TO CHRONIC INFECTION

4.1.1 Course of Malarial Infection

Throughout the course of infection, trends in parasitemia were asynchronous in all four animals, although the relative degree of parasite burden varied. Parasites were detected in the blood on day 1 PI. Parasitemia grew exponentially in the following days until reaching >5% (5.6-7-8%, corresponding to 236,320-290,160 parasites/μL) for each animal on day 8 PI (Figure 5). All four animals' parasitemias peaked on day 8 PI, with the exception of M2198, which had a 6.2% parasitemia on day 8 PI, decreased to 3.05% on day 9, followed by a hyperparasitemia on day 10 PI of 8.3%. Animals were treated with chloroquine (150mg PO) and quinine (150mg PO) on days 8 and 10 PI. The animals' parasitemias declined after the two days of treatment. Treatment did not fully clear the infection. Parasitemia was undetectable by day 16 PI, however, all four animals relapsed, and parasites were present again by day 20 PI. This event enabled for comparison of an acute primary malarial infection with a more chronic secondary malarial infection. For this study, acute infection was defined as days 0-15 PI, while chronic phase of infection encompassed the reminder of the infection through day 150 PI.

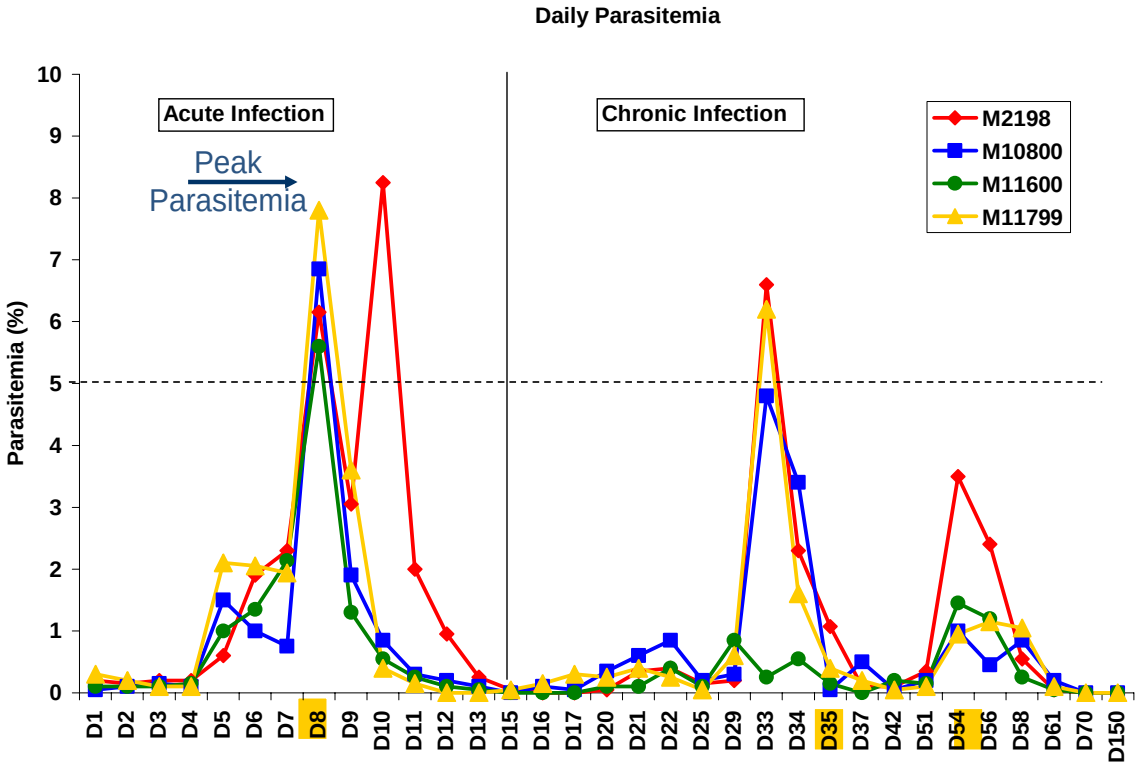


Figure 5: Daily Parasitemia in Rhesus Macaques Infected with *P. coatneyi*

During the chronic phase of infection, there were two distinct increases in parasitemia. The first occurred on day 33 PI. Two animals, M2198 and M11799, had parasitemias of >6% on day 33 PI, while M11799 had a parasitemia of 4.8%. M11600 had an increase in parasitemia to 0.85% on day 29, but was able to control parasitemia to 0.25% by day 33 PI. The animals were monitored and treated on day 34 with combination therapy of quinine (20mg/kg IM SID at 0 and 48 hours) and chloroquine (7mg/kg, IM SID x 5 days). During this time parasitemia had decreased and was clearing around day 37 PI. However, parasitemia re-emerged in all four animals, and by day 54 PI, all four animals had parasitemias between 1-3.5%. Mefloquine can be used to treat *P. coatneyi*, especially if an isolate is suspected to be chloroquine-resistant. On

day 55 PI, a single dose of mefloquine (20 mg/kg SID PO) was administered. The malarial infection cleared completely by day 70 PI, and remained clear through day 150 PI

At each peak in parasitemia, with the exception of day 8 PI, M2198 had the highest parasite load among the four animals. M2198 was also the only animal to rebound with a hyperparasitemia after the initial treatments on day 10 PI, with a parasitemia of 309,375 parasites/μL, which falls in the lower range of susceptibility for CM. M11799 had the most severe parasite burden on day 8 (peak parasitemia) at 290,160 parasites/μL (7.8%). M11799 had a high parasitemia during the chronic infection on day 33 PI (6.2%), whereas the final increase in parasitemia during the chronic infection on day 54 did not increase above 1.0% in this animal. M10800 reached a 5% parasitemia or greater in both the acute and chronic phases of infection, but had a slightly lower parasite burden on each of the three peaks in parasitemia than two of the other three animals. The animal that was best able to control parasite burden was M11600. In the acute infection, M11600 peaked at 236,320 parasites/μL (5.6%) on day 8 PI. During the chronic phase of infection, M11600's parasitemia did not elevate above 2%.

4.1.2 Clinical Outcomes

Body temperature and weight were recorded at each time the animal was anesthetized for venepuncture and/or treatment. Pre-infection temperature ranged from (37.72-38.5°C) (Table 3). Fever occurred on days 8 (peak parasitemia) and day 22, which was the first re-emergence of parasitemia ($p<0.05$ by Mann Whitney U). M2198 and M11799 also had fevers on day 34 PI. There was an increase in body temperature on days 35 and 56 PI, both days where parasitemia had peaked again, but was not statistically significant.

Table 3: Clinical Observations in Rhesus Macaques Infected with *P. coatneyi*

Clinical Observations													
Parasitemia	**(/mL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
M2198		0	7,020**	32,700*	99,820*	286,590**	309,375	1,410	14,560*	94,990*	3,930	129,360	0
M10800		0	4,140**	75,750*	34,725*	274,000**	31,790	0	35,360*	107,100*	1,400	23,130	0
M11600		0	4,350**	52,600*	87,740*	236,320**	21,230	0	18,720*	19,635*	6,880	61,800	0
M11799		0	7,540**	104,790*	66,348*	290,160**	12,080	1,435	10,400*	68,960*	1,990	50,600	0
Weight	**(kg)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
M2198		7.2	7.3	7.1	7.3	7.2	6.9	6.9	6.2	6.1	6.1	6.0	6.6
M10800		4.8	5.0	4.8	5.0	4.9	4.8	5.1	4.6	4.7	4.6	4.9	5.1
M11600		6.3	6.8	6.6	6.7	6.6	6.5	6.9	6.4	6.5	6.5	6.7	7.8
M11799		6.6	6.7	6.5	6.6	6.5	6.4	6.5	6.0	5.8	5.8	5.8	6.8
Temperature	**(°C)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
M2198		37.9	38.2	37.8	38.2	38.4*	36.6	37.8	39.9*	39.8	38.4	38.5	38.7
M10800		37.4	38.4	38.4	37.8	38.8*	38.5	38.6	38.6*	38.3	38.7	38.1	38.2
M11600		38.5	38.5	38.5	37.9	39.7*	38.5	38.2	39.2*	38.8	38.5	39.2	38.1
M11799		37.7	38.6	38.2	38.2	40.2*	37.9	38.5	40.0*	40.4	38.5	39.7	38.8

**** Denotes P<0.05 (by Mann-Whitney U) relative to baseline levels (Day 0)***
***** Denotes P<0.01 (by Mann-Whitney U) relative to baseline levels (Day 0)***

Overall, M11799 showed the highest fever at the four different timepoints, with a 7.1% increase in temperature above baseline during the chronic phase of disease, and 6.7% increase during the acute phase (Table 4). Upon parasite clearance and post-treatments body temperature decreased to normal range. Parasite load was not a significant predictor of body temperature.

Table 4: Magnitude of Weight Loss and Fever in Acute and Chronic Stages of Malarial Infection

Percent Change over Baseline		
Weight Loss	**ACUTE**	**CHRONIC**
M2198	-4%	-17%
M10800	0%	-4%
M11600	0%	0%
M11799	-3%	-12%
Temperature Increase	**ACUTE**	**CHRONIC**
M2198	1%	5%
M10800	4%	4%
M11600	3%	2%
M11799	7%	7%

Weight loss varied from 0-17% during the course of infection, with the largest decline in weight occurring in the chronic phase of infection on days 22, 34, and 42 PI, again following with the re-emergence of parasitemia. The two animals that had the highest parasite burdens during the infection, M2198 and M11799, also had the largest decline in body weight during the

chronic infection at 17% and 12%, respectively. However, on day 10 (hyperparasitemia) when the parasitemia was over 300,000 parasites/μL in M2198, fever was reduced, due to the treatment with anti-malarials on day 8 PI. M10800 had a 4% drop in weight during the chronic phase of disease. M11600 had the lowest parasite loads and had no decline in body weight through the course of infection, but rather weight gain.

4.1.3 Hematological Outcomes

Along with presence of parasitemia, Hb and Hct were used to define the level of anemia. Baseline levels of Hb ranged from 10.2-11.2 g/dL (mean = 10.7g/dL), and baseline Hct ranged from 31.7-34.9% (mean = 33.2%). Anemia was defined as Hb and Hct two standard deviations below the mean, <9.9g/dL, or <30.5%, respectively. As there was a small cohort of animals used in the calculation of normal ranges, a more severe anemia of four standard deviations below the mean (Hb, Hct), and a 25% and 50% reduction in Hb below the mean were characterized (TABLE 2).

Mann-Whitney U was used to determine significant changes from baseline ($p<0.05$, 95% CI) (Table 5). Hct, Hb, and RBC were all positively correlated ($p<0.01$). Randomization tests were used to compute p-values for Pearson correlations, and used to determine the significance between parasitemia and hematological outcomes, anemia, and other hematological parameters. The timepoint at which the animals had the highest parasite burden was positively correlated with level of Hb ($p<0.05$).

Hb, Hct, and RBC levels significantly increased above baseline ($p<0.01$) in all four animals on day 5 PI, also the first exponential increase in parasitemia (Table 5), which could be due to the absence of a robust early cytokine response, which would negatively regulate Hb

levels. Anemia was defined as [Hb] <9.9g/dL, or [Hct] <30.5%. In Table 5, anemia was categorized as one of the following: two standard deviations below the mean (yellow), four standard deviations below the mean (blue), or 25% reduction below the mean (green). None of the animals had an anemia with 50% reduction in Hb or Hct below the mean (gray). All four animals were anemic on days 10, 15, and 42 PI. At least one of the animals was considered anemic days 2, 7, 8, 22 and 34 PI.

Table 5: Red Blood Cell Indices in Rhesus Macaques Infected with *P. coatneyi*

Red Blood Cell Indices													
RBC	**(x10^6/mL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	4.89	4.68	5.45**	4.34	4.66*	3.75*	2.82*	3.64	4.13*	3.93*	5.39	5.43*
	M10800	4.47	4.14	5.05**	4.63	4*	3.74*	3.45*	4.16	3.15*	2.8*	5.14	4.82*
	M11600	4.5	4.35	5.26**	4.1	4.22*	3.86*	3.84*	4.68	3.57*	3.44*	5.15	4.9*
	M11799	4.47	3.77	4.99**	3.42	3.72*	3.02*	2.87*	4.16	4.31*	3.98*	4.4	5.24*
Hb	**(g/dL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	11.2	10.9	12.3**	9.9	10.6*	8.7*	6.9*	8.8	9.7	8.9*	12	12*
	M10800	10.2	9.7	11.6**	10.7	9.2*	8.7*	8.2*	9.4	6.7	5.9*	10.8	11*
	M11600	10.7	10.5	12.4**	10.1	10*	9.1*	9.3*	11.1	8.2	7.8*	11.7	11.5*
	M11799	10.6	9.2	12**	8.1	8.8*	7.1*	7.5*	10.1	10.4	9.3*	10.1	12.5*
Hct	**(%)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	34.9	33.1	37.6**	30.2	31.6*	24.6*	21.7*	27.4	30.1	28.4*	37.2	37.6*
	M10800	31.7	29.4	35.4**	32.3	27.5*	26.5*	25.5*	29	20.9	18.8*	34.3	34.5*
	M11600	33.5	32.4	38.4**	29.9	30.2*	27*	28.9*	34.1	25.3	24.6*	36.6	35.7*
	M11799	32.8	28.1	35.6**	24.5	26.3*	20.6*	22.1*	31.6	31.6	29*	31.5	38.5*
MCV	**(fL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	71.3	70.8	69*	69.5**	67.8**	65.5	76.8*	75.1	72.6	72.4	69.1*	69.4
	M10800	70.8	71	69.9*	69.7**	68.6**	71.25	73.9*	69.6	66.4	67.2	66.6*	71.6
	M11600	74.3	74.4	72.9*	72.8**	71.5**	69.8	75.2*	72.8	70.7	71.4	71.1*	72.8
	M11799	73.3	74.5	71.2*	71.5**	70.5**	67.9	76.9*	75.9	73.4	72.7	71.5*	73.4
MCH	**(pg)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	22.9	23.2*	22.6	22.7	22.7	23.1	24.4*	24.2	23.5	22.5	22.2*	22.2
	M10800	22.9	23.4*	23	23	23	23.4	23.8*	22.5	21.3	21	21.1*	22.9
	M11600	23.8	24.2*	23.7	24.6	23.7	23.5	24.3*	23.8	23	22.5	22.7*	23.4
	M11799	23.8	24.5*	24.1	23.8	23.7	23.4	26.2*	24.9	24.1	23.3	23.1*	23.8
MCHC	**(g/dL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	32.1	32.8**	32.7*	32.7*	33.5**	35.2**	31.8	32.2	32.3	31.1*	32.1	32
	M10800	32.3	33**	32.8*	33*	33.6**	35**	32.2	32.4	32	31.3*	31.6	32
	M11600	32	32.5**	32.4*	33.7*	33.2**	33.6**	32.3	32.6	32.5	31.5*	31.9	32.4
	M11799	32.5	32.9**	33.8*	33.2*	33.6**	34.4**	34	32.7	32.8	32.1*	32.2	33.4
RDW	**(%)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	14.1	13.3	13.7	13.5*	13.4	13	15.3	21.4	17.6*	18.8*	14.8*	14.2
	M10800	12.8	12.7	12.7	12*	12.4	12.7	9.5	13.5	16*	16.8*	15.4*	12.8
	M11600	13.3	13.7	13.4	13.2*	13.5	13.9	17.5	14.6	14.6*	15.5*	14.7*	13.4
	M11799	12.3	12.2	12.1	11.5*	12.1	11.9	20.3	17.8	14*	14.8*	13.6*	12.3

**** Denotes P<0.05 (by Mann-Whtiney U) relative to baseline levels (Day 0)***
***** Denotes P<0.01 (by Mann-Whitney U) relative to baseline levels (Day 0)***

Anemia defined by 2 STDEV below Mean
[Hb] <9.9g/dL, [Hct] <30.5%

4 STDEV below mean
[Hb] <9.0g/dL, [Hct] <27.9%

25% reduction below Mean
[Hb] <8.0g/dL, [HCT] <24.9%

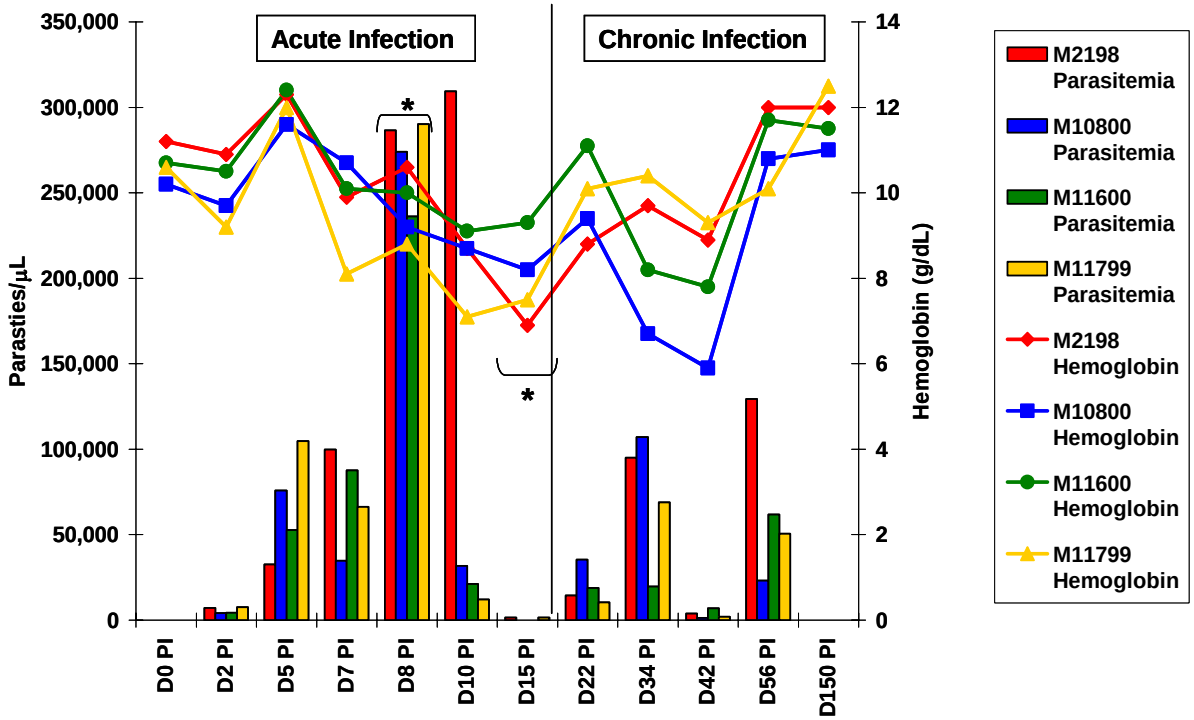
50% reduction below Mean
[Hb] <5.3g/dL, [HCT] <16.6%

During the acute infection, Hb, Hct and RBCs were lowest on days 8, 10, and 15 ($p<0.05$). By day 22 they began to increase closer to baseline levels. During the chronic infection, on day 34 (RBC decrease only) and 42 PI, there was again a significant decrease in Hb, Hct and RBC counts ($p<0.05$). Hb decreased 15-38% from baseline during the acute infection, and 12-42% during the chronic infection, while Hct decreased 19-38% decrease in the acute infection and a 12-41% decrease in the chronic infection (Table 6). The average percent change in Hb, Hct and RBC did not significantly change between acute and chronic phases of disease in the four animals.

Table 6: Red Blood Cell Indices in the Acute and Chronic Stages of Malaria in Rhesus Macaques (Percent Change from Baseline)

Red Blood Cell Indices	ACUTE (% Decrease)	CHRONIC (% Decrease)	ACUTE (% Increase)	CHRONIC (% Increase)
RBC				
M2198	-42	-26	11	11
M10800	-23	-37	13	15
M11600	-15	-24	17	14
M11799	-36	-11	12	17
AVERAGE	**-29**	**-24**	**13**	**14**
Hb				
M2198	-38	-21		
M10800	-20	-42		
M11600	-15	-27		
M11799	-33	-12		
AVERAGE	**-26**	**-26**		
Hct				
M2198	-38	-21		
M10800	-20	-41		
M11600	-19	-27		
M11799	-37	-12		
AVERAGE	**-28**	**-25**		
MCV				
M2198	-8	-3	8	5
M10800	-3	-6	4	1
M11600	-6	-5	1	-2
M11799	-7	-2	5	4
AVERAGE	**-6**	**-4**	**5**	**2**
MCH				
M2198	-1	-2	7	6
M10800	0	-8	4	-2
M11600	-1	-5	3	0
M11799	-2	-3	10	5
AVERAGE	**-1**	**-5**	**6**	**2**
MCHC				
M2198	-1	-3	10	1
M10800	0	-3	8	0
M11600	0	-2	5	2
M11799	0	-1	6	3
AVERAGE	**0**	**-2**	**7**	**1**
RDW				
M2198	-8	-5	9	52
M10800	-26	-5	0	31
M11600	1	-10	32	17
M11799	-7	-11	65	45
AVERAGE	**-10**	**-8**	**26**	**36**

During the acute primary infection, parasite burden at peak parasitemia (day 8 PI) was found to be a significant predictor of acute anemia, (Hb levels on day 15 ($p<0.05$)). Parasite burden at peak parasitemia was also correlated with the level of RBCs at acute anemia (day 15 PI) (Figure 6). However, Hct was not predicted by parasite burden. During the chronic infection, peaks in parasitemia did not significantly predict the subsequent decreases in Hb, Hct or RBCs.



**Denotes p<0.025 by randomization tests used to determine p-values for Pearson correlations*

Figure 6: Parasitemia Relative to Hemoglobin Concentrations during Malarial Infection in Rhesus Macaques

MCHC significantly increased on days 2, ($p<0.01$), 5 ($p<0.05$), 7 PI ($p<0.05$) 8 ($p<0.01$) and 10 ($p<0.01$) PI. Both intra- and extracellular Hb are measured in the MCHC, giving a false high value. RBCs can not have supersaturation of Hb, therefore MCHC is not normally elevated. It is possible the increase in MCHC could be due to the hemolysis of RBCs with increased Hb in

circulation, and Hz deposition in the RBC and circulation. MCHC significantly decreased during the chronic infection on day 42 PI. Decreased in MCHC can be due to reticulocytosis, as reticulocytes do not have their full Hb component.

MCH significantly increased on days 2, 15, and 56 PI. Elevated MCH index is seen with increases with hemolysis. There was no significant change in the average percent change from baseline in these parameters in the acute relative to the chronic stages of infection. The MCV was significantly decreased on day 5 ($p<0.05$) and the decrease was highly significant on days 7 and 8. MCV significantly increase on day 15 in the acute infection and significantly decreased in the chronic infection on day 56 PI. The RDW significantly decreased on day 7 in the acute infection and significantly increased on days 34, 42, and 56 in the chronic infection. Increased RDW has been shown to be an indicator of increased hemtaopoiesis, and is correlated with the degree of macrocytosis (increases MCV) (115). During the *P. coatneyi* infection, the increased RDW was correlated with MCV ($p<0.05$), and RDW was correlated with the increase in hemtaopoiesis (RBC) ($p<0.05$). There was an average increase of 26% in RDW in the acute stage, compared with a 36% average increase in the chronic infection.

Examining the disease severity profiles for each animal individually, M2198 had the worst parasitic burden (highest overall parasitemia, and two strongest rebounds in parasitemia), yet did not succumb to the most severe anemia during the chronic stage of disease. M2198 showed a 38% reduction in Hb (6.9 g/dL) and Hct (21.7%) levels relative to baseline, and a 42% reduction in RBC count ($2.82 \times 10^6/\mu L$) during the acute infection (Table 6). During the chronic infection, M2198 had a 21% reduction in Hct and Hb, a 26% decrease in RBCs and a 52% increase in RDW (21.4% on day 22 PI).

Over the duration of the malarial infection, M10800 became the most anemic of all four monkeys on day 42 PI during the chronic infection (11 days following a peak parasitemia of almost 5%), with a Hb score of 5.9g/dL, Hct of 18.8%, and a RBC count of (2.8 x 10^6/μL) which is a 37-42% reduction in these parameters below baseline for this animal (Table 6). During the acute infection in M10800, there was a 20-23% decrease in red blood Hct, Hb and RBC count. M10800 had a 26% decrease (Day 15 – 9.5%) in RDW in the acute infection and a 31% increase (Day 42 -16.8%) over baseline in RDW during the chronic infection.

M11600 had the least evidence of anemia both in the acute and chronic phases of infection, with a 14-20% ([Hb] 9.1g/dL and [Hct] 27%, day 10 PI) and a 27% ([Hb] 7.8 g/dL and [Hct] 24.6%, day 42 PI) reduction in overall Hb and Hct, respectively. M11600 controlled its' parasitemia better than the other three animals, having the lowest overall mean in parasite load. The RDW in M11600 increased during the acute infection to 32% above baseline (day 15, [RDW] 17.5%) and 17% above baseline (day 42, [RDW] 15.5%) during the chronic infection.

M11799 had the highest parasite burden during the acute infection on day 8 (peak parasitemia, 290,160 parasites/μL). During the acute infection Hb and Hct was lowest at day 10 PI ([Hb] 7.1g/dL, [Hct] 20.6%) and a 33-37% reduction in over baseline respectively. RBC count was decreased by 36% below baseline in the acute phase of infection. During the chronic stage of disease, M11799 did not become anemic with the lowest Hb (day 42 PI) decreasing only to 9.3 g/dL, and Hct to 29%, and RBCs to 3.98 x 10^6/μL.

Normal published values for PLT in healthy adult rhesus macaques are 260-361 x 10^3/μL (shown in Table 1) (136). In all four animals, there was a significant thrombocytopenia (PLT < 260) during the acute infection on days 2 and 8 PI, and on day 34 PI during the chronic infection (Table 7).

Table 7: Leukocyte and Platelet Indices in Rhesus Macaques infected with *P. coatneyi*

Leukocyte Indices													
WBC	**(x10^3/mL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	6.8	6.6	4.9*	4.3	5.8	9.1	26	6	9.9	6.8	10	10.4
	M10800	6.7	4.6	5.2*	6.2	6.4	9.2	11.2	8.7	9.7	8.4	8.9	28.4
	M11600	6.8	5.6	5.9*	5.1	3.5	3.8	6.7	7.1	4.5	5.8	7.7	16.8
	M11799	7	5.3	4.3*	3	4.1	4.3	11.7	7.4	7.5	4.8	7.5	6.6
LY	**(x10^3/mL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	3.5	1.4*	2.1**	2	1.1*	11.6	11.6	1.8*	2	3.8	5.2*	5.1
	M10800	3.3	1.7*	1.8**	3.7	1.8*	6.3	6.3	2*	4.4	4.9	6.7*	24.3
	M11600	3.2	2.3*	1.7**	1.9	1*	6.0	6	1.8*	1.5	3.3	4.8*	9
	M11799	4.3	1.8*	2.1**	2.1	0.9*	7.0	7	3.9*	1.2	1.9	6*	3.9
MO	**(x10^3/mL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	0.5	0.3	0.5	0.4	0.6	0.4	0.1	0.9	0.8	1.2*	0.6	0.4
	M10800	0.2	0.1	0.2	0.7	0.5	0.6	0.6	0.4	1.5	1.1*	0.3	0.9
	M11600	0.3	0.4	0.1	0.4	0.2	0.3	0.3	0.1	0.5	1.2*	0.2	0.8
	M11799	0.6	0.2	0.3	0.4	0.3	0.3	0.2	0.4	0.8	0.8*	0.2	0.6
NEU	**(x10^3/mL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	2.5	4.7	2.3	1.8*	4.1	9.1	14	3.3	6.8	1.5	4	4.8
	M10800	3.2	2.1	2.8	1.7*	4.1	3.9	3.6	6	3.7	2.2	1.8	3.2
	M11600	3.2	2.7	4.1	2.6*	2.3	1.4	0.4	5.2	2.5	1.2	2.5	6.9
	M11799	2	3.1	1.8	0.5*	2.7	3.5	4.2	2.4	5.4	2.1	1.3	2
Platlet Indices													
Plt	**(x10^3/mL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	332	289*	329	375	259*	139	474*	299	270*	282	318	405*
	M10800	316	194*	282	257	240*	439.5	639*	216	193*	242	357	387*
	M11600	273	156*	281	278	205*	257	703*	211	173*	247	299	288*
	M11799	483	377*	462	353	270*	197	584*	520	284*	236	374	520*
MPV	**(fL)**	**D0**	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**	**D34**	**D42**	**D56**	**D150**
	M2198	7.6	7.7	8.6**	8.4*	8	9.3*	9.5	9**	10.2**	10.7*	9*	9.2**
	M10800	8.8	8.7	10.3**	10*	10.4	9.3*	9.5	10.1**	11.4**	10*	10*	11.4**
	M11600	7.3	7.2	8.5**	8.1*	7.7	8*	7.3	8.2**	8.8**	8.7*	7.6*	8.8**
	M11799	6.9	6.5	8.1**	8.6*	8.5	8.9*	9.5	8.6**	8.7**	9.7*	8*	8.7**
	**** Denotes P<0.05 (by Mann-Whtiney U) relative to baseline levels (Day 0)***												
	***** Denotes P<0.01 (by Mann-Whitney U) relative to baseline levels (Day 0)***												

Thrombocytopenia was most severe during the acute infection, with a 39-59% reduction (mean = 50% reduction) during the acute infection, and 19-51% (mean = 37% reduction) in the chronic infection (Table 8). PLT were significantly elevated on both days 15 and 150 PI. The increase in PLT on day 150 PI may be due to an infection in the animals, as they all had a strong inflammatory immune response on this day though parasitemia was cleared. On day 15 PI during the chronic infection, PLT significantly peaked to levels as high as 700, and the increase over baseline ranged from 21-158 (mean = 81% increase).

Table 8: Leukocyte and Platelet Indices in Acute and Chronic Stages of Malaria (Percent Change Relative to Baseline)

Leukocyte Indices	ACUTE	CHRONIC	ACUTE	CHRONIC
	(% Decrease)	(% Decrease)	(% Increase)	(% Increase)
WBC				
M2198	-37	-12	282	53
M10800	-31	-25	67	324
M11600	-49	-34	0	147
M11799	-57	-31	67	7
AVERAGE	**-43**	**-26**	**104**	**133**
LY				
M2198	-69	-49	231	49
M10800	-48	-39	91	636
M11600	-69	-53	88	181
M11799	-79	-72	63	7
AVERAGE	**-66**	**-53**	**118**	**218**
MO				
M2198	-80	-20	20	80
M10800	-50	-50	250	650
M11600	-67	-67	33	300
M11799	-67	-67	0	33
AVERAGE	**-66**	**-51**	**76**	**266**
NEU				
M2198	-28	-40	460	172
M10800	-47	-44	28	88
M11600	-88	-63	28	116
M11799	-75	-35	110	170
AVERAGE	**-59**	**-45**	**157**	**136**
Platlet Indices	**ACUTE**	**CHRONIC**	**ACUTE**	**CHRONIC**
	(% Decrease)	(% Decrease)	(% Increase)	(% Increase)
Plt				
M2198	-58	-19	43	22
M10800	-39	-39	102	22
M11600	-43	-37	158	10
M11799	-59	-51	21	8
AVERAGE	**-50**	**-36**	**81**	**15**
MPV				
M2198	0	0	25	41
M10800	0	0	18	30
M11600	-1	0	16	21
M11799	-6	0	38	41
AVERAGE	**-2**	**0**	**24**	**33**

During the chronic infection, the mean PLT increase was 15%. Parasitemia negatively correlated with level of PLT ($p<0.05$, by Pearson correlation) (Figure 7). PLT were positively correlated with both WBC and LY through the course of infection ($p<0.05$). PLT were also associated with MCV and MCH ($p<0.01$).

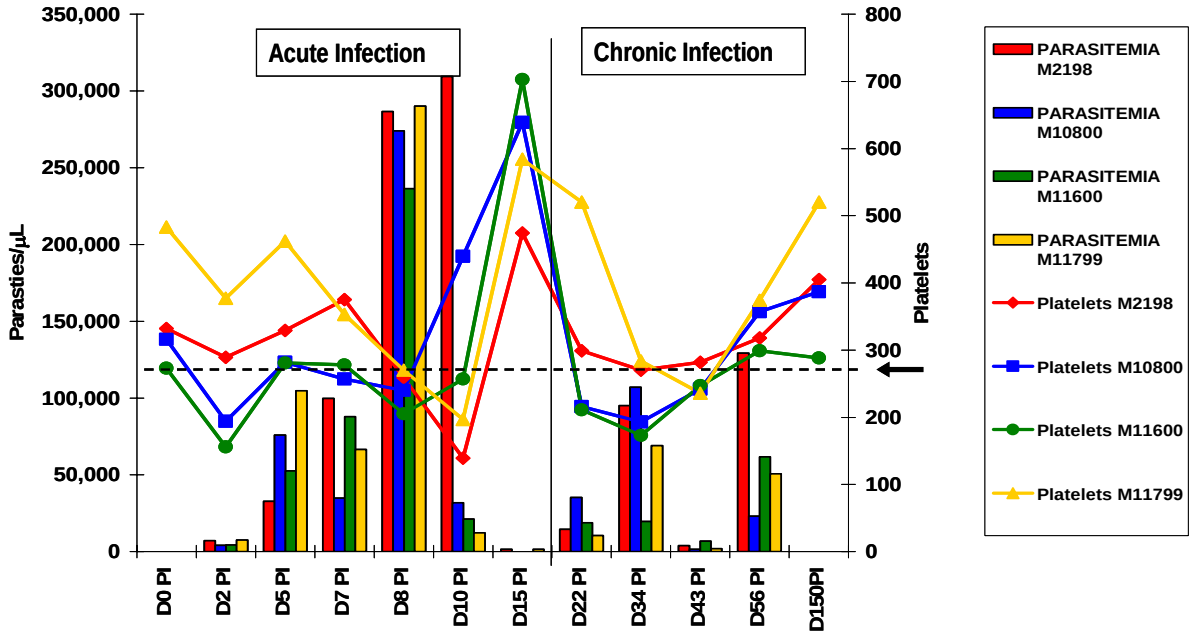


Figure 7: Parasitemia and Thrombocytopenia in Rhesus Macaques infected with *P. coatneyi*

Lowest levels of Hb during the acute infection were correlated with magnitude of PLT rebound after thrombocytopenia at parasite clearance ($p<0.05$) by randomization tests to obtain p-values for Pearson correlation (Figure 8).

MPV was examined as an indicator of PLT production in bone marrow. MPV in malaria is thought to decrease with increasing parasitemia (107). However, this was contradictory to the findings in our study. MPV was not positively correlated with PLT production, nor negatively correlated with increased parasitemia. There was an average 24% increase in the acute infection and 33% mean increase in the chronic infection. MPV was significantly elevated above baseline on days 5, 22, 34, and 150 PI ($p<0.01$), and was significantly higher on days 7, 10, 42, and 56 PI ($p<0.05$), which encompassed most of the days during the malarial infection.

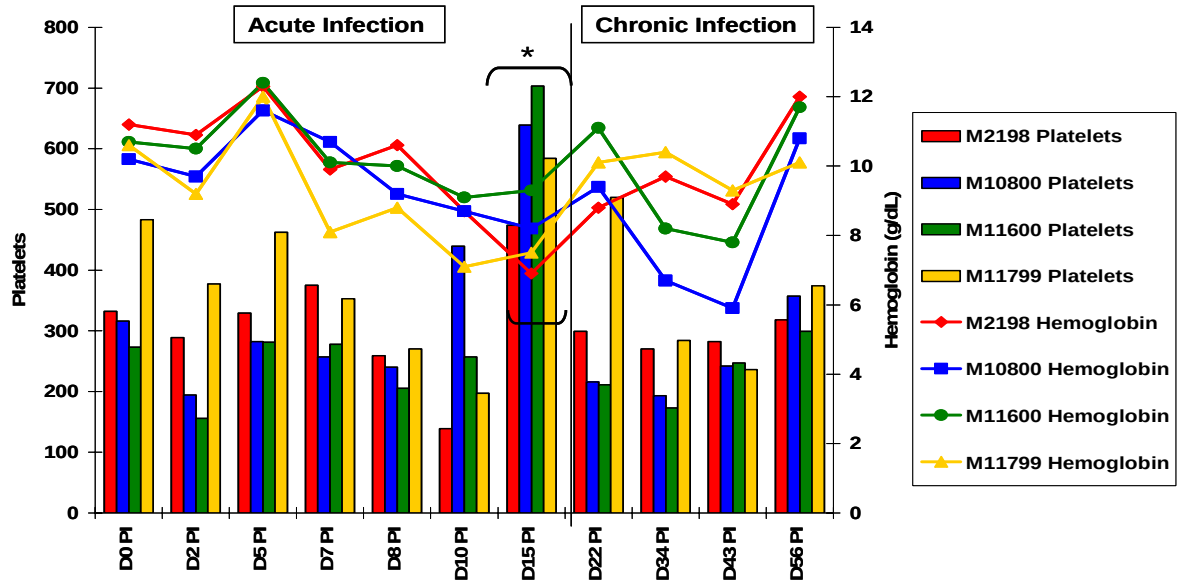


Figure 8: Thrombocytopenia and Anemia in Rhesus Macaques Infected with *P. coatneyi*

No significant association existed between parasite clearance and rebound in MPV, counterbalancing the loss of PLT during parasitemia (Table 9). MPV was found to be positively associated with both Hb and Hct ($p<0.01$), and negatively associated with both MCV and MCH ($p<0.01$). Lastly, there was a positive correlation between MPV and MO, LY, and WBC ($p<0.01$).

Table 9: Correlations among Hematological Parameters during *P. coatneyi* Malaria

	PARA	RBC	HB	HCT	MCV	MCH	MCHC	RDW	WBC	LY	MO	NEU	PLT	MPV	TEMP
PARA	x				-0.0005								-0.0115		
RBC		x	0.0000	0.0000				-0.0385							
HB	0.0000		x	0.0000				-0.0360			-0.0230			-0.0220	
HCT		0.0000	0.0000	x										-0.0345	
MCV	-0.0005				x	0.0000		0.0245					0.0000	-0.0095	0.0155
MCH					0.0000	x	0.0020				0.0015		0.0020	-0.0015	
MCHC						0.0020	x	0.0075	-0.0405		0.0045				
RDW		-0.0385	-0.0360		0.0245		0.0075	x			0.0145				0.0210
WBC							-0.0405		x	0.0000		0.0000	0.0300	0.0010	
LY									0.0000	x		0.0410	0.0130	0.0040	-0.0425
MO			-0.0230			0.0015	0.0045	0.0145			x			0.0000	
NEU									0.0000	0.0410		x			
PLT	-0.0115				0.0000	0.0020			0.0300	0.0130			x		
MPV			-0.0220	-0.0345	-0.0095	-0.0015			0.0010	0.0040	0.0000			x	
TEMP					0.0155			0.0210		-0.0425					x

WBC, LY and NEU were all positively correlated ($p<0.05$), and none were associated with MO. WBC were also correlated with MPV ($p<0.01$), and negatively correlated with MCHC ($p<0.05$). During the acute infection, there was a significant leukopenia in the four animals just prior to peak parasitemia, on day 7 PI ($p<0.05$). Leukopenia is common in *falciparum* malaria in non-immune adults and is usually reflective of neutropenia. (116). As predicted, neutrophils were significantly decreased on day 7 PI ($p<0.05$). There was a monocytosis on day 22 PI, which is seen in *falciparum* malaria in children and non-immune adults (116). MO were positively associated with MCH ($p<0.01$), MPV ($p<0.01$), MCHC ($p<0.01$) and RDW ($p<0.05$). Hb and monocytes were found to be inversely correlated ($p<0.05$). LY were positively associated with MPV ($p<0.01$). There was a significant lymphopenia on days 2 ($p<0.05$), 5, ($p<0.01$) 8 ($p<0.05$) and 22 ($p<0.05$) PI, which is seen in acute malaria in the non-immune (116), with a lymphocytosis rebound on day 56 PI ($p<0.05$). Lymphopenia was found to be correlated with body temperature ($p<0.05$).

M2198 had a 37% decrease in WBC on day 7 PI of the acute infection (4.3 x 10^3/μL) and then a 282% increase in WBC on day 15 PI (26 x 10^3/μL), also the day of lowest Hb during the acute infection (Table 7). During the chronic stage of infection, there was a 53% increase in WBC during the chronic infection on day 150 (10.4 x 10^3/μL). LY were lowest in the acute infection on day 8 (peak parasitemia) at 1.1 x 10^3/μL. On day 10, when a hyperparasitemia occurred, LY increased to 11.6 x 10^3/μL (231% above baseline) and remained at this level on day 15 PI. During the chronic infection stage, LY dropped to 1.8 x 10^3/μL on day 22 PO (49% reduction) and increased again on day 56 PI to 5.2 x 10^3/μL (49% increase). MO decreased by 80% in the acute phase of infection (day 15 PI, 0.1 x 10^3/μL) and increased 80 % in the chronic stage (day 42 PI, 1.2 x 10^3/μL) (Table 8). NEU were significantly lower on day 7 PI, one day

prior to peak parasitemia (1.8 x 10^3/μL). NEU were elevated to 9.1 x 10^3/μL and increased by 460% from baseline in the acute infection (day 15 PI, 14 x 10^3/μL) and 172% in the chronic infection (day 34 PI, 6.8 x 10^3/μL).

M10800 had a 31% decrease in WBC on day 2 PI, and then a 67% increase from baseline on day 15 PI, with a WBC of 11.2 x 10^3/μL (Table 7). During the chronic infection WBC increased by 324% on day 150 PI, where they were at 28.4 x 10^3/μL. On day 2 PI, M10800 also had the lowest level of LY at 1.7 x 10^3/μL, which was a 48% decrease from baseline. On days 10 and 15, LY had increased by 91% above baseline to 6.3 x 10^3/μL, decreasing on day 22 PI to 1.8 x 10^3/μL, a 39% reduction. On day 150 PI, the animal had a 636% increase in LY to 24.3 x 10^3/μL, which follows the increase in WBC. MO increased by 250% during the acute infection (day 7 PI, 0.7 x 10^3/μL) and by 650% during the chronic infection on day 34 PI to 1.5 x 10^3/μL. NEU were lowest in the acute infection on day 7 PI (1.7 x 10^3/μL, 47% reduction). On day 8 PI, NEU increased to 4.1 x 10^3/μL (28% increase), the peaked again on day 22 PI (88% increase, 6 x 10^3/μL) (Table 8). NEU were lowest during the chronic infection on day 56 PI with a 44% reduction to 1.8 x 10^3/μL.

M11600 had the best control over both parasitic and anemic conditions. WBC were elevated on day 150 PI to 16.8 x 10^3/μL (147% increase) (Table 7). WBC were not significantly elevated during the other timepoints. WBC were lowest at peak parasitemia in the acute phase (day 8 PI, 3.5 x 10^3/μL, 57% reduction), and day 34 PI during the chronic infection (4.5 x 10^3/μL, 34% reduction). LY were highest in on day 150 PI (9 x 10^3/μL, 181% increase), and also elevated in day 56 PI (4.8 x 10^3/μL). There was a lymphopenia at peak parasitemia during the acute infection (day 8, 1 x 10^3/μL, 69% reduction), and on day 34 in the chronic phase of

infection (1.5 x 10^3/μL, 53% reduction). During the chronic infection on day 42 PI, there was a monocytosis at 1.2 x 10^3/μL (a 300% increase). MO were lowest on days 5 and 22 PI (0.1 x 10^3/μL, 67% reduction). M11600 had a neutropenia on day 15 PI (0.4 x 10^3/μL, 88% reduction). NEU were highest on day 150 PI, at 6.9 x 10^3/μL, and also elevated on day 22 PI ay 5.2 x 10^3/μL.

M11799 showed the largest fluctuations in WBC during the acute phase of infection. WBC increased on day 15 PI to 11.7 x 10^3/μL (67% increase) and were lowest on day 7 PI at 3 x 10^3/μL (57% decrease) (Table 7). LY were lowest on day 8 PI (0.9 x 10^3/μL, a 79% decrease) and highest on day 10 and 15 at 7 x 10^3/μL (63% increase). MO were highest on days 34 and 56 PI at 0.8 x 10^3/μL. NEU were lowest on day 7 PI at 0.5 x 10^3/μL (75% decrease) and highest on days 15 (4.2 x 10^3/μL, 110% increase) and 34 PI (5.4 x 10^3/μL, 170% increase).

4.2 CYTOKINE AND EFFECTOR MOLECULE PROFILES

4.2.1 Plasma Cytokine Levels

Cytokines were measured at each of the eight timepoints for each monkey. Plasma cytokine levels at each timepoint, varied among the four animals. As such cytokine levels were examined in the group, and then individual profiles were also addressed. Plasma levels of IL-12, IL-10, TNF-α and NOx were all positively associated ($p<0.01$ by Pearson correlation) (Table 10). However, none of these cytokines were correlated with plasma levels of IFN-γ, released from CD4+ T cells. Associations among IL-12, TNF-α, IL-10 and NOx have been shown in human malaria (108), (141).

Table 10: Correlation Among Plasma Cytokine Levels during *P. coatneyi* Malaria

Pearson Correlations Among Plasma Cytokine & Effector Molecule										
PLASMA LEVELS	IL-12		IFN-γ		TNF-α		IL-10		NO	
	r	(p-value)	r	(p-value)	r	(p-value)	r	(p-value)	r	(p-value)
IL-12	X	X	0.284	(0.058)	0.543	(0.001)**	0.567	(0.001)**	0.679	<0.001**
IFN-γ	0.284	(0.058)	X	X	0.146	0.213	0.164	0.185	0.174	0.170
TNF-α	0.543	(0.001)**	0.146	(0.213)	X	X	0.585	<0.001**	0.498	(0.002)**
IL-10	0.567	(0.001)**	0.164	(0.185)	0.585	<0.001**	X	X	0.426	(0.008)**
NO	0.679	<0.001**	0.174	(0.170)	0.498	(0.002)**	0.426	(0.008)**	X	X

Denotes (p<0.05) by 1-tailed randomization-based Pearson correlations
****Denotes (p<0.01) by 1-tailed randomization-based Pearson correlations***

Plasma cytokine levels at each timepoint were compared relative to baseline for significance using a Mann-Whitney U test. (Table 11) IFN-γ plasma levels were significantly elevated above baseline in all four animals on day 22 PI ($p<0.05$). IL-12p40 cytokine levels were not significantly augmented above baseline at any timepoints in all four animals. TNF-α plasma levels were significantly augmented on day 8 PI (peak parasitemia). Plasma levels of NOx were significantly elevated on day 2 PI. Plasma levels of anti-inflammatory IL-10 were significantly elevated above baseline on days 5 and 7 PI in all four animals. ($p<0.05$)

Table 11: Plasma Cytokine Levels Relative to Baseline

	p-values						
Plasma	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**
IFN-γ	0.262	0.071	0.249	0.166	0.053	0.053	0.014**
IL-12p40	0.209	0.109	0.063	0.160	0.192	0.110	0.259
TNF-α	0.389	0.989	0.382	0.013*	0.072	0.072	0.081
IL-10	0.136	0.007*	0.022*	0.100	0.130	0.081	0.131
NO	0.038*	0.082	0.225	0.255	0.053	0.420	0.070

**** Denotes P<0.05 (by Mann-Whitney U) relative to baseline levels (Day 0)***
***** Denotes P<0.01 (by Mann-Whitney U) relative to baseline levels (Day 0)***

Plasma levels of IL-12 were shown as percent change relative to baseline (Figure 9). IL-12 plasma levels in the four animals were positively associated with parasitemia ($p<0.01$ by

Pearson correlation). The degree of peak parasitemia on day 8 PI was predicted by plasma levels of IL-12 on day 5 PI ($p<0.05$).

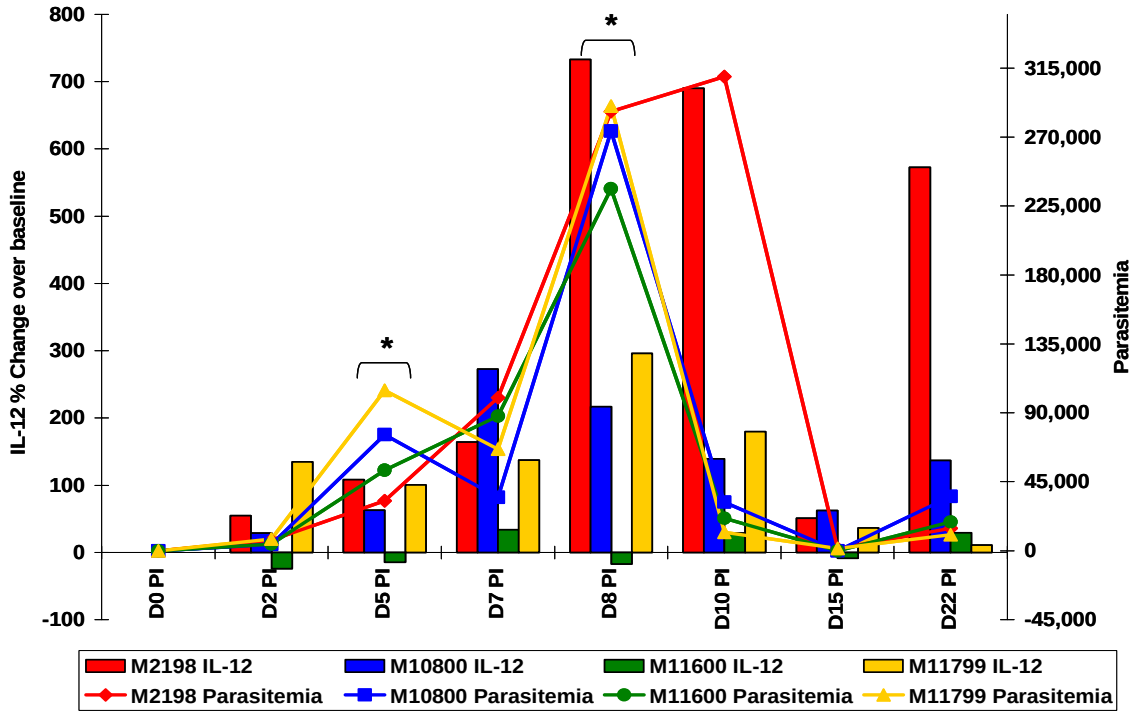


Figure 9: Plasma IL-12 Relative to Parasitemia during *P. coatneyi* Malaria

M2198 had the highest IL-12 response of all four animals, increasing 573-733% above baseline on days with high parasite burden (Figure 10). Plasma IL-12 levels increased with increasing parasitemia in M2198 and were highest on days 8 (peak parasitemia), 10 (hyperparasitemia), and 22 PI (re-emergence of parasites). In M10800, levels of IL-12 were highest on day 7 (273% increase), one day prior to peak parasitemia. IL-12 plasma levels decreased with clearance of parasitemia and increased with the re-emergence of parasites on day 22 PI (137% increase). M11600 showed a reduction in plasma levels of IL-12 on days 2, 5, 8, and 15 PI. Plasma IL-12 levels increased slightly on days 7 and 10 PI in M11600 (29% increase). M11799 had a high IL-

12 response at peak parasitemia (day 8 PI, 296% increase). IL-12 response did not increase significantly on day 22 PI with the re-emergence of parasitemia (11% increase).

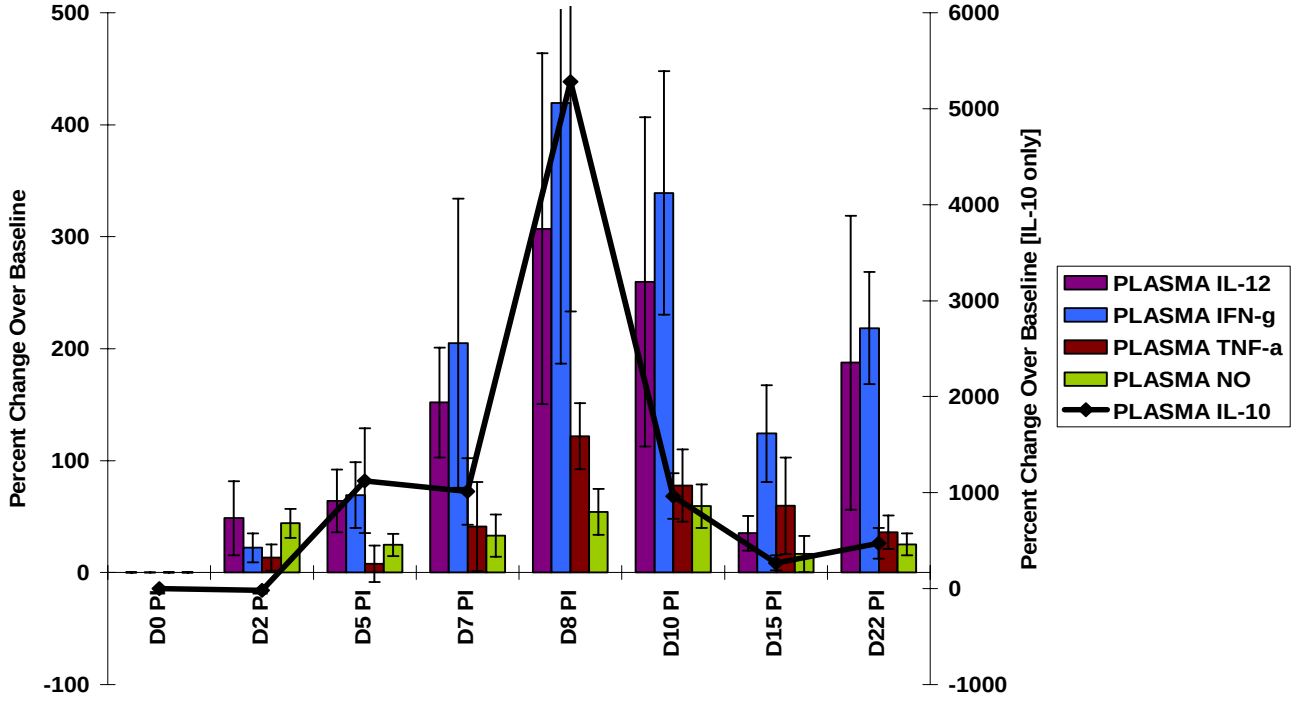


Figure 10: Magnitude of Plasma Cytokine Response during Malaria

Plasma IFN-γ levels in the four animals were shown to be positively correlated with parasitemia ($p<0.05$) (Figure 11). Plasma IFN-γ response in M2198 increased with parasitemia on days 8 (peak parasitemia, 302%) and 10 PI (hyperparasitemia, 388%), then decreased on day 15 PI, when parasitemia had cleared (32% increase). Plasma levels increased on day 22 PI with the re-emergence of parasitemia (339 % above baseline) (Figure 10). M10800 had a decrease in plasma IFN-γ levels below baseline on days 2 and 7 PI. Plasma levels of IFN-γ increased dramatically in M10800 at peak parasitemia (1106% on day 8 PI), and remained elevated at day

10 PI (626% above baseline). Plasma IFN-γ levels in M10800 were similar on days 15 and 22, with 146 and 125% increase respectively. IFN-γ plasma levels in M11600 were highest on days 7 (177% increase), 10 (157% increase) and 22 PI (262 % increase). Plasma IFN-γ levels in M11799 were highest on day 7 PI (568%) and on parasite clearance, day 15 PI (233%).

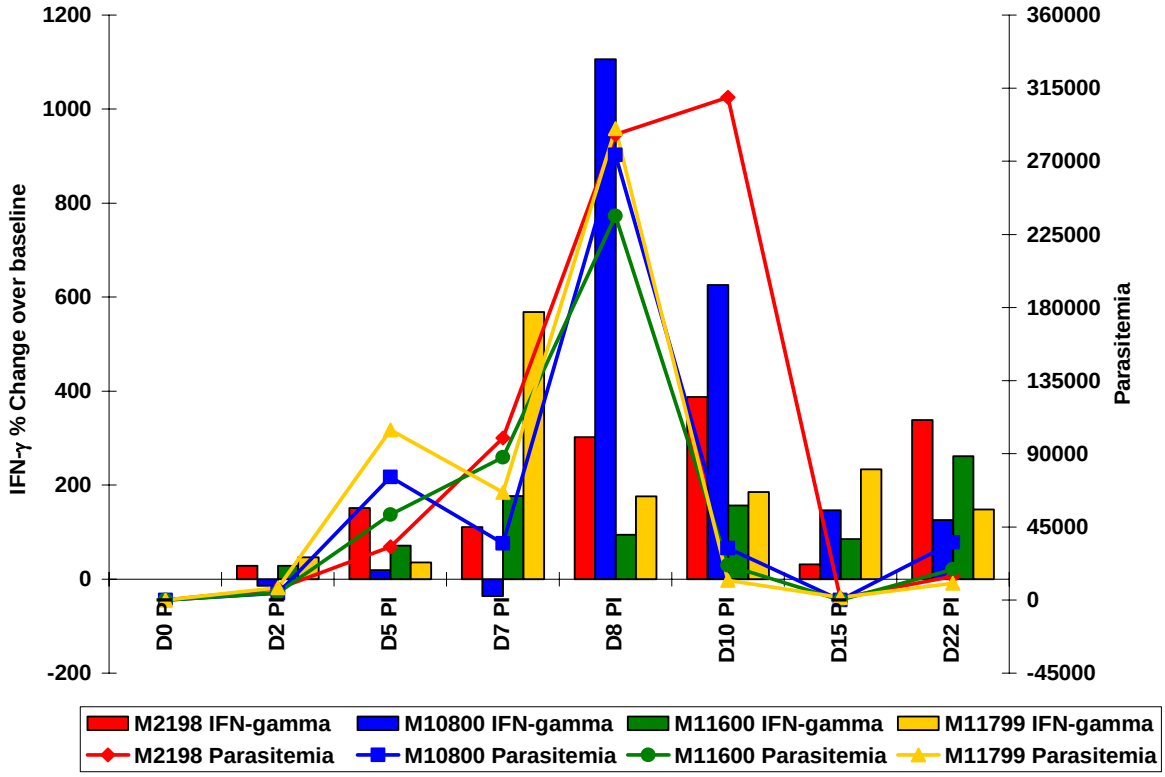
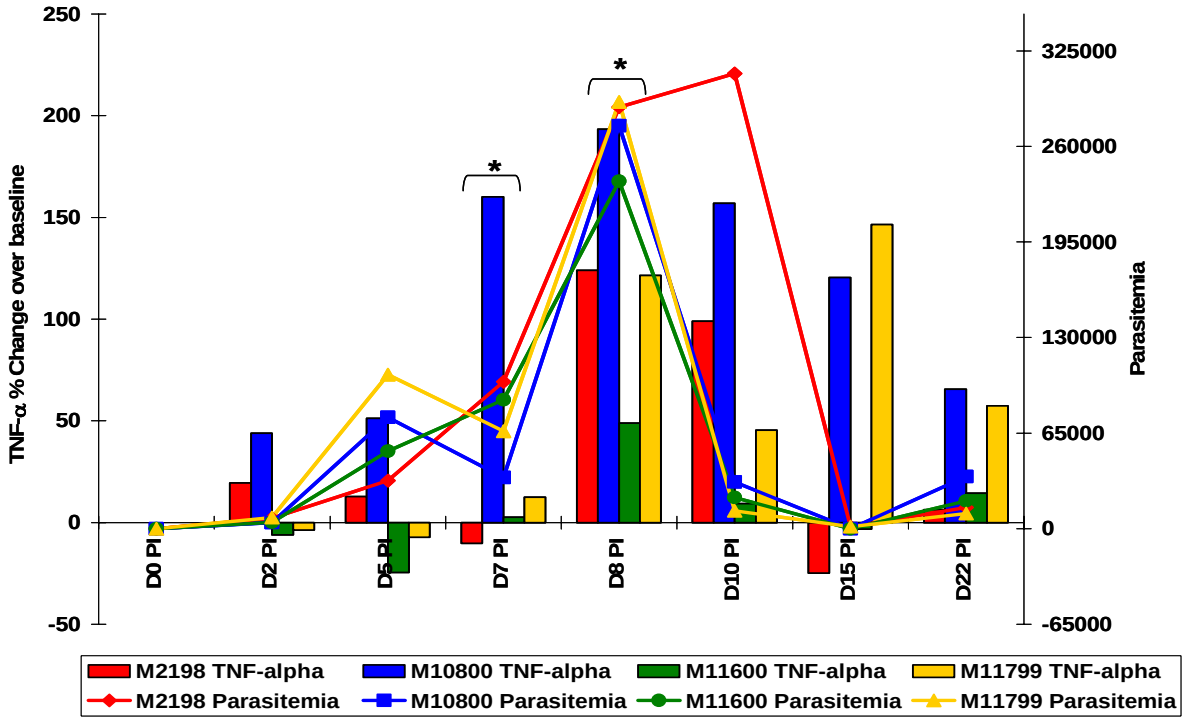


Figure 11: Plasma IFN-γ Levels Relative to Parasitemia

Plasma TNF-α levels were positively correlated with parasitemia in all four animas ($p<0.01$). (Figure 12). TNF-α plasma levels on day 7 PI were a negative predictor of peak parasitemia on day 8 PI ($p<0.05$, Pearson). Plasma TNF-α in M2198 was highest at peak parasitemia (124% increase) and hyperparasitemia on day 10 PI (99% increase), and decreased below baseline on days 7 PI and day 15 PI, just prior to increases in parasitemia. M10800 had an elevated plasma TNF-α response on days 7, 8, and 10 PI (>150% increases above baseline). At

parasite clearance on day 15 PI, TNF-α plasma levels remained elevated (120% above baseline, and then declined at the re-emergence of parasitemia on day 22 PI (66% increase over baseline). M11600 had a decrease in plasma TNF-α levels on days 2, 5 and 15 PI, following the same trends as plasma IL-12 reduction. TNF-α plasma levels were highest at peak parasitemia in M11600 (49% increase above baseline). M11799 had low plasma levels of TNF-α relative to baseline on days 2, 5, and 7 PI. Plasma TNF-α levels in M11799 were highest at parasite clearance, day 15 PI (146% increase) and at peak parasitemia, day 8 PI (121% increase).



**Level of plasma TNF-α on day 7 PI is negatively correlated with parasitemia on day 8 PI (p<0.05)*

Figure 12: Plasma Levels of TNF-α Relative to Parasitemia

Parasitemia and plasma levels of IL-10 were positively correlated in the four animals ($p<0.01$) (Figure13). Plasma IL-10 levels in M2198 increased with parasitemia, and were highest on peak parasitemia (3162% increase) and hyperparasitemia day 10 (1662% increase).

Levels of IL-10 decreased at parasite clearance (369% increase from baseline) and increased with the re-occurrence of parasites on day 22 PI (763% increase) (Figure 10). Plasma levels of IL-10 followed parasitemia in M10800, and increased to 733% above baseline on day 5 PI, and then decreased to 276% on day 7 PI, when parasitemia decreased. In M10800, plasma IL-10 levels increased to 988% above baseline at peak parasitemia, decreased to 169% at parasite clearance, and then increase to 732% at the re-occurrence of parasites on day 22 PI (Figure 10). M11600 had high plasma IL-10 levels on day 5 PI (2740% increase) and peak parasitemia (4930% increase). Plasma IL-10 levels decreased at parasite clearance on day 15 PI (107%) and rebounded (237% above baseline) on day 22 PI when parasitemia increased. M11799 had increased plasma IL-10 levels at peak parasitemia (12046% increase), and decreased plasma IL-10 levels as parasitemia decreased. Plasma IL-10 levels in M11799 did not increase significantly as parasitemia re-emerged on day 22 PI (155% increase).

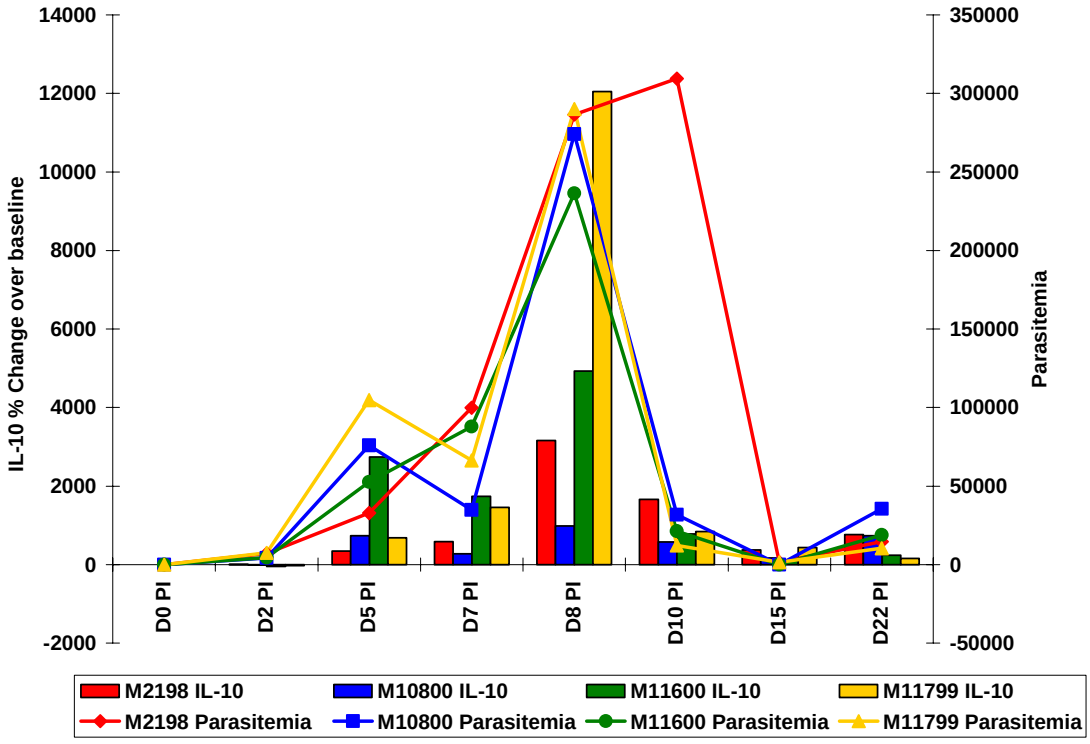


Figure 13: Plasma Levels of IL-10 Relative to Parasitemia

4.2.2 Effector Molecule Profiles

Parasitemia was positively correlated with NOx plasma levels in all four animals ($p<0.01$ by Pearson correlation) (Figure 14). NOx plasma levels increased with parasitemia and were elevated during peak parasitemia (112% increase) and hyperparasitemia (117% increase) in M2198. Levels of NOx did not increase significantly in M2198 with the reemergence of parasites on day 22 PI (16% increase). NOx plasma levels in M10800 did not follow trends in parasitemia. Plasma NOx levels were highest on day 2, 5, 8, 10, and 22 PI (47-57% increases). Levels of plasma NOx in M11600 were highest on days 2, 7, and 10 PI (34-36% increases). NOx plasma levels declined in M11600 at parasite clearance on day 15 PI and were at 24%

below baseline. Plasma levels of NOx in M11799 did not follow trends in parasite burden. M11799 had the highest plasma NOx levels on day 2 PI (72% increase) and on days 8, 10 and 15 PI (38-43% increases).

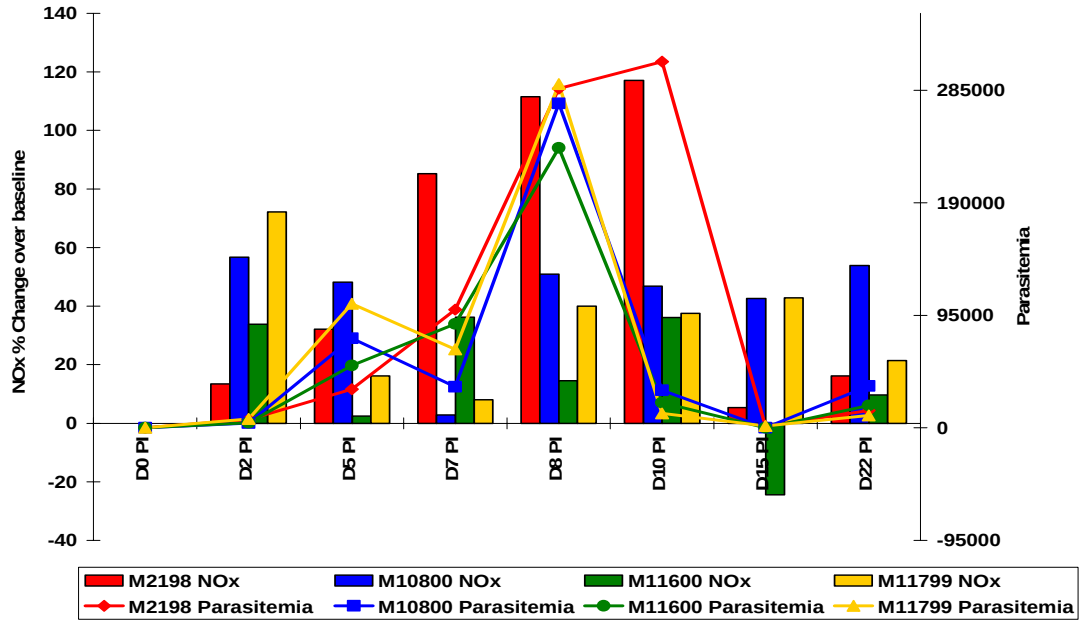


Figure 14: Plasma NOx Levels Relative to Parasitemia

4.2.3 Transcript Profiles

To determine if plasma cytokine levels were regulated at the transcriptional level in PBMC, transcript levels of each cytokine were measured at each of the eight timepoints. Pearson correlation was performed to determine if there was an association between plasma cytokine levels and PBMC transcripts. IL-12 transcript levels significantly decreased on days 10 ($p<0.05$), 15 ($p<0.05$), and 22 PI ($p<0.01$). IFN-γ transcript levels significantly increased above baseline on days 2 ($p<0.01$), 5 ($p<0.05$), and 7 ($p<0.05$), 10 ($p<0.01$), and 15 PI ($p<0.01$) (Table 12).

Table 12: Statistical Significance of Cytokine Transcript Levels Relative to Baseline

	p-values						
Transcripts	**D2**	**D5**	**D7**	**D8**	**D10**	**D15**	**D22**
IFN-γ	0.0003**	0.016*	0.018*	0.000**	0.000**	0.000**	0.202
IL-12p40	0.296	0.235	0.173	0.856	0.007*	0.033*	0.001**
TNF-α	0.378	0.305	0.281	0.039*	0.263	0.024*	0.121
IL-10	0.320	0.324	0.195	0.339	0.252	0.432	0.833

**** Denotes P<0.05 (by Mann-Whitney U) relative to baseline levels (Day 0)***
***** Denotes P<0.01 (by Mann-Whitney U) relative to baseline levels (Day 0)***

TNF-α transcript levels were significantly elevated on days 8 ($p<0.05$) and 15 PI ($p<0.05$). IL-10 transcript levels were not significantly elevated over baseline at any of the timepoints during the study. Due to the variation among animals and the small sample size, transcript profiles were examined in each monkey, and compared to plasma levels. IL-12 transcripts were positively correlated with IFN-γ transcripts ($p<0.05$), which were positively correlated with TNF-α transcripts ($p<0.05$), which were positively correlated with IL-10 transcripts ($p<0.01$) (Table 13). This coincides with the mechanism of regulation, where IL-12 induced protection in malaria is related to the ability of IL-12 to promote secretion of IFN-γ from Th1 cells. IFN-γ stimulates monocytes to release TNF-α that can induce cytotoxic free radicals, such as NO (60).

Table 13: Correlation Among Cytokine Transcripts during *P. coatneyi* Malaria

Pearson Correlations Among Cytokine Transcripts								
TRANSCRIPTS	**IL-12**		**IFN-γ**		**TNF-α**		**IL-10**	
	r	**(p-value)**	**r**	**(p-value)**	**r**	**(p-value)**	**r**	**(p-value)**
IL-12	X	X	0.371	(0.018)*	-0.068	0.356	-0.003	(0.493)
IFN-γ	0.371	(0.018)*	X	X	-0.055	0.382	0.091	(0.310)
TNF-α	-0.068	(0.356)	-0.055	(0.382)	X	X	0.592	<0.001**
IL-10	-0.003	(0.493)	0.091	(0.310)	0.592	<0.001**	X	X

****Denotes (p<0.05) by 1-tailed randomization-based Pearson correlations***
*****Denotes (p<0.01) by 1-tailed randomization-based Pearson correlations***

IL-12 increased in the early infection as parasitemia began to increase (Figure 15). M2198 had a 2-fold increase on days 5 and 7, and then transcript levels decreased at peak parasitemia, and remained relatively unchanged for the other timepoints. M10800 had high levels on days 2 (4-fold increase), 5(10-fold increase), and 7 (6-fold increase) PI. M11600 had an increase in IL-12 transcripts on days 2 (3-fold), 5 (4-fold), 7 (9-fold) and 8 (4-fold) PI. IL-12 transcript levels M11799 showed little fluctuation during the entire infection. Plasma levels of IL-12 were not significantly associated with transcript levels in any of the animals, suggesting that plasma levels of IL-12 are from other sources than solely PBMC.

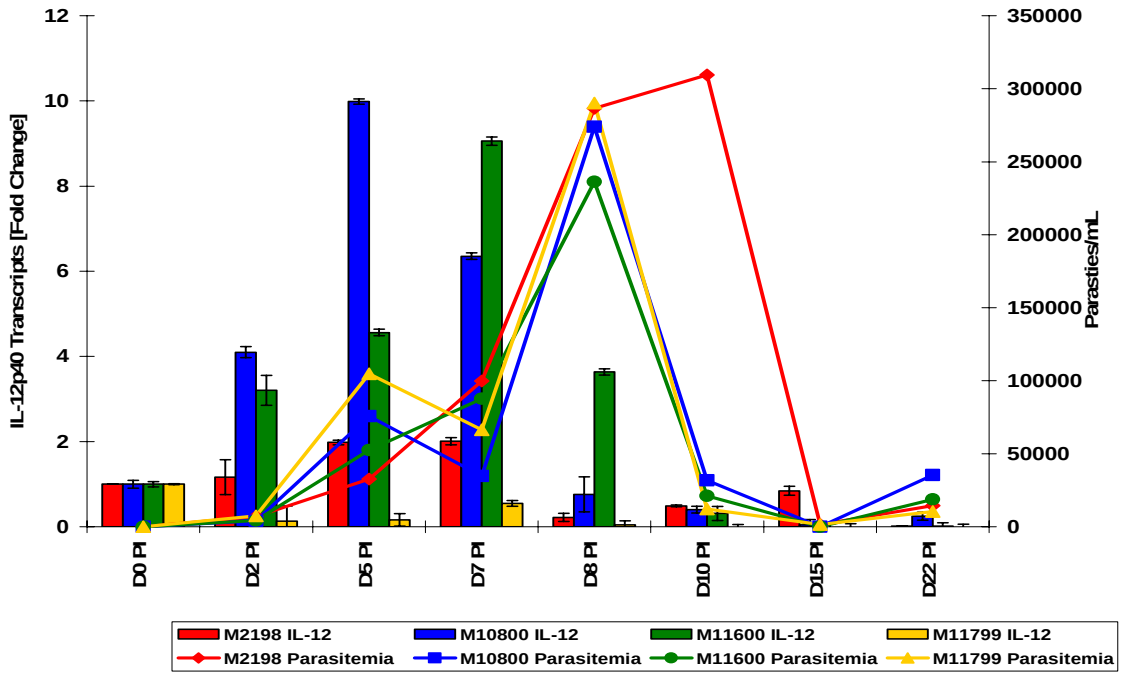


Figure 15: IL-12p40 Transcript Levels Relative to Parasitemia

M2198 had a 2-fold increase in IFN-γ on day 5, followed by a 6-fold IFN-γ increase on day 10 PI (Figure 16). M10800 showed a 2-fold increase in IFN-γ early in the infection on days 2 and 5, and little IFN-γ transcription activity on any other days. In M11600, there was a strong early IFN-γ transcription on days 2 and 5 PI (above a 10-fold increase), followed by a 4-fold

increase on day 7 PI and 2-fold increase on day 15 PI. M11799 did not have much fluctuation in IFN-γ transcription. IFN-γ transcript and plasma levels were not correlated by regression analysis, demonstrating that plasma IFN-γ is not solely derived from PBMC in rhesus infected with *P. coatneyi*.

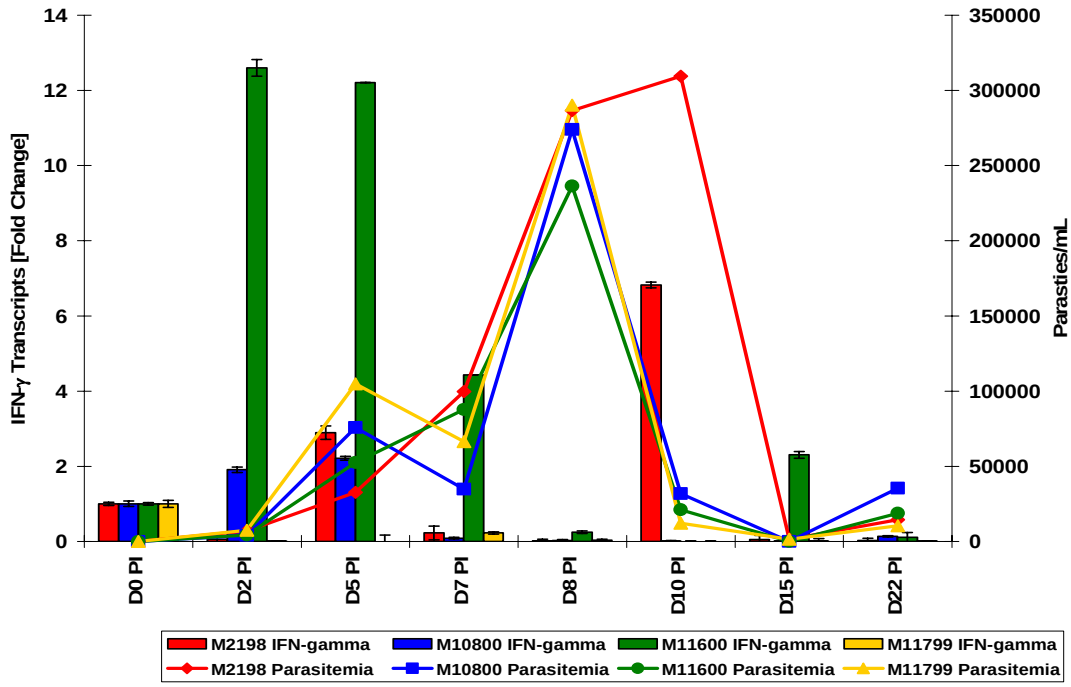


Figure 16: IFN-γ Transcript Levels Relative to Parasitemia

There was a strong increase in TNF-α transcription after peak parasitemia (Figure 17). M2198 had increasing TNF-α levels with the initial peak in parasitemia, where they increased 2-fold on day 8, and 3-fold on day 10 PI. M10800 had the highest TNF-α response, which increased with initial peak parasitemia, and elevated through day 15 (9-fold increase) until day 22, where levels dropped to 7-fold increase. TNF-α transcripts in M11600 increased 5-fold at peak parasitemia, increased 10-fold at parasite clearance on day 15, and then decreased to 5-fold

on day 22 PI. In animal M11799, TNF-α transcripts increased 3-fold at peak parasitemia, and then increased 5-fold at re-emergence of parasitemia on day 22 PI. TNF-α transcript and plasma levels were not correlated in any of the four animals.

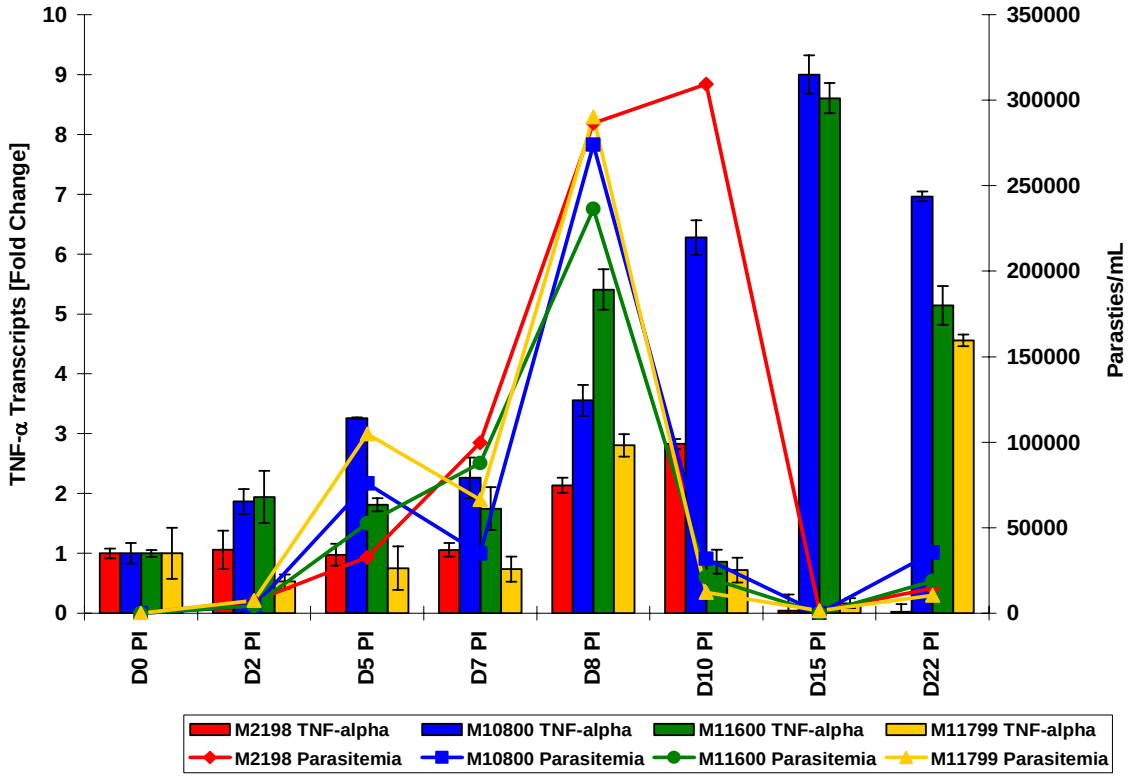


Figure 17: TNF-α Transcript Levels Relative to Parasitemia

IL-10 transcripts did not fluctuate in the four animals with the exception of M11600, which had an increase in IL-10 transcription in day 7, 8 and 15 (12-fold increase) and 22. (Figure18). M11799 also had an increase in IL-10 transcripts at the re-emergence of parasitemia on day 22 PI (4-fold increase). Plasma levels of IL-10 did not follow transcript levels in any of the animals and therefore plasma IL-10 not likely from PBMC.

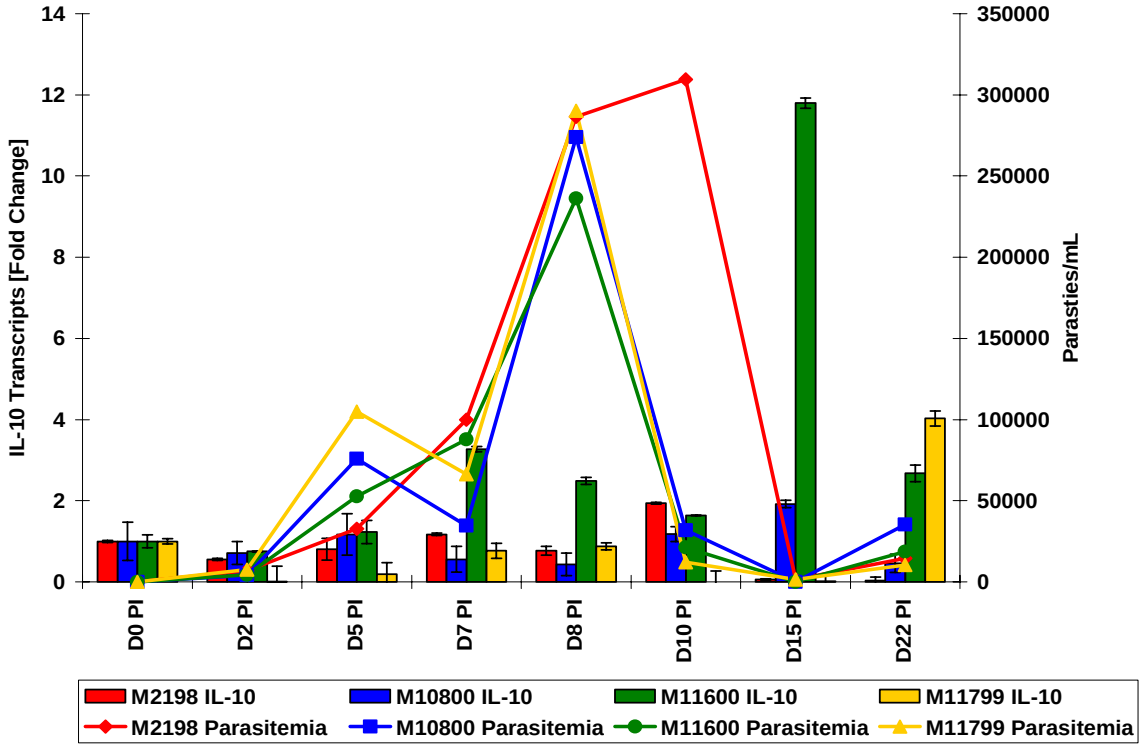


Figure 18: IL-10 Transcript Levels Relative to Parasitemia

4.3 CORRELATION OF CYTOKINE LEVELS WITH HEMATOLOGICAL OUTCOMES

Both plasma cytokine and transcript cytokine levels were compared to Hb and Hct to determine in there was a correlation between pro- and anti-inflammatory cytokine production and transcription as predictors of anemia. Randomization tests to determine p-values for Pearson-correlations were performed at each timepoint, and also compared variables at different timepoints to determine if there was a correlation between anemia and cytokine production. Small sample size accompanied by the level of variation among the four animals made it difficult to model and achieve significance.

4.3.1 Cytokine and Effector Molecule Regulation during Anemia

IL-12 transcript levels were positively correlated with Hb and Hct and RBCs ($p<0.05$), which supports that IL-12 does play a protective role in malarial anemia in a *P. coatneyi* infection in rhesus macaques, just as it does in human malaria (32), (142) (Table 14). IL-12 may play a protective role in *coatneyi* malaria through PLT (106).

Table 14: Correlation Among Plasma Cytokine Levels and Clinical Parameters of Disease Severity in *P. coatneyi* Malaria

Pearson Correlations Among Plasma Cytokine & Effector Molecule and Clinical Parameters of Disease Severity										
PLASMA LEVELS	IL-12		IFN-γ		TNF-α		IL-10		NO	
	r	(p-value)	r	(p-value)	r	(p-value)	r	(p-value)	r	(p-value)
PARASITEMIA	0.612	(0.000)**	0.399	(0.012)*	0.605	(0.000)**	0.759	(0.000)**	0.561	(0.001)**
HB	-0.169	(0.177)	-0.307	(0.044)*	-0.209	(0.126)	-0.137	(0.227)	-0.076	(0.340)
HCT	-0.229	(0.104)	-0.328	(0.034)*	-0.279	(0.061)	-0.185	(0.155)	-0.146	(0.213)
PLATELETS	-0.280	(0.605)	-0.105	(0.283)	-0.089	(0.313)	-0.229	(0.104)	-0.287	(0.056)
**Denotes (p<0.05) by 1-tailed randomization-based Pearson correlations* ***Denotes (p<0.01) by 1-tailed randomization-based Pearson correlations*										

Animals that had a lower Hb in the acute infection had increased IL-12 plasma levels during the higher levels of parasitemia (Figure 19). At peak parasitemia (day 8 PI), plasma levels of IL-12 followed the decrease in Hb until day 15 PI, when both increased through day 22 PI. IL-12 transcripts on days 7 and 8 PI were positive predictors of anemia during the acute infection ($p<0.05$). M2198 had the lowest Hb during the acute infection and also the highest levels of IL-12. Animals that had the least change in Hb during the acute phase of disease had the highest increase in IL-12 transcript levels in the early infection on days 2, 5, 7 PI (M10800, M11600). M2198 and M11799 had the lowest Hb levels in the acute phase of infection with a low level of IL-12 transcription in the early infection.

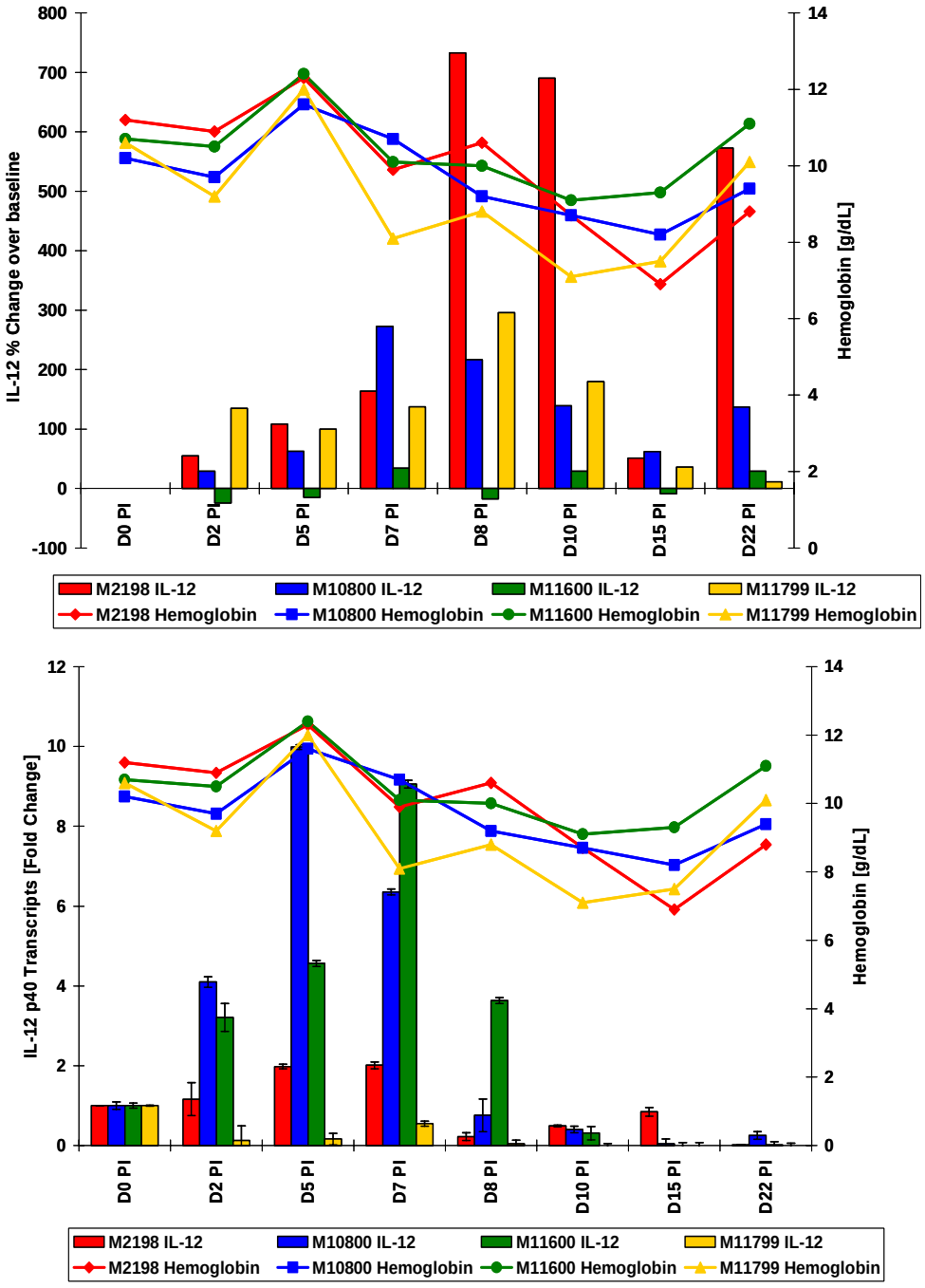


Figure 19: IL-12 (Plasma and Transcript Levels) Relative to Anemia

IFN-γ transcript levels were positively correlated with Hb, Hct and RBC counts and IL-12 transcript levels, implicating that IL-12 induced IFN-γ could play a protective role in anemia

(Table 14). Both plasma levels of IFN-γ and Hb decrease at parasite clearance (day 15 PI) (Figure 20) and increase with the reoccurrence of parasites on day 22 PI, though results were not significant. Plasma levels of IFN-γ on day 10 PI were positively correlated with level of Hb on day 42 PI ($p<0.05$) (Figure 20). The degree of Hb loss during day 42 in the acute infection was in part predicted by the level of IFN-γ response during day 10 in the acute infection ($p<0.05$).

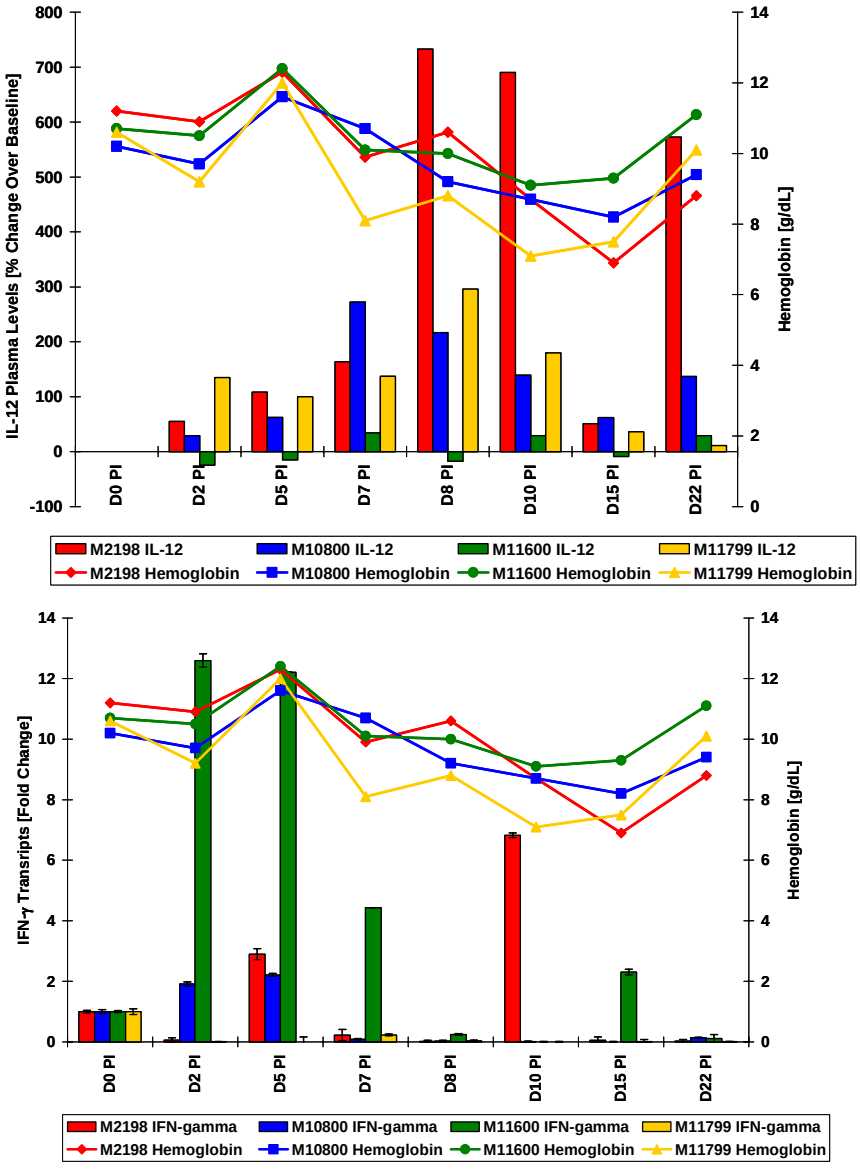


Figure 20: IFN-γ (Plasma and Transcript Levels) Relative to Anemia

TNF-α levels in plasma increased at peak parasitemia and decreased with the decline in Hb, with the exception of M11799, increased on day 15 PI. Transcript levels of TNF-α were highest in animals that were able to control anemia during the acute phase of infection (M10800, M11600) (Figure 21). TNF-α transcripts were highest in these animals at peak parasitemia and parasite clearance. TNF-α transcript levels at levels on day 15 PI were significantly associated with level of Hb decline ($p<0.05$) by Pearson-correlation.

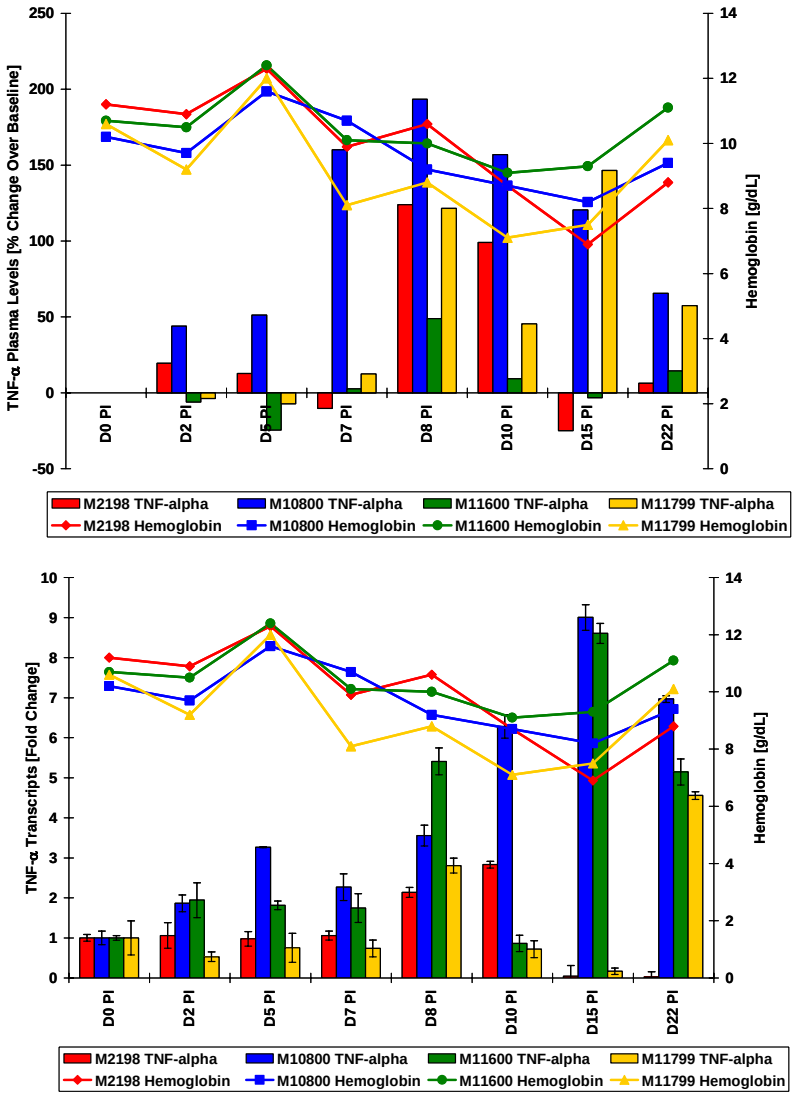


Figure 21: TNF-α (Plasma and Transcript Levels) Relative to Anemia

IL-10 plasma levels were highest at peak parasitemia, and correlated negatively with level of Hb (Figure 21). Higher plasma level of IL-10 on day 7 PI correlated with the lowest Hb levels ($p<0.05$). Transcript levels of IL-10 were highest following parasite clearance, and were higher in the animals that had elevated Hb during the acute phase of infection (M10800 and M11600). Increased IL-10 transcripts at parasite clearance (day 15 PI) were significantly associated with having a less severe anemia ($p<0.05$).

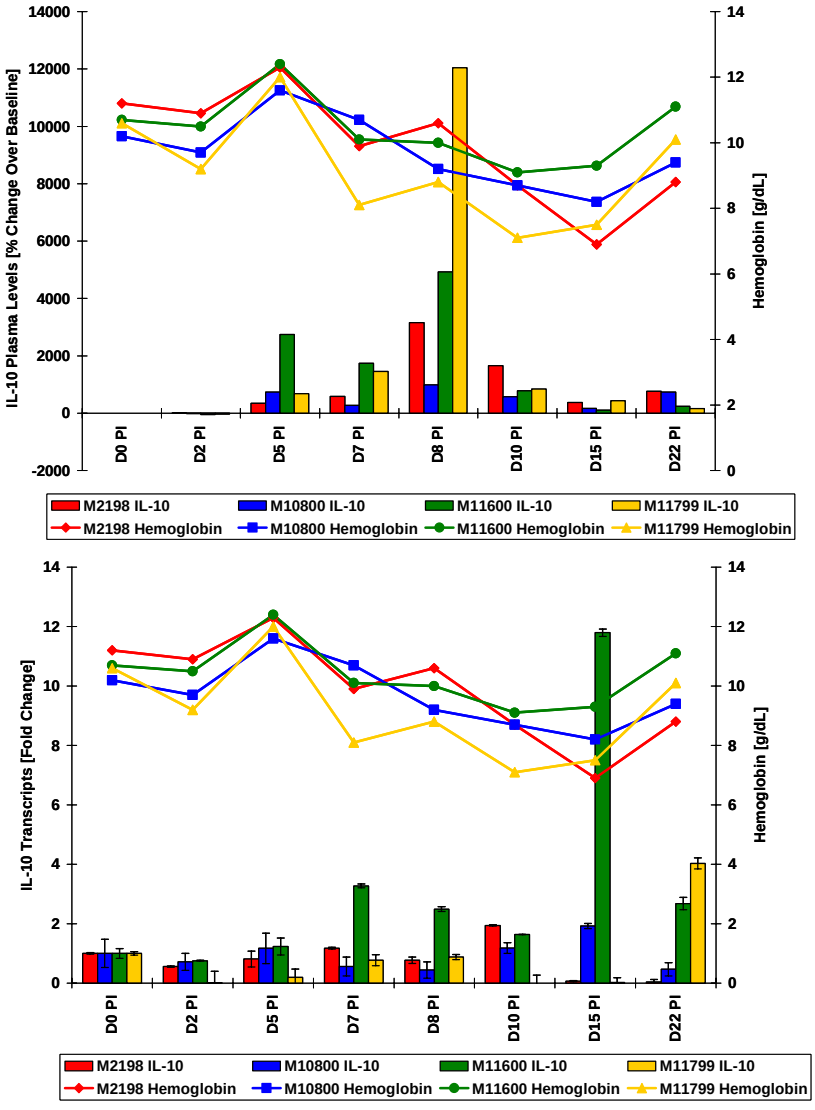


Figure 22: IL-10 (Plasma and Transcript Levels) Relative to Anemia

Plasma levels of total NO were highest in the animal that had the lowest Hb in the acute infection (M2198) at peak parasitemia and hyperparasitemia (Figure 23). Transcript levels of NO were undetectable by real time RT-PCR. Levels of NO production were not associated with Hb in all four animals.

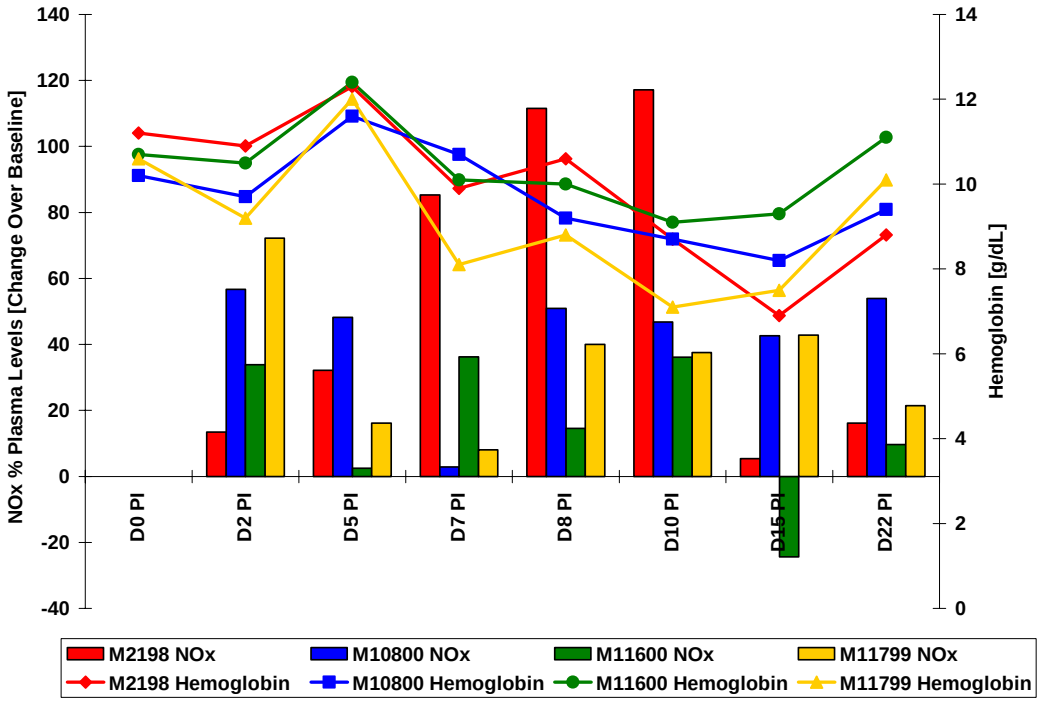


Figure 23: NOx (Plasma Levels) Relative to Anemia

4.3.2 Regulation of Platelets

Although thrombocytopenia can be harmful, having a reduced PLT count during *falciparum* malaria has also been shown to be protective as it reduces parasite sequestration (106). IL-12 transcripts were negatively correlated with PLT levels during the malarial infection ($p<0.05$). High plasma levels of IL-12 at peak parasitemia on day 8, were significantly inversely associated with PLT levels at parasite clearance on day 15 ($p<0.05$) (Figure 24). M11600 and

M10800 had the highest early levels of IL-12 transcription and had the highest levels of PLT production at parasite clearance.

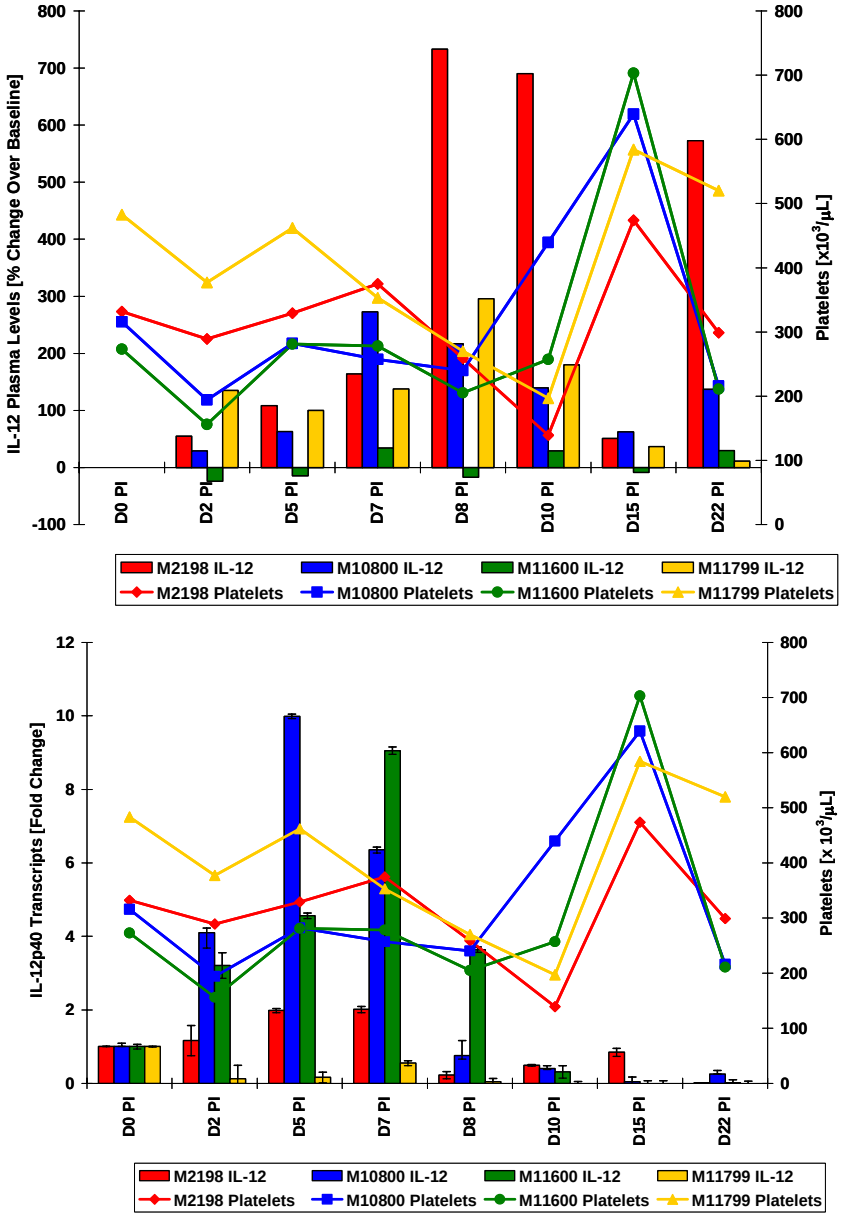


Figure 24: IL-12 (Plasma and Transcript Levels) Relative to Thrombocytopenia

PLT followed inverse trends with plasma IFN-γ levels during the malarial infection, but were not statistically significant (Figure 25). Early IFN-γ transcript levels were highest in the

animals that had the highest levels of PLT at parasite clearance, but again, there was no statistical correlation.

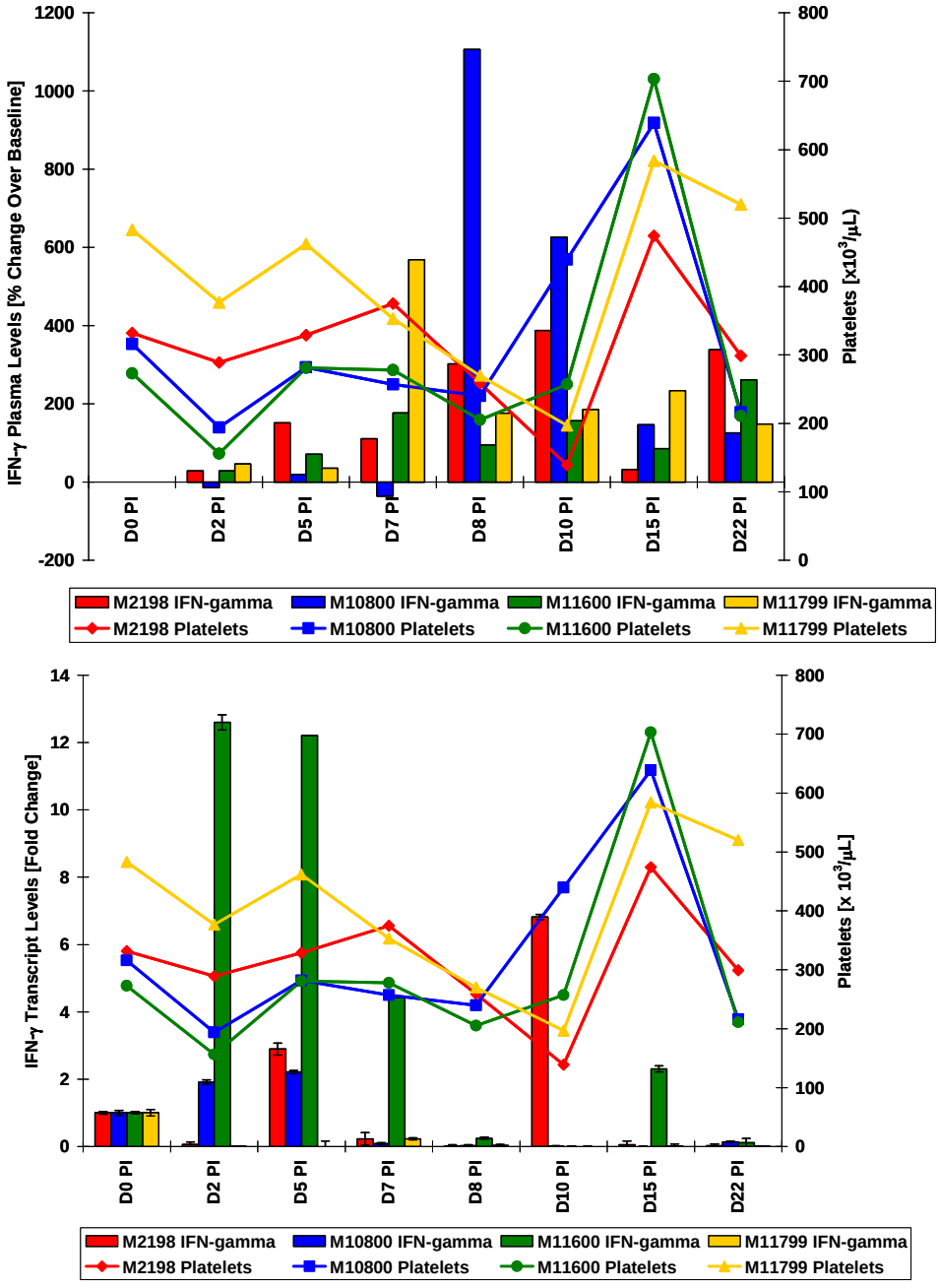


Figure 25: IFN-γ (Plasma and Transcript Levels) Relative to Thrombocytopenia

Through the course of infection, PLT decreased as TNF-α plasma levels increased (Figure 26). Plasma TNF-α levels prior to peak parasitemia on day 7 PI were significantly correlated with high levels of PLT production at parasite clearance on day 15 PI ($p<0.05$). TNF-α transcription was highest at parasite clearance in the animals that produced the highest levels of PLT. High levels of TNF-α at peak parasitemia on day 8 PI corresponded significantly with high levels of PLT production at parasite clearance on day 15 PI ($p<0.05$).

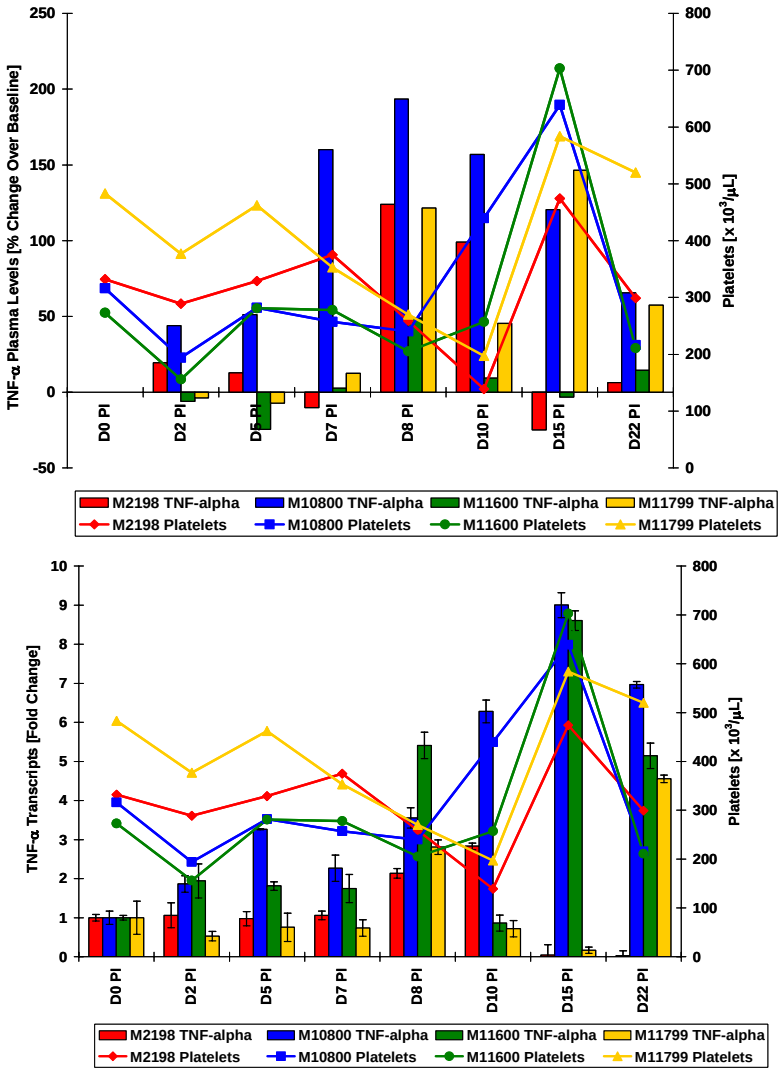


Figure 26: TNF-α (Plasma and Transcript Levels) Relative to Thrombocytopenia

PLT levels were found to be positively associated with IL-10 transcripts ($p<0.01$). Levels of plasma IL-10 were lowest at parasite clearance, which is when PLT were highest (Figure 27). There was a significant correlation between higher IL-10 plasma levels and lower PLT production at parasite clearance ($p<0.05$). Transcript levels of IL-10 were highest in M11600, which had the highest level of PLT at parasite clearance and the lowest level of PLT at the onset of parasitemia (day 2 PI), though these results were not significantly correlated.

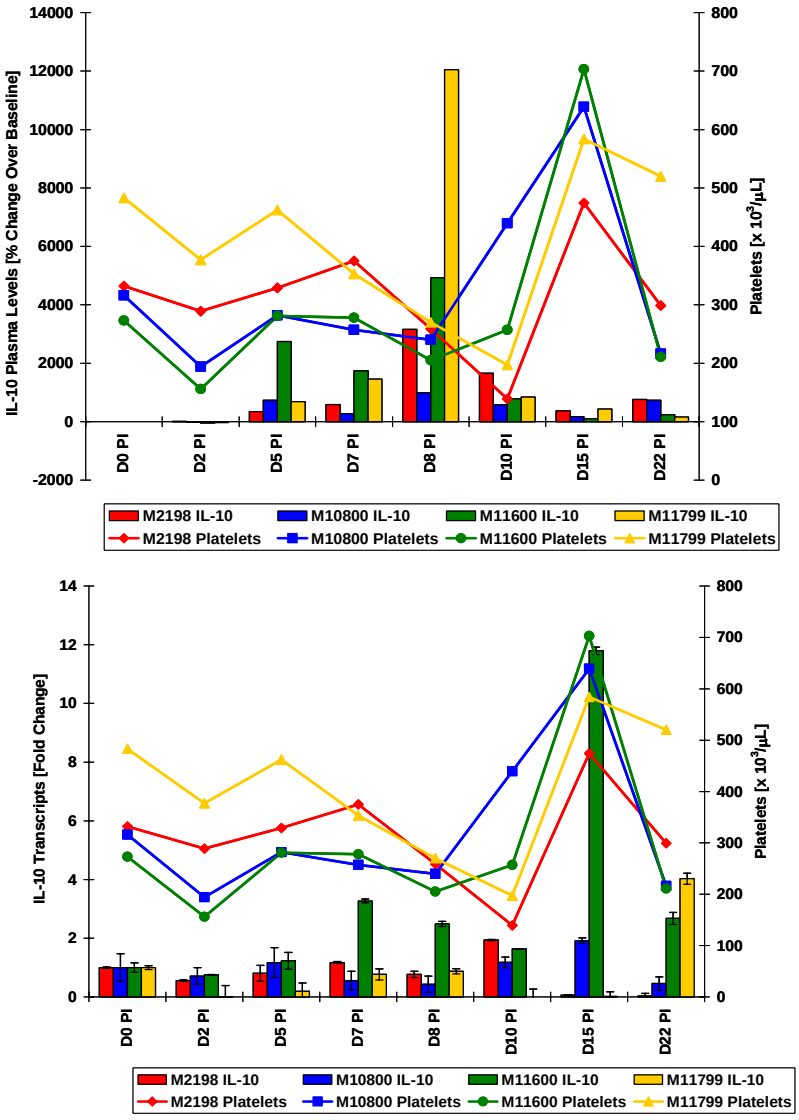


Figure 27: IL-10 (Plasma and Transcript Levels) Relative to Thrombocytopenia

The ability to produce highest levels of NO during acute malaria was highly significantly associated with high PLT levels at parasite clearance ($p<0.01$) (Figure 28). Transcript levels of NO were undetectable by real time RT-PCR. Production of high plasma levels of NO at peak parasitemia were significantly correlated with high PLT at parasite clearance ($p<0.05$).

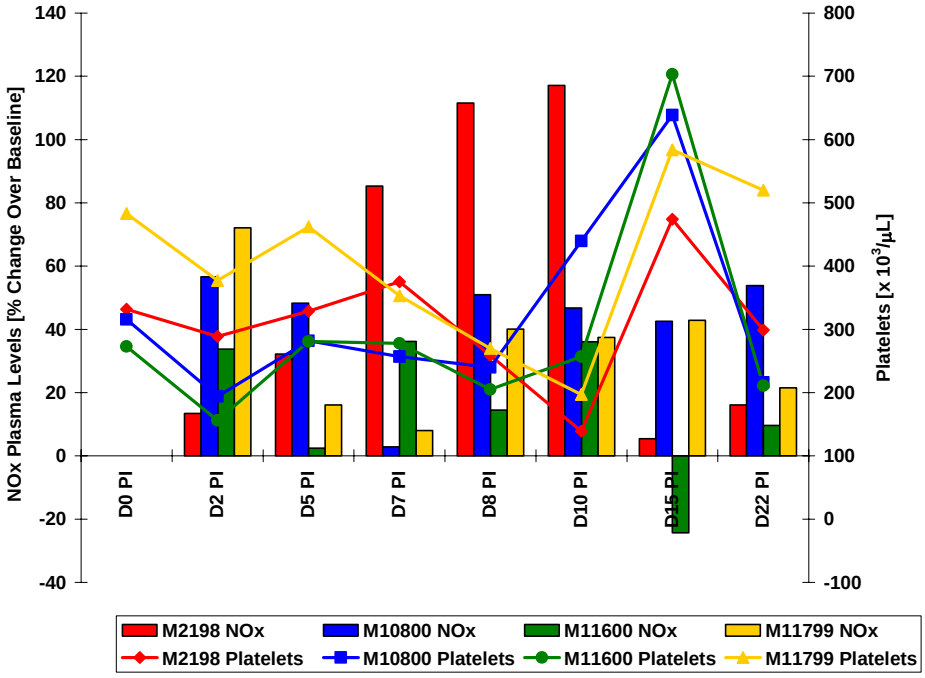


Figure 28: Plasma NOx Levels Relative to Thrombocytopenia

4.3.3 Anti-/Pro-Inflammatory Cytokine Profiles during Hyperparasitemia and Anemia

Plasma ratios of anti-inflammatory and pro-inflammatory cytokines were examined at levels of peak parasitemia, anemia and highest and lowest PLT levels. Statistical significance was determined using Mann-Whitney U tests (95% Confidence Interval) among the different conditions, and randomization tests to compute p-values for Pearson-correlations were used to

determine significance between ratios and markers of diseases severity (parasitemia, anemia, PLT production).

During the acute infection, M2198 and M11799 had the most severe parasitemias and anemias. M11600 and M10800 were able to control the degree of anemia in the acute infection. Interestingly, both animals had the quickest resolution of parasitemia in the acute infection (day 15 PI) and the highest level of PLT. For the duration of this study, M11600 had the least severe disease. In addition, this M11600 presented with the lowest anti-/pro-inflammatory cytokine ratios overall (Table 15).

Table 15: Plasma Cytokine Ratios during Peak Parasitemia, Anemia and Thrombocytopenia

	Baseline				
	[Hb]	IL-10/TNF–α	IL-10/IL-12	IL-10/IFN-γ	IL-10/NOx
M2198	**10.6**	**0.19**	**0.15**	**0.17**	**0.95**
M10800	**9.2**	**0.42**	**0.14**	**0.09**	**0.85**
M11600	**10**	**0.02**	**0.02**	**0.02**	**0.11**
M11799	**8.8**	**0.10**	**0.08**	**0.08**	**0.42**
	Peak Parasitemia				
	[PARA]	IL-10/TNF–α	IL-10/IL-12	IL-10/IFN-γ	IL-10/NOx
M2198	**286,590**	**2.58***	**0.57***	**1.31***	**10.25***
M10800	**274,000**	**1.57***	**0.48***	**1.31***	**6.11***
M11600	**236,320**	**1.13***	**1.88***	**0.60***	**6.97***
M11799	**290,160**	**5.47***	**2.43***	**3.55***	**36.76***
	Lowest Hemoglobin				
	[Hb]	IL-10/TNF–α	IL-10/IL-12	IL-10/IFN-γ	IL-10/NOx
M2198	**6.9**	**1.60**	**0.33****	**0.60****	**7.43**
M10800	**8.2**	**1.12**	**0.40****	**0.11****	**3.94**
M11600	**9.1**	**0.06**	**0.06****	**0.02****	**0.39**
M11799	**7.1**	**0.23**	**0.34****	**0.14****	**1.72**
	Lowetst Platelets				
	[PLT]	IL-10/TNF–α	IL-10/IL-12	IL-10/IFN-γ	IL-10/NOx
M2198	**139**	**1.60**	**0.33**	**0.60**	**7.43**
M10800	**194**	**0.26**	**0.10**	**0.09**	**0.47**
M11600	**156**	**0.01**	**0.01**	**0.00**	**0.03**
M11799	**197**	**0.69**	**0.29**	**0.29**	**3.12**

Denotes P<0.05 by Mann-Whitney U relative to baseline

****Denotes P<0.05 by Mann-Whitney U relative to peak parasitemia***

Plasma levels of IL-10/TNF-α were not significantly associated with Hb levels at peak parasitemia, nor were they significantly associated with lowest Hb levels (anemia). However, plasma levels of IL-10/TNF-α decreased in each animal by at least 40% (and up to>2,000%) in all four animals from peak parasitemia, to the day in the acute infection that the animal had the

lowest Hb. Low IL-10/TNF-α level when PLT were lowest was significantly associated with the magnitude of PLT increase at parasite clearance ($p<0.01$).

Levels of IL-10/IL-12 were significantly lower at anemia than at peak parasitemia ($p<0.05$). Levels of IL-10/IL-12 were significantly lower when PLT counts peaked and were lowest, than at parasite clearance than at peak parasitemia ($p<0.05$). When levels of peak PLT were compared to cytokine ratios, IL-10/IL-12 levels were significantly associated with highest levels of PLT ($p<0.05$, Pearson).

IL-10/IFN-γ were significantly lower at acute anemia than at peak parasitemia ($p<0.05$) Levels of IL-10/IFN-γ were significantly lower when PLT were highest, than at parasite clearance than at peak parasitemia ($p<0.05$). Levels of peak PLT were compared to cytokine ratios and it was determined that IL-10/IFN-γ levels were significantly associated with highest levels of PLT ($p<0.05$, Pearson).

Lastly, levels of IL-10/NOx were examined at peak parasitemia, anemia, and highest and lowest PLT. There was a significant decrease in the IL-10/NOx ratios from peak parasitemia to parasite clearance, which is the same day PLT peaked ($p<0.05$). Increased levels of PLT on parasite clearance (day 15 PI) correlated with low levels of IL-10/NOx ($p<0.05$).

5.0 DISCUSSION

P. coatneyi infection in rhesus macaques represents a valid model system for investigating malarial anemia. Cytokines are key determinants of malaria severity and outcome (143), (33) (144), (145), (35), (146), (147) and are potential targets for therapeutic interventions if their effects can be better understood. The balance between pro-and anti-inflammatory cytokines can determine the degree of malaria parasitemia the level of anemia, the clinical severity and disease outcome. Cytokine production and regulation in *P. coatneyi*-infected rhesus macaques are similar to those observed in children with malaria anemia. In addition, a characteristic of malarial infection is the effect of parasitemia on vital immune response parameters. The present study underlies pathophysiological effects of malaria in the rhesus macaque model. As such, parameters of infection determined during malarial infection in rhesus were consistent with that observed in humans. This study is the first to examine vital parameters associated with anemia implicated during malaria pathogenesis in the rhesus macaque model.

5.1 EFFECT OF *P. COATNEYI*-INFECTION ON HEMATOLOGICAL INDICES – ACUTE RELATIVE TO CHRONIC INFECTIONS

Plasmodium coatneyi infection in rhesus macaques includes clinical symptoms such as jaundice, anorexia, listlessness, fever, anemia, and splenomegally (130). During the course of

the malarial infection, fever occurred on days of peak parasitemia, but the overall burden of parasitemia did not determine the highest increase in body temperature. Fever was reduced in M2198 at hyperparasitemia, which could be a result of treatment with anti-malarials at peak parasitemia. As expected, body temperature decreased to normal range post-treatments and upon parasite clearance. The largest decline of weight loss occurred in the chronic phase of infection. The two animals that had the highest parasite burdens during the infection, M2198 and M11799 also had the largest decline in body weight during the chronic infection at 17% and 12% respectively. This is a critical maker of disease progression in monkey model systems; in that regulations require that if the animal is assessed at 20% weight loss in a study, this is possible grounds for early termination.

Reference values for Hb and Hct vary, and there is no standard definition of malarial anemia in rhesus macaques. Therefore, in this study, we defined malarial anemia as Hb concentration two standard deviations below the mean. The mean was derived from baseline values for the four animals, as their baseline Hb levels fell below the single source of "normal" published values (136). Anemia associated with malaria is complex, not resulting from a single source, and there is a wide spectrum of clinical manifestations. As such, we attempted to characterize the immune response in these animals relative to important markers of malaria pathogenesis seen in humans.

We have shown that Hb scores began to decrease after peak parasitemia and were at lowest levels at 1 week post-parasitemia. After the destruction of RBC by the parasite and/or the spleen, the decrease in Hb is also one of the hallmarks in malarial anemia cause by *P. falciparum* in humans. The decrease in Hb and Hct could be due to the decline in parasitemia post-treatment, or from the destruction of uninfected RBCs (43). Hb, Hct, and RBC count, levels

significantly increased above baseline ($p<0.01$) in all four animals on day 5 PI, when parasitemia values increased exponentially. High plasma levels of pro-inflammatory cytokines that would suppress hemtaopoiesis did not peak until after this timepoint. At peak parasitemia, Hct, Hb and RBCs were significantly decreasing and continued though the acute infection to parasite clearance. Levels increased during the chronic infection, though not to baseline levels, and decreased again at the second onset of parasitemia. During *falciparum* malaria, there is a decrease in Hb after treatment of acute infection, that has been shown to correlate with the level of parasitemia when the patient was admitted to the hospital (91). Likewise, we found that during the acute infection, day 8 PI parasitemia was a significant predictor of Hb on day 15 PI. However, this was not the case during the chronic infection, where peaks in parasitemia did not significantly predict the subsequent decrease in Hb. Peripheral parasitemia can underestimate parasite burden due to parasite sequestration (89), which could explain why the relationship between level of parasitemia and degree of anemia are not always correlated during malaria.

The MCV significantly decreased as parasitemia began to increase in the acute infection, indicating a microcytosis ($p<0.05$). However, when the animals were the most anemic (lowest Hb) in the acute infection, they demonstrated a high MCV but with a decrease in MCHC (hypochromia). This clinical profile is typical of a highly regenerative anemia, where increased number of reticulocytes with low Hb concentration will bring down MCHC. Regenerative anemia would be more probable during an acute malarial anemia rather than in chronic anemia. As well, the RDW significantly decreased on day 7 PI during the acute infection. Low RDW signifies a normal, homogeneous population of cells, and can also be used as a marker of release of early erythrocytes and reticulocytes. During the chronic infection RDW significantly increased on days 34, 42, and 56. Increased RDW signifies active hemtaopoiesis due to large

immature red cells and has been correlated in malaria with the degree of macrocytosis (114), but were not significantly associated in this study.

Thrombocytopenia is a decreased production of PLT, though the mechanism during malaria is unclear (104). Thrombocytopenia may due to suppression of erythropoiesis by pro-inflammatory cytokine oversecretion (104). Acute infections in non-immune people present in mild to moderate thrombocytopenia, less frequent in immune and semi-immune populations (117). In all four animals, PLT counts significantly decreased during the acute infection. Highest levels of PLT on day 15 PI were positively correlated with lowest Hb levels during the acute infection ($p<0.05$). PLT have also been shown to be a factor in pathogenesis of malaria (105), and have been shown to aggregate with parasitized RBCs, impairing circulation and increasing sequestration even further (106). At the timepoint when Hb was lowest during the acute infection, PLT counts were highest. This is possibly due to the compensation for loss of RBC, as RBC and PLT both originate from the same hematopoietic progenitor cells. An increased MPV occurs when increased numbers of PLT are being produced. At levels of highest PLT production MPV was highest as well. Both PLT and MPV decrease with increasing parasitemia, and after parasite clearance, increased significantly to counterbalance the loss during parasitemia. This profile is similar to what has been demonstrated in *Aotus* monkeys infected with *falciparum* malaria (107). Leukopenia during acute *falciparum* malaria. is common in non-immune adults. The infiltration of leukocytes was highest at peak parasitemia which may subsequently induce dysregulation of other immune parameters leading to an exacerbation of disease.

5.2 CYTOKINE AND EFFECTOR MOLECULE PROFILES

The regulation of cytokine profiles during malaria in rhesus was similar to that in humans (141), (124), (122). The timing, degree and balance of the cytokine response has been associated with clinical outcomes of disease severity in malaria (26), (141), (148), (29). The protective immune response to malaria is associated with alterations in pro- and anti-inflammatory cytokines that may determine disease severity by regulating NO production from blood mononuclear cells. Results presented here show that circulating levels of IL-12, IFN-γ, TNF-α, IL-10 and NO increased with disease severity and were positively correlated with parasitemia. NO in plasma increased with disease severity and was correlated with circulating levels of TNF-α and IL-10. In contrast, plasma NO was inversely correlated with IFN-γ. Plasma levels of IL-12 (day 5 PI) and TNF-α (day 7 PI) in the days prior to a peak parasitemia, were positive indicators of parasite burden at peak parasitemia. High levels of immune complexes have been documented in severe *falciparum* malaria (149). The magnitude of plasma cytokine response was greatest in IL-10, with up to 5000% increase over baseline at peak parasitemia. Pro-inflammatory IL-12 and IFN-γ were also considerably increased over the course of infection.

IL-12 transcripts were highest in the early infection at the occurrence of parasitemia and at the approach of peak parasitemia. In malaria, IL-12 is an important initiator of the inflammatory cascade, activating monocytes-macrophages (28). In three of the four animals, plasma levels of IL-12 were highest when close to or on the days when parasite burden was very high. The animal with the least severe disease had reduced plasma levels of IL-12 on days 2, 5, 8, and 15 PI, but also had high levels of IL-12 transcripts in the early acute infection. Plasma IFN-γ levels increased with increasing parasitemia, and were significantly elevated above baseline in all four animals when parasites reoccurred on day 22 PI. Transcript levels also were

highest at higher parasitemias. TNF-α levels were significantly augmented with peak parasitemia. Cytokine levels showed considerable variation in the four animals on each of the different timepoints, making it difficult to show significant correlations. The cytokine profiles from this study indicate that the rhesus model is similar to those observed in humans (26), (150), (56), (151), (141). The results of this study reveal differences in transcriptional response of cytokines during malaria infection. PBMC transcript levels did not correlate with plasma levels of cytokines, suggesting that the cytokines might be from different sources other than solely circulating monocyte-macrophages, such as spleenic macrophages and lymphocytes.

In the present studies, we have shown that rhesus macaques infected with malaria cause an enhanced dysregulation of cytokine production. Differential expression of IFN-γ and IL-10 correlated with peak parasitemia. This is consistent with previous reports which indicate a significant inverse relationship between production of IFN-γ and IL-10 with respect to enhanced disease severity during malaria (152). Additionally, over-production of TNF-α is associated with promotion of anemia in a variety of chronic and acute inflammatory diseases (45), (44). Therefore, since pro-inflammatory responses are implicated in malaria pathogenesis, we have shown that the production of TNF-α transcripts correlated with enhanced disease severity, while IL-12 transcripts were relatively low and were suppressed with disease progression. Taken together, the data presented here elucidates the response of plasma cytokines during malaria infection at whereby peak parasitemia is associated with enhanced production.

5.3 CORRELATION OF CYTOKINE AND EFFECTOR MOLECULE PRODUCTION WITH HEMATOLOGICAL OUTCOMES

Plasma cytokine and transcript cytokine levels were compared to Hb and Hct to determine if there was a correlation between pro- and anti-inflammatory cytokine production and transcription as predictors of anemia. In the acute infection, plasma IL-10/TNF-α decreased in each animal by at least 40% (and up to>2,000%) in all four animals from peak parasitemia to the timepoint when the animal had the lowest Hb. A low IL-10/TNF-α ratio has been shown to be associated with decrease in Hb in malarial anemia (55), (56), (29), (57).

A low IL-10/TNF-α ratio at thrombocytopenia was significantly associated with the magnitude of PLT increase at parasite clearance ($p<0.01$). Though high levels of PLT production during parasitemia have been correlated with disease severity (106), it appears that PLT production later in the infection as parasitemia is resolving, is beneficial. Animals that had decreased Hb during the acute infection, showed elevated IL-12 plasma levels with higher levels of parasitemia. In acute malaria, the suppression of bone marrow is partly in response to a decrease in EPO (90), which is downregulated by pro-inflammatory cytokines. As well, non-immune individuals have suppression of erythropoiesis in acute malaria (119), which could be due to overproduction of TNF-α (93). We found that plasma levels of TNF-α on day 7 PI are positive predictors of anemia in acute malarial infection**.** The ability to produce high levels of NO during acute malaria was significantly associated with high PLT levels at parasite clearance. Production of high plasma levels of NO at peak parasitemia were significantly correlated with high PLT at parasite clearance. iNOS, expressed by primarily MO, has been shown to be protective during malaria, and has also been shown to be expressed by platelets (64). NO also plays a critical role in the adhesion of pRBC, MO, LY and PLT to the endothelial wall in CM,

leading to enhanced disease severity (153). Increased plasma NO during high parasitemia was associated with a thrombocytopenia, and high rebound in platelets at parasite clearance.

Elimination of intracellular pathogens requires cell-mediated immunity generated by pro-inflammatory molecules such as IL-12 (154). Induction of immunity in susceptible A/J mice following administration of recombinant (r) IL-12 strongly supports the necessity of a Th1 cytokine response for controlling a malarial infection (31). Protection against the blood stage of murine *P. chabaudi* and *P. berghei* requires upregulation of IFN-γ and TNF-α, cytokines that presumably promote anti-parasitic properties through the generation of high levels of NO (31), (155). In addition, administration of rIL-12 before inoculation of mice with *P. yoelii* or rhesus monkeys with *P. cynomolgi* provided 100% protection in both animal models of malaria through an IFN-γ-dependent (and perhaps NO-dependent) anti-plasmodial mechanism (32), (142). Enhanced IFN-γ,-responsiveness to malarial antigens has been associated with protection from severe malaria (152). Taken together, previous studies in experimental malaria suggest that protective immunity in malaria requires a cascade of events that involve increased production of IL-12, IFN-γ, TNF-α, and NO. The balance between Th1 and Th2 cytokines is an essential component in the development of severe malaria. This study demonstrated that high IL-10/ IL-12, IFN-γ, TNF-α, and NO ratios were associated with increased parasite burden, whereas low IL-10/pro-inflammatory ratios were associated with decreased hemoglobin.

6.0 CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

In conclusion, we have found that both cytokine profiles and hematological parameters in rhesus macaques infected with *P. coatneyi* show many similarities to *falciparum* malarial anemia in humans. Rhesus macaques develop malaria-induced anemia and thrombocytopenia when infected with *P. coatneyi*-. If the animals are minimally treated during the acute infection, allowing for the controlled persistence of the parasite, but preventing CM, they can develop a more severe and persistent anemia than in the acute phase of disease. Therefore, the primate model could be used for investigation of molecular- and mechanistic-based immunologic studies for vaccine and pharmacologic trials. However, as in humans, there is considerable variation in levels of responses in macaques, so a larger sample size would be required to confer statistical significance.

6.2 FUTURE DIRECTIONS

Future studies of the model should include examining profiles of rhesus macaques infected with *P. coatneyi* over a long-term chronic infection, and examining inflammatory mediators of disease severity (cytokine and effector molecules) at different time intervals during a

longitudinal chronic malarial anemia study. Also, it would be interesting to look at *in vitro* studies taking cells from monkeys infected with *P. coatneyi,* and determining cytokine profiles. Likewise it would be advantageous to compare those results with *in vitro* stimulation of non-infected, malaria-naïve rhesus macaques with parasite antigen and malarial pigment and determine levels of cytokine production.

Since malaria immunity requires both a humoral and cellular response, it would be beneficial to examine both the anti-parasite and parasite antigen antibody response during first a primary, then secondary *P. coatneyi* malarial infection in relation to cytokine production.

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