IMPACT OF HEMOZOIN ON HEMATOLOGICAL OUTCOMES AND INNATE INFLAMMATORY MEDIATOR PRODUCTION IN INFANTS AND YOUNG CHILDREN WITH MALARIAL ANEMIA

by

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Douglas J. Perkins, Ph.D.

IMPACT OF HEMOZOIN ON HEMATOLOGICAL OUTCOMES AND INNATE INFLAMMATORY MEDIATOR PRODUCTION IN INFANTS AND YOUNG CHILDREN WITH MALARIAL ANEMIA

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Malaria causes 300-500 million clinical cases and 1-3 million deaths per year, the majority of which occur in young African children. Ingestion of hemozoin by peripheral blood mononuclear cells (PBMC) initiates the cytokine-mediated cascade of immune dysregulation in malaria. Innate immunity plays a critical role in protective responses against infection, which are determined by an appropriate balance between pro- and anti-inflammatory mediators. Pro-inflammatory mediators (TNF-α, IL-12, IFN-γ) that control parasitemia also contribute to pathology. Over-production of immunomodulatory cytokines (TGF-β1, IL-10) suppresses the protective pro-inflammatory immune response. The effects of hemozoin on hematological outcomes and inflammatory mediator production in children with malarial anemia and the temporal kinetics of hemozoin-induced cytokine dysregulation in cultured PBMC were investigated in this study. Hematological analyses of healthy control (HC), uncomplicated malaria (UM), mild malarial anemia (MlMA), moderate malarial anemia (MdMA), and severe malarial anemia (SMA) groups revealed that age, temperature, and all erythrocyte, leukocyte, and platelet indices were significantly different between clinical categories. Neither parasitemia nor the prevalence of high density parasitemia (HDP) differed significantly between clinical groups. Stratification of study participants according to proportion of pigment-containing monocytes (PCM) demonstrated that parasitemia, HDP, temperature, and most leukocyte, erythrocyte, and platelet indices were significantly different between 0%, ≤ 10%, and > 10% PCM categories. The > 10% PCM category contained children with the lowest hemoglobin, hematocrit, and erythrocyte counts and the greatest proportion of children with SMA. Plasma IFN-α, IL-4, IL-6, IL-10, and IL-12p40/p70 levels differed significantly between clinical categories but not between UM and SMA groups. Plasma IFN-α, IFN-γ, TNF -α, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12p40/p70

were not significantly associated with % PCM. β-hematin-induced IL-1β, IL-2, IL-6, IL-12p35, IL-12p40, IL-18, IFN-α, IFN-γ, TNF-α, LT-α, NOS-2A, COX-1, COX-2, IL-4, IL-10, and TGF-β1 expression in cultured PBMC revealed a pro-inflammatory response that varied in magnitude among individuals. Importantly, innate inflammatory mediators modulate the adaptive immune response to *Plasmodium* parasites. Of public health significance, a better understanding of the molecular mechanisms governing these responses will facilitate the development of more immunogenic vaccines, through inclusion of cytokines or other compounds that activate the innate immune system.

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1.0 INTRODUCTION

In humans, the protozoan parasites *Plasmodium vivax, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium falciparum* cause malaria, a disease transmitted by the bite of an infected female *Anopheles* mosquito. Endemic to tropical and subtropical regions of the world, malaria causes 300-500 million clinical cases resulting in one to three million deaths per year (Stevenson and Riley, 2004; Struik and Riley, 2004; Snow *et al.*, 1999; Zambrano-Villa, 2002). Whereas adults living in hyperendemic areas typically develop semi-immunity following frequent episodes of childhood malaria, children may develop severe malaria with hyperparasitemia and life-threatening complications such as severe anemia, hypoglycemia, and cerebral malaria (Perkins *et al.*, 1999). *Plasmodium falciparum* produces the majority of severe malaria cases and deaths, including up to 35% of infant and child mortality in some areas of Africa (Korenromp *et al.*, 2003). Children less than five years of age are at greatest risk of mortality due to their non-immune status (Perkins *et al.*, 1999). Malaria control methods including the use of insecticide-treated bed nets, vector control, and chemotherapy have not achieved substantial reductions in morbidity and mortality (Struik and Riley, 2004). In addition, resistance against anti-malarial drugs such as chloroquine and sulphadoxine-pyrimethamine has developed in recent years (Ridley, 2003). Currently, the only effective treatment for multidrug-resistant malaria are the sesquiterpene lactone artemisinin drugs (derived from the Chinese herb qinghao) (Eckstein-Ludwig *et al.*, 2003; Ridley, 2003). Development of an effective vaccine against *P. falciparum* malaria has been slow, due in part to numerous species- and stage-specific parasite target antigens and to the lack of long-term immunological memory responses in the human host (Struik and Riley, 2004; Stevenson and Riley, 2004). For these reasons, understanding the innate immune responses that mediate protection against malaria will be critical in designing alternative therapies. The purpose of this study is to investigate the impact of naturally

acquired *Plasmodium falciparum* hemozoin on hematological outcomes and inflammatory mediator production in infants and young children with malarial anemia, and the temporal kinetics of hemozoin-induced cytokine transcript dysregulation in cultured human peripheral blood mononuclear cells.

1.1 *P. falciparum* Life Cycle

The life cycle of *P. falciparum* is complex, having intra- and extracellular stages (Figure 1). During a bloodmeal, the female Anopheles mosquito injects haploid sporozoites into the host's bloodstream. These sporozoites migrate to the liver and invade hepatocytes. Following five to seven days of replication by asexual mitosis, these sporozoite-infected hepatocytes produce exoerythrocytic schizonts that rupture and release thousands of merozoites into the bloodstream (Struik and Riley, 2004). Merozoites invade erythrocytes and develop into erythrocytic schizonts via asexual mitotic replication. Each erythrocytic schizont contains up to 20 daughter merozoites, which upon schizont rupture invade new erythrocytes and give rise to new schizonts. The rupture of erythrocytic schizonts is characterized by episodes of fever, rigors, nausea, and headaches that result from the systemic pro-inflammatory cytokine cascade initiated by cells of the innate immune system (Miller *et al.*, 2002). This cyclical pattern of blood-stage infection continues for 48-72 hours (Struik and Riley, 2004). During intraerythrocytic infection, parasites catabolize hemoglobin as a source of amino acids and transform toxic heme released during hemoglobin catabolism into the insoluble, inert crystal hemozoin (malarial pigment) (Chong and Sullivan, 2003). In some instances, merozoites do not invade erythrocytes, but instead differentiate into male and female gametocytes. Following ingestion by a mosquito, these gametocytes develop into extracellular gametes. Male and female gametes then fuse during sexual replication in the mosquito midgut to produce motile zygotes that penetrate the midgut wall and form oocysts. Following meiosis, oocysts develop into haploid sporozoites that migrate to the mosquito salivary glands and can be transmitted to the next host, beginning the cycle anew.

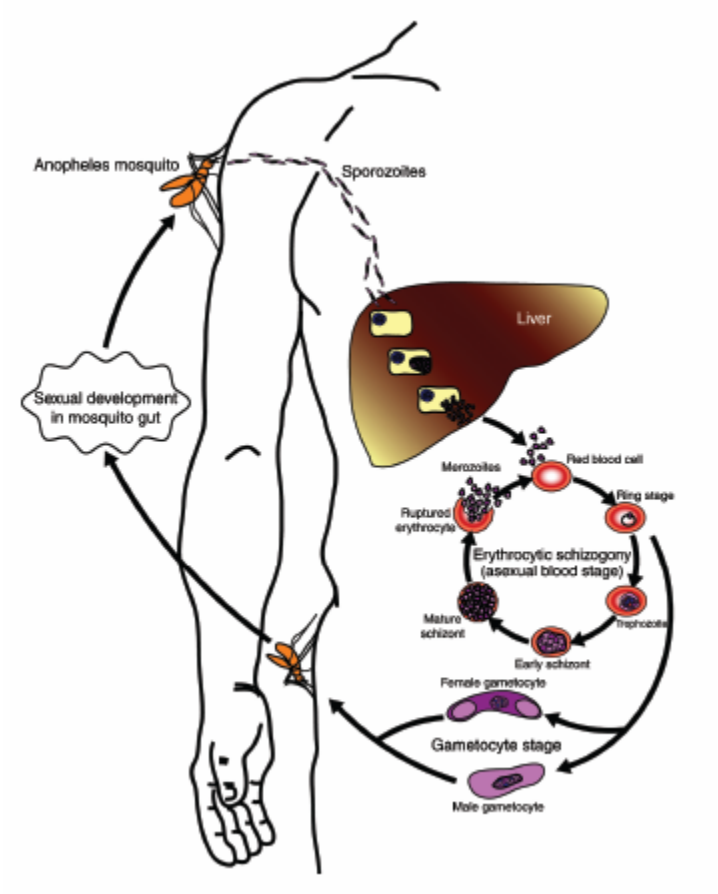

Figure 1. Plasmodium falciparum life cycle in the human host and mosquito vector. The female *Anopheles* mosquito injects haploid sporozoites into the host's bloodstream, where they migrate to the liver to invade hepatocytes. Sporozoites undergo 5-7 days of replication by asexual mitosis to produce exoerythrocytic (liver) schizonts. This stage of infection is asymptomatic. Upon rupture, liver schizonts release thousands of merozoites into the bloodstream. Merozoites invade erythrocytes and develop into erythrocytic schizonts via asexual mitotic replication. Erythrocytic schizonts rupture, releasing up to 20 daughter merozoites that invade new erythrocytes and give rise to new schizonts. The rupture of erythrocytic schizonts triggers a cascade of pro-inflammatory cytokines leading to episodes of fever, rigors, nausea, and headaches. This cyclical pattern of blood-stage infection lasts for 48-72 hours. Some merozoites do not invade erythrocytes, but instead differentiate into male and female gametocytes that develop into extracellular gametes following ingestion by a mosquito. Male (micro) and female (macro) gametes fuse during sexual reproduction in the mosquito midgut, producing motile zygotes (ookinetes) that penetrate the midgut wall and form oocysts. Oocysts develop into haploid sporozoites via meiosis. Infective sporozoites migrate to the mosquito salivary glands, where they can be transmitted to the next host. (adapted with permission from Struik and Riley, 2004)

1.2 Properties of Hemozoin and β-hematin

Hemozoin (malarial pigment) is formed in the digestive vacuoles of late trophozoites and schizonts during intraerythrocytic development as the end product of host hemoglobin digestion (Day *et al.*, 1996). During this process, heme released as a

byproduct is autoxidized to toxic hematin [aquaferriprotoporphyrin IX, H_2O-Fe(III)PPIX], which is subsequently detoxified by conversion to hemozoin (Ladan *et al.*, 1993; Scheibel and Sherman, 1988). Heme polymerase catalyzes the polymerization of hemozoin through linkage of heme groups via iron carboxylate bonds (Slater and Cerami, 1992; Day *et al.*, 1996). The core Fe(III)PPIX structure of hemozoin (Figure 2) is surrounded by proteins, carbohydrates, lipids, and nucleic acids (Goldie *et al.*, 1990). Hemozoin has been shown to induce both pro-inflammatory and anti-inflammatory cytokines in cultured peripheral blood mononuclear cells (PBMC) (Pichyangkul *et al.*, 1994; Mordmüller *et al.*, 1998). Human monocyte-derived macrophages that phagocytose trophozoite-parasitized red blood cells (RBCs) or free pigment are unable to degrade the ingested pigment heme, repeat phagocytosis, and generate the oxidative burst (Schwarzer *et al.*, 1992). As such, pigment formation not only protects the parasite from toxic heme, but also from the host immune reponse. The toxicity of hemozoin may be attributed to the covalent iron carboxylate bonds that contribute to its stability and indigestibility in both the phagolysosome and the parasite food vacuole (Slater *et al.*, 1991).

β-hematin (synthetic hemozoin) is a crystalline cyclic dimer of Fe(III)PPIX that is chemically, crystallographically, and spectroscopically identical to hemozoin (Egan *et al.*, 2001; Fitch and Kanjananggulpan, 1987; Slater *et al.*, 1991; Bohle *et al.*, 1994,1997). β-hematin (Figure 2) is formed by reciprocal iron-carboxylate bonds that link the ionized heme-propionate side chain of one Fe(III)PPIX to the central Fe(III) of another (Pagola *et al.*, 2000; Egan *et al.*, 2001). Fe(III)PPIX dimers stack in the crystal lattice to form parallel iron porphyrin rings. Studies in our laboratory have demonstrated that similar to hemozoin, β-hematin elicits pro- and anti-inflammatory cytokine synthesis in cultured peripheral blood mononuclear cells (PBMC) (Keller *et al.*, 2004a, 2006, manuscript submitted).

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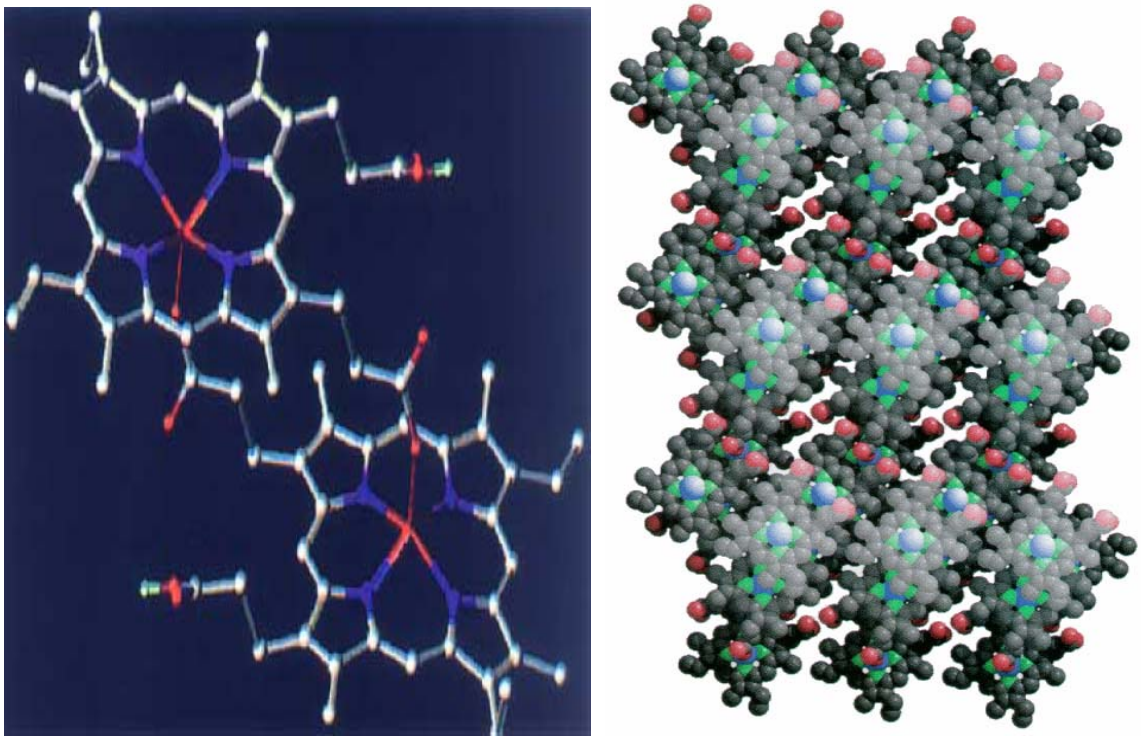

Figure 2. Structure of hemozoin and β-hematin. (A) Reciprocal iron-carboxylate Fe(III)PPIX dimer (adapted with permission from Tekwani and Walker, 2005). (B) Packing diagram of the crystal structure of β-hematin (core structure of hemozoin). C atoms are illustrated in grey; O, red; N, green; Fe, blue. (adapted with permission from Pagola *et al.*, 2000)

1.3 Inflammatory Response/Pathology

Plasmodium evades the immune response by altering its surface antigens throughout the various stages of its life cycle (Bull *et al.*, 1998; Zambrano-Villa *et al.*, 2002). Each phase of the life cycle is characterized by the transient expression of species-specific proteins that are highly polymorphic and antigenically variable. The erythrocytic stage of *P. falciparum* infection is responsible for all disease pathology in malaria and is characterized by dysregulatory processes that prevent the induction of robust immune responses (Luke and Hoffman, 2003). During schizogony, parasitized red blood cells (pRBC) burst, creating RBC ghosts and releasing daughter merozoites and hemozoin into the blood stream (Phu *et al.*, 1994). Scavenger monocytes and neutrophils ingest malarial pigment through phagocytosis of mature pRBCs, RBC ghosts, living or dead pigment-containing phagocytes, and particulate material released at schizogony

(Day *et al.*, 1996). Ingestion of opsonized hemozoin by monocytes impairs their expression of MHC class II molecules, CD54, and CD11c, as well as generation of the oxidative burst, protein kinase C activity, and their ability to repeat phagocytosis (Arese and Scwarzer, 1997; Schwarzer *et al.*, 1992, 1993, 1998). Intracellular hemozoin released into the circulation upon schizont rupture becomes concentrated in resident macrophages of the reticuloendothelial system (Schwarzer *et al.*, 1998). Impaired antigen presentation to T cells results from the suppression of dendritic cell maturation by parasitized red blood cells (Urban *et al.*, 1999). Parasitized red blood cells can also cause apoptosis of malaria-specific B cells and T cells (Good, 2005).

P. falciparum infection is characterized by anemia, metabolic acidosis, and/or cerebral malaria (Stevenson and Riley, 2004). Disease pathology is caused by the destruction of erythrocytes, bone marrow suppression, and impaired circulation resulting from peripheral hypotension and the adherence of infected erythrocytes to the vascular endothelium (Stevenson and Riley, 2004). Hypoxia, hypoglycemia, and lactic acidosis resulting from the increased metabolic demands of the parasite are associated with erythrocyte destruction and bone marrow suppression (Stevenson and Riley, 2004).

1.4 Malarial Anemia

Malarial anemia (MA) is complex and results not only from immune destruction of parasitized erythrocytes, but also from excess removal of nonparasitized erythrocytes and bone marrow dysfunction (Ekvall, 2003). The severity of anemia in acute malaria most likely depends on the degree of RBC destruction, whereas ineffective erythropoiesis and dyserythropoiesis play a greater a role in chronic infection with low parasitemia. Severe malarial anemia (SMA) is the predominant clinical manifestation of severe falciparum malaria in areas of holoendemic (high/sustained) transmission, whereas cerebral malaria and metabolic acidosis are more prevalent in areas of lower/seasonal transmission (Day, 1996; Ekvall, 2003). In addition, children aged less than 24 months are more susceptible to malarial anemia, whereas cerebral malaria peaks in children approximately 3 years of age (Ekvall, 2003). The leading cause of death among children with malaria, SMA accounts for 190,000-974,000 deaths per year in children less than five years of age (WHO, Children and malaria, *http://www.who.int/cmcupload/0/000/*

015/367/RBMInfosheet6.htm). Due to antimalarial drug resistance, many children fail to clear parasitemia after treatment and remain anemic; others who require blood transfusions to survive are at risk of contracting HIV and other blood-borne diseases.

Destruction of uninfected erythrocytes accounts for more than 90% of erythrocyte loss in adults with acute uncomplicated malaria, and is the primary cause of severe malarial anemia (SMA) (Stoute *et al.*, 2003; Ekvall, 2003). Several mechanisms are thought to play a role in this destruction. For example, alterations in the membranes of non-parasitized RBCs may target them for removal from circulation and destruction in the spleen. Oxygen and nitrogen radicals released from macrophages as a result of pro-inflammatory cytokine secretion cause oxidative damage to non-parasitized RBCs in the form of membrane disruption, which leads to enhanced RBC removal (Griffiths *et al.*, 2001; Perkins *et al.*, 2000; Greve *et al.*, 1999). Reduced RBC deformability, which correlates with disease severity and degree of anemia, may play a role in non-parasitized RBC destruction by immune effector cells in malaria (Dondorp *et al.*, 1999). Reduced RBC deformability is also thought to be responsible for the removal of normal senescent erythrocytes from the circulation. In addition, parasite-induced complement activation may contribute to RBC destruction. Complement can act synergistically with Ig to induce phagocytosis or may cause direct lysis of non-parasitized RBCs (Ekvall, 2003). Furthermore, deficiencies in surface complement regulatory proteins (such as CR1 and CD55) that protect RBCs from complement-mediated lysis have been implicated in the pathogenesis of malarial anemia (Devine, 1991; Waitumbi *et al.*, 2000).

Bone marrow dysfunction in malarial anemia results from the decreased responsiveness of erythroid progenitor cells to erythropoietin and to the impaired production of erythropoietin, both of which are regulated by inflammatory cytokines (Ekvall, 2003). Elevated plasma TNF and IL-10 levels are found in children with malarial anemia and high-density parasitemia, and a low IL-10/TNF ratio is specifically associated with malarial anemia in these children (Kurtzhals *et al.*, 1998; Mordmüller *et al.*, 1997; Othoro et. al., 1999; Shaffer *et al.*, 1991). Since IL-10 inhibits *P. falciparum*-induced TNF production, low levels of IL-10 may facilitate high levels of TNF, resulting in anemia (Ho *et al.*, 1995, 1998). Acute childhood malaria is characterized by bone marrow suppression, sometimes in conjunction with decreased erythropoiesis (Kurtzhals

et al., 1997). Hyperactive bone marrow, accompanied by morphologic dyserythropoiesis and the absence of reticulocyte release, is more common in chronic malaria (Wickramasinghe and Abdalla, 2000). Though elevated erythropoietin levels in African children with malaria would seem to indicate increased erythropoiesis, bone marrow suppression still occurs (Nussenblatt *et al.*, 2001; Menendez *et al.*, 2001; Verhoef *et al.*, 2001). Possible mechanisms for this inhibition include the direct effect of parasites/parasite products on the bone marrow, macrophage dysfunction, secretion of cytokines such as TNF-α, IFN-γ, and MIF (macrophage migration inhibitory factor) by activated T cells, and the local release of reactive oxygen and nitrogen species (Clark and Chaudri, 1988; Martiney *et al.*, 2000; Kremsner *et al.*, 2000; Kun *et al.*, 2001; Schwarzer *et al.*, 1992; Wickramasinghe and Abdalla, 2000).

1.5 Cytokine Dysregulation

While the destruction of erythrocytes contributes significantly to *P. falciparum* malaria, the same pro-inflammatory mediators that contribute to the control of parasite replication also increase the severity of the disease (Kwiatkowski *et al.*, 1997; Hunt and Grau, 2003; Artavanis-Tsakonas *et al.*, 2003a; Riley, 1999). Like many infectious and noninfectious acute diseases, malaria may be characterized as a cytokine release syndrome (Clark *et al.*, 2004). The rupture of schizont-infected erythrocytes triggers a cascade of inflammatory cytokines that can result in death (Artavanis-Tsakonas *et al.*, 2003; Riley, 1999). As illustrated in Figure 3, the differential effects of hemozoin ingestion by phagocytes on cytokine production play a critical role in this dysregulation. For example, the pro-inflammatory cytokine IL-12 is required during the early cell-mediated immune response to malaria infection (Crutcher *et al.*, 1995; Mosmann and Coffman, 1989; Trinchieri, 1993). IL-12-induced up-regulation of TNF-α and IFN-γ facilitate parasite killing, partially due to increased production of nitric oxide (NO) (Stevenson *et al.*, 1995; Yoshimoto *et al.*, 1998). However, IL-12 production by monocytes is suppressed following the ingestion of hemozoin, whereas TNF-α and IL-10 production are enhanced (Mordmüller *et al.*, 1998). Anti-inflammatory IL-10 inhibits IL-12 production by monocytes, and elevated levels of TNF-α promote the development of severe anemia (Luty *et al.*, 2000; Storkus *et al.*, 1998). Increased NO production has

been associated with accelerated parasite clearance in Gabonese children and adults with malaria, but prolonged overproduction of NO can lead to SMA through suppression of erythropoiesis and induction of apoptosis in CD34+ hematopoietic stem cells (Kremsner *et al.*, 1996; Reykdal *et al.*, 1999; Shami and Weinberg, 1996). Furthermore, low IL-12 levels have been associated with increased susceptibility to severe *P. falciparum* malaria (Luty *et al.*, 2000).

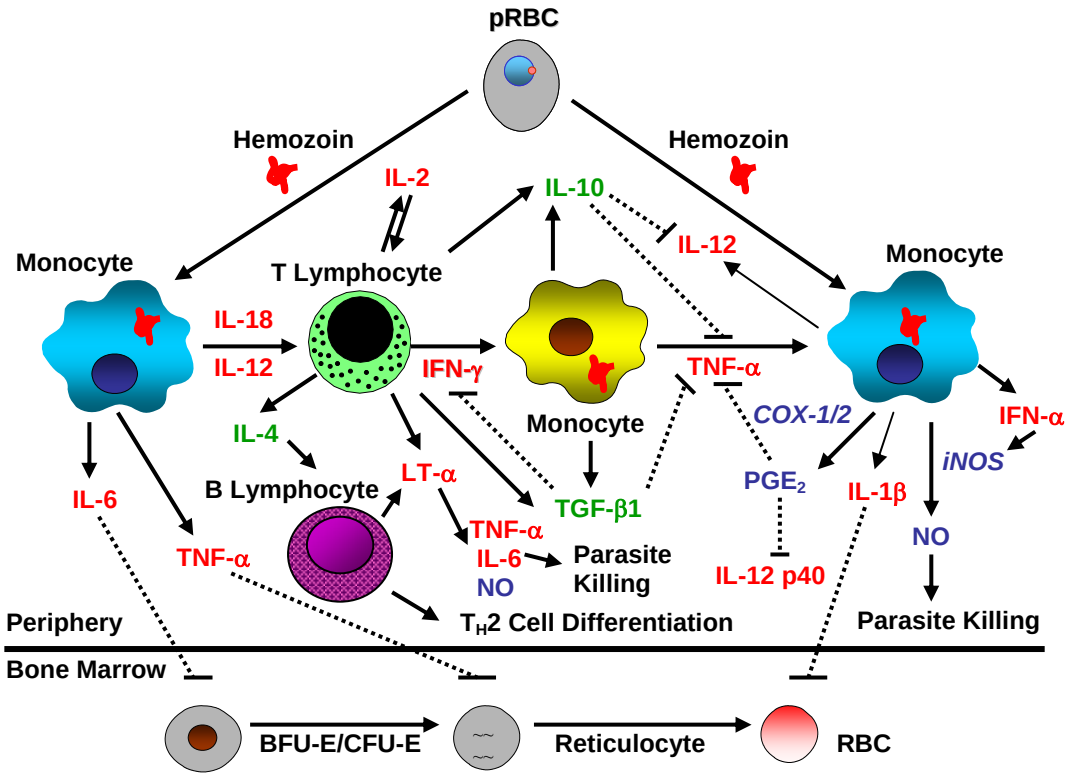

Figure 3. Model of innate immune response to malaria. Protective immunity against malaria is regulated by both pro- and anti-inflammatory cytokines, the balance of which determines disease outcome. Pro-inflammatory mediators are illustrated in red text, and anti-inflammatory cytokines are illustrated in green text. Enzymes and effector molecules are shown in blue. Solid lines represent positive signaling, whereas dashed lines represent suppression. Exposure of monocytes/macrophages to parasitized red blood cells (pRBC) or hemozoin initiates the inflammatory cascade through release of IL-12. Alone or in conjunction with IL-18, IL-12 promotes IFN-γ secretion from T helper 1 cells. Activated T lymphocytes secrete IL-2, which acts in an autocrine fashion to promote T cell growth and differentiation. IL-12 and IFN-γ stimulate monocytes/macrophages to produce TNF-α, which facilitates parasite killing through generation of toxic free radicals such as nitric oxide (NO), via the enzymatic activity of inducible nitric oxide synthase (iNOS). Production of IFN-α by monocytes and of LT-α by T- or B lymphocytes also induces NO-mediated parasite killing. While pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α control parasitemia, elevated levels of these cytokines promote the development of severe malarial anemia (SMA) through suppression of erythropoiesis. In response to parasitic products, cyclooxygenase-2 (COX-2) is inducibly expressed to enhance prostaglandin E_2 (PGE_2) synthesis. TNF-α-induced PGE_2 negatively regulates production of TNF-α and of the IL-12p40 subunit. Induction of anti-inflammatory mediators is required to counteract the pathological effects of prolonged pro-inflammatory cytokine production during acute malaria. Monocytic release of anti-inflammatory cytokines IL-10 and TGF-β1 down-regulates the pro-inflammatory response through suppression of IL-12, IFN-γ, and TNF-α. In addition, IL-4 production by T cells activates B lymphocytes and induces T_H2 cell differentiation.

However, the ability of immunoregulatory cytokines to control circulating levels of pro-inflammatory cytokines may facilitate parasite clearance without triggering pathology (Stevenson and Riley, 2004). In mice and humans, IL-10 and TGF-β (transforming-growth factor-β) negatively regulate both innate and adaptive immune responses by inhibiting pro-inflammatory cytokine responses (IFN-γ, IL-12) (Omer and Riley, 1998; Omer *et al.*, 2003a; Li *et al.*, 2003; Kurtzhals *et al.*, 1998; Dodoo *et al.*, 2002; Tsutsui and Kamiyama, 1999). As in the case of IL-12, reduced plasma levels of TGF-β are characteristic of severe malaria (Perkins *et al.*, 2000). IL-10 also suppresses TNF-α and IL-1β production through inhibition of NF-κB (Wang *et al.*, 1995). NO serves a homeostatic role in falciparum malaria by down-regulating TNF-α (which generates NO via the enzymatic activity of inducible nitric oxide synthase, iNOS/NOS-2A) through inhibition of NF-κB (Iuvone *et al.*, 1996; Otterbein *et al.*, 2000; Sarady *et al.*, 2002). NO further reduces TNF-α levels by inhibiting the adhesion of pRBCs to the vascular endothelium (which is required for maximal TNF production) (Serirom *et al.*, 2003; O'Dea and Pasvol, 2003). The balance of pro- and anti-inflammatory cytokines and effector molecules determines whether a protective or a pathogenic response develops (Perkins *et al.*, 2000). The mechanism by which this balance is achieved is unknown, but has been linked to naturally acquired immunity to malaria in endemic areas, whereby chronic, low-grade infections allow the host to remain asymptomatic (premunition) (Stevenson and Riley, 2004; Struik and Riley, 2004; Targett, 2005).

1.6 Hypothesis and Objectives

Since the ingestion of hemozoin by peripheral blood mononuclear cells (PBMC) initiates the cytokine-mediated cascade of immune dysregulation in malaria, we decided to investigate the temporal effects of malarial pigment on cytokine production *in vitro*. In addition, as MA is the most prevalent clinical outcome of pigment-induced immune dysregulation in areas of holoendemic *P. falciparum* transmission (Bloland *et al.*, 1999), and SMA is the leading cause of morbidity and mortality among children under 5 years of age in these areas (WHO, 2000; Breman *et al.*, 2001), we studied the impact of hemozoin on hematological indices and plasma cytokine protein levels in children with varying degrees of parasitemia and malarial anemia.

The main hypothesis of this study is that dysregulation in innate immune responses to malaria occurs at the level of *de novo* cytokine transcription. Furthermore, malarial pigment-induced changes in hematological parameters lead to MA. While level of parasitemia is not necessarily predictive of MA or disease severity (Ong'echa *et al.*, 2006; McElroy *et al.*, 2000), we propose that the percent of pigment-containing monocytes in the blood of infected children correlates with the severity of anemia.

To illustrate the effect of hemozoin on hematological outcomes, blood was drawn from parasitemic and aparasitemic children with/without varying degrees of MA. Complete blood counts were performed, and study participants (n = 648) were stratified by clinical (anemia) category to examine the category-specific effects of malarial pigment on hematological measurements. Parasitemic study participants were then stratified according to percent of pigment-containing monocytes (% PCM) to determine if pigment content was related to severity of anemia. To determine the relationship between *in vivo* innate inflammatory mediator production and monocytic acquisition of hemozoin, plasma levels of IL-12p40/p70, IFN-α, IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, and IL-10 from children (n = 189) with/without varying degrees of MA were determined by multiplex assay. Parasitemic study participants were further stratified according to blood content of pigment-containing monocytes to examine whether plasma cytokine levels were correlated with % PCM.

To investigate the temporal effect of pigment on *in vitro* cytokine transcript production, β-hematin (synthetic hemozoin), which is chemically, crystallographically, and spectroscopically identical to the core ferriprotoporphyrin IX [Fe(III)PPIX] of hemozoin (Fitch *et al.*, 1987; Slater *et al.*, 1991; Bohle *et al.*, 1994,1997), was used to stimulate cultured PBMC isolated from the blood of malaria-nave donors. RNA was isolated from harvested cells, reverse transcribed into cDNA, and analyzed by quantitative real time PCR for expression of the following cytokines: TNF-α, IL-12A (p35), IL-12B (p40), IL-10, TGF-β1, IFN-α, IFN-γ, IL-2, IL-4, IL-6, IL-18, IL-1β, LT-α, NOS-2A, COX-1, and COX-2.

2.0 MATERIALS AND METHODS

2.1 ***PBMC Culture and Isolation:*** Venous blood (70 mL) from malaria-naïve human donors (n = 9) was drawn into heparin-containing vials. PBMC were isolated using ficoll/Hypaque and plated at a density of 1 x 10^6 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) containing 10% human serum (heat-inactivated at 56°C for 30 min). Cultures were stimulated with media alone (controls) or β-hematin (10 μg/mL). β-hematin preparations were sonicated an average of 25 times prior to addition to the cultures and were vortexed periodically during addition. Concentrations of β-hematin used in the present study were comparable to physiological levels of hemozoin measured in children with severe malaria (Keller *et al.*, 2004b).

2.2 ***β-hematin Preparation:*** β-hematin was formed according to the method of Egan *et al.* (Egan *et al.*, 2001) by dissolving hemin chloride (bovine, ICN Biomedicals, Aurora, OH) in 0.1 M NaOH, followed by the addition of HCl and acetate stock solution (12.8 M, pH 5) at 60°C. After incubation at 60°C for 150 min in the absence of stirring, the mixture was centrifuged at 10,000 rpm for 10 min, washed with filter-sterilized H_2O, and dried on a heat block at 65°C. The resulting pellet was weighed and resuspended at 1.0 mg/mL in filter-sterilized H_2O. The resuspended β-hematin was sonicated thoroughly before freezing at -20°C. Endotoxin levels were determined to be less than 0.25 EU/mL (0.05 ng/mL) using the Limulus Amebocyte Lysate test (Cambrex Bio Science, Walkersville, MD).

2.3 ***RNA Isolation:*** Following the removal of culture supernatants, 1.0 mL of Solution D per 2 x 10^6 PBMC was added to culture plates, which were stored at -20°C. Total RNA, was isolated by the GITC method (Chomczynski and Sacchi, 1987). Briefly, thawed PBMC in Solution D were transferred to sterile baked glass tubes.

Following addition of 100 μL of 2M sodium acetate (pH 4.0), 1 mL phenol, and 200 μL chloroform/isoamyl alcohol (49:1), glass tubes were covered in parafilm and vortexed at high speed for 10 min. Samples were incubated on ice for 30 min, and then centrifuged at 3750 rpm for 30 min at 4°C. Three quarters (750 μL) of the upper aqueous phase of each centrifuged sample was transferred to a sterile micro-centrifuge tube. An equal volume of cold isopropanol was added to the extracted RNA in each tube, after which the solution was mixed thoroughly and precipitated at -20°C for a minimum of 2 days. Precipitated RNA was pelleted by centrifugation at 10,000 rpm for 30 min at 4°C. Isopropanol was decanted, and tubes were centrifuged repeatedly to remove residual isopropanol with a pipette. Open tubes were placed upside-down under a fume hood for 15-30 min to allow isopropanol to evaporate, without complete drying of the RNA pellet. RNA was subsequently dissolved on ice with a minimal (15 μL) volume of DEPC-treated water. RNA samples were then tritriated, held on ice for 15 min, and heated at 65°C for 10 min to solubilize the RNA. Samples were stored at -20°C.

2.4 ***Quantitative Real Time RT-PCR:*** Total RNA (1 μg/(L) was reverse-transcribed into cDNA using a PTC-100 Peltier Thermal Cycler (MJ Research, Inc., Waltham, MA). Cytokine gene expression was analyzed by quantitative real time PCR on either an ABI Prism 7700 Sequence Detection System, an Applied Biosystems 7500 Real Time PCR System, or an ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA). All experiments involving use of the ABI Prism 7900HT instrument were conducted by the TaqMan® core facility of the University of Pittsburgh Genomics and Proteomics Core Laboratories. cDNA was amplified in duplicate with the following primer/probe sets (Applied Biosystems, Foster City, CA): TNF-α (Assay ID Number Hs00174128m1), IL-12A (p35) (Hs00168405m1), IL-12B (p40) (Hs00233688m1), IL-10 (Hs00174086m1), TGF-β1 (Hs00171257m1), IFN-γ (Hs00174143), IFN-α (Hs00256882s1), IL-2 (Hs00174114m1), IL-4 (Hs00174122m1), IL-6 (Hs00174131m1), IL-18 (Hs00155517m1), IL-1β (Hs00174097m1), LT-α (Hs00236874), NOS-2A (Hs00167248m1), COX-1 (Hs00377721m1), and COX-2 (Hs00153133m1). The endogenous control gene β-actin [Ascession Number NM001101 (Applied Biosystems,

Foster City, CA)] was used as a reference gene to normalize the expression levels of target genes by correcting differences in the amount of cDNA loaded between samples. No template controls were included in quadruplicate for each gene to control for nonspecific background fluorescence. Data were analyzed using the comparative C_T method ($\Delta\Delta C_T$) (Keller *et al.*, 2004b). The change in C_T (ΔC_T) for each experimental sample was derived by subtracting the endogenous control gene (β-actin) C_T from the experimental gene C_T. The ΔC_T for each experimental sample was then subtracted from the ΔC_T of the control (medium alone) sample. Fold change was expressed as $2^{-\Delta\Delta C_T}$ relative to control conditions.

2.5 ***Hematology/Malarial Pigment Data:*** The ongoing hospital-based study is being conducted at the pediatric ward of the Siaya District Hospital in Siaya District, Nyanza Province, western Kenya, and at the University of Pittsburgh/KEMRI clinical laboratories (Ong'echa *et al.*, 2006). Study participants (n = 648) included children from 0 to 3 years of age, who had visited the hospital with symptoms of malaria. Heel/finger prick blood (< 100μL) was used to screen for hemoglobin (Hb) levels and to prepare blood smears for the determination of parasitemia. Hb levels were determined using a HemoCue system (HemoCue AB, Angelholm, Sweden). Geimsa-stained blood smears were examined under oil immersion for malaria parasites, which were counted against 300 leukocytes. Parasite densities (/μL of blood) were estimated using leukocyte counts (/μL of blood) for the same samples. Complete blood counts were performed with a Beckman Coulter® AcT diff2™ coulter counter (Beckman-Coulter Corporation, Miami, USA). Reticulocyte counts were determined from thin blood films stained with new methylene blue. Reticulocyte index (RI) was calculated as reticulocyte count (%) x (hematocrit/0.36) (Ong'echa *et al.*, manuscript submitted). Reticulocyte production index (RPI) was derived as RI/maturation factor (MF), where MF = b + (m)(x), b = 1, m = 0.05, and x = (average normal population hematocrit – patient's hematocrit) (Turgeon, 1993). Our measurements of Hb levels and hematocrit values in age-matched healthy children in Siaya revealed a normal hemoglobin value of 12.0 g/dL and an average normal population hematocrit value of 36.0 (Ong'echa *et al.*, manuscript submitted). Thus, based on age- and geographically-matched normal Hb and hematocrit values obtained

from over 1000 children enrolled in this study, a hematocrit value of 36.0 was used to normalize the reticulocyte counts (Ong'echa *et al.*, manuscript submitted). This correction takes into account the increased shift in reticulocyte extravasation from the bone marrow (reticulocyte shift) in anemic patients and the dependence of reticulocyte maturation time on the degree of anemia (Clark and Hippel, 2002). The percent of pigment-containing monocytes (% PCM) was calculated as the number of monocytes containing malarial pigment (hemozoin)/30 x 100, where 30 is the number of monocytes counted from a blood film.

Based on Hb and parasitemia test results, children were placed into one of six clinical categories: Healthy Controls (HC), Hospitalized Controls (HosC), Uncomplicated Malaria (UM), Mild Malarial Anemia (MlMA), Moderate Malarial Anemia (MdMA), and Severe Malarial Anemia(SMA). Anemia was defined as Hb < 11.0 g/dL, according to standard guidelines for children under 5 years of age in developing nations (Murray and Lopez, 1996). The HC group included aparasitemic children with Hb ≥ 11.0 g/dL, no prior hospitalizations, and absence of fever or diarrhea in the last 14 days (Ong'echa et. al, 2006). HosC included aparasitemic children with Hb < 11.0 g/dL, who were treated for non-malarial diseases. Children with UM were defined as having parasitemia (*P. falciparum*) of any density, Hb < 11.0 g/dL, and no symptoms of severe malaria (such as hypoglycemia). Children in the MlMA group had parasitemia of any density, 11.0 g/dL > Hb ≥ 8.00 g/dL, and no symptoms of severe malaria. MdMA subjects were defined by parasitemia of any density, 8.00 g/dL > Hb ≥ 6.00 g/dL, and no symptoms of severe malaria. Children with SMA were defined as having parasitemia of any density and Hb < 6.00 g/dL. Children with cerebral malaria and children who had received antimalarial treatment within two weeks prior to visiting the hospital were excluded from the study (Ong'echa *et al.*, 2006). Due to our particular interest in malarial anemia, the HosC group was excluded from these analyses.

2.6 ***Multiplex Assay:*** Plasma cytokine protein levels (IFN-α, IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12p40/p70) of the study participants described above (n = 189) were determined using the BioSource Human Cytokine 25-Plex Antibody Bead Kit (BioSource, International, Inc., Camarillo, CA). The assay was performed according to

the manufacturers specifications, using the Luminex® 100™ System analyzer (Luminex Corporation, Austin, TX). Briefly, wells of filter bottom microplates were pre-wetted with Working Wash Solution (200 μL) and then aspirated with a vacuum manifold. Followed by a second wash, incubation buffer (50 μL) was pipetted into the wells. Beads (25 μL) of defined spectral properties conjugated to analyte-specific capture antibodies, standards (100 μL) of known analyte concentration, control samples (100 μL), and unknown (plasma) samples (100 μL) were pipetted into designated wells. Standards, controls, and unknown samples were assayed in duplicate. Plasma samples (50 μL) were centrifuged at 14,000 rpm (and/or filtered) and diluted 1:1 with Assay Diluent (50 μL) prior to addition to the wells. Sealed, foil-wrapped plates were incubated at room temperature for 2 hrs on an orbital shaker, after which wells were aspirated and washed twice with Working Wash Solution (200 μL). Following addition of analyte-specific Biotinylated Detector Antibody (100 μL) to each well, the plates were incubated at room temperature for 1 hr on an orbital shaker. Wells were then aspirated and washed twice, before addition of Streptavidin-R-Phycoerythrin (100 μL/well). After a 30-min incubation at room temperature on an orbital shaker, wells were aspirated, and the beads were washed three times. 100 μL of Working Wash Solution was dispensed to each well, and the plates were placed on an orbital shaker for 2-3 min to resuspend the beads. Analyte concentrations were determined by analyzing the spectral properties of the capture beads and the amount of associated R-Phycoerythrin fluorescence with a Luminex® 100™ instrument.

2.7 ***Statistical Analyses:*** Data were analyzed using the Minitab statistical program (Minitab Release 14 for Windows, Minitab, Inc.). β-actin, TNF-α, IL-12A (p35), IL-12B (p40), IL-10, TGF-β1, IFN-α, IFN-γ, IL-2, IL-4, IL-6, IL-18, IL-1β, LT-α, NOS-2A, COX-1, and COX-2 mRNA transcripts were measured in duplicate. Pairwise comparisons between conditions were performed using the paired Student's *t* test, with statistical significance set at $P < 0.05$. To adjust for multiple comparisons, a Bonferroni correction was applied, and $P < 0.01$ was considered statistically significant.

For Tables 1-4, multiple group comparisons were performed by Kruskal-Wallis tests, and differences between proportions were determined by Chi-square analysis. Pairwise analyses of UM vs. SMA groups (Table 3) were performed by Mann-Whitney U tests for variables that were significantly different by Kruskal-Wallis tests. Statistical significance was set at $P < 0.05$ for all tests.

3.0 RESULTS

Specific Aim 1: ***To determine the effect of naturally acquired Plasmodium falciparum hemozoin on hematological outcomes in infants and young children with malarial anemia***

3.1 Results: Hematology and Malarial Pigment Data

3.1.1 *Clinical and laboratory parameters:*

Due to the importance of elucidating the pathological effects of malarial pigment (hemozoin) on hematopoietic development and its role in malarial anemia (MA), we chose to examine hematological data obtained from healthy children and children with varying degrees of MA. Study participants included infants and children aged 0-3 years from an area of holoendemic *Plasmodium falciparum* transmission (Siaya District, Nyanza Province, western Kenya) (Ong'echa *et al.*, 2006). These children were chosen as participants in the hospital-based study, since malaria is the primary cause of morbidity and mortality in African children in this age group (WHO, 2000). Furthermore, MA causes the majority of malaria-related morbidity and mortality among children less than five years of age (Breman *et al.*, 2001; WHO, 2000). In western Kenya, the prevalence of malaria in children 1-4 years of age is 83%, and the prevalence of anemia in children under 5 years of age ranges from 60-90% (Bloland *et al.*, 1999; McElroy *et al.*, 2000).

Heel/finger prick blood (< 100μL) was obtained from subjects to screen for hemoglobin (Hb) levels and parasitemia, and to perform complete blood counts. Based on Hb and parasitemia (*P. falciparum*) test results, children (n = 648) were placed into one of six clinical categories: Healthy Controls (HC), Hospitalized Controls (HosC), Uncomplicated Malaria (UM), Mild Malarial Anemia (MlMA), Moderate Malarial

Anemia (MdMA), and Severe Malarial Anemia (SMA). Anemia was defined as Hb < 11.0 g/dL, according to standard guidelines for children under 5 years of age in developing nations (Murray *et al.*, 1996). The HC group included aparasitemic children with Hb ≥ 11.0 g/dL, no prior hospitalizations, and absence of fever or diarrhea in the last 14 days (Ong'echa *et. al*, 2006). HosC included aparasitemic children with Hb < 11.0 g/dL, who were treated for non-malarial diseases. This group was excluded from the analyses, which focus on malarial anemia. Children with UM were defined as having parasitemia of any density, Hb < 11.0 g/dL, and no symptoms of severe malaria (such as hypoglycemia). Children in the MlMA group had parasitemia of any density, 11.0 g/dL > Hb ≥ 8.00 g/dL, and no symptoms of severe malaria. MdMA subjects were defined by parasitemia of any density, 8.00 g/dL > Hb ≥ 6.00 g/dL, and no symptoms of severe malaria. Children with SMA were defined as having parasitemia of any density and Hb < 6.00 g/dL. Multiple group comparisons were performed by Kruskal-Wallis tests, and differences between proportions were determined by Chi-square analysis, with statistical significance set at $P < 0.05$ for all tests.

Table 1 summarizes the clinical and demographic characteristics of study participants (n = 648), stratified according to clinical category. Specifically, leukocytic, erythrocytic, platelet, and parasitological indices were analyzed. The distribution of study participants among the five clinical categories was as follows: HC, n = 63; UM, n = 36; MlMA, n = 146; MdMA, n = 167; and SMA, n = 236. Enrolled subjects included 327 males and 314 females, and the proportion of males and females in the different clinical categories was not significantly different ($P = 0.079$). Multiple group comparisons revealed that age was significantly associated with disease severity ($P < 0.0001$). Children in the HC group were the youngest, with a mean enrollment age of 9.35 ± 0.85 months (mean ± SEM), followed by children with SMA (10.76 ± 0.45 months). Mean enrollment ages of subjects in the UM, MlMA, and MdMA categories were 12.24 ± 1.51, 12.87 ± 0.58, and 12.38 ± 0.53 months, respectively. Thus, among children with malaria, those with SMA were the youngest.

Mean axillary temperature (°C) differed significantly among the clinical groups ($P < 0.0001$), but parasitemia was not significantly associated with disease severity ($P = .358$). The UM group exhibited the highest mean parasitemia (76,737 ± 15,948/μL),

followed by those children in the SMA group (63,245 ± 6,148/μL). The MdMA group had the lowest mean parasitemia (51,074 ± 5,638/μL). High density parasitemia (HDP, ≥ 10,000 parasites/μL) was also not significantly different among the groups ($P = 0.764$). The UM group contained the greatest proportion of subjects with HDP (77.78%), whereas the MdMA group contained the least (69.46%).

In Table 1, all leukocyte indices [white blood cell (WBC), lymphocyte (LY), monocyte (MO), and granulocyte (GR) counts] were significantly associated with disease severity ($P < 0.0001$). Mean WBC counts were highest in the SMA group (15.04 ± 0.57 $x10^3$ cells/μL) and lowest in the MdMA group (11.65 ± 0.36 $x10^3$ cells/μL). Children with SMA also had the highest mean lymphocyte (7.62 ± 0.31 $x10^3$ cells/μL) and monocyte counts (1.55 ± 0.06 $x10^3$ cells/μL), whereas those with UM had the lowest (4.82 ± 0.42 $x10^3$ and 0.76 ± 0.42 $x10^3$ cells/μL, respectively). Mean granulocyte number ($x10^3$ cells/μL) was highest among those in the UM category (6.81 ± 0.60) and lowest among study participants in the HC group (3.96 ± 0.39).

Table 1. Clinical and demographic characteristics of study participants

	HC	UM	MlMA	MdMA	SMA	*P*
No. of subjects	63	36	146	167	236	
Demographic Factors						
Age, *mos.*	9.35 (0.85)	12.24 (1.51)	12.87 (0.58)	12.38 (0.53)	10.76 (0.45)	**<0.0001**[a]
Sex						
Female, *n (%)*	38 (60.32)	20 (58.82)	59 (40.97)	80 (48.48)	117 (49.79)	0.079[b]
Male, *n (%)*	25 (39.68)	14 (41.18)	85 (59.03)	85 (51.52)	118 (50.21)	
Clinical/Parasitological Factors						
Temperature, *˚C*	36.77 (0.10)	37.66 (0.19)	37.60 (0.09)	37.73 (0.09)	37.56 (0.06)	**<0.0001**[a]
Parasitemia, /µL	0 (0)	76,737 (15,948)	51,172 (5,091)	51,074 (5,638)	63,245 (6,148)	0.358[c]
HDP, *n (%)*	0 (0)	28 (77.78)	106 (72.60)	116 (69.46)	169 (71.61)	0.764[d]
Leukocytic Factors						
WBC, $x10^3$ *cells/µL*	11.88 (0.57)	12.39 (0.72)	11.65 (0.36)	12.02 (0.39)	15.04 (0.57)	**<0.0001**[a]
LY, $x10^3$ *cells/µL*	7.05 (0.38)	4.82 (0.42)	5.52 (0.23)	6.03 (0.22)	7.62 (0.31)	**<0.0001**[a]
MO, $x10^3$ *cells/µL*	0.89 (0.05)	0.76 (0.07)	0.96 (0.05)	1.12 (0.05)	1.55 (0.06)	**<0.0001**[a]
GR, $x10^3$ *cells/µL*	3.96 (0.39)	6.81 (0.60)	5.17 (0.24)	4.88 (0.24)	5.87 (0.29)	**<0.0001**[a]

Table 1 Continued.

	HC	UM	MlMA	MdMA	SMA	*P*
Erythrocytic Factors						
RBC, *x10^6 cells/ μL*	5.01 (0.07)	4.72 (0.12)	4.17 (0.06)	3.20 (0.05)	2.21 (0.04)	**<0.0001**[a]
Hb, *g/dL*	11.70 (0.07)	11.68 (0.14)	9.28 (0.07)	6.85 (0.05)	4.80 (0.06)	**<0.0001**[a]
Hct, *%*	35.51 (0.25)	34.26 (0.86)	28.19 (0.30)	21.81 (0.19)	15.35 (0.22)	**<0.0001**[a]
RPI < 2	0.82 (0.06)	0.86 (0.08)	0.62 (0.03)	0.74 (0.04)	0.76 (0.04)	**0.021**[a]
RPI ≥ 3	5.16 (1.15)	6.40 (2.40)	-	3.59 (0.36)	4.07 (0.30)	0.168[a]
MCV, *fL*	71.32 (0.74)	73.10 (1.21)	68.39 (0.63)	69.43 (0.74)	71.42 (0.66)	**0.002**[a]
MCH, *pg/cell*	23.54 (0.29)	24.30 (0.43)	22.26 (0.24)	21.94 (0.26)	22.45 (0.23)	**<0.0001**[a]
MCHC, *g/dL*	32.97 (0.13)	33.19 (0.21)	32.51 (0.13)	31.61 (0.14)	31.43 (0.15)	**<0.0001**[a]
RDW, *%*	16.71 (0.43)	17.08 (0.51)	19.31 (0.27)	22.17 (0.26)	23.27 (0.28)	**<0.0001**[a]
Platelet Factors						
Plt, *x10^3 cells/ μL*	432.57 (20.18)	239.69 (20.15)	203.54 (11.34)	175.62 (7.96)	165.31 (6.10)	**<0.0001**[a]
MPV, *fL*	7.40 (0.12)	7.81 (0.20)	8.37 (0.12)	8.66 (0.10)	8.71 (0.09)	**<0.0001**[a]
Pct, *%*	0.32 (0.01)	0.18 (0.01)	0.17 (0.01)	0.15 (0.01)	0.14 (0.01)	**<0.0001**[a]
PDW, *%*	16.95 (0.12)	17.01 (0.17)	17.23 (0.08)	17.61 (0.09)	17.71 (0.10)	**<0.0001**[a]

Values represent mean (SEM) unless otherwise noted.
[a]Multiple group comparisons were analyzed by Kruskal-Wallis tests.
[b]Chi-square analysis was performed for multiple group comparisons of proportions.
[c]Multiple group comparisons were analyzed by Kruskal-Wallis tests and included all parasitemic groups.
[d]Chi-square analysis was performed for multiple group comparisons of proportions and included all parasitemic groups.

Abbreviations: HC, healthy controls; UM, uncomplicated malaria; MlMA, mild malarial anemia; MdMA, moderate malarial anemia; SMA, severe malarial anemia; Parasitemia, parasites/μL; HDP, high density parasitemia (≥ 10,000 parasites/μL); Hb, hemoglobin concentration; RPI, reticulocyte production index; WBC, white blood cell; LY, lymphocyte count; MO, monocyte count; GR, granulocyte count; RBC, red blood cell count; Hct, hematocrit (relative volume of erythrocytes); MCV, mean corpuscular (erythrocyte) volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell (erythrocyte volume) distribution width; Plt, platelet/thrombocyte count; MPV, mean platelet(thrombocyte) volume; Pct, plateletcrit; PDW, platelet distribution width.

All erythrocyte parameters were significantly different among clinical categories at $P < 0.0001$, with the exception of mean corpuscular volume (MCV), which was significant at $P = 0.002$. The mean hemoglobin (Hb, g/dL) concentrations for the groups were 11.70 ± 0.07 (HC), 11.68 ± 0.14 (UM), 9.28 ± 0.07 (MlMA), 6.85 ± 0.05 (MdMA), and 4.80 ± 0.06 (SMA). As expected, mean red blood cell (RBC) counts ($x10^6$ cells/μL) were highest in the HC group (5.01 ± 0.07) and lowest in the SMA category (2.21 ± 0.04). In agreement with this finding, mean hematocrit (Hct, %) was highest among healthy controls (35.51 ± 0.25) and lowest among children with SMA (15.35 ± 0.22). Reticulocyte production index (RPI), which is a function of reticulocyte maturation time and dependent on the degree of anemia (Clark and Hippel, 2002), was stratified as RPI < 2 or RPI ≥ 3. A reticulocyte production index less than 2 (RPI < 2), indicative of an inadequate bone marrow proliferative response or ineffective erythropoiesis, differed significantly among HC, UM, MlMA, MdMA, and SMA groups ($P = 0.021$). An RPI less than 2 was lowest in children with mild MA (0.62 ± 0.03) and highest among those with uncomplicated malaria (0.86 ± 0.08). A reticulocyte production index greater than or equal to 3 (RPI ≥ 3), which indicates an erythropoietic response appropriate for the degree of anemia, was not significantly different between clinical categories ($P = 0.168$). MCV (mean corpuscular/erythrocyte volume, fL) correlated with RPI, being lowest in the MdMA category (68.39 ± 0.63) and highest in the UM category (73.10 ± 1.21). Mean corpuscular hemoglobin (MCH, pg/cell) was greatest in the UM group and lowest in the MdMA group. Mean corpuscular hemoglobin concentration (MCHC, g/dL) was also highest in the UM group, but lowest in children with SMA. Red cell distribution width (RDW, %), which represents the coefficient of variation of the RBC size, was lowest in HC subjects (16.71 ± 0.43) and highest in the SMA group (23.27 ± 0.28).

Platelet indices were also significantly different among the clinical categories ($P < 0.0001$), with the most extreme values occurring between the HC and SMA groups. The HC group had the highest mean platelet counts (432.57 ± 20.18 $x10^3$ cells/μL) and mean plateletcrit (0.32 ± 0.01%), and the SMA group had the lowest (165.31 ± 6.10 and 0.14 ±0.01, respectively). Conversely, mean platelet volume (MPV, fL) was highest in the SMA clinical category (8.71 ± 0.09) and lowest in healthy control subjects (7.40 ±

0.12). Platelet distribution width (PDW, %), which represents the coefficient of variance of platelet size, was highest in the SMA group (17.71 ± 0.10) and lowest in healthy children (16.95 ± 0.12).

Table 2 summarizes the clinical and demographic characteristics of parasitemic study participants (UM, MlMA, MdMA, and SMA groups, n = 555), stratified according to percent of pigment-containing monocytes (% PCM). Children were grouped as such, since the presence of intraleukocytic pigment is a useful prognostic indicator of severe malaria infection after clearance of parasites from the peripheral blood (Phu *et al.*, 1994; Day *et al.*, 1996). Furthermore, hemozoin is present in the peripheral blood phagocytes of > 90% of patients with severe malaria (Day *et al.*, 1996).

Monocyte pigment categories were designated as 0% PCM, ≤ 10% PCM, and > 10% PCM. Distribution of study participants among the different pigment categories was as follows: 0% PCM, n = 284; ≤ 10% PCM, n = 110; and > 10% PCM, n = 161. The proportion of males and females in each group was not significantly different ($P = 0.068$). Multiple group comparisons showed that age was not significantly associated with blood content of pigment-containing monocytes ($P = 0.216$), though children with > 10% PCM were the youngest (11.15 ± 0.54 months). Mean axillary temperature (°C) was significantly different among pigment categories ($P = 0.034$). Parasitemia was significantly associated ($P = 0.002$) with percent of pigment-containing monocytes and increased with increasing % PCM. The > 10% PCM group contained subjects with the highest mean parasitemia (76,133 ± 7,983 parasites/μL), followed by the ≤ 10% and 0% PCM groups (56,348 ± 7,505 and 45,337 ± 3,532, respectively). However, the ≤ 10% PCM group contained the greatest proportion of subjects with HDP (78%), which also differed significantly between groups ($P = 0.032$).

All leukocyte indices were significantly different between pigment categories ($P < .0001$), with the exception of granulocyte count ($P = 0.441$). WBC, lymphocyte, and monocyte counts increased with the percent of pigment-containing monocytes.

Table 2. Clinical and demographic characteristics of parasitemic study participants stratified according to percent of pigment-containing monocytes

Monocyte Pigment Categories	**0%**	**≤ 10%**	**> 10%**	***P***
No. of subjects	284	110	161	
Demographic Factors				
Age, *mos.*	12.17 (0.41)	12.44 (0.74)	11.15 (0.54)	0.216[a]
Sex				
Female, *n (%)*	123 (43.62)	62 (56.36)	83 (51.88)	0.068[b]
Male, *n (%)*	159 (56.38)	48 (43.64)	77 (48.13)	
Clinical/Parasitological Factors				
Temperature, *˚C*	37.74 (0.07)	37.60 (0.10)	37.44 (0.08)	**0.034[a]**
Parasitemia, */µL*	45,337 (3,532)	56,348 (7,505)	76,133 (7,983)	**0.002[a]**
HDP, *n (%)*	192 (67.61)	86 (78.00)	124 (77.00)	**0.032[b]**
Leukocytic Factors				
WBC, $x10^3$ *cells/µL*	12.17 (0.35)	12.92 (0.60)	14.95 (0.66)	**<0.0001[a]**
LY, $x10^3$ *cells/µL*	5.82 (0.21)	6.52 (0.37)	7.46 (0.33)	**<0.0001[a]**
MO, $x10^3$ *cells/µL*	1.07 (0.04)	1.22 (0.08)	1.49 (0.07)	**<0.0001[a]**
GR, $x10^3$ *cells/µL*	5.29 (0.18)	5.19 (0.34)	6.00 (0.37)	0.441[a]
Erythrocytic Factors				
RBC, $x10^6$ *cells/ µL*	3.54 (0.06)	2.90 (0.09)	2.64 (0.07)	**<0.0001[a]**
Hb, *g/dL*	7.77 (0.13)	6.57 (0.20)	5.83 (0.16)	**<0.0001[a]**
%SMA, *n (%)*	64 (22.54)	56 (50.91)	99 (61.49)	**<0.0001[b]**
Hct, *%*	24.05 (0.39)	20.30 (0.58)	18.23 (0.49)	**<0.0001[a]**
RPI < 2	0.80 (0.03)	0.71 (0.05)	0.69 (0.04)	**0.023[a]**
RPI ≥ 3	3.96 (0.20)	4.01 (0.41)	4.99 (1.44)	0.862[a]
MCV, *fL*	69.21 (0.55)	71.27 (0.94)	70.21 (0.67)	0.103[a]
MCH, *pg/cell*	22.21 (0.20)	22.62 (0.32)	22.44 (0.25)	0.407[a]
MCHC, *g/dL*	32.06 (0.11)	31.73 (0.17)	31.96 (0.16)	0.243[a]
RDW, *%*	21.17 (0.26)	21.96 (0.41)	22.09 (0.30)	**<0.006[a]**

Table 2 Continued.

Monocyte Pigment Categories	**0%**	**≤ 10%**	**> 10%**	***P***
Platelet Factors				
Plt, *$x10^3$ cells/ μL*	201.87 (7.21)	164.01 (8.52)	156.78 (6.26)	**<0.0001**[a]
Pct, *%*	8.34 (0.08)	8.61 (0.14)	8.90 (0.11)	**<0.0001**[a]
MPV, *fL*	0.17 (0.01)	0.14 (0.01)	0.14 (0.01)	**0.004**[a]
PDW, *%*	17.44 (0.07)	17.42 (0.10)	17.74 (0.08)	**0.014**[a]

Values represent mean (SEM) unless otherwise noted.
[a]Multiple group comparisons were analyzed by Kruskal-Wallis tests.
[b]Chi-square analysis was performed for multiple group comparisons of proportions.

Abbreviations: Parasitemia, parasites/μL; HDP, high density parasitemia (≥ 10,000 parasites/μL); Hb, hemoglobin concentration; %SMA, percent severe malarial anemia; RPI, reticulocyte production index; WBC, white blood cell; LY, lymphocyte count; MO, monocyte count; GR, granulocyte count; RBC, red blood cell count; Hct, hematocrit (relative volume of erythrocytes); MCV, mean corpuscular (erythrocyte) volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell (erythrocyte volume) distribution width; Plt, platelet/thrombocyte count; MPV, mean platelet(thrombocyte) volume; Pct, plateletcrit; PDW, platelet distribution width.

Among erythrocyte indices, RBC count, Hb, and hematocrit were significantly different ($P < 0.0001$) between pigment categories. The 0% PCM category contained children having the highest RBC counts (3.54 ± 0.06 x10^6 cells/μL), Hb (7.77 ± 0.13 g/dL), and hematocrit (24.05 ± 0.39 %), whereas the > 10% PCM category contained children with the lowest (2.62 ± 0.07 x10^6 cells/μL, 5.83 ± 0.16 g/dL, and 18.23 ± 0.49 %, respectively). Notably, the percent of study participants diagnosed with SMA was significantly different among PCM categories ($P < 0.0001$), and the greatest proportion of children with SMA (61.49%) were included in the > 10% PCM group. RPI < 2 (suppression of erythropoiesis) was significantly associated with pigment category ($P = .023$), decreasing with increasing % PCM. RPI ≥ 3 (appropriate erythropoietic response for the degree of anemia) did not differ significantly between pigment categories. Taken together, these results demonstrate that percent of pigment-containing monocytes may be predictive of the severity of MA. However, erythrocytic factors that were not significantly associated with pigment category included MCV ($P = 0.103$), MCH ($P = 0.407$), and MCHC ($P = 0.243$). RDW was significantly different between groups ($P = 0.006$) and increased with increasing % PCM.

In addition, all platelet indices were significantly different among monocyte pigment categories. Mean platelet count ($P < 0.0001$) was inversely associated with percent of pigment-containing monocytes. Mean platelet volume ($P < 0.0001$) increased with increasing % PCM. Plateletcrit ($P = 0.004$) was highest in the 0% PCM category and equivalent in the ≤ 10% PCM and > 10% PCM categories. Platelet distribution width ($P = 0.014$) was greatest in the > 10% pigment group (17.74 ± 0.08).

In summary, results presented in Tables 1 and 2 indicate that monocytic acquisition of hemozoin is significantly associated with hematological parameters in children with varying severities of malarial anemia. Leukocyte, erythrocyte, and platelet indices were significantly different between HC, UM, MlMA, MdMA, and SMA clinical groups. The majority of leukocyte, erythrocyte, and platelet indices were also significantly different between pigment-containing monocyte categories. Temperature was significantly associated with clinical category and with proportion of pigment-

containing monocytes. Age was significantly different between clinical groups but not between PCM categories. However, among children with malaria, those with SMA and those with > 10% pigment-containing monocytes were the youngest. Parasitemia (/μL) and high density parasitemia (%) were significantly associated with proportion of pigment containing monocytes but not with clinical group. Suppression of erythropoiesis, as indicated by a reticulocyte production index less than 2, was greatest (lowest RPI) in children with mild MA (Table 1) and in those children with the greatest proportion (> 10%) of pigment-containing monocytes (Table 2). Children in the > 10% pigment-containing monocyte group exhibited the lowest Hb, hematocrit, and erythrocyte counts. In addition, the > 10% PCM group contained the greatest proportion of children with SMA. Taken together, these results suggest that the development of SMA is mediated, at least in part, by the phagocytosis of hemozoin by monocytes. Of relevance, malarial immunity is defined by concomitant interactions between a large number of different innate inflammatory mediators, and ingestion of hemozoin by peripheral blood mononuclear cells promotes dysregulation of inflammatory mediator production in children with malaria. Since these measurements have not been performed previously in children with malaria, we decided to measure numerous innate inflammatory mediators in children with varying severities of malarial anemia using a cytokine multiplex assay.

Specific Aim 2: ***To determine the relationship between in vivo innate inflammatory mediator production and monocytic acquisition of Plasmodium falciparum hemozoin in infants and young children with malarial anemia***

3.2 Results: Multiplex Assay

3.2.1 *Clinical and laboratory parameters:*

In order to compare cytokine protein levels among children with (and without) varying degrees of malarial anemia (MA), plasma levels of IL-12p40/p70, IFN-α, IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, and IL-10 from study participants described previously (n = 189) were determined by multiplex assay. Plasma cytokine levels of parasitemic study participants (UM, MlMA, MdMA, and SMA groups) were further stratified according to blood content of pigment-containing monocytes to examine whether cytokine production was positively correlated with percent of pigment-containing monocytes (% PCM). Multiple group comparisons were performed by Kruskal-Wallis tests, with statistical significance set at $P < 0.05$ for all analyses. Due to our interest in understanding the role of these cytokines in mediating the development of uncomplicated versus severe disease (anemia), pairwise comparisons of UM versus SMA groups were performed by Mann-Whitney U tests for variables that were significantly different by Kruskal-Wallis tests.

Table 3 illustrates the plasma cytokine profiles of study participants, stratified by clinical category (HC, n = 34; UM, n = 20; MlMA, n = 38; MdMA, n = 13; and SMA, n = 84). Multiple group comparisons revealed that plasma levels (pg/mL) of IFN-γ (P = 0.138), TNF-α (P = 0.465), IL-1β (P = 0.816), and IL-2 (P = 0.673) were not significantly different between clinical groups. Plasma IFN-α was significantly different among clinical categories ($P < 0.0001$). The HC group had the highest mean (mean ± SEM) IFN-α levels (93.71 ± 14.95), and those subjects with moderate MA had the lowest IFN-α levels (9.97 ± 4.04). Pairwise comparison of UM (43.77 ± 12.63) versus SMA (71.56 ± 32.47) groups showed that mean plasma IFN-α levels were not significantly

different (P = 0.586) between the groups. Mean plasma levels of IL-4 and IL-6 were significantly different among clinical categories (P = 0.004 and P < 0.0001, respectively). Consistent with IFN-α levels, plasma IL-4 was highest among healthy controls (34.26 ± 8.53) and lowest in the MdMA group (4.68 ± 1.16). Pairwise comparison of UM (10.42 ± 2.72) versus SMA (13.17 ± 2.15) groups indicated that plasma IL-4 levels were not significantly different between these clinical categories (P = 0.745). Plasma IL-6 levels were highest in children with severe malarial anemia (273.09 ± 56.64) and lowest in healthy control subjects (56.24 ± 13.06), but were not significantly different (P = 0.289) between UM and SMA groups. Study participants with uncomplicated malaria had the highest plasma levels of IL-10 (978.82 ± 478.34), which were determined to be significantly different among clinical groups (P < 0.0001). The HC group contained those subjects with the lowest levels of IL-10 (118.03 ± 24.82). However, pairwise comparison of UM versus SMA clinical groups revealed that plasma IL-10 levels were not significantly different (P = 0.539) between the two groups. Plasma IL-12p40/p70 also differed significantly among clinical categories (P = 0.009). Children in the MlMA group had the highest levels of IL-12p40/p70 (564.68 ± 69.93), whereas plasma IL-12p40/p70 was lowest among those with moderate malarial anemia (309.18 ± 32.91). IL-12 levels were not significantly different between UM and SMA clinical categories (P = 0.911).

Table 3. Plasma cytokine levels of study participants

	HC	UM	MlMA	MdMA	SMA	*P*	*P (UM vs. SMA)*
No. of subjects	34	20	38	13	84		
Cytokine *(pg/mL)*							
IFN-α	93.71 (14.95)	43.77 (12.63)	52.42 (13.47)	9.97 (4.04)	71.56 (32.47)	**<0.0001**[a]	0.568[b]
IFN-γ	35.22 (7.28)	46.36 (20.59)	18.93 (5.58)	15.46 (5.06)	19.09 (4.50)	0.138[a]	
TNF-α	103.45 (57.48)	36.18 (7.19)	54.85 (12.48)	27.23 (7.63)	48.77 (5.26)	0.465[a]	
IL-1β	300.08 (73.63)	169.25 (46.17)	278.59 (71.67)	272.67 (86.68)	234.57 (28.77)	0.816[a]	
IL-2	117.52 (39.31)	41.03 (14.08)	114.53 (52.12)	81.67 (38.36)	90.91 (32.18)	0.673[a]	
IL-4	34.26 (8.53)	10.42 (2.72)	17.89 (7.14)	4.68 (1.16)	13.17 (2.15)	**0.004**[a]	0.745[b]
IL-6	56.24 (13.06)	159.95 (53.96)	248.23 (65.10)	121.37 (48.26)	273.09 (56.64)	**<0.0001**[a]	0.289[b]
IL-10	118.03 (24.82)	978.82 (478.34)	926.72 (253.57)	302.40 (103.82)	538.96 (76.16)	**<0.0001**[a]	0.539[b]
IL-12p40/p70	557.39 (59.51)	400.48 (45.81)	564.68 (69.93)	309.18 (22.91)	418.95 (26.73)	**0.009**[a]	0.911[b]

Values represent mean (SEM) unless otherwise noted.
[a]Multiple group comparisons were analyzed by Kruskal-Wallis tests.
[b]Pairwise analyses of UM vs. SMA groups were performed by Mann-Whitney U tests for variables that were significantly different by Kruskal-Wallis tests.

Abbreviations: HC, healthy controls; UM, uncomplicated malaria; MlMA, mild malarial anemia; MdMA, moderate malarial anemia; SMA, severe malarial anemia.

Table 4 summarizes the plasma cytokine protein levels of parasitemic study participants (UM, MlMA, MdMA, and SMA groups; n = 155), stratified according to percent of pigment-containing monocytes (0%, ≤ 10%, and > 10%). Distribution of study participants among the three pigment categories was as follows: 0% PCM, n = 73; ≤ 10% PCM, n = 32; and > 10% PCM, n = 50. Multiple group comparisons showed that when subjects were grouped according to percent of pigment-containing monocytes (% PCM), mean plasma levels of IFN-γ ($P = 0.211$), IL-1β ($P = 0.761$), and IL-2 ($P = 0.509$) were not significantly different among pigment categories. Plasma IFN-γ was lowest among children in the ≤ 10% PCM category and highest among those with 0% pigment-containing monocytes. Relative to IL-1β levels of subjects with 0% pigment-containing monocytes, plasma IL-1β was elevated in the ≤ 10% PCM group and suppressed in the > 10% PCM group. Children with 0% pigment-containing monocytes exhibited the lowest levels of IL-2, whereas children in the ≤ 10% PCM category had the highest levels of IL-2.

Although IFN-α, IL-4, IL-6, IL-10 and IL-12p40/p70 levels had been determined to differ significantly between clinical groups (Table 3), these cytokines were also not significantly associated with the proportion of pigment-containing monocytes ($P = 0.579$, $P = 0.300$, $P = 0.542$, and $P = 0.172$, respectively). Mean plasma IFN-α and IL-4 were highest among children in the > 10% PCM category and lowest in the ≤ 10% PCM group. Relative to children with 0% pigment-containing monocytes, those in the ≤ 10% PCM category had elevated levels of IL-6 and IL-12p40/p70, and those in the > 10% PCM category exhibited decreased levels of IL-6 and IL-12p40/p70. Plasma levels of IL-10 were inversely associated with proportion of pigment-containing monocytes.

Differences in plasma TNF-α between pigment-containing monocyte categories bordered statistical significance at $P = 0.051$. Parasitemic children in the ≤ 10% PCM group exhibited the highest levels (pg/mL) of TNF-α (61.42 ± 13.06), whereas those with 0% PCM had the lowest plasma TNF-α (39.92 ± 6.21).

Table 4. Plasma cytokine levels of parasitemic study participants stratified according to percent of pigment-containing monocytes

Monocyte Pigment Categories	**0%**	**≤ 10%**	**> 10%**	***P***
No. of subjects	73	32	50	
Cytokine *(pg/mL)*				
IFN-α	45.22 (9.62)	32.04 (9.06)	93.64 (53.67)	0.579[a]
IFN-γ	26.60 (6.62)	9.72 (3.14)	23.97 (6.91)	0.211[a]
TNF-α	39.92 (6.21)	61.42 (13.06)	47.57 (5.28)	0.051[a]
IL-1β	239.54 (32.00)	300.48 (89.98)	202.35 (25.12)	0.761[a]
IL-2	69.07 (12.69)	131.15 (65.01)	92.64 (50.67)	0.509[a]
IL-4	13.79 (3.83)	9.27 (2.16)	15.04 (3.29)	0.300[a]
IL-6	228.35 (48.66)	342.13 (118.02)	190.61 (37.63)	0.221[a]
IL-10	835.29 (195.56)	611.96 (108.12)	468.73 (74.69)	0.542[a]
IL-12p40/p70	454.50 (37.00)	502.20 (58.61)	388.60 (33.45)	0.172[a]

Values represent mean (SEM) unless otherwise noted.
[a]Multiple group comparisons were analyzed by Kruskal-Wallis tests.

Taken together, results presented in Tables 3 and 4 demonstrate that plasma levels of IFN-α, IL-4, IL-6, IL-10, and IL-12p40/p70 are significantly different among HC, UM, MlMA, MdMA, and SMA clinical categories. Differences in plasma levels of these inflammatory mediators between children with uncomplicated malaria and children with severe MA are not statistically significant. Furthermore, among parasitemic children, plasma IFN-α, IL-4, IL-6, IL-10, and IL-12p40/p70 are not significantly associated with percent of pigment-containing monocytes. Differences in plasma TNF-α are not significant between clinical groups but approach statistical significance between 0%, ≤ 10%, and > 10% PCM categories. Mean plasma levels of IFN-γ, IL-1β, and IL-2 are not significantly different among clinical groups or pigment-containing monocyte categories.

In summary, stratification of children according to the proportion of pigment-containing monocytes revealed distinct patterns of cytokine production that differed substantially for the different inflammatory mediators. In children with and without varying degrees of malarial anemia, hemozoin produced a dose-response effect on plasma cytokine levels, differentially up-regulating production of some inflammatory mediators while down-regulating production of others. These results suggest that malarial pigment plays an important role in promoting changes in cytokine production during malaria infection which contribute to the development of severe malarial anemia. Importantly, malarial immunity is determined by concomitant interactions between numerous innate inflammatory mediators, and the role of hemozoin in regulating the temporal kinetics of cytokine production has not been previously investigated concomitantly for a large number of innate immune response genes. For this reason, we next sought to determine the temporal kinetics of several innate immune response genes thought to be important mediators of the pathogenesis of malarial anemia.

Specific Aim 3: ***To determine the temporal kinetics of hemozoin-induced dysregulation in innate inflammatory mediators in cultured human peripheral blood mononuclear cells***

3.3 Results: Quantitative Real Time RT-PCR

3.3.1 *Laboratory parameters:*

PBMC isolated from malaria-nave donors (n = 9) were stimulated with media alone or a high dose of β-hematin (10 μg/mL), and cells were harvested at 4, 8, 24, 48, and 72 hrs. To investigate the temporal effect of β-hematin on *de novo* cytokine transcription, RNA was extracted from cells and reverse transcribed for real time RT-PCR analysis. Results are expressed as fold change ± SEM relative to control (media alone) conditions. Statistical significance of the difference in means between baseline and stimulated conditions or between fold change at each time point and 4 hrs (time zero) was determined by paired *t*-tests. $P < 0.05$ was considered statistically significant for stimulated versus control conditions. To adjust for multiple comparisons of fold change at 8, 24, 48, and 72 hrs relative to fold change at 4 hrs, a Bonferroni correction was applied, and $P < 0.01$ was deemed statistically significant.

3.3.2 *Pro-inflammatory mediator production*

3.3.2.1 *Temporal effect of β-hematin on IL-1β transcripts:*

IL-1β mRNA was detectable in stimulated cells at 4 hrs and continued to increase at 8 and 24 hrs (Figure 4). The difference in means between transcript production under stimulated versus baseline conditions was statistically significant at all time points (indicated by asterisks). In the majority of donors, transcript production peaked at 48 hrs, as evidenced by a > 300- to 1300-fold increase relative to untreated cells. β-hematin significantly augmented donor 5 IL-1β mRNA at 24 hrs, producing a > 2400-fold increase over unstimulated conditions. By 72 hrs, IL-1β transcripts began to decrease to levels below 600-fold over baseline. Relative to the 4 hr time point (time zero), IL-1β mRNA production was significantly elevated at 48 ($P = 0.001$) and 72 hrs ($P = 0.003$).

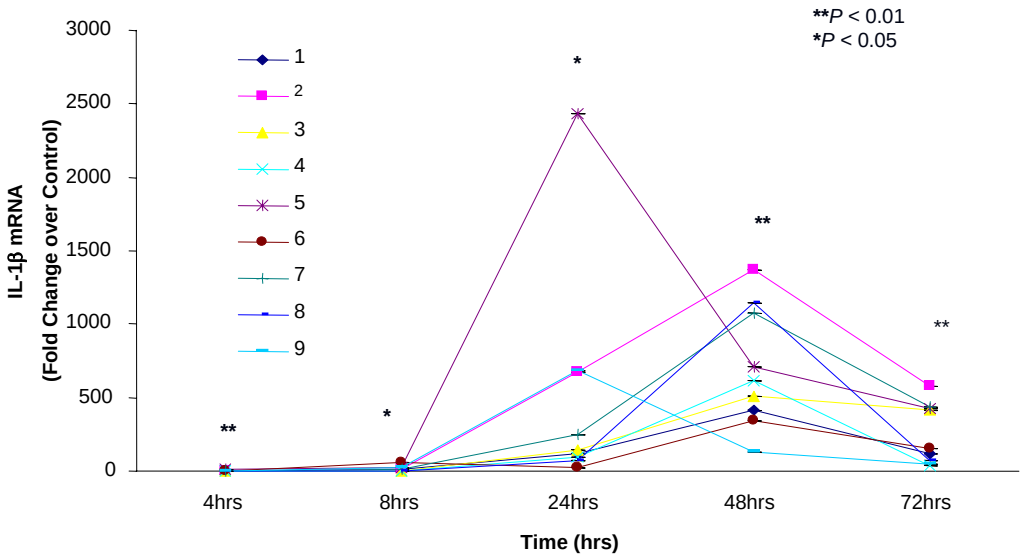

Figure 4. Temporal effect of β-hematin on IL-1β transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IL-1β mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.2 *Temporal effect of β-hematin on IL-2 transcripts:*

In the presence of β-hematin, IL-2 transcript production at 4, 8, and 24 hrs fluctuated at or slightly above baseline levels in most donors (Figure 5). IL-2 mRNA increased between 24 and 48 hrs, peaking in the majority of subjects at 48 hrs. Donors 6 and 9 exhibited a sharp increase in IL-2 transcript production at 8 (> 18-fold) and 24 hrs (> 17-fold), respectively. The difference in means between IL-2 transcript production under stimulated versus control conditions was statistically significant ($P < 0.05$) only at 48 hrs. IL-2 mRNA decreased by 72 hrs in 5 of 9 donors. After correcting for multiple comparisons, it was determined that fold change in IL-2 was not significantly augmented at any subsequent time point, relative to 4 hrs.

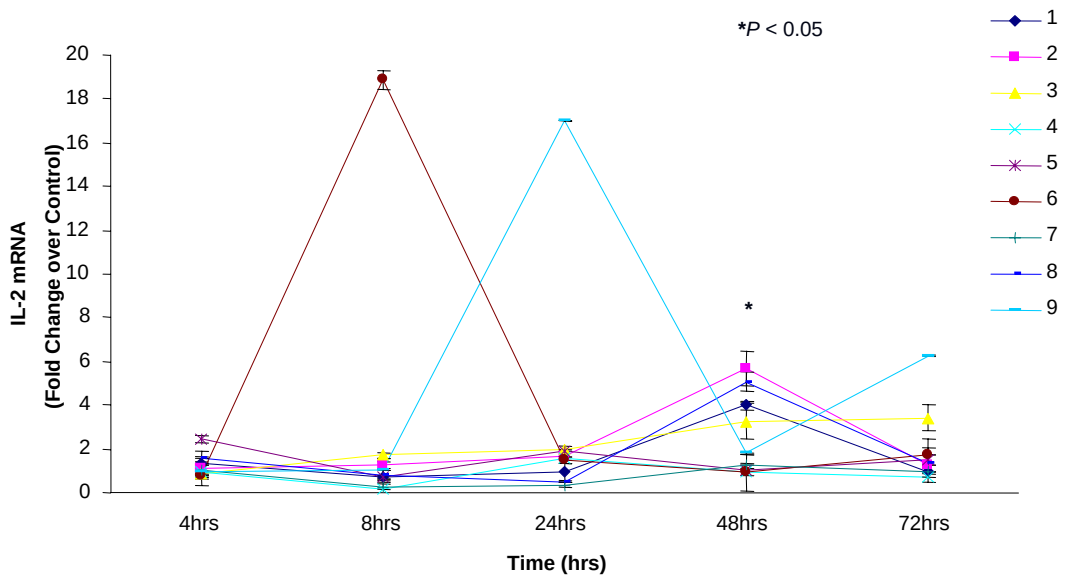

Figure 5. Temporal effect of β-hematin on IL-2 transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IL-2 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.3 *Temporal effect of β-hematin on IL-6 transcripts:*

Relative to baseline conditions, mean IL-6 transcript production in cells stimulated with a high dose of β-hematin was statistically significant at all time points (Figure 6). IL-6 mRNA peaked in the majority of subjects at 24 hrs, reaching levels > 12 to 1300 times those measured in unstimulated cells. Fold change in IL-6 transcript production increased significantly between 4 and 48 hrs ($P = 0.008$). β-hematin-induced IL-6 mRNA production decreased in 8 of 9 donors by 72 hrs.

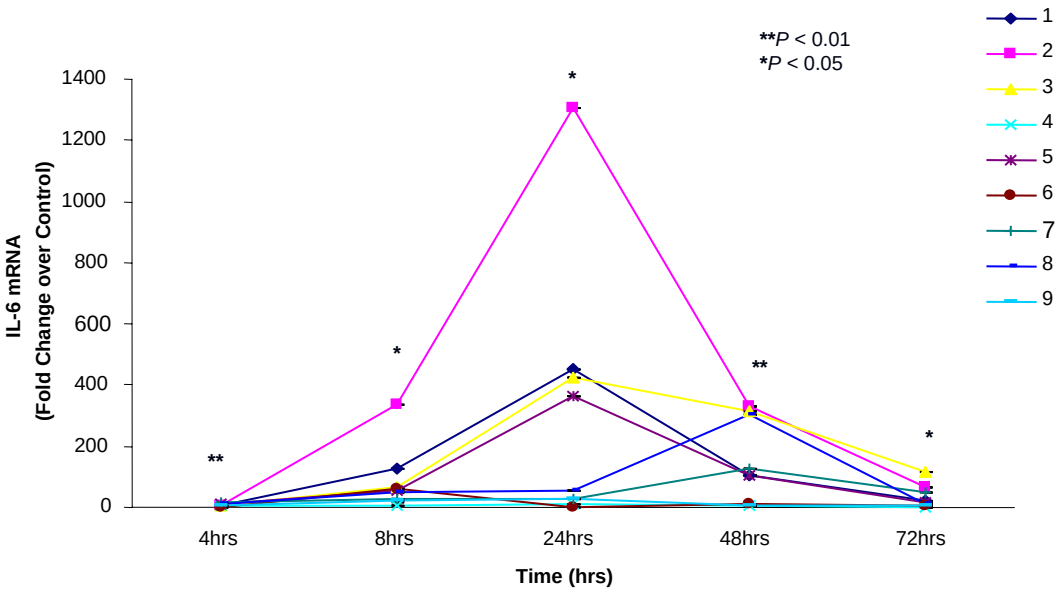

Figure 6. Temporal effect of β-hematin on IL-6 transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IL-6 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.4 *Temporal effect of β-hematin on IL-12p35 transcripts:*

After 4 hrs in culture, cells stimulated with β-hematin produced IL-12p35 transcripts at near baseline levels (Figure 7). IL-12p35 mRNA synthesis began to increase by 8 hrs post-stimulation, peaking at 72 hrs in most donors. Fold change over unstimulated conditions was statistically significant at 24, 48, and 72 hrs. Relative to fold change at 4 hrs, a significant increase in β-hematin-induced IL-12p35 transcript production occurred by 72 hrs post-stimulation ($P = 0.006$).

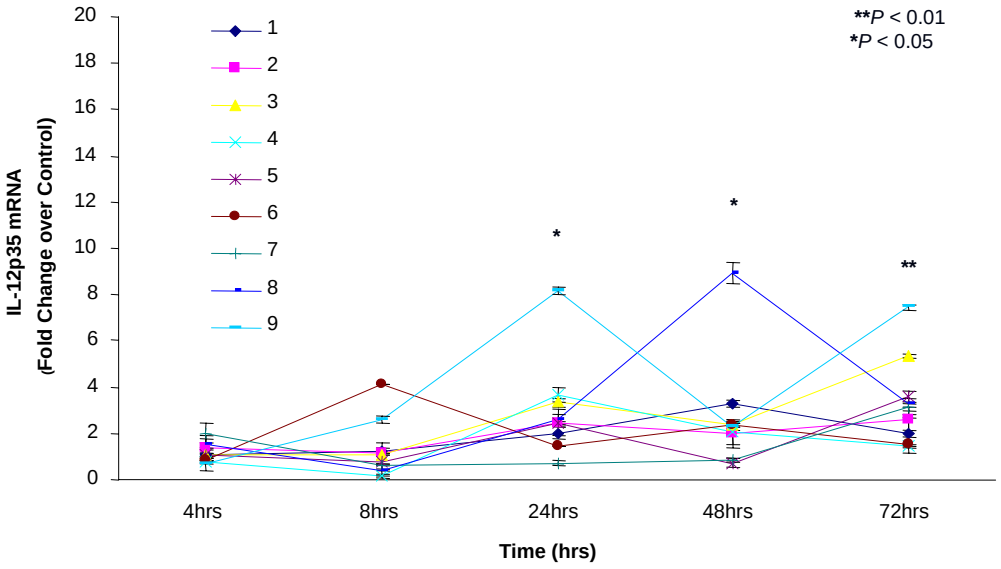

Figure 7. Temporal effect of β-hematin on IL-12p35 transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IL-12p35 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.5 *Temporal effect of β-hematin on IL-12p40 transcripts:*

In the presence of β-hematin, IL-12p40 mRNA was detectable in most subjects at 4 hrs (Figure 8). Peak IL-12p40 transcript production occurred at 24 hrs post-stimulation in the majority of donors and varied markedly in magnitude (> 4- to 850-fold over unstimulated cells). IL-12p40 mRNA decreased by 48 hrs and continued to decrease at 72 hrs in 8 of 9 subjects. Donor 7 IL-12p40 transcript production peaked at 4 hrs and fluctuated over the course of 72 hrs. Mean fold change in IL-12p40 transcript production under stimulated versus baseline conditions was statistically significant at all time points ($P < 0.05$).

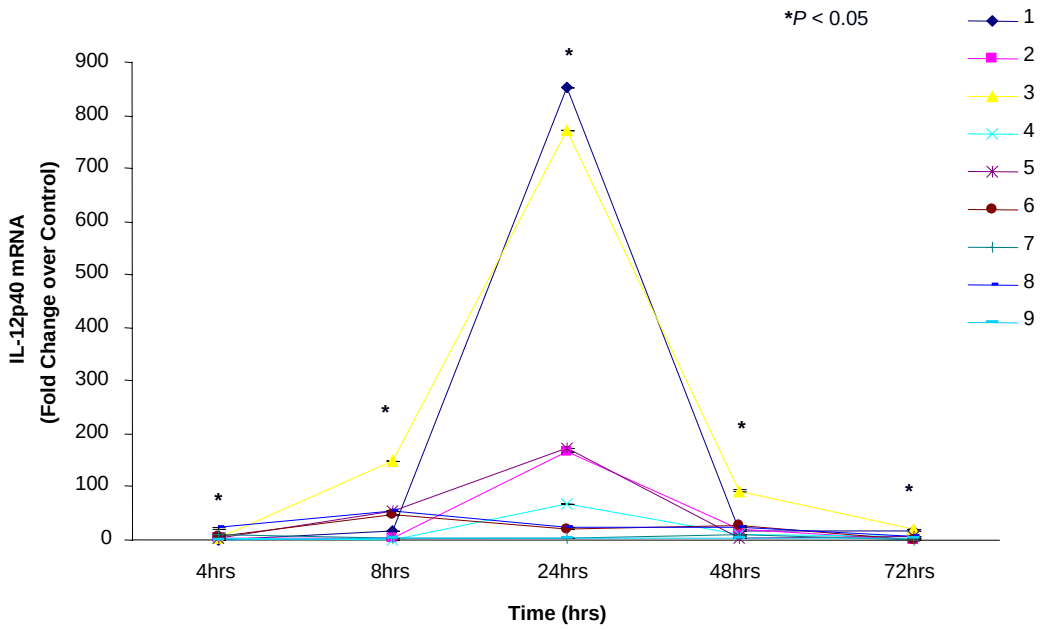

Figure 8. Temporal effect of β-hematin on IL-12p40 transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IL-12p40 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.6 *Temporal effect of β-hematin on IL-18 transcripts:*

Fold change in β-hematin-induced IL-18 transcript production was statistically significant ($P < 0.05$) at 4 hrs post-stimulation (Figure 9). Peak IL-18 mRNA production was divided among donors between 4 and 8 hrs. Whereas peak transcript production ranged from levels slightly above baseline to approximately 6-fold over control in most subjects, donor 6 exhibited a > 57-fold change in IL-18 mRNA. With the exception of one donor, IL-18 transcripts decreased by 24 hrs and were suppressed from this time onward.

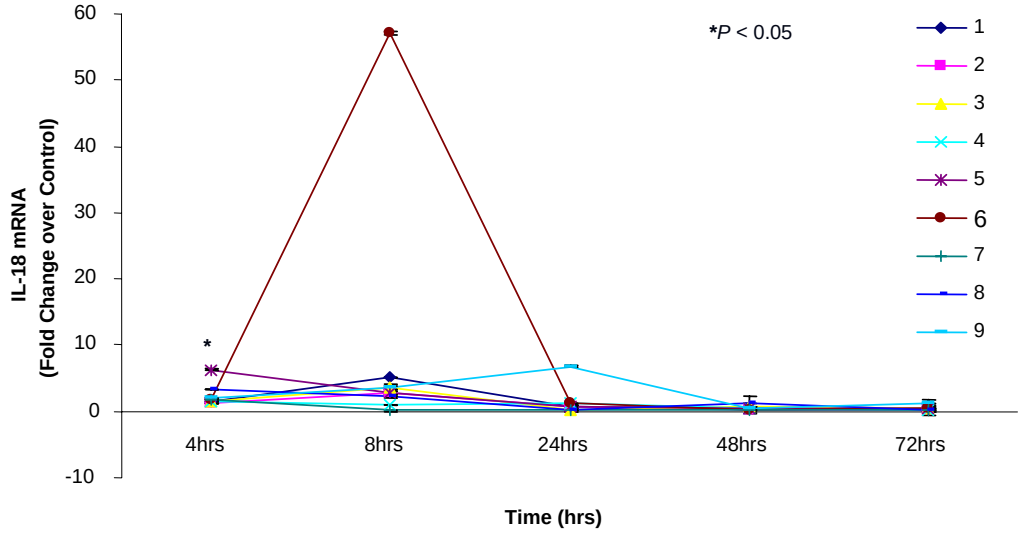

Figure 9. Temporal effect of β-hematin on IL-18 transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IL-18 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.7 *Temporal effect of β-hematin on IFN-α transcripts:*

β-hematin-induced IFN-α mRNA was detectable near baseline levels at 4 hrs in most subjects and fluctuated above and below baseline throughout the time course (Figure 10). Peak transcript production was distributed among the five time points but occurred at 48 hrs in the majority of donors (3). While most subjects showed a 2- to 13-fold increase in IFN-α mRNA at peak transcript production, donor 9 exhibited a > 120-fold increase in IFN-α mRNA. The difference in means between IFN-α transcript production under stimulated versus baseline conditions was statistically significant at 48 hrs post-challenge ($P < 0.05$).

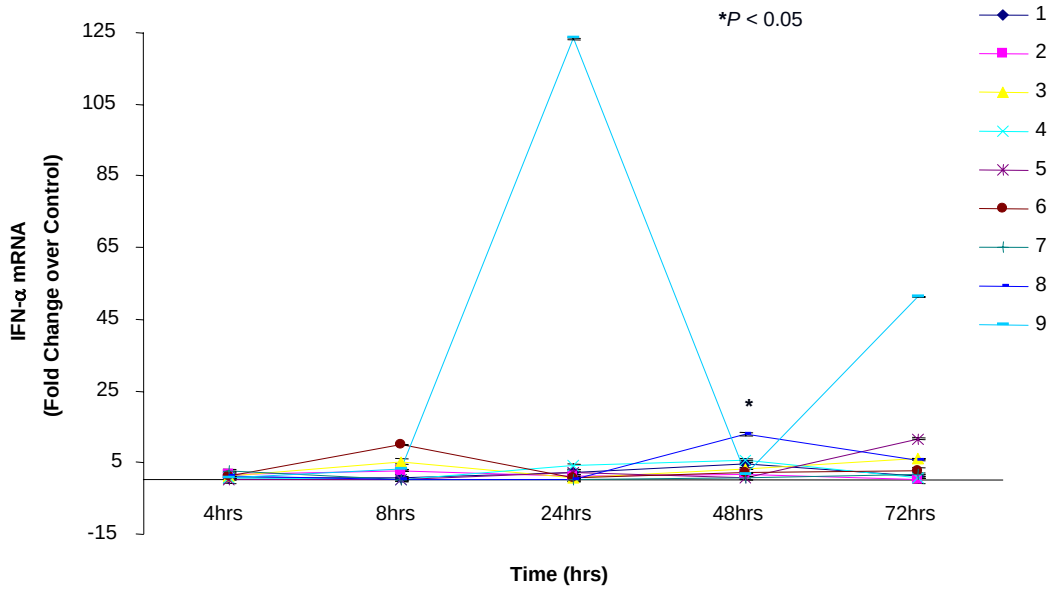

Figure 10. Temporal effect of β-hematin on IFN-α transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 µg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IFN-α mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.8 *Temporal effect of β-hematin on IFN-γ transcripts:*

β-hematin produced IFN-γ mRNA expression patterns that varied significantly among donors (Figure 11). IFN-γ transcripts were detectable at 4hrs post-stimulation and fluctuated over time. Peak transcript production occurred at 24 hrs in the majority of subjects (3) but was observed at 4, 8, 48, and 72 hrs, as well. Peak levels of IFN-γ mRNA ranged from 4 to 22 times those measured in unstimulated cells. Fold change in IFN-γ mRNA was statistically significant at 4, 24, and 72 hrs.

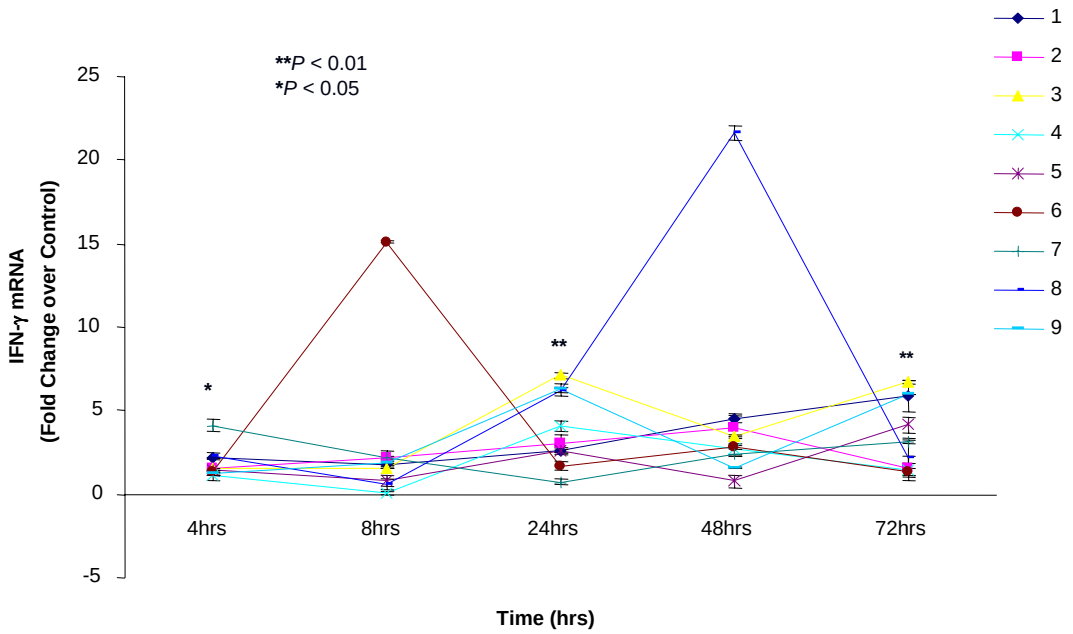

Figure 11. Temporal effect of β-hematin on IFN-γ transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IFN-γ mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.9 *Temporal effect of β-hematin on TNF-α transcripts:*

After 4 hrs in culture, cells stimulated with β-hematin produced a 3- to 10-fold increase in TNF-α transcripts (Figure 12). In most subjects, mRNA expression continued to increase at 8 hrs post-challenge, peaking at levels > 3- to 14-fold over baseline. TNF-α mRNA decreased by 24hrs and peaked slightly at 48 hrs in 5 of 9 donors. By 72 hrs, TNF-α transcripts had decreased to levels < 3-fold over baseline. Fold change in β-hematin-induced TNF-α production was statistically significant at all time points ($P < 0.01$).

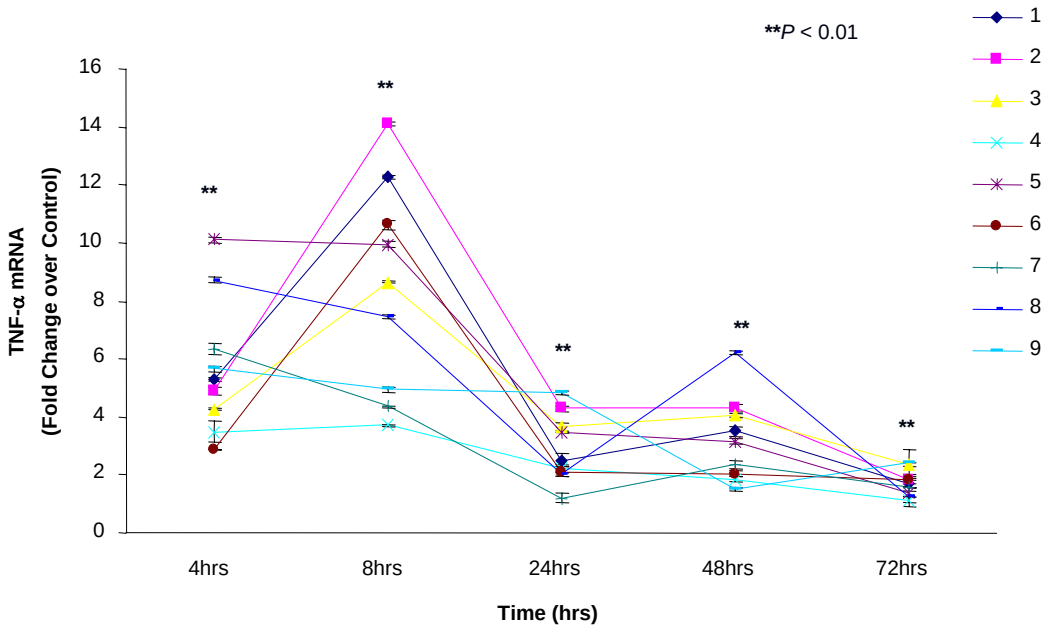

Figure 12. Temporal effect of β-hematin on TNF-α transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for TNF-α mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.10 *Temporal effect of β-hematin on LT-α transcripts:*

At 4 hrs, β-hematin induced LT-α mRNA at levels slightly above baseline (Figure 13). LT-α transcripts increased gradually over time, peaking in 7 of 9 donors at 72 hrs. Peak mRNA expression was measured at levels > 2 to 8 times those observed in unstimulated cells. The difference in means between LT-α mRNA expression under stimulated versus baseline conditions was statistically significant at all time points ($P < 0.01$). Relative to fold change at 4 hrs, fold change in LT-α mRNA increased significantly at 48 ($P = 0.007$) and 72 hrs ($P = 0.001$).

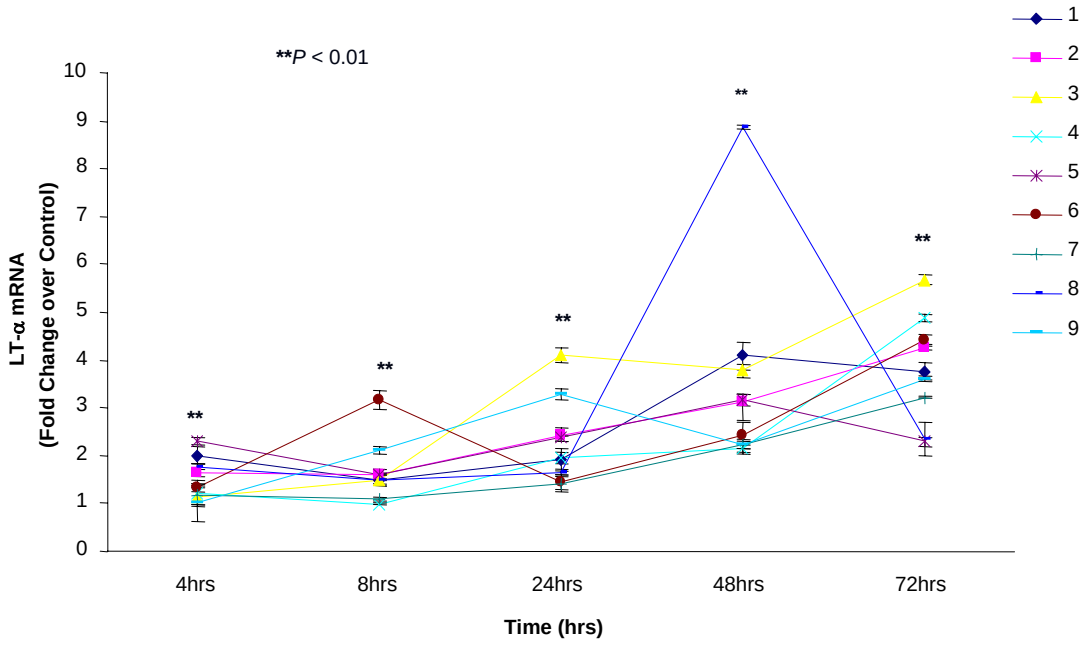

Figure 13. Temporal effect of β-hematin on LT-α transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for LT-α mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.11 *Temporal effect of β-hematin on NOS-2A transcripts:*

Patterns of NOS-2A transcript production varied temporally and in magnitude among donors (Figure 14). In response to β-hematin, NOS-2A mRNA fluctuated throughout the time course, peaking at levels > 3- to 17-fold over baseline. Peak transcript production was divided among the majority of subjects between 24 and 48 hrs. The difference in means between transcript production under stimulated versus control conditions was statistically significant at 24, 48, and 72 hrs post-stimulation. Fold change in NOS-2A mRNA expression increased significantly between 4 and 72 hrs ($P = 0.006$).

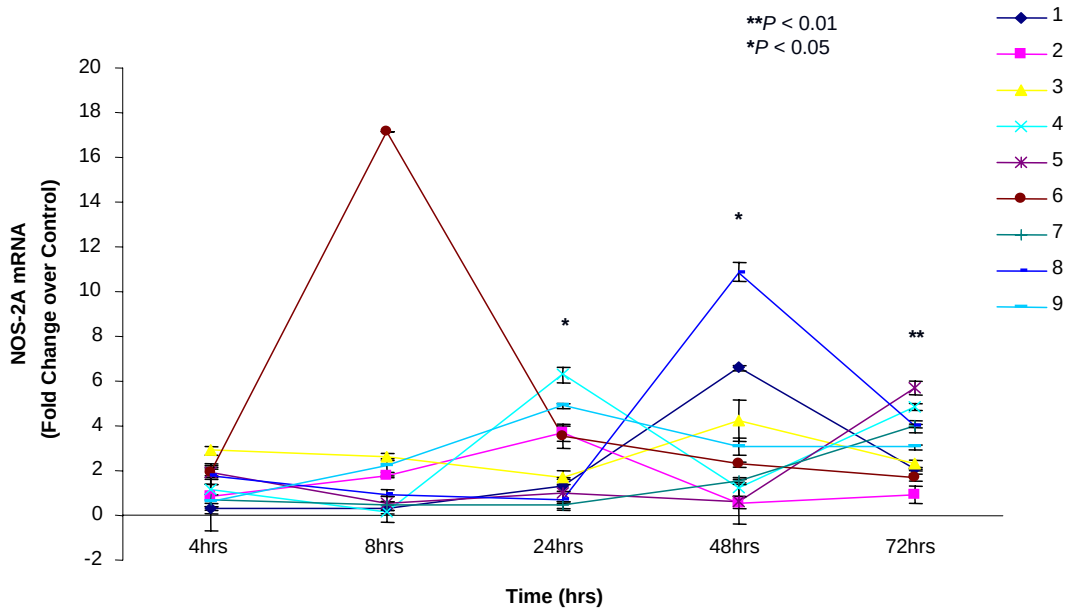

Figure 14. Temporal effect of β-hematin on NOS-2A transcripts. PBMC were isolated from venous blood (70 mL) obtained from nine healthy, malaria-nave donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for NOS-2A mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired *t*-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.12 *Temporal effect of β-hematin on COX-1 transcripts:*

In the presence of β-hematin, COX-1 transcripts were produced slightly above or below baseline levels throughout the 72 hr-time course (Figure 15). Peak transcript production was detected in 6 of 9 subjects at 4 hrs post-stimulation, at which time fold change over control conditions was statistically significant ($P < 0.01$). With the exception of one donor, fold change in COX-1 mRNA did not exceed 2 times that observed in unstimulated cells.

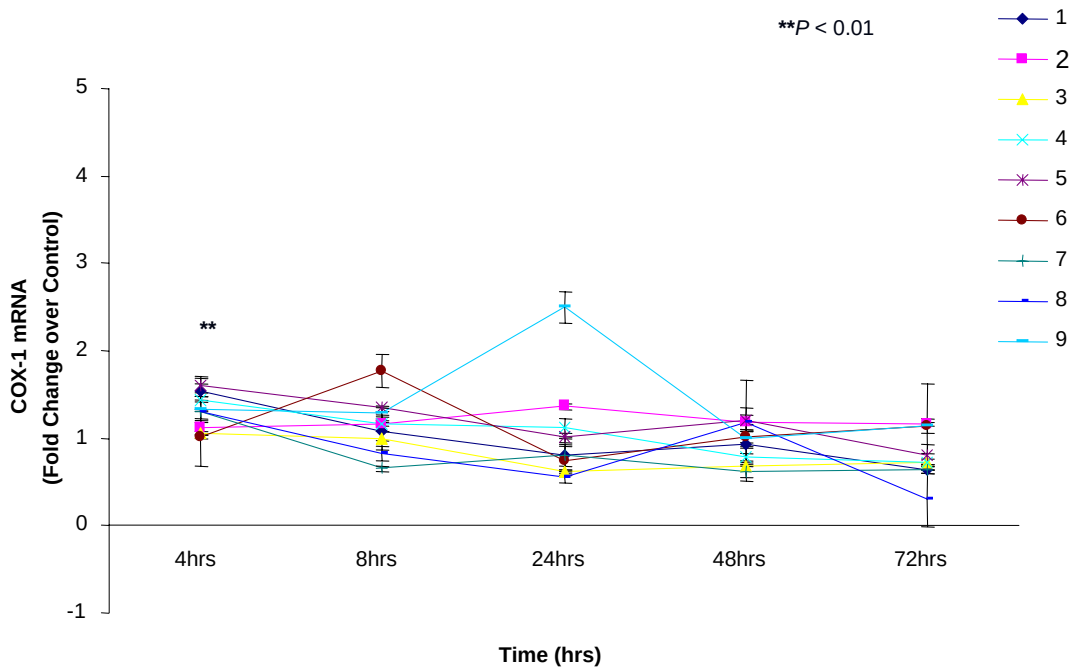

Figure 15. Temporal effect of β-hematin on COX-1 transcripts. PBMC were isolated from venous blood (70 mL) obtained from three healthy, malaria-naïve donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for COX-1 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired t-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.2.13 *Temporal effect of β-hematin on COX-2 transcripts:*

COX-2 mRNA expression was detectable at 4 hrs post-stimulation, progressively increasing at 8 and 24 hrs (Figure 16). Transcript production peaked in the majority of subjects at 24 hrs, reaching levels from > 17- to 180-fold over those observed under unstimulated conditions. β-hematin induced a progressive decrease in COX-2 transcripts at 48 and 72 hrs in these individuals. The difference in means between COX-2 mRNA expression under stimulated versus baseline conditions was statistically significant at all time points. Relative to the 4-hr time point, fold change in COX-2 transcript production was significantly elevated at 8 ($P < 0.0001$) and 48 hrs ($P = 0.003$).

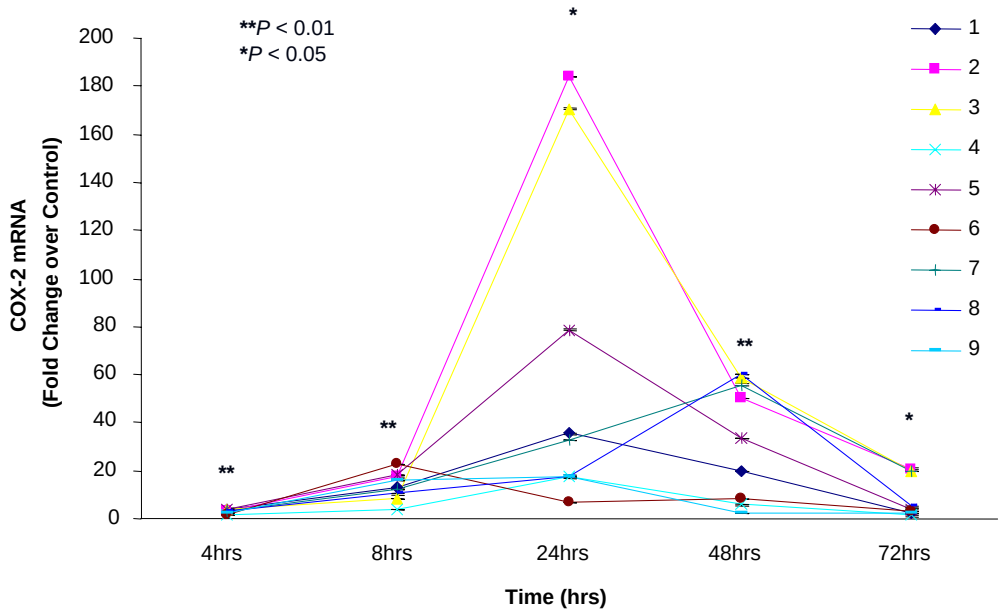

Figure 16. Temporal effect of β-hematin on COX-2 transcripts. PBMC were isolated from venous blood (70 mL) obtained from three healthy, malaria-naïve donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for COX-2 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired t-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.3 *Anti-inflammatory mediator production*

3.3.3.1 *Temporal effect of β-hematin on IL-4 transcripts:*

β-hematin induced patterns of IL-4 mRNA expression that varied markedly among donors (Figure 17). At 4 hrs, IL-4 transcripts were detectable at levels near baseline. IL-4 mRNA levels began to peak at 8 hrs post-stimulation and fluctuated over time. β-hematin produced a 3- to 26-fold increase in IL-4 mRNA at 72 hrs, at which time the majority of donors exhibited peak transcript production. IL-4 fold change over control was statistically significant at 4, 48, and 72 hrs post-stimulation.

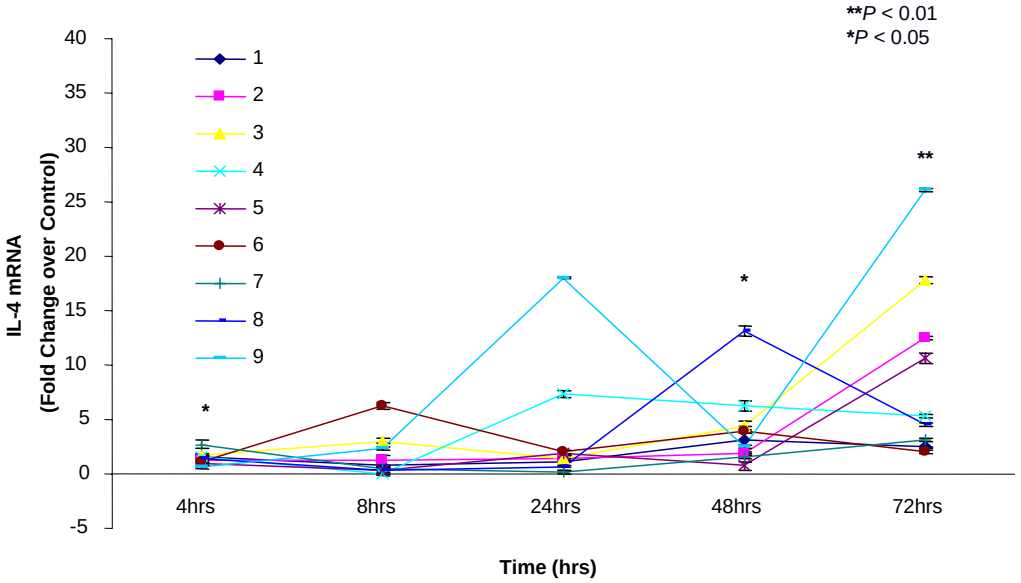

Figure 17. Temporal effect of β-hematin on IL-4 transcripts. PBMC were isolated from venous blood (70 mL) obtained from three healthy, malaria-naïve donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IL-4 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired t-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.3.2 *Temporal effect of β-hematin on IL-10 transcripts:*

β-hematin-induced IL-10 mRNA was detectable at 4 hrs post-stimulation in most subjects (Figure 18). 5 of 9 donors exhibited a decrease in IL-10 mRNA expression at 8hrs, while the remainder showed an increase in transcript production. Peak IL-10 transcript production occurred at 24 hrs, at levels ranging from near baseline to > 13-fold over unstimulated cells. IL-10 mRNA synthesis decreased at 48 hrs in the majority of donors, becoming suppressed by 72 hrs. Fold change in IL-10 mRNA expression was statistically significant at 4, 24, and 48 hrs ($P < 0.05$).

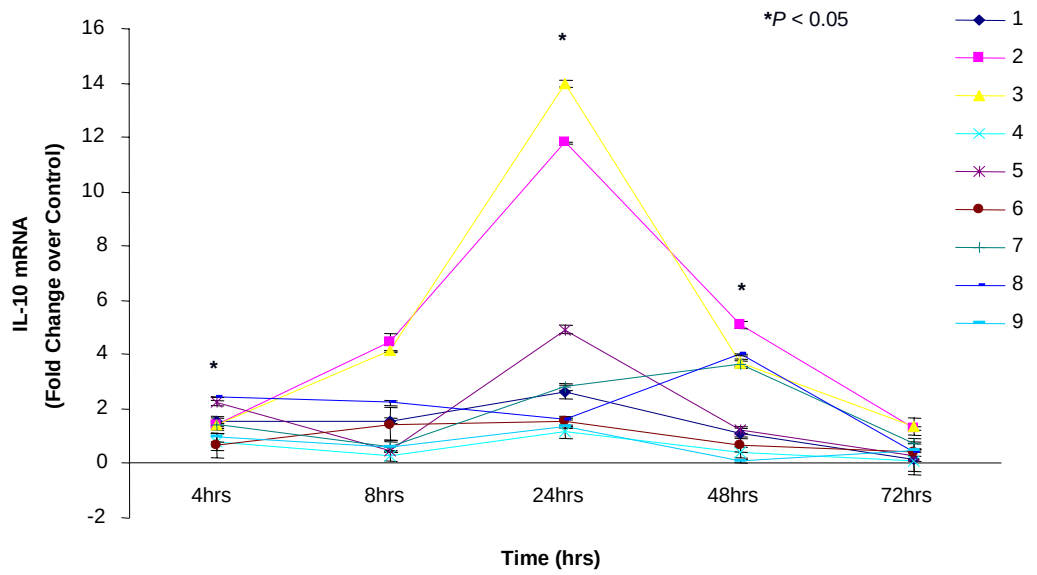

Figure 18. Temporal effect of β-hematin on IL-10 transcripts. PBMC were isolated from venous blood (70 mL) obtained from three healthy, malaria-naïve donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for IL-10 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired t-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

3.3.3.3 *Temporal effect of β-hematin on TGF-β1 transcripts:*

In the presence of β-hematin, TGF-β1 transcript production fluctuated near baseline levels at 4, 8, and 24 hrs in the majority of malaria-naïve donors (Figure 19). Peak TGF-β1 transcript production occurred at 72 hrs in most subjects (3), but was equally distributed between the 8-, 24-, and 48-hr time points among the remaining 6 donors. TGF-β1 mRNA expression peaked at levels approximately 2 to 4 times greater than those detected in unstimulated cells. The difference in means between TGF-β1 transcript production under stimulated versus baseline conditions was statistically significant at all time points. Overall, fold change in TGF-β1 mRNA increased significantly between 4 and 72 hrs ($P = 0.004$).

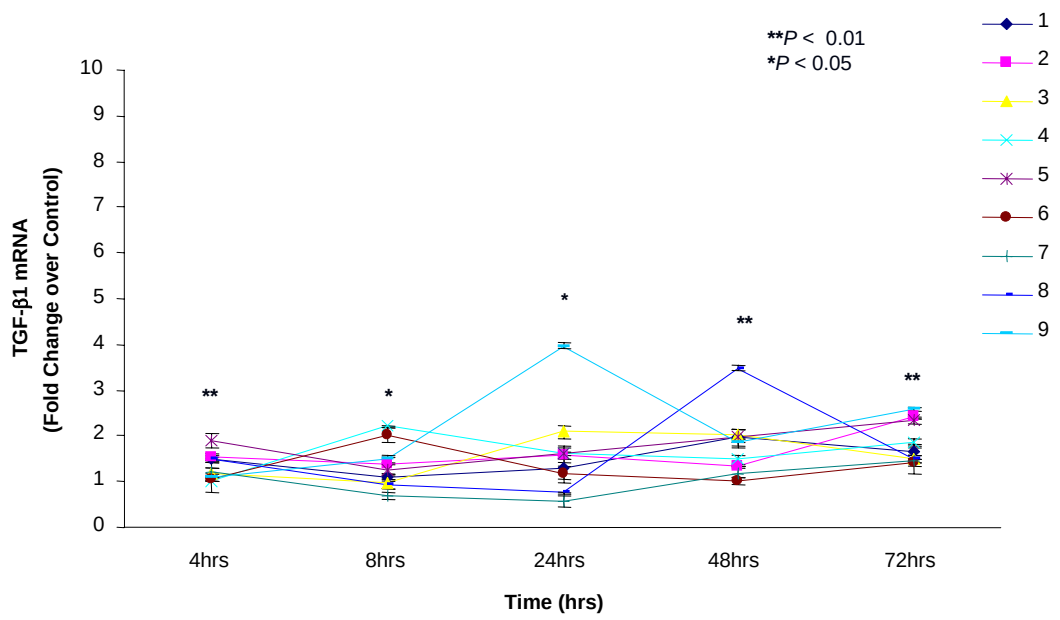

Figure 19. Temporal effect of β-hematin on TGF-β1 transcripts. PBMC were isolated from venous blood (70 mL) obtained from three healthy, malaria-naïve donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for TGF-β1 mRNA quantification by real-time RT-PCR. Results are expressed as fold change relative to unstimulated (baseline) conditions. Values shown are the means ± SEM of results from duplicate samples at each time point. Statistical significance was determined by paired t-tests. * denotes statistical significance of stimulated versus baseline conditions at $P < 0.05$. ** denotes statistical significance of stimulated versus baseline conditions at $P < 0.01$.

Overall, real time RT-PCR analyses revealed an early and predominantly pro-inflammatory response to β-hematin that varied in magnitude among malaria-naïve individuals. Temporal patterns of β-hematin-induced transcript production were similar among individuals for IL-1β, IL-6, IL-12p40, IL-18, TNF-α, LT-α, COX-1, COX-2, IL-4, and IL-10. IL-2, IL-12p35, IFN-α, IFN-γ, NOS-2A, and TGF-β1 mRNA expression were more variable among donors. Although IL-10 was also induced, it did not suppress pro-inflammatory mediator production within 72 hrs of β-hematin stimulation. TGF-β1 was expressed at near baseline levels in the majority of individuals and did not appear to serve an immunoregulatory role. In addition, increases in IL-4 mRNA expression at 72 hrs post-stimulation may indicate a switch from a pro-inflammatory (Th1) to an anti-inflammatory (Th2) immune response.

4.0 DISCUSSION

4.1 Effect of hemozoin on hematological outcomes in infants and children with malarial anemia

One aim of this study was to examine the *in vivo* effects of hemozoin on hematological indices, as a measure of the physiological dysregulatory processes that lead to malarial anemia. Analyses of complete blood counts obtained from healthy children, children with uncomplicated malaria, and children with mild, moderate, or severe malarial anemia, revealed that several factors were significantly associated with disease status (Table 1). Age was determined to be significantly different among clinical groups, and those subjects in the SMA group were the youngest at enrollment. Mean axillary temperature was significantly associated with clinical category, although further analyses must be performed to determine whether temperature is predictive of disease severity. However, previous studies in African children residing in areas of holoendemic malaria transmission have demonstrated that while fever is associated with parasitemia, it is not correlated with MA (Ong'echa *et al.*, 2006; Smith *et al.*, 1995).

Results of these analyses indicated that neither parasitemia nor the prevalence of high-density parasitemia (HDP) were significantly different among clinical groups. This finding is in agreement with previous studies showing that concurrent parasitemia levels are not predictive of malaria disease severity in this region (McElroy *et al.*, 2000; Ong'echa *et al.*, 2006). Multiple group comparisons of leukocyte, erythrocyte, and platelet indices revealed that all of these factors differed significantly between HC, UM, MlMA, MdMA, and SMA groups. Taken together, these findings are in support of the hypothesis that ingestion of malarial pigment by mononuclear phagocytes creates pathological changes in hematological parameters that contribute to malaria disease severity.

Since MA is the most common clinical manifestation of hemozoin-induced immune dysregulation among infants and young children in areas of holoendemic malaria transmission (such as western Kenya), we sought to determine the relationship between prevalence of pigment-containing monocytes in the blood of parasitemic children and severity of anemia. Following stratification of study participants according to percent of pigment-containing monocytes (0% PCM, ≤ 10% PCM, and > 10% PCM), hematological parameters analyzed in Table 1 were compared across pigment categories. Age was not significantly different between pigment categories, but mean axillary temperature was still significantly different between groups. Although an association between parasitemia and clinical category or between HDP and clinical category could not be found in previous analyses (Table 1), both mean parasitemia and prevalence of HDP were significantly associated with % PCM categories (Table 2). Interestingly, children in the > 10% PCM group had the highest mean parasitemia, whereas HDP was most prevalent in the ≤ 10% PCM group. In addition to platelet indices, all leukocyte indices except granulocyte count were significantly different between pigment categories. As when compared across clinical categories, most erythrocytic factors differed significantly between monocyte pigment categories. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were not, however, significantly different between pigment groups. Consistent with the hypothesis that pigment-induced phagocyte incapacitation leads to MA, mean RBC counts, hematocrit, and Hb levels were lowest in the > 10% PCM category, and proportion of subjects with SMA were highest. Taken together, these results suggest that the dysregulatory processes involved in the development of MA are mediated, at least in part, by the ingestion of hemozoin by peripheral blood monocytes.

4.2 Effect of hemozoin on inflammatory mediator production in infants and children with malarial anemia

The second aim of this study was to determine whether innate inflammatory mediator production in infants and children with varying degrees of malarial anemia is related to disease severity and/or to percent of pigment-containing monocytes. Specifically, differences between the plasma cytokine levels of children with

uncomplicated malaria (UM) and children with severe malarial anemia (SMA) were examined to elucidate the potential involvement of these cytokines in the development of uncomplicated versus severe disease.

Results detailed in Table 3 revealed that plasma levels of IFN-α, IL-4, IL-6, IL-10, and IL-12p40/p70 were significantly different among HC, UM, MlMA, MdMA, and SMA clinical categories. IFN-α has been shown to induce NOS-2 and NO production, which are essential for an effective pro-inflammatory response to malaria parasites (Sharara *et al.*, 1997). Likewise, IL-12 production is inversely associated with risk of severe malaria and positively associated with hemoglobin levels (implying protection from MA) (Dodoo *et al.*, 2002; Luty *et al.*, 2000; Perkins *et al.*, 2000). Equally important is the role of IL-10 in regulating the production of pro-inflammatory cytokines that contribute to malarial pathogenesis. Therefore, it is not surprising that plasma IFN-α, IL-10, and IL-12p40/p70 levels would differ among individuals with varying degrees of disease severity. Due to its function in fever induction and acute phase protein secretion, IL-6 might also be expected to play a central role in the early pro-inflammatory response that determines protection against malaria, and therefore, to differ among disease categories. Furthermore, since IL-4 enhances IL-12 production by amplifying transcription of the genes encoding the IL-12p35 and p40 subunits (D'Andrea *et al.,* 1995; Marshall *et al.,* 1997), it follows that plasma levels of IL-4 may differ between HC, UM, MlMA, MdMA, and SMA groups if IL-12 levels were determined to be statistically significant between these clinical groups. Differences in IL-4 levels may also reflect a shift in the balance between Th1 and Th2 cells that has been shown to exist among malaria-exposed individuals in *P. falciparum*-endemic areas (Elghazi *et al.*, 1997). In these individuals, increased ratios of IL-4/IFN-γ-producing cells facilitate production of elevated plasma IgE, which is thought to be required for complete elimination of parasitemia (Elghazi *et al.*, 1997; Stevenson and Riley, 2004).

However, multiple group comparisons indicated that plasma levels of IFN-γ, TNF-α, IL-1β, and IL-2 were not significantly different between clinical categories. It is unexpected that plasma TNF-α, IL-1β and IFN-γ levels did not differ significantly between clinical categories, since these cytokines mediate both protective and pathological immune responses in malaria. For example, while TNF-α is required for an

effective pro-inflammatory response, high levels of TNF promote development of severe pathology and MA (Clark and Chaudri, 1988; Grau *et al.*, 1989; Keller *et al.*, 2006; Kwiatkowski *et al.*, 1990). Overproduction of IL-1β, which produces the same clinical symptoms as TNF-α, has also been linked to malaria disease severity (Kwiatkowski *et al.*, 1990; Walsh *et al.,* 1992). Furthermore, due to its ability to activate macrophages and increase NO production, IFN-γ plays a central role in the early pro-inflammatory response to malaria infection. As such, the ability to mount a rapid IFN-γ response has been linked to a more favorable clinical outcome in most animal models of malaria and in humans (Deloron *et al.*, 1991; Dodoo *et al.*, 2002; Jacobs *et al.*, 1996; Luty *et al.*, 1999; Sedegah *et al.*, 1994). IL-2, which is known to induce TNF-α, might also be expected to differ between HC, UM, MlMA, MdMA, and SMA clinical categories. However, data presented here suggest that IL-2 may not be as essential in the early pro-inflammatory response.

Pairwise comparisons of plasma IFN-α, IL-4, IL-6, IL-10, and IL-12p40/p70 levels in the UM and SMA groups were performed, since differences in levels of these cytokines were found to be statistically significant between the five clinical groups. Statistical analyses demonstrated that levels of none of these inflammatory mediators were significantly different between children with uncomplicated malaria and children with severe malarial anemia (Table 3). This finding suggests that although plasma IFN-α, IL-4, IL-6, IL-10, and IL-12p40/p70 differ significantly between healthy children and those with varying degrees of MA, the development of uncomplicated versus severe disease is not necessarily mediated by these cytokines. Further analyses are required to clarify the specific involvement of these cytokines in the progression to MA.

Stratification of parasitemic study participants (UM, MlMA, MdMA, and SMA groups; n = 155) according to percent of pigment-containing monocytes (%PCM) was performed to determine whether plasma cytokine levels were altered by increased loading of monocytes with hemozoin (Table 4). The presence of phagocyte pigment has been used as a prognostic indicator of severe malaria infection and an alternative for diagnosis (Phu *et al.*, 1994). In a study by Day *et al.*, 88% of patients treated for severe falciparum malaria exhibited symptoms of the disease that persisted after parasite clearance but before the clearance of intraphagocytic pigment (Day *et al.*, 1996). Since the immune

dysregulation caused by monocytic ingestion of hemozoin is cytokine-mediated, one would expect the degree of cytokine imbalance to be positively correlated with the number of hemozoin-containing monocytes. Accordingly, one might expect that plasma cytokine levels, which modulate such clinical symptoms of malaria as fever, rigors, nausea, headache, and fatigue, would vary according to monocyte pigment content.

Multiple group comparisons revealed that statistical differences in plasma TNF-α levels between 0% PCM, ≤ 10% PCM, and > 10% PCM categories were borderline significant (P = .051), indicating that monocytic acquisition of hemozoin may impact production of this inflammatory mediator. Children in the ≤ 10% PCM category exhibited the highest levels (pg/mL) of TNF-α, whereas plasma TNF-α was lowest among children in the 0% PCM group. TNF-α production was suppressed in the > 10% PCM group (47.57 ± 5.28) relative to children with ≤ 10% PCM (61.42 ± 13.06), although it was elevated relative to the 0% PCM group (39.92 ± 6.21). This trend suggests that elevated production of TNF-α is required in early infection to limit parasitemia but may become impaired as blood content of pigment-containing monocytes surpasses 10%. As such, TNF-α production may be suppressed by increased loading of monocytes with hemozoin.

Plasma levels of IFN-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12p40/p70 were not significantly different between 0% PCM, ≤ 10% PCM, and > 10% PCM categories. Results presented here suggest that plasma levels of these cytokines are not a function of the blood content of hemozoin-containing monocytes in parasitemic individuals. However, it is important to note that murine and human dendritic cells (DCs) can be activated by hemozoin (as well as synthetic hemozoin) through TLR-9 (toll-like receptor 9) and are capable of producing cytokines that influence both innate and adaptive immune responses (Pichyangkul *et al.*, 2004; Janeway, 2005). DCs produce TNF-α, IL-1, IL-12, IL-6, IL-18, IFN-γ, and IFN-α (Janeway, 2005), but IFN-α is the principal cytokine produced by plasmacytoid DCs in response to TLR-9 ligand (Hemmi *et al.*, 2000). Furthermore, whereas hemozoin has been shown to enhance human DC maturation (Coban *et al.*, 2002), *P. falciparum*-infected erythrocytes inhibit the maturation of DCs (Urban *et al.*, 1999). As such, the potential contribution of DCs to cytokine dysregulation in malaria warrants further investigation.

4.3 Temporal effect of β-hematin on cytokine and effector molecule transcript production in cultured human peripheral blood mononuclear cells

The final part of this study focused on determining the temporal kinetics of *in vitro* cytokine and effector molecule mRNA expression in severe *P. falciparum* malaria. PBMC isolated from healthy, malaria-naïve donors were stimulated with a physiologically relevant dose of β-hematin in order to investigate its impact on *de novo* inflammatory mediator production. Figure 20 depicts the temporal profile of peak cytokine mRNA production among nine donors for those transcripts detected by real time RT-PCR.

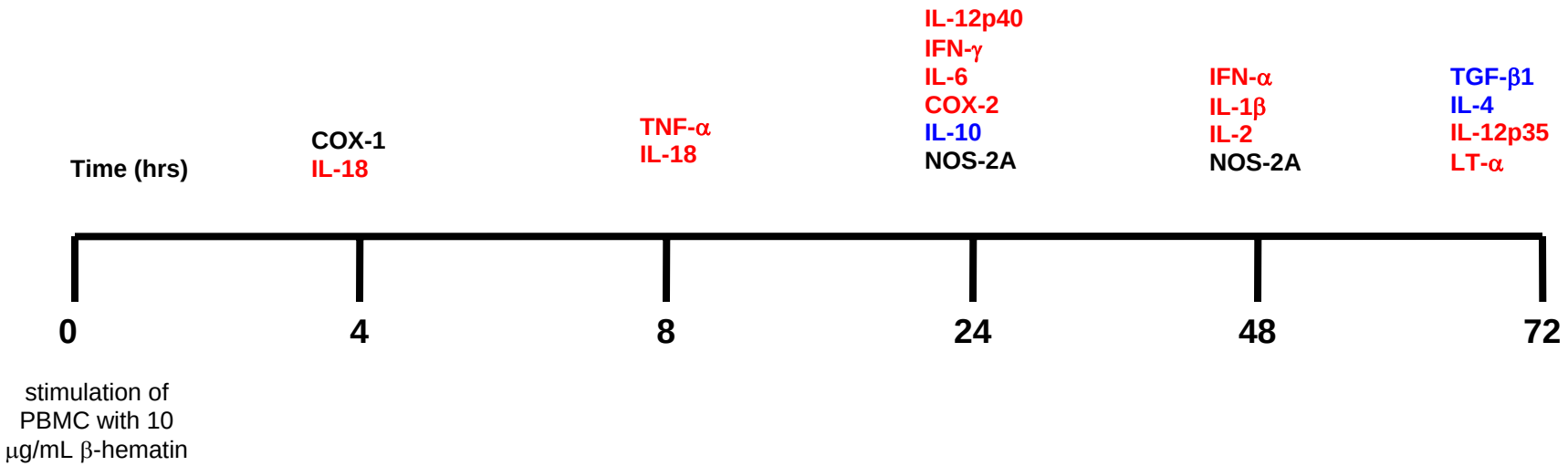

Figure 20. Temporal model of peak β-hematin-induced cytokine and effector molecule transcript production. PBMC were isolated from nine healthy, malaria-naïve donors. Cultured PBMC (10^6 cells/mL) were stimulated with media alone or with β-hematin (10 μg/mL). RNA was isolated from harvested cells at 4, 8, 24, 48, and 72 hrs for mRNA quantification by real-time RT-PCR. Peak mRNA production of cytokines/effector molecules listed occurred at the time points indicated. Peak IL-18 transcript production was divided among donors between 4 and 8 hrs. Peak NOS-2A mRNA expression was divided between 24 and 48 hrs. Pro-inflammatory cytokines are illustrated in red type, and anti-inflammatory cytokines are illustrated in blue type.

4.3.1 *Effect of β-hematin on IL-1β transcripts:*

β-hematin induced patterns of IL-1β transcript production that were temporally similar but differed markedly in magnitude among donors (Figure 4). Peak IL-1β mRNA expression occurred at 48 hrs in the majority of subjects, reaching levels > 300- to 2400-fold over those measured under unstimulated conditions. Like TNF-α, IL-1 (produced in α and β forms) is a pro-inflammatory cytokine that is released by murine macrophages and by human monocytes, macrophages, and PBMC in response to malaria infection, and has been shown to induce NO generation *in vivo* (Pichyangkul *et al.*, 1994; Rockett *et al.*, 1992; Schofield *et al.*, 1993; Wahlgren *et al.*, 1995; Wood and Clark, 1984). Formerly known as "endogenous pyrogen," IL-1 (in its recombinant form) shares with recombinant TNF the ability to generate fever, rigors, nausea, headache, and fatigue in human subjects (Clark *et al.*, 2004; Walsh *et al.*, 1992). Furthermore, high serum levels of IL-1β have been associated with the severity of *P. falciparum* malaria in West Africa (Kwiatkowski *et al.*, 1990). As such, results presented here are not surprising, since IL-1β would play an equally important role in the early pro-inflammatory immune response to malaria infection. The magnitude of the observed *in vitro* responses to a high dose of β-hematin may mimic the high levels of IL-1β produced in severe falciparum malaria.

4.3.2 *Effect of β-hematin on IL-2 transcripts:*

IL-2 is synthesized by activated T cells and acts in an autocrine fashion to drive their proliferation and differentiation into armed effector T cells (Janeway, 2005). Th1 cells secrete IL-2 to induce the proliferation of CD8 T cells. IL-2 also acts synergistically with IL-12 to induce the proliferation of lymphokine-activated killer (LAK) cells and the production of IFN-γ (Chan *et al.*, 1991, 1992). However, the IL-2-induced proliferation of NK and T cells can also be inhibited by IL-12 (Perussia *et al.*, 1992). In murine malaria, macrophages, B cells, and bone-marrow-derived dendritic cells (DCs) are capable of processing and presenting malaria antigens to T cells and of promoting T cell IFN-γ production, but these antigen presenting cells (APCs) have been shown to inhibit the production of IL-2 by T cells (Luyendyk *et al.*, 2002; Quin *et al.*, 2001).

Furthermore, inhibition of IL-2 production may account for the inadequate responses to unrelated antigens that have been observed during acute malaria (Greenwood *et al.*, 1972; McGregor and Barr, 1962).

Results presented here indicated a peak in IL-2 mRNA production at 24 to 48 hrs post-stimulation with β-hematin (Figure 5). IL-2 transcripts increased approximately 2- to 18-fold over control conditions at these time points. This pattern may represent the induction of T cell proliferation in response to β-hematin, which would be characteristic of the early Th1 type, cell-mediated response that is required for protective immunity to malaria during natural infection.

4.3.3 *Effect of β-hematin on IL-6 transcripts:*

β-hematin-induced IL-6 mRNA expression patterns were temporally similar among donors but differed in magnitude (Figure 6). IL-6 transcript production peaked at 24 hrs, showing a 12- to 1300-fold increase over unstimulated conditions. Levels of IL-6 mRNA detected were similar to levels of IL-1β induced by β-hematin (although IL-1β transcript production peaked at 48 hrs post-stimulation). As IL-6 is also an endogenous pyrogen and can be induced by TNF-α, LT-α, and IL-1β (Clark *et al.*, 1992; Janeway, 2005) it may be expected that high levels of IL-6 mRNA would be produced during the early phase of the immune response to β-hematin. The increase in TNF-α and IL-1β transcript production that occurred in all donors prior to and at 24 hrs could have contributed to the up-regulation of IL-6 mRNA expression observed at 24 hrs.

4.3.4 *Effect of β-hematin on IL-12p35 and IL-12p40 transcripts:*

As mentioned previously, protective immunity against malaria requires an early Th1-type, pro-inflammatory response characterized by enhanced production of IL-12 (Crutcher *et al.*, 1995; Mosmann and Coffman, 1989; Trinchieri, 1993), whereas IL-12 suppression increases susceptibility to severe malaria (Luty *et al.*, 2000; Perkins *et al.*, 2000). The protective effects of IL-12 have been supported by data obtained from prospective epidemiological studies in an area of holoendemic *P. falciparum* transmission (Dodoo *et al.*, 2002). These studies demonstrated that IL-12 production is inversely associated with risk of clinical infection and positively associated with

hemoglobin concentration and TNF-α and IFN-γ production (Dodoo *et al.*, 2002). This positive association between IL-12 production and hemoglobin levels indicates a possible role for IL-12 in protection from malarial anemia as well, a finding that has also been supported by rodent studies (Dodoo *et al.*, 2002; Mohan and Stevenson, 1998).

Biologically active IL-12 (IL-12p70) is a heterodimeric cytokine comprised of IL-12p35 and IL-12p40 subunits that are encoded by separate genes (Kobayashi *et al.*, 1989; Trinchieri, 1998). IL-12p35 mRNA is present in numerous cell types, whereas IL-12p40 mRNA is restricted to cells that produce the bioactive heterodimer (D'Andrea *et al.*, 1992; Trinchieri, 2003). Transcription of both genes is induced following cell activation by inflammatory stimuli, though fewer p35 transcripts are synthesized, even in activated inflammatory cells (Ma and Trinchieri, 2001; Snidjers *et al.*, 1996). Although IL-12p40 is produced in excess over IL-12p70, IL-12p35 is secreted only in association with the p40 subunit (Kobayashi *et al.*, 1989; Stern *et al.*, 1990; Trinchieri, 2003; Wysocka *et al.*, 1995). IFN-γ and the anti-inflammatory cytokines IL-4 and IL-13 augment IL-12 production in various cells through enhancement of IL-12p35 and IL-12p40 gene transcription (D'Andrea *et al.*, 1995; Hayes *et al.*, 1998; Ma *et al.*, 1996). However, unlike IFN-γ, IL-4 and IL-13 act by initially inhibiting transcription of the p40 gene in activated cells, and later, by strongly enhancing it (D'Andrea *et al.*, 1995). IL-10 is the most effective suppressor of IL-12p40 gene transcription (Aste-Amezaga *et al.*, 1998).

Results presented here indicate that individual IL-12p35 transcripts fluctuated throughout the time course, but overall, showed a gradual increase to levels 2- to 9-fold greater than those observed under unstimulated conditions by 72 hrs (Figure 7). An approximate 5- to 850-fold increase in IL-12p40 mRNA expression was detected in the majority of malaria-naïve donors at 24 hrs, after which β-hematin-induced IL-12p40 transcripts dramatically decreased (Figure 8). This is consistent with previous findings that IL-12p40 mRNA is restricted to cells that produce biologically active, heterodimeric IL-12 in response to an inflammatory event and not present in cultured PBMC from healthy donors (D'Andrea *et al.*, 1992; Keller *et al.*, 2005; Ma and Trinchieri, 2001; Trinchieri, 2003), and that physiological conditions simulating severe malaria (such as exposure to a high dose of β-hematin) further down-regulate IL-12 production (Luty *et al.*, 2000). Taken together, these results suggest an early increase in β-hematin-induced

IL-12p40 expression that may have been subsequently suppressed/downregulated by IL-10.

4.3.5 *Effect of β-hematin on IL-18 transcripts:*

Structurally related to IL-1β, IL-18 is a pro-inflammatory cytokine that is produced by the same cell populations and under the same activation conditions as IL-12 (Bazan *et al.*, 1996; Dinarello, 1999). IL-18 acts synergistically with IL-12 to up-regulate IFN-γ production by macrophages, T cells and NK cells, and to enhance the cytotoxic functions of NK and T cells (Hunter *et al.*, 1997; Micallef *et al.*, 1996; Robinson *et al.*, 1997). Alone, IL-18 is ineffective at inducing IFN-γ, does not induce a Th1 type immune response, and may promote Th2 responses (Barbulescu *et al.*, 1998; Nakanishi *et al.*, 2001; Walker *et al.*, 2001). As in the case of IL-12, low levels of IL-18 have been associated with severe malarial pathology and anemia (Chaisavaneeyakorn *et al.*, 2003; Malaguarnera *et al.*, 2002a, 2002b).

Results of this study show a temporal pattern of β-hematin-induced IL-18 transcript production similar to that observed with TNF-α (Figure 9). IL-18 mRNA peaked at 4 to 8 hrs and became suppressed in most donors by 24 hrs. This pattern suggests that IL-18 transcription may have been up-regulated concomitantly with IL-12 mRNA expression (which peaked at 8 and 24 hrs) for the induction of IFN-γ. Consistent with this hypothesis, the majority of subjects exhibited a peak in IFN-γ transcript production at 24 to 72 hrs post-stimulation.

4.3.6 *Effect of β-hematin on IFN-α transcripts:*

In addition to its anti-viral properties, IFN-α has been shown to induce increased expression of human monocyte NOS activity, NOS-2 antigen and mRNA content, and NO production, both *in vitro* and *in vivo* (Sharara *et al.*, 1997). These findings have important implications in malaria, since NOS activity and NO production serve protective roles in infection by facilitating parasite clearance (Gyan *et al.*, 1994; Kremsner *et al.*, 1992; Mellouk *et al.*, 1994; Nussler *et al.*, 1991; Rockett *et al.*, 1991; Stevenson *et al.*, 1995). Furthermore, the ability to produce NO is a key factor in the development of mild

versus severe malaria (Anstey *et al.*, 1996; Kremsner *et al.*, 1996; Perkins *et al.*, 1999). Much like IL-12, IFN-α induces IFN-γ production and Th1 responses, yet is capable of suppressing IL-12 (Cousens *et al.*, 1999). IFN-α can also activate NK cells, which have been shown to interact with parasitized erythrocytes and to play a role in the innate immune response to blood-stage parasites (Artavanis-Tsakonas *et al.*, 2002, 2003b).

Findings of this study indicate peaks in IFN-α mRNA expression throughout the 72-hr time course, but occurring at 48 hrs post-stimulation in the majority of donors (Figure 10). β-hematin-induced IFN-α transcripts fluctuated above and below baseline levels, peaking at levels 2- to 13-fold greater than those detected under unstimulated conditions. Donor 9 showed a > 120-fold increase in IFN-α mRNA. Elevated IFN-α transcript production (along with that of other pro-inflammatory cytokines) may have contributed to corresponding peaks in NOS-2A mRNA exhibited by donors 1, 5, 6, 8, and 9. These results suggest that individuals differ in their abilities to produce IFN-α, and that perhaps the role of IFN-α in protective immunity against malaria may not be as essential as that of other cytokines. IFN-α is typically induced by viral infection or by the recognition of double-stranded RNA (characteristic of many viruses) (Janeway, 2005), and since the molecular mechanisms that regulate IFN-α production in malaria are unknown at present, the current findings warrant further investigation.

4.3.7 *Effect of β-hematin on IFN-γ transcripts:*

Production of IFN-γ by innate and adaptive immune cells plays a critical role in the early pro-inflammatory response to malaria infection. IFN-γ synergizes with TNF-α to optimize NO production, which facilitates parasite killing in both mice and humans (Jacobs *et al.*, 1996; Kremsner *et al.*, 1995, 1996; Rockett *et al.*, 1991). In humans, IFN-γ production is correlated with protection from clinical attacks of malaria and with resistance to reinfection with *P. falciparum* (Deloron *et al.*, 1991; Dodoo *et al.*, 2002; Luty *et al.*, 1999). Recent studies have shown that NK cells are the first producers of IFN-γ following *in vitro* exposure of malaria-naïve PBMC to *P. falciparum*-infected erythrocytes (Artavanis-Tsakonas and Riley, 2002). These NK cell-mediated IFN-γ responses are IL-12-dependent, partially IL-18-dependent, and do not occur in all donors

(Artavanis-Tsakonas and Riley, 2002). Despite the use of β-hematin (instead of live parasitized RBCs) to stimulate PBMC, results presented here support such heterogeneity in the magnitude of IFN-γ responses between donors (Figure 11). β-hematin-induced IFN-γ mRNA expression fluctuated throughout the time course and peaked at levels approximately 4- to 21-fold over baseline conditions at 24 to 48 hrs in the majority of individuals. However, peak transcript production was observed at each of the five time points. This variation in timing and magnitude of IFN-γ responses may reflect differences in each donor's ability to mount a prompt pro-inflammatory response to simulated infection. In addition, peak IFN-γ mRNA production corresponded to peak IL-12p40 expression in 5 of 9 subjects (donors 3, 4, 6, 7, and 9), suggesting that increased IL-12p40 production at these time points augmented IFN-γ gene expression.

4.3.8 *Effect of β-hematin on TNF-α transcripts:*

Patterns of TNF-α transcript production were consistent among donors, showing a peak in TNF-α mRNA at 4 to 8 hrs post-stimulation that diminished by 72 hrs (Figure 12). It is possible that peaks in IL-10 transcript production at 24 hrs (Figure 18) may have down-regulated TNF-α gene expression between 24 and 72 hrs. Consistent with experiments in cultured monocytes (Pichyangkul *et al.*, 1994; Keller *et al.*, 2006), these results demonstrate that malarial pigment augments TNF-α production. This ability to mount a rapid pro-inflammatory response is required for protection against malaria in both mice and humans, since TNF-α acts synergistically with IFN-γ to promote phagocytosis of pRBCs and NO release by macrophages (Doolan and Good, 1999; Kremsner *et al.*, 1995, 1996; Dodoo *et al.*, 2002). Consequently, TNF-α serves a protective role by limiting parasitemia, but has the potential to produce severe pathology and anemia at high levels that are characteristic of acute *P. falciparum* malaria (Clark and Chaudri, 1988; Grau *et al.*, 1989; Keller *et al.*, 2006; Kwiatkowski *et al.*, 1990).

4.3.9 *Effect of β-hematin on LT-α transcripts:*

Lymphotoxin-α (LT-α) is another pro-inflammatory cytokine that plays an important role in both murine and human models of malaria and shares several functions

in common with TNF. Previously labeled TNF-β, LT (which is divided into α and β categories) has the ability to up-regulate TNF expression and shares a receptor with TNF (Owen-Schaub *et al.*, 1989; Schoenfeld *et al.*, 1991). Like TNF, LT increases cellular adhesion to the endothelium through up-regulation of ICAM-1 and VCAM-1 (intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, respectively) and in its recombinant form, induces high levels of IL-6 and hypoglycemia in malaria-primed mice (Clark *et al.*, 1992; Husson *et al.*, 2000). Similar to TNF and IL-1, LT can be induced by the malaria toxin GPI (glycosylphosphatidylinositol) and has the ability to induce NO production *in vivo* (Ferrante *et al.*, 1990; Rockett *et al.*, 1992; Tachado *et al.*, 1996). In addition, up-regulated LT has been identified as the primary mediator of murine cerebral malaria, and a TNF promoter polymorphism that increases LT transcription is associated with increased risk of cerebral malaria in African children (Engwerda *et al.*, 2002; Knight *et al.*, 1999, 2003; McGuire *et al.*, 1994). Results of this study showed an overall increase in LT-α mRNA expression throughout the time course (Figure 13). By 72 hrs, β-hematin produced a > 2- to 8-fold increase in LT-α transcripts, consistent with an increasing pro-inflammatory response. Of interest, expression of LT-α, which is also a product of B lymphocytes, peaked at the same time as expression of IL-4, an activator of B cells.

4.3.10 *Effect of β-hematin on NOS-2A transcripts:*

In response to hemozoin, cells of the innate immune system produce pro-inflammatory cytokines such as IL-12, IFN-γ, and TNF-α, which increase NO production, and anti-inflammatory cytokines such as IL-10 and TGF-β, which down-regulate NO synthesis (Oswald *et al.*, 1994; Vodovotz *et al.*, 1993). NO is formed from arginine via the action of nitric oxide synthase (NOS) (Moncada and Higgs, 1993; Nathan and Xie, 1994). The NOS family of enzymes includes two forms of NOS that are constitutively expressed and activated by high levels of local calcium to maintain biological homeostasis (Clark *et al.*, 2004). Inducible nitric oxide synthase type 2 (NOS-2, or iNOS) is another form of NOS that operates independently of local calcium concentration to generate high levels of NO during an inflammatory event (Clark *et al.*, 2004; Nathan and Xie, 1994).

Several studies support the role of NO as molecular determinant that regulates disease severity in malaria. For example, levels of NO metabolites in plasma and urine and expression of NOS-2 in PBMC were shown to be inversely related to disease severity in *P. falciparum*-infected Tanzanian children (Anstey *et al.*, 1996). In a similar study, plasma levels of NO metabolites in Gabonese adults and children were shown to increase with disease severity and were related to accelerated parasite clearance and clinical cure (Kremsner *et al.*, 1996). Protection against severe malaria in this same population of Gabonese children was later determined to be related to a promoter polymorphism in the NOS-2 gene, which greatly enhanced NO production in response to inflammatory stimuli (Kun *et al.*, 1998, 2001). More recently, elevated NOS-2 activity and NO production have been implicated in the pathogenesis of malarial anemia (Keller *et al.*, 2004b).

Results of this study illustrate inter-individual variation in the ability to generate NO following exposure to β-hematin (Figure 14). Peaks in NOS-2A transcript production were detected at 24 to 48 hours post-stimulation in most donors, at levels 3 to 17 times those observed under unstimulated conditions. However, peaks in NOS-2A mRNA synthesis also occurred as early as 8 hrs and as late as 72 hrs, which indicates that genetic variation among individuals (such as promoter polymorphisms) may account for their ability to mount a more rapid inflammatory response. In addition, timing of NOS-2A transcript production may have been influenced by levels of both pro- and anti-inflammatory cytokines being generated in these individuals. For example, elevated levels of IL-12, TNF-α, IFN-α, or IFN-γ mRNA would have augmented NOS-2A expression. Conversely, increased IL-10 transcript production could have down-regulated NOS-2A gene expression through suppression of IL-12 or TNF-α. Likewise, TGF-β1 may have elicited decreases in NOS-2A transcripts via inhibition of TNF-α or IFN-γ mRNA synthesis.

4.3.11 *Effect of β-hematin on COX-1 and COX-2 transcripts:*

Cyclooxygenase-1 (COX-1) and cyclooxegenase-2 (COX-2) are enzymatic mediators of prostaglandin synthesis (Appleton *et al.*, 1996; Vane *et al.*, 1998). Prostaglandins (such as prostaglandin E_2, PGE_2) are effector molecules that regulate macrophage function, cytokine production, fever, vascular permeability, and adhesion

molecule expression (Vane *et al.*, 1998). COX-1, which is constitutively expressed in numerous tissues, is involved in the generation of prostaglandins for homeostatic purposes (Seibert and Masferrer, 1994). COX-2 is inducibly expressed for the generation of high levels of prostaglandins in response to inflammatory stimuli (Seibert and Masferrer, 1994). Previous studies in our lab have demonstrated that reduced plasma levels of PGE_2, reduced PBMC COX-2 gene expression, and increased plasma levels of IL-10 are associated with enhanced severity of *P. falciparum* malaria (Perkins *et al.*, 2001). Although IL-10 can suppress COX-2 gene expression (Niiro *et al.*, 1995), subsequent investigations confirmed that the molecular mechanisms by which hemozoin-induced suppression of COX-2 transcription and PGE_2 production occur are independent of increased IL-10 production (Keller *et al.*, 2004a). Results of the present study indicate a pattern of COX-2 mRNA expression that was temporally similar among malaria-naive donors but varied in magnitude (Figure 16). β-hematin produced a peak in COX-2 transcripts at 24 hrs that ranged in magnitude from > 17- to 180-fold over baseline conditions. Elevated levels of COX-2 transcripts at 24 hrs may have contributed to the decrease in TNF-α and IL-12p40mRNA expression observed among donors at 24 and at 48 to 72 hrs, respectively, through increased PGE_2 production. COX-2 mRNA expression was subsequently downregulated at 48 to 72 hrs post-stimulation. The temporal pattern of this pro-inflammatory response is similar to that observed in previous studies in our lab, in which cultured PBMC were stimulated with hemozoin (Keller *et al.*, 2004a). COX-1 transcripts fluctuated slightly above or below baseline conditions throughout the time course (Figure 15). This pattern is consistent with the homeostatic role of COX-1 in prostaglandin synthesis, since COX-1 transcripts would not be expected to increase in response to β-hematin.

4.3.12 *Effect of β-hematin on IL-4 transcripts:*

Similar to IL-10, IL-4 is an anti-inflammatory cytokine that polarizes the immune response toward a Th2-type response by activating B lymphocytes to up-regulate the production of malaria-specific antibodies (Luoni *et al.*, 2001). Anti-malaria antibodies function to block invasion of sporozoites into hepatocytes and invasion of merozoites into erythrocytes, prevent binding of infected erythrocytes to the vascular endothelium and

subsequent sequestration, inhibit induction of the inflammatory cytokine cascade by neutralizing parasite GPI, and prevent zygote development by mediating complement-dependent lysis of gametes (Good, 2001; Good and Doolan, 1999; Schofield *et al.*, 2002; Sherman, 1998). During liver-stage and blood-stage malaria, activated NKT cells respond rapidly to antigen-specific or polyclonal stimulation by producing high levels of both IL-4 and IFN-γ, which modulate adaptive immunity and the polarization of Th cells (Hansen *et al.*, 2003; Roura-Mir and Moody, 2003; Schmieg *et al.*, 2003; Sköld and Behar, 2003). Winkler *et al.* have shown that IFN-γ, rather than IL-4, plays a predominant role in parasite clearance during uncomplicated *P. falciparium* malaria (Winkler *et al.*, 1998). However, others believe that while innate immune mechanisms may limit parasite density, adaptive immune mechanisms ultimately are required for complete parasite elimination (Stevenson and Riley, 2004). In support of this notion, other studies have demonstrated a shift in the balance between Th1 and Th2 cells in malaria-primed individuals residing in *P. falciparum*-endemic areas (Elghazi *et al.*, 1997). Compared to malaria-naïve persons, these individuals have elevated anti-*P. falciparum* plasma IgE levels, which correlate with an increased ratio of IL-4/IFN-γ-producing cells (Elghazi *et al.*, 1997). In another study, iron deficiency and high IL-4 mRNA expression levels in Kenyan children were associated with protection from clinical malaria (Nyakeriga *et al.*, 2005).

Results presented here show that patterns of IL-4 gene expression varied greatly among malaria-naïve individuals (Figure 17). Detectable at near baseline levels at 4 hrs post-stimulation, β-hematin-induced IL-4 transcripts fluctuated throughout the time course, peaking at 72 hrs in the majority of donors. Overall, these findings suggest a predominantly pro-inflammatory early immune response to β-hematin, and the absence of an IL-4-induced anti-inflammatory (Th2) response within 3 days of stimulation. This pattern might be expected in malaria-naïve donors lacking the high number of IL-4-producing cells present in malaria-exposed individuals. Furthermore, β-hematin lacks the parasite antigens present in live parasites and probably would not elicit an antibody-mediated immune response. However, the 3- to 26-fold increase in IL-4 mRNA observed at 72 hrs may be indicative of the switch to a Th2 immune response.

4.3.13 *Effect of β-hematin on IL-10 transcripts:*

Due to its anti-inflammatory properties, IL-10 serves an immunomodulatory role in malaria infection by inhibiting the production of pro-inflammatory cytokines that may cause pathology in the host. IL-10 limits TNF-α production, which can promote anemia at high levels (Ho *et al.*, 1995, 1998). Murine models of malaria and experiments in cultured monocyte-derived macrophages have demonstrated that IL-10 negatively regulates IL-12 production (Aste-Amezaga *et al.*, 1998; Xu *et al.*, 2001). Studies in our lab have shown that children with malarial anemia and hyperparasitemia residing in an area of hyperendemic *P. falciparum* transmission (Gabon) have decreased plasma levels of IL-12 and increased levels of both TNF-α and IL-10 (Luty *et al.*, 2000; Perkins *et al.*, 2000). More recent investigations in an area of holoendemic malaria transmission (western Kenya) have confirmed that hemozoin-induced over-production of IL-10 is responsible for suppression of IL-12 (Keller *et al.*, manuscript submitted).

Results of this study indicate a peak in β-hematin-induced IL-10 transcript production among malaria-naïve individuals at 24 hrs post-stimulation (Figure 18). Though individual responses differed in magnitude, this increase in IL-10 mRNA suggests an attempt at immunoregulation through down-regulation of pro-inflammatory cytokine production (TNF-α, IL-12) and a switch to a Th2 type immune response. This hypothesis is supported by the fact that a peak in IL-10 transcript production followed surges in TNF-α mRNA at 4 to 8 hrs and in IL-12 mRNA from 4 to 24 hrs.

4.3.14 *Effect of β-hematin on TGF-β1 transcripts:*

Known for its immunoregulatory properties, TGF-β maintains the balance between protection from and progression toward severe malaria by negatively regulating both innate and adaptive immune responses (Perkins *et al.*, 2000; Stevenson and Riley, 2004). Produced by macrophages, NK cells, T cells, and B cells, TGF-β possesses concentration-dependent pro- and anti-inflammatory properties (Wahl, 1994). At low concentrations, TGF-β has pro-inflammatory properties, whereas high levels of TGF-β produce anti-inflammatory effects (Omer *et al.*, 2000). Among its pro-inflammatory properties is the ability to increase the expression of adhesion molecules that attract

immune cells to sites of inflammation (Omer *et al.*, 2000). Anti-inflammatory functions of TGF-β include the suppression of TNF and NO production from macrophages, suppression of TNF and IFN-γ production from NK cells, and up-regulation of IL-10 (Omer *et al.*, 2000). In addition, IL-10 may synergize with TGF-β to suppress IL-2, IL-4, and IL-12 production, facilitating the shift from a Th1-type immune response to a Th2-type response (Omer *et al.*, 2000). However, unlike IL-10, TGF-β is also capable of inhibiting IL-12 through destabilization of IL-12p40 mRNA (Du and Sriram, 1998). Whereas TGF-β initially up-regulates cell-mediated Th1-type responses that control parasitemia, it later suppresses these responses to reduce the pathological effects of inflammation.

Of interest, one murine model of malaria infection has demonstrated that a surge of TGF-β within the first two days of *P. yoelii* blood-stage infection inhibits early pro-inflammatory cytokine responses to infection, thus leading to uncontrolled parasite growth and death (Omer *et al.*, 2003a). A more recent study illustrated the ability of malaria parasites to activate latent, endogenous TGF-β to its bioactive form, thereby modulating the host immune response (Omer *et al.*, 2003b). In support of results obtained from mouse models, human studies have shown an inverse relationship between plasma levels of TGF-β and malaria disease severity (Chaiyaroj *et al.*, 2004; Malaguarnera *et al.*, 2002a; Musumeci *et al.*, 2003). Thus, favorable clinical outcomes to infection depend not only on an appropriate balance between TGF-β and pro-inflammatory cytokines such as IL-12 and IL-18, but also on the timing of TGF-β induction in the host (Ndungu *et al.*, 2005; Omer *et al.*, 2003a).

Results presented here demonstrate that in the presence of β-hematin, TGF-β1 mRNA fluctuated at levels near and slightly above baseline conditions, increasing to approximately 2- to 4-fold over control between 8 and 72 hrs (Figure 19). This pattern of low-level TGF-β1 expression in the first 1 to 3 days post-stimulation would be consistent with the desired pattern of TGF-β production during natural infection. At low concentrations, TGF-β would have a protective pro-inflammatory effect (needed to control parasitemia) and would not inhibit synthesis of other pro-inflammatory cytokines. Variation in the timing of increased TGF-β1 transcript production among individuals

may represent each donor's unique response to the β-hematin-induced increase in synthesis of pro-inflammatory cytokines.

Taken together, results presented here illustrate that the temporal kinetics of β-hematin-induced cytokine transcript production appear to be similar among individuals for IL-1β, IL-6, IL-12p40, IL-18, TNF-α, LT-α, COX-1, COX-2, IL-4, and IL-10. IL-2, IL-12p35, IFN-α, IFN-γ, NOS-2A, and TGF-β1 mRNA expression were more variable among donors. In summary, these findings support well-established patterns of cytokine production during malaria infection. Although the complex interplay between pro-inflammatory and anti-inflammatory cytokines cannot be determined from the current data, it appears that in malaria-naive donors, a physiologically relevant dose of malarial pigment induces an early and robust pro-inflammatory immune response. As expected, IL-10 was also induced, but seemed not to suppress pro-inflammatory cytokine transcript production within 72 hrs of β-hematin stimulation. TGF-β1 mRNA was produced near baseline levels throughout the time course, which suggests that it did not serve an immunoregulatory role within the first 72 hrs of simulated infection. IL-4 transcripts fluctuated at low levels but increased at 72 hrs post-stimulation, possibly indicating the switch to a Th2 immune response. Importantly, the temporal sequence of cytokine induction observed differs from the predicted model of innate immune responsiveness to malaria (Figure 3), in which TNF-α is induced by IL-12 and IFN-γ. Results of the present *in vitro* study indicate that peak β-hematin-induced TNF-α transcript production precedes IL-12 and IFN-γ expression (Figure 20). In addition, data presented here reflect heterogeneity in the magnitude of cytokine responses between individuals, perhaps due to genetic polymorphisms.

5.0 CONCLUSIONS

In conclusion, results of this study confirm that malarial pigment modifies hematological parameters, thereby promoting pathological changes that result in malarial anemia and severe disease. The development of MA is mediated, at least in part, by the phagocytosis of hemozoin by peripheral blood monocytes. Plasma levels of IFN-α, IL-4, IL-6, IL-10, and IL-12p40/p70 were found to differ significantly between healthy children, children with uncomplicated malaria, and children with mild, moderate or severe MA, but not specifically between UM and SMA clinical groups. Such findings imply that these cytokines do not necessarily mediate the development of uncomplicated versus severe disease. Although they have been shown to play prominent protective and pathogenic roles in malaria infection, plasma IFN-γ, TNF-α, IL-1β, and IL-2 were not significantly different between clinical categories. Among parasitemic children, plasma levels of neither IFN-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, nor IL-12p40/p70 were significantly associated with percent of pigment-containing monocytes. Differences in plasma TNF-α between 0%, ≤ 10%, and > 10% PCM categories approached statistical significance at $P = .051$. Ongoing studies in our laboratory are aimed at investigating the relationship between monocytic acquisition of hemozoin and inflammatory mediator production in the development of MA.

Based on further results of this study, temporal profiles of β-hematin-induced IL-1β, IL-2, IL-6, IL-12p35, IL-12p40, IL-18, IFN-α, IFN-γ, TNF-α, LT-α, NOS-2A, COX-1, COX-2, IL-4, IL-10, and TGF-β1 mRNA expression were established. Overall, patterns of cytokine transcript production revealed an early pro-inflammatory response to malarial pigment that varied in magnitude among malaria-naïve individuals. Additional studies are required to address the role of genetic variation in shaping individual cytokine responses to *P. falciparum.* Findings presented here are significant, in that inflammatory mediators produced by innate immune cells shape the adaptive immune response to

Plasmodium parasites. A better understanding of the molecular mechanisms governing these responses will facilitate the development of more effective vaccines and therapeutic strategies.

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