

CYTOKINE AND EFFECTOR MOLECULE DYSREGULATION IN  
*PLASMODIUM FALCIPARUM* MALARIA

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Childhood malarial anemia (MA) remains a global health burden with the vast morbidity and mortality occurring mostly in sub-Saharan Africa. Although design and testing of malaria vaccines is currently underway, the pattern of inflammatory mediator production that predicts a protective immune response against severe malaria, which would dramatically enhance vaccine testing, is largely unknown. Protective malarial immunity is regulated in part by cytokines, such as interleukin (IL)-12, IL-10, and tumor necrosis factor (TNF)- $\alpha$ , and effector molecules, such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and nitric oxide (NO). Previous studies have illustrated that children with severe MA have lower levels of circulating IL-12p70 and PGE<sub>2</sub>, and increased plasma levels of IL-10, TNF- $\alpha$ , and NO relative to children with mild malaria, however, the mechanism(s) responsible for this pattern of immune production is unknown. Phagocytosis of parasitic products, such as hemozoin, by cultured peripheral blood mononuclear cell (PBMC) elicits dysregulation of inflammatory mediator production, therefore, the regulation and interactions of cytokines and effector molecules was investigated during acute childhood malaria and in cultured PBMC stimulated with *Plasmodium falciparum*-derived hemozoin. Children with high-density parasitemia had decreased IL-12p70 and increased levels of IL-10 and TNF- $\alpha$ . Experiments in cultured PBMC from malaria-naïve donors revealed that hemozoin suppressed IL-12p70 through induction of IL-10, but not over-expression of TNF- $\alpha$  transcripts and protein, which was independent of suppressor of cytokine signaling (SOCS)-3 induction. Hemozoin

suppressed cyclooxygenase (COX)-2-dependent PGE<sub>2</sub> production through reductions in COX-2 transcript and protein formation, and inhibition of COX-2 enzymatic activity. Suppression of PGE<sub>2</sub>, which was independent of hemozoin-induced IL-10, resulted in over-production of TNF- $\alpha$ . The ratio of plasma PGE<sub>2</sub>/TNF- $\alpha$  was decreased in children with severe disease. Cultured PBMC from children with severe malaria had elevated nitric oxide synthase (NOS)2 enzyme activity, which occurred at least in part through PBMC ingestion of hemozoin. Thus, ingestion of hemozoin by PBMC elicits a similar pattern of inflammatory mediator production to that observed in children with severe MA. Results presented here are of significant public health relevance in that understanding the regulation of cytokine and effector molecule production during severe malaria will vastly improve vaccine design and testing.

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## FOREWORD

The majority of the work presented in this dissertation has been either published or submitted for publication in peer-reviewed scientific journals. Manuscripts that have been prepared as a direct result of the work presented in this dissertation include:

1. **Keller CC**, Hittner JB, Misukonis MA, Weinberg JB, Kremsner PG, and DJ Perkins. (2004). Elevated nitric oxide production in children with malarial anemia: Hemozoin-induced nitric oxide type 2 transcripts and nitric oxide in blood mononuclear cells. *Infection and Immunity*. 72:4868-73.
2. **Keller CC**, Hittner JB, Nti BK, Weinberg JB, Kremsner PG, and DJ Perkins. (2004). Reduced peripheral PGE<sub>2</sub> biosynthesis in *Plasmodium falciparum* malaria occurs through hemozoin-induced suppression of blood mononuclear cell cyclooxygenase-2 gene expression via an interleukin-10 independent mechanism. *Molecular Medicine*. 10:1-6, 45-54.
3. **Keller CC**, Hittner JB, Weinberg JB, Kremsner PG, and DJ Perkins. (2005). Hemozoin-induced suppression of COX-2 and PGE<sub>2</sub> promotes overproduction of TNF- $\alpha$ : Implications for the pathogenesis of severe malaria. *Journal of Infectious Diseases*. Manuscript Submitted.
4. **Keller CC**, Ong'echa JM, Obuya CO, Hittner JB, and DJ Perkins. (2005). Hemozoin-induced IL-10 overproduction suppresses IL-12 independent of upregulated SOCS-3 expression. *Journal of Immunology*. Manuscript Submitted.

In addition, portions of this work have been selected for presentation at the following international conferences:

1. **Keller CC**, Nti BK, Udhayakumar V, and DJ Perkins. (2002). Cyclooxygenase-2 and prostaglandin-E<sub>2</sub> down-regulation by hemozoin is associated with overproduction of TNF-alpha: Implications for the pathogenesis of severe malarial anemia. Presented at the 51<sup>st</sup> annual American Society of Tropical Medicine and Hygiene conference.
2. **Keller CC**, Nti BK, Udhayakumar V, and DJ Perkins. (2002). Hemozoin-induced suppression of cyclooxygenase-2 and prostaglandin-E<sub>2</sub> promotes overproduction of TNF- $\alpha$ : Implications for the pathogenesis of severe malarial anemia. Presented at the 3<sup>rd</sup> annual Multilateral Initiative on Malaria Pan-African Conference on Malaria.
3. **Keller CC**, Kremsner PG, Weinberg JB, and DJ Perkins. (2003). Hemozoin suppresses IL-12 production through over-expression of IL-10. Presented at the 52<sup>nd</sup> annual American Society of Tropical Medicine and Hygiene conference.
4. **Keller CC**, Misukonis MA, Kremsner PG, Weinberg JB, and DJ Perkins. (2003). Ingestion of hemozoin by PBMC induces dysregulation in PGE<sub>2</sub> and NO: Association of effector molecule ratios with malarial anemia. Presented at the 52<sup>nd</sup> annual American Society of Tropical Medicine and Hygiene conference.
5. **Keller CC**, Hittner JB, Kremsner PK, Misukonis MA, Weinberg JB, and DJ Perkins. (2004). Elevated nitric oxide production in children with malarial anemia: Hemozoin-induced NOS2 transcripts and nitric oxide in blood mononuclear cells. Presented at the 53<sup>rd</sup> annual American Society of Tropical Medicine and Hygiene conference.

## ABBREVIATION LIST

6-carboxy-fluorescein (FAM); arachidonic acid (AA); Asembo Bay Cohort Project (ABCP); burst forming unit (BFU); Centers for Disease Control and Prevention (CDC); cerebral malaria (CM); confidence interval (CI); cyclooxygenase (COX); dendritic cell (DC); Dulbecco's modified Eagle's medium (DMEM); endotoxin unit (EU); enzyme immunoassay (EIA); enzyme linked immunosorbent assay (ELISA); ethylenediaminetetraacetic acid (EDTA); extracellular regulated kinase (ERK); ferriprotoporphyrin (FP); glucocorticoid response (GR); glyceraldehydes-6-phosphate dehydrogenase (GAPDH); glycosylphosphatidylinositol (GPI); healthy control (HC); guanidinium isothiocyanate (GITC); hemoglobin (Hb); high phase liquid chromatography (HPLC); high-density parasitemia (HDP); human immunodeficiency virus (HIV); immunoglobulin (Ig); inhibitory concentration (IC); interferon (IFN); interleukin (IL); intervillous blood mononuclear cell (IVBMC); lipophosphoglycan (LPG); lipopolysaccharide (LPS); low density parasitemia (LDP); macrophage chemoattractant protein (MCP); macrophage inflammatory protein (MIP); macrophage migration inhibitory factor (MIF); major histocompatibility complex (MHC); malarial anemia (MA); methylthiazol tetrazolium (MTT); mild malaria (MM); nitric oxide (NO); nitric oxide synthase (NOS); nuclear factor (NF); odds ratio (OR); parasitized red blood cell (pRBC); peripheral blood mononuclear cell (PBMC); peroxisome proliferator-activator receptor (PPAR); phosphate buffered saline (PBS); phospholipase A<sub>2</sub> (PLA<sub>2</sub>); preoptic area of the anterior hypothalamus (POAH); prostaglandin



(PG); protein kinase C (PKC); red blood cell (RBC); restriction fragment length polymorphism (RFLP); severe malaria (SM); severe malarial anemia (SMA); single nucleotide polymorphism (SNP); small interfering RNA (siRNA); sodium dodecyl sulphate (SDS); standard error of the mean (SEM); suppressor of cytokine signaling (SOCS); systemic lupus erythrematosus (SLE); toll-like receptor (TLR); transcription factor (TF); transforming growth factor (TGF); tumor necrosis factor (TNF); World Health Organization (WHO).

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## **1. CHAPTER 1: INTRODUCTION**

### **1.1. MALARIA PATHOGENESIS**

Malaria continues to represent a global health threat, with current estimates of clinical infections at 300-500 million per year, resulting in 1.5-2.7 million deaths (World Health Organization 1997). In areas of hyperendemic malaria transmission, children under the age of 5 are most affected by malaria, mainly due to a lack of malarial immunity, and in these areas, mortality rates can reach 20% (Breman *et. al.* 2001). Due to overwhelming antimalarial drug resistance, current efforts involve design of vaccines and novel therapeutic strategies to combat malaria. Despite these efforts, our current understanding of the development of immunity to malaria, and the immune response(s) that leads to pathogenesis and/or protection is largely unknown. As such, immune markers and/or mediators which define protection to malaria are needed to determine the efficacy of vaccines and antimalarial compounds.

The clinical manifestations of severe malaria are disparate in different geographical areas, due to factors such as the intensity of transmission (Cook *et. al.* 1996), and include; severe anemia, hyperparasitemia, hypoglycemia, respiratory distress, renal failure, and cerebral malaria (CM) (Cook *et. al.* 1996). In areas of hyperendemic and holoendemic malaria transmission, such

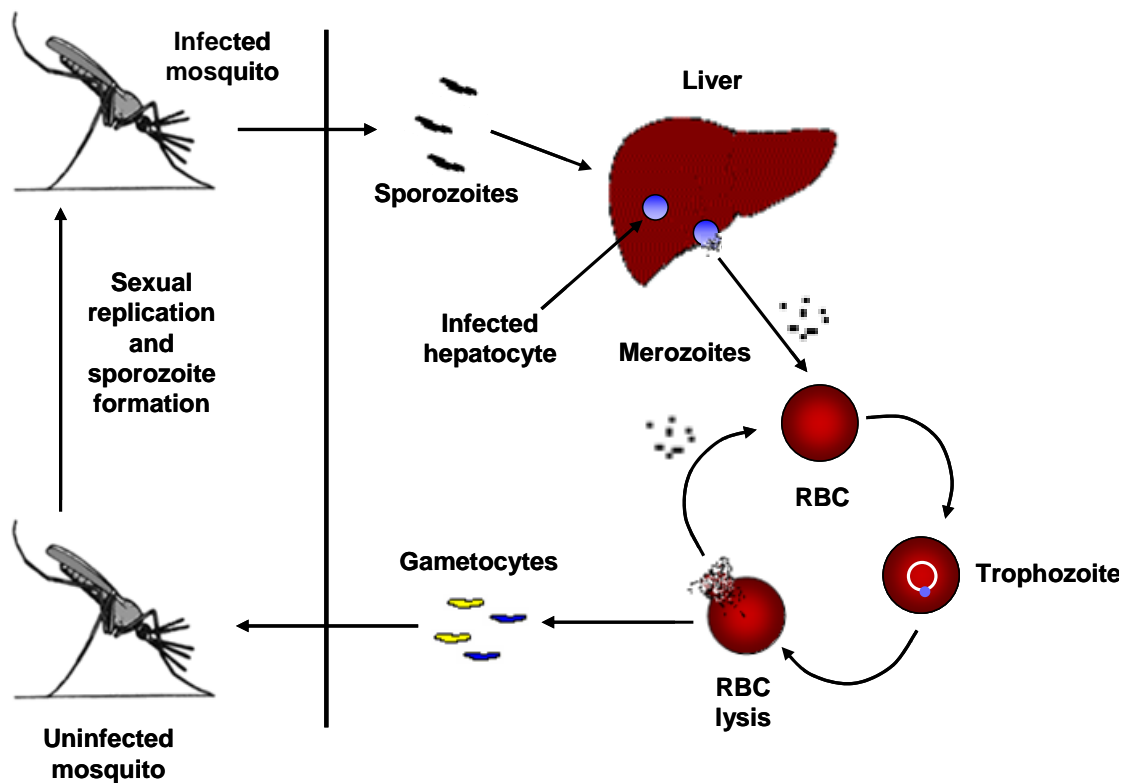
as Gabon and Kisumu, Kenya, respectively, the primary clinical manifestations of severe disease are hyperparasitemia and severe anemia, with CM occurring rarely (Bloland *et. al.* 1999, Breman *et. al.* 2001, Kun *et. al.* 1998). Although the clinical manifestations of severe malaria are similar in hyper- and holoendemic areas, in Gabon, children under the age of 5 years suffer the most malaria-related morbidity and mortality, whereas in Kisumu, severe malarial anemia [SMA, defined by hemoglobin (Hb) < 5.0 g/dL in the presence of any density parasitemia, (World Health Organization 1990)] occurs mainly in children less than 2 years of age (Bloland *et. al.* 1999, Kun *et. al.* 1998). The underlying causes which contribute to anemia in these children are multifactorial and include: maternal deficiency states resulting in low birth weight infants, iron and folate deficiency, nutritional deficiencies, presence of parasitic infections such as hookworm, bacterial infections with post-infective malabsorption, and the presence of hemoglobinopathies (Hillman *et. al.* 1996). Although the above mentioned factors contribute to the development of malarial anemia [MA, defined by Hb < 8.0 g/dL in the presence of any density parasitemia, (Abdallah 1990)], acute loss of red blood cells (RBC) from repeated cycles of parasite invasion and replication is one of the primary determinants of MA. This is compounded by intravascular and extravascular hemolysis of both parasitized RBC (pRBC) and non-pRBC (Hillman *et. al.* 1996). Due to the loss of RBC in these already anemic children, increases in erythropoiesis are essential for recovery from MA.

Resolution of MA in children who survive infection typically occurs about seven to fourteen days following initiation of antimalarial administration. During this time, immature hematopoietic stem cells are mobilized and differentiate into burst forming unit-erythroid (BFU-E) cells, and ultimately into mature RBC (Hillman *et. al.* 1996). Although parasitemia is

resolved during this time, parasitic products released during acute malaria, such as hemozoin (malarial pigment), can persist in mononuclear cells for prolonged periods (Day *et. al.* 1996). Hemozoin is formed during the erythrocytic stage of the malaria lifecycle, when parasite-specific enzymes, plasmepsin and falcipain, digest host RBC Hb in order to obtain essential amino acids from globin [for review see (Francis *et. al.* 1997)]. The process of Hb digestion causes the release of heme, which is highly toxic to parasites through inhibition of enzyme function (Ginsburg *et. al.* 1992, Vander Jagt *et. al.* 1986), peroxidation of membranes (Tappel 1953), and production of free radicals (Atamna *et. al.* 1996). To avoid the toxic effects of heme, parasites polymerize heme into a non-toxic insoluble compound, hemozoin (Slater 1992). Upon rupture of infected erythrocytes, hemozoin is released into the blood stream and avidly phagocytosed by circulating monocytes and tissue macrophages (Schwarzer *et. al.* 1998). Hemozoin is also acquired by monocytes/macrophages through phagocytosis of parasitized erythrocytes (Arese *et. al.* 1997). *In vivo*, hemozoin exists as a coordinated polymer of ferriprotoporphyrin-IX (FP-IX) subunits containing a conglomeration of host- and parasite-derived lipids and proteins (Ashong *et. al.* 1989, Goldie *et. al.* 1990), however, the core FP-IX structure of protease- and acetone-purified preparations of hemozoin are structurally identical to synthetically prepared  $\beta$ -hematin (Egan *et. al.* 2001). Previous studies in malaria-infected adults showed evidence of dyserythropoiesis and dysplastic erythroid maturation in bone marrow, in association with high levels of hemozoin deposition (Abdallah 1990), suggesting that hemozoin may negatively impact the formation of new erythrocytes. Furthermore, circulating levels of cytokines and effector molecules released during the inflammatory response(s) to malaria and malarial products, such as hemozoin, may contribute to the regulation of erythropoiesis during childhood malaria.

## 1.2. MALARIA LIFECYCLE

As shown in Figure 1, *P. falciparum* infection in humans begins when an infected female Anopheles mosquito injects sporozoites into the host during a blood meal. The sporozoites rapidly migrate to the liver and infect hepatocytes where they undergo asexual replication into merozoites. After a period of about 5 days, the merozoites burst from the infected hepatocyte and enter the circulating blood where they rapidly infect RBC. This process involves attachment and orientation of the merozoite to the RBC surface, and active invasion into the host cell. The parasites mature into ring forms (trophozoites) within the first 12 hours of the erythrocytic stage, digesting host RBC hemoglobin for essential nutrients, forming hemozoin to detoxify the released heme. After 36 hrs from the initial merozoite invasion of RBC, the parasite forms a schizont containing between 6 and 36 merozoites. The pRBC then ruptures after 48 hrs, releasing the merozoites, as well as hemozoin, into circulation. The released merozoites rapidly invade new RBC to start a new asexual phase of replication. After several asexual cycles (approximately 5 cycles, or 10 days of infection), a small number of parasites develop into gametocytes. These forms are ingested by uninfected female mosquitoes during a blood meal, and migrate to the midgut where they fuse into a zygote. Within 24 hrs of the sexual phase of replication, the zygote pierces the midgut wall, forming an oocyst where thousands of sporozoites are formed. The oocyte then bursts from the midgut, releasing the sporozoites. When the female Anopheles mosquito takes a subsequent blood meal, these sporozoites are inoculated into the vertebrate host.



*Figure 1. Lifecycle of P. falciparum.* An infected female *Anopheles* mosquito injects sporozoites into the host, which infect hepatocytes and undergo asexual replication into merozoites. Merozoites burst from the liver and infect circulating RBC. The parasites mature into ring forms (trophozoites), and then schizonts that contain new merozoites. The infected RBC ruptures, releasing the merozoites into circulation which infect new RBC. After several asexual cycles a few parasites develop into gametocytes which are ingested by an uninfected female mosquito during a blood meal. The gametocytes undergo sexual replication, forming sporozoites which are inoculated into the vertebrate host.

### 1.3. HOST INNATE IMMUNE RESPONSES TO MALARIA

Host immunoprotection against malaria is regulated by the production of pro-inflammatory molecules, such as interferon (IFN)- $\gamma$ , interleukin (IL)-12, tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$ , and nitric oxide (NO), as well as anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)- $\beta$ , which down-regulate the pro-inflammatory response [for review see (Stevenson *et. al.* 2004)]. Initiation of the immune cascade in murine models of malaria is dependent on IL-12 production from monocytes and other cells types (Crutcher *et. al.* 1995). A protective role for IL-12 is further evidenced by the sterile protection of mice and monkeys from malaria infection following treatment with recombinant IL-12 prior to infection (Hoffman *et. al.* 1997, Sedegah *et. al.* 1994). IL-12 then activates T helper cells to secrete IFN- $\gamma$  driving immunity towards a type 1 (pro-inflammatory) response. IFN- $\gamma$  in turn promotes secretion of TNF- $\alpha$  from monocytes, which can act in an autocrine and paracrine fashion to stimulate the production of effector molecules, such as nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). NO has potent antiplasmodial properties through inhibition of parasite growth *in vitro* and *in vivo* (Anstey *et. al.* 1996, Gyan *et. al.* 1994, Mellouk *et. al.* 1994, Rockett *et. al.* 1991). Although the protective versus pathogenic role of PGE<sub>2</sub> is largely unknown in the context of malaria, PGE<sub>2</sub> regulates macrophage function, vascular permeability, extracellular adhesion molecules, fever, and cytokine production (Vane *et. al.* 1998).

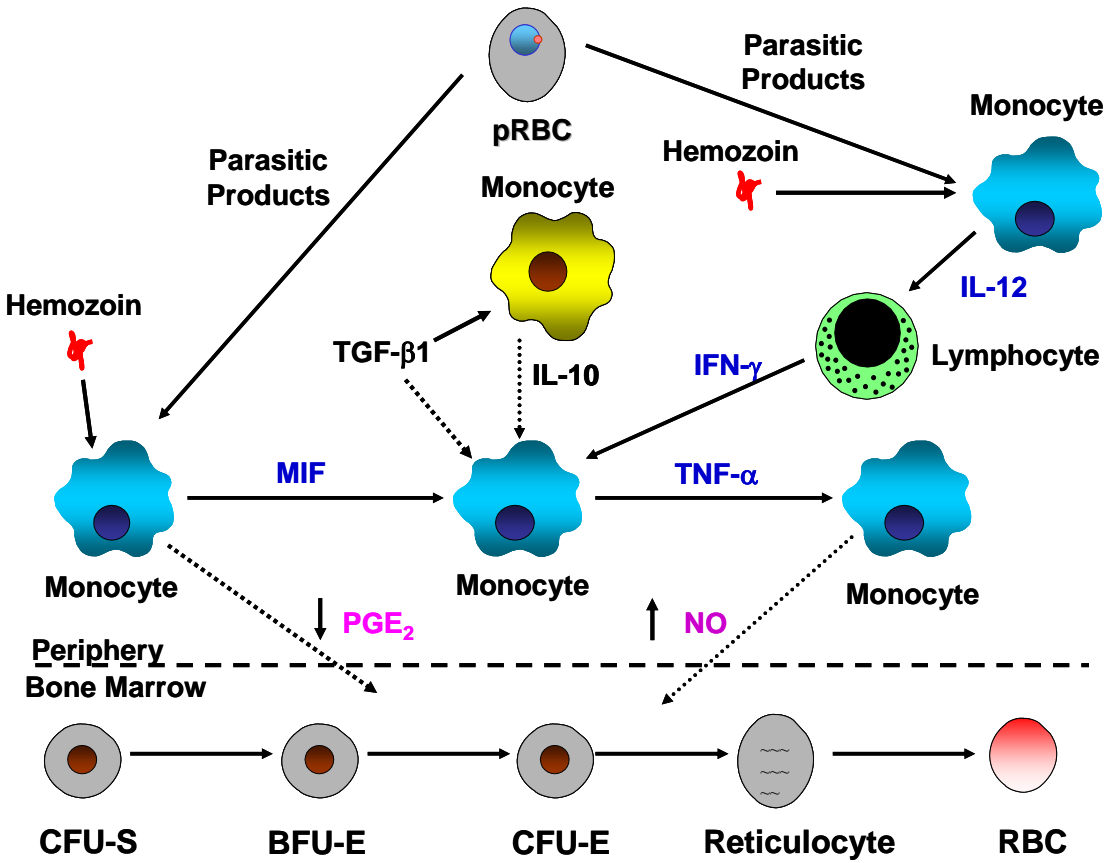


#### 1.4. CYTOKINE AND EFFECTOR MOLECULE DYSREGULATION IN MALARIA

Despite the identification of protective immune responses in animal models of malaria, little is known about the development of protective immune responses in children with SMA. Our previous studies (Luty *et. al.* 2000), as well as those of others (Grau *et. al.* 1989, Kwiatkowski *et. al.* 1990), have shown that children with SMA have high systemic production of TNF- $\alpha$ , which can serve as a protective factor by decreasing parasite clearance time (Kremsner *et. al.* 1995), but can also be pathogenic by promoting anemia (Clark *et. al.* 1988). TNF- $\alpha$  production during malaria is influenced by IL-10, which we and others have shown to be increased in plasma from children with MA (Perkins *et. al.* 2001, Perkins *et. al.* 2000, Peyron *et. al.* 1994). Previous studies in our laboratory show that, relative to children with mild MA, children with SMA have suppressed plasma levels of IL-12 (Luty *et. al.* 2000), which may limit the immune response to blood-stage malaria. Our previous results show that healthy, malaria-exposed Gabonese children with a history of mild malaria have significantly elevated levels of peripheral blood mononuclear cell (PBMC) NO production and nitric oxide synthase (NOS) enzyme activity compared to their age-matched cohorts with a history of severe malaria (Perkins *et. al.* 1999), suggesting a protective role of elevated NO production in MA. The protective role of increased NO during acute malaria, however, does not appear to be universal, since previous reports in Tanzanian children with CM showed that PBMC NOS2 expression and peripheral levels of NO metabolites (NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup>, total NO: NO<sub>x</sub>) are inversely related to disease severity (Anstey *et. al.* 1996). Although the role of PGE<sub>2</sub> in malaria remains to be defined, elevated production of PGE<sub>2</sub> may

serve as a protective factor in malaria as evidenced by our previous results illustrating that children with MA have suppressed PBMC COX-2 transcript and protein levels, and lower plasma PGE<sub>2</sub> relative to healthy children (Perkins *et. al.* 2001). Thus, although protective immunity against malaria appears to require the appropriate timing and level of cytokine (i.e. TNF- $\alpha$ , IL-10, and IL-12) and effector molecule (NO and PGE<sub>2</sub>) release, particularly in animal models, protective immune responses in human malaria are largely unknown.

As part of our ongoing studies on the genetic basis of SMA in Gabonese and Kenyan children, we have developed a model to investigate the role of immune regulation as a determinant of MA. As shown in Figure 2, parasites and parasitic products, such as hemozoin, stimulate monocytes to release IL-12, which induces IFN- $\gamma$  production from lymphocytes. IFN- $\gamma$  stimulates monocytes to release both pro-inflammatory cytokines, such as TNF- $\alpha$ , and anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$ . This cytokine network regulates the production of effector molecules, such as NO and PGE<sub>2</sub>, which may influence erythropoiesis, and ultimately, malaria disease outcomes.



*Figure 2. Regulation of erythropoiesis by the immune cascade to malaria.* In response to pRBC or hemozoin, monocytes secrete IL-12, which activates CD4<sup>+</sup> T cells to produce IFN- $\gamma$ , a cytokine that promotes monocytes to secrete pro-inflammatory cytokines, such as TNF- $\alpha$ , and anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$ . This network of cytokine production regulates effector molecules, such as NO and PGE<sub>2</sub>, which influence malaria disease outcomes through modulation of erythropoiesis.

## 1.5. ROLE OF HEMOZOIN IN SEVERE MALARIA

Hemozoin was first described by Laveran in 1880, which enabled the diagnosis of malaria infection in humans. As mentioned previously, parasite-derived hemozoin is phagocytosed by circulating monocytes upon rupture of infected erythrocytes (Schwarzer *et. al.* 1998). As shown in Figure 3, once hemozoin is phagocytosed, it remains within human mononuclear cells for extended periods of time (i.e., 48 hrs). In adult patients with repeated exposure to malaria, accumulation of hemozoin in the reticuloendothelial system has been associated with slate-gray to black appearance of the liver and spleen (Edington 1967) and bone marrow (Abdallah 1990). In Gabonese children diagnosed with severe malaria, 24% of monocytes were reported to contain hemozoin, compared to 7% pigmented monocytes in children with mild disease (Metzger *et. al.* 1995). Previous studies demonstrate that both plasma and mitogen-stimulated IL-12 levels are suppressed in children with elevated numbers of pigmented monocytes and neutrophils, suggesting that acquisition of hemozoin can suppress the formation of some immuno-regulatory cytokines (Luty *et. al.* 2000). Dysregulation of innate immunity in children with SMA may, therefore, be a consequence of mononuclear cell ingestion of parasitic products such as hemozoin.

Although historically, ingestion of hemozoin by circulating phagocytes served as a marker of malaria infection, and was believed to have no impact on cellular function, previous *in vitro* studies have shown that hemozoin causes dysregulation of monocyte/macrophage cytokine production. For example, experiments in cultured human blood mononuclear cells illustrated that hemozoin augments the release of cytokines and chemokines, such as IL-1 $\beta$  (Biswas *et. al.*

2001, Pichyangkul *et. al.* 1994), IL-10 (Mordmuller *et. al.* 1998), IL-12p70 (Mordmuller *et. al.* 1998), MIP-1 $\alpha$  (Sherry *et. al.* 1995), MIP-1 $\beta$  (Sherry *et. al.* 1995), and TNF- $\alpha$  (Biswas *et. al.* 2001, Mordmuller *et. al.* 1998, Pichyangkul *et. al.* 1994, Sherry *et. al.* 1995), and decreased mononuclear cell oxidative burst (Schwarzer *et. al.* 1993) (Table 1). Similar experiments in cultured murine macrophages illustrated that hemozoin did not alter IL-1 $\beta$  production (Taramelli *et. al.* 1995), decreased IL-6 (Prada *et. al.* 1995) and NO production (Prada *et. al.* 1996, Taramelli *et. al.* 1995), and increased MIF (Martiney *et. al.* 2000). Furthermore, the effects of hemozoin on TNF- $\alpha$  production have yielded conflicting results (Prada *et. al.* 1995, Taramelli *et. al.* 1995), suggesting an anti-inflammatory affect of hemozoin ingestion in murine malaria (Table 1). Additional effects of hemozoin on human blood mononuclear cells include; inhibition of oxidative burst and protein kinase C activity (Schwarzer *et. al.* 1993), reduction of microbicidal and anti-tumor capabilities (Fiori *et. al.* 1993), impaired expression of MHC class II and CD54 expression in monocytes (Schwarzer *et. al.* 1998), and an inability of monocytes to undergo repeated phagocytosis (Schwarzer *et. al.* 1992).



*Figure 3. Phagocytosed hemozoin in cultured monocytes.* Cultured monocytes were treated with hemozoin for 48 hrs and then stained with Giemsa. Left panel: 60X magnification, black arrows indicate monocytes which have ingested hemozoin. Right panel: 100X magnification, black arrows indicate hemozoin within a monocyte.

Table 1. Effect of hemozoin ingestion on cytokine and effector molecule production in cultured mononuclear cells

Cytokine	Cell Type	Effect of Hz	Reference
IL-1 $\beta$	Murine	No affect	(Taramelli <i>et. al.</i> 1995)
	Human	Increase	(Pichyangkul <i>et. al.</i> 1994)
	Human	Increase	(Biswas <i>et. al.</i> 2001)
IL-6	Murine	Decrease	(Prada <i>et. al.</i> 1995)
IL-10	Human	Increase	(Mordmuller <i>et. al.</i> 1998)
IL-12p70	Human	Increase	(Mordmuller <i>et. al.</i> 1998)
MIF	Murine	Increase	(Martiney <i>et. al.</i> 2000)
MIP-1 $\alpha$	Human	Increase	(Sherry <i>et. al.</i> 1995)
MIP-1 $\beta$	Human	Increase	(Sherry <i>et. al.</i> 1995)
NO	Murine	Decrease	(Taramelli <i>et. al.</i> 1995)
	Murine	Decrease	(Prada <i>et. al.</i> 1996)
Oxidative burst	Human	Decrease	(Schwarzer <i>et. al.</i> 1993)
TNF- $\alpha$	Murine	Increase	(Prada <i>et. al.</i> 1995)
	Murine	Decrease	(Taramelli <i>et. al.</i> 1995)
	Human	Increase	(Sherry <i>et. al.</i> 1995)
	Human	Increase	(Mordmuller <i>et. al.</i> 1998)
	Human	Increase	(Pichyangkul <i>et. al.</i> 1994)
	Human	Increase	(Biswas <i>et. al.</i> 2001)

## 2. CHAPTER 2: SPECIFIC AIMS

Malaria is responsible for 300-500 million new clinical cases and 1.5 to 2.7 million deaths each year. In areas where *Plasmodium falciparum* transmission is high, the primary clinical manifestations of severe malaria are malarial anemia (MA) and hyperparasitemia. Children under the age of 5 are most affected by severe malaria, mainly due to a lack of acquired immunity, and in holoendemic transmission areas, the mortality rate attributed to severe malaria can be as high as 25-30%. Since *P. falciparum* is an intracellular pathogen, cell-mediated immunity is essential for protection from severe disease. Although previous studies in our laboratory have shown that children with severe malaria have dysregulation of cytokines, such as interleukin (IL)-12, IL-10, and tumor necrosis factor (TNF)- $\alpha$ , and effector molecules, such as nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) during acute disease, the underlying molecular basis responsible for this dysregulation is largely unknown. Ingestion of parasitic products, such as hemozoin (malarial pigment), is known to influence mononuclear cell activities, such as impairment of phagocytosis, inhibition of oxidative burst and protein kinase C activity, and reduced microbial killing. Furthermore, experiments in cultured human mononuclear cells indicate that hemozoin enhances the release of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ . We therefore postulate that ingestion of hemozoin by peripheral blood mononuclear cells

(PBMC) is a major contributory factor of immune dysregulation through altered production of cytokines and effector molecules during childhood *P. falciparum* malaria.

**Specific Aim 1: To determine if hemozoin alters IL-12, IL-10, and TNF- $\alpha$  production in cultured human PBMC, and to determine if increased IL-10 and/or TNF- $\alpha$  are responsible for suppression of IL-12.**

***Hypothesis 1:*** Acquisition of hemozoin suppresses IL-12 production and augments both IL-10 and TNF- $\alpha$  production in cultured human PBMC.

Cultured PBMC from healthy, malaria-naïve U.S. donors were stimulated with LPS and IFN- $\gamma$ , to induce IL-12, IL-10, and TNF- $\alpha$  production, in the absence or presence of hemozoin or  $\beta$ -hematin. IL-12p70, IL-10, and TNF- $\alpha$  production was determined by ELISA. Temporal profiles of gene expression for IL-12p35, IL-12p40, IL-10, and TNF- $\alpha$  were quantified by real time RT-PCR at 2, 4, 8, 24, and 48 hrs.

***Hypothesis 2:*** Hemozoin-induced IL-10 and/or TNF- $\alpha$  production is responsible for suppression of IL-12 production in PBMC.

To investigate the effect of IL-10 and TNF- $\alpha$  on IL-12 production, cultured PBMC from healthy, malaria-naïve U.S. donors were stimulated with LPS and IFN- $\gamma$ , to induce IL-12, IL-10, and TNF- $\alpha$  production, in the presence or absence of hemozoin. Cultures were incubated with



varying concentrations of neutralizing IL-10 or TNF- $\alpha$  antibodies, or exogenous addition of recombinant IL-10 or TNF- $\alpha$  followed by measurement of IL-12p70 by ELISA.

**Hypothesis 3:** Hemozoin suppresses IL-12 production through dysregulation of JAK/STAT signaling.

Induction of IL-12 gene expression is enhanced by JAK/STAT signaling. The suppressor of cytokine signaling (SOCS) gene family consists of nine currently identified genes which suppress the JAK/STAT signaling cascade. To determine if hemozoin suppresses IL-12 production through dysregulation of JAK/STAT signaling, we investigated the effects of hemozoin on cultured PBMC on the temporal profile of SOCS-3 gene expression using real time RT-PCR, since SOCS-3 is induced by IL-10 and TNF- $\alpha$ . To determine if SOCS-3 is responsible for inhibition of IL-12, cultured PBMC from healthy, malaria-naïve U.S. donors were stimulated with hemozoin, and cells were nucleofected with SOCS-3 short interfering RNA (siRNA) to ablate SOCS-3 signaling. IL-12p40 transcripts were determined by real time RT-PCR in PBMC in which SOCS-3 has been suppressed by SOCS-3-specific siRNA.

**Specific Aim 2: To determine if hemozoin decreases COX-2 gene expression and the subsequent production of PGE<sub>2</sub> in cultured human PBMC.**

**Hypothesis 1:** Hemozoin suppresses COX-2-derived PGE<sub>2</sub> production in cultured human PBMC by suppressing *de novo* COX-2 transcripts and protein, and/or inhibiting COX-2 enzymatic activity.

To investigate the effects of hemozoin on mononuclear cell PGE<sub>2</sub> production, PBMC from healthy, malaria-exposed children and children with mild MA were cultured under baseline- and stimuli-induced conditions, followed by measurement of PGE<sub>2</sub> using an enzyme immunoassay (EIA). To examine the direct effect(s) of hemozoin and  $\beta$ -hematin on COX-2 and PGE<sub>2</sub> production, PBMC from healthy, malaria-naïve U.S. adults were stimulated with hemozoin or  $\beta$ -hematin. COX-2 protein was determined by immunoblot and COX-2 transcripts were quantified by Taqman real time RT-PCR. PBMC PGE<sub>2</sub> production was determined by EIA. The effect of hemozoin on cell viability was examined by the MTT assay. The effects of hemozoin-induced anti-inflammatory cytokines (*i.e.*, IL-10), which are known to decrease COX-2 gene expression, were determined by examining PGE<sub>2</sub> formation in the presence of IL-10 neutralizing antibodies. The effect of hemozoin or  $\beta$ -hematin on COX-2 enzymatic activity was examined using a COX inhibitor screening assay.

***Hypothesis 2:*** Acquisition of hemozoin by mononuclear phagocytes causes overproduction of TNF- $\alpha$  through suppression of COX-2 gene expression.

Since PGE<sub>2</sub> can inhibit TNF- $\alpha$  transcription, we examined the effect of hemozoin on TNF- $\alpha$  production in the presence of exogenous PGE<sub>2</sub>. PBMC from healthy, malaria-naïve U.S. adults were stimulated with hemozoin or  $\beta$ -hematin, and PGE<sub>2</sub> and TNF- $\alpha$  production was determined by EIA and enzyme-linked immunosorbent assay (ELISA), respectively. The effects of exogenous PGE<sub>2</sub> on TNF- $\alpha$  production was determined in cultured PBMC stimulated with hemozoin or  $\beta$ -hematin.

**Hypothesis 3:** The relative production of PGE<sub>2</sub> to TNF- $\alpha$  (PGE<sub>2</sub>/TNF- $\alpha$  ratio) is associated with disease severity in children with MA.

The relative production of cytokines and effector molecules can serve as correlates of disease severity. Therefore, we examined the plasma PGE<sub>2</sub>/TNF- $\alpha$  ratio in healthy, malaria-exposed children, and children with mild and severe MA. To examine the association of the PGE<sub>2</sub>/TNF- $\alpha$  ratio with MA, the PGE<sub>2</sub>/TNF- $\alpha$  ratio was correlated with hemoglobin concentrations in children with MA. To determine if ingestion of hemozoin by mononuclear cells suppresses the PGE<sub>2</sub>/TNF- $\alpha$  ratio, PBMC from healthy, malaria-naïve U.S. adults were stimulated with hemozoin, and PGE<sub>2</sub> and TNF- $\alpha$  production was determined by ELISA.

**Hypothesis 4:** Polymorphisms in the promoter of COX-2 condition the clinical outcomes in children with MA.

We have recently identified a novel polymorphism in the COX-2 promoter (COX-2 -512 C $\rightarrow$ T) in children with MA. The prevalence of the COX-2 C-512T polymorphism was determined in DNA from a large cohort of children with varying degrees of malaria disease severity using a novel restriction fragment length polymorphism (RFLP) assay. Using multivariate logistic regression analyses, we examined the association of the COX-2 C-512T polymorphism with high-density parasitemia (HDP), MA, or SMA.

**Specific Aim 3: To determine if children with MA have altered NO production and to determine if ingestion of malarial products (hemozoin) regulates NO production through changes in induction of *de novo* NOS2 transcripts.**

***Hypothesis 1:*** Increased production of NO from circulating mononuclear cells is associated with increasing severity of MA.

To determine the effect of acute malaria on mononuclear cell NO production, NOS enzyme activity was measured in *ex vivo* isolated PBMC from healthy, malaria-exposed children and children with mild or severe MA. To investigate the association between NO production and anemia, *ex vivo* PBMC NOS enzyme activity was compared with hemoglobin measurements (MA outcomes) in children with mild and severe MA. Furthermore, the cellular source of NO was examined by measuring NOS enzyme activity in cultured PBMC from healthy children and children with acute MA.

***Hypothesis 2:*** Hemozoin induces NO production through *de novo* induction of NOS2 transcripts in cultured human PBMC.

Since parasitic products may influence PBMC NO production, the effect of hemozoin on NO production was determined. Cultured PBMC from healthy, malaria-naïve U.S. adults were stimulated with hemozoin followed by measurement of total NO ( $\text{NO}_x$ :  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ) by the Griess reaction and *de novo* NOS2 transcript formation by Taqman real time RT-PCR.

### 3. CHAPTER 3: SPECIFIC AIM 1

**Specific Aim 1: To determine if hemozoin alters IL-12, IL-10 and TNF- $\alpha$  production in cultured human PBMC, and to determine if increased IL-10 and/or TNF- $\alpha$  are responsible for suppression of IL-12.**

Previous observations in our laboratories have shown that Gabonese children with severe malaria have suppressed plasma levels of IL-12 relative to children with mild disease (Perkins *et. al.* 2000). Although several studies have examined the cellular mechanism of this suppression in murine models of malaria (Stevenson *et. al.* 1995, Xu *et. al.* 2001), no studies to date have been performed in human malaria. The aim of the experiments described in this chapter was to determine the cellular source and/or mechanism responsible for suppression of IL-12 production in human malaria. To accomplish Hypothesis 1, plasma levels of IL-12p70, IL-10, and TNF- $\alpha$  were examined from children with varying degrees of malaria disease severity, which was defined according to the density of parasitemia. To examine the cellular sources involved in IL-12p70, IL-10, and TNF- $\alpha$  production, the effect of *P. falciparum* malaria and malaria products (hemozoin) on mononuclear cell IL-12p70, IL-10, and TNF- $\alpha$  mRNA and soluble protein production was determined. To examine Hypothesis 2, the ability of endogenously produced IL-10 and TNF- $\alpha$  to suppress IL-12p70 production was examined using neutralizing antibodies

against IL-10 and TNF- $\alpha$ , respectively. Hypothesis 3 was examined by investigating the contribution of JAK/STAT signaling in the malaria-associated suppression of IL-12 using SOCS-3-specific siRNA. The Hypotheses of Specific Aim 1 have been addressed in the following manuscript which has been submitted to the *Journal of Immunology*.

**3.1. PRESENTATION OF MANUSCRIPT: HEMOZOIN-INDUCED IL-10 OVERPRODUCTION SUPPRESSES IL-12 INDEPENDENT OF UPREGULATED SOCS-3 EXPRESSION.**

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### 3.1.1. Abstract

Protective immunity to malaria is regulated in part by cytokines, such as interleukin (IL)-12, which is a pro-inflammatory cytokine important for initiating cell-mediated immunity. We have previously shown that children with severe malaria have suppressed systemic IL-12 production, and elevated levels of cytokines known to inhibit IL-12 such as, IL-10 and tumor necrosis factor (TNF)- $\alpha$ . Therefore, the production of IL-12, IL-10, and TNF- $\alpha$  in children with high-density parasitemia was investigated. Children with high-density parasitemia had decreased IL-12p70 and increased levels of IL-10 and TNF- $\alpha$ . Parasitic products such as hemozoin (malarial pigment) are known to increase monocyte-derived TNF- $\alpha$  and IL-10 production *in vitro*. We therefore investigated if ingestion of hemozoin by monocytes suppresses IL-12p70 through increased synthesis of IL-10 and/or TNF- $\alpha$ . Peripheral blood mononuclear cells (PBMC) and CD14<sup>+</sup> monocytes from healthy donors were stimulated with media alone (controls), lipopolysaccharide (LPS) and interferon (IFN)- $\gamma$ , or LPS and IFN- $\gamma$  in the presence of hemozoin (malarial pigment) or a synthetically prepared hemozoin,  $\beta$ -hematin. Ingestion of hemozoin and  $\beta$ -hematin by LPS- and IFN- $\gamma$ -stimulated cells suppressed soluble IL-12p70 and augmented soluble IL-10 and TNF- $\alpha$ . Real time RT-PCR showed that hemozoin suppressed induction of IL-12p40 transcripts, and increased IL-10 and TNF- $\alpha$  gene expression. Addition of IL-10 neutralizing antibodies restored hemozoin-induced suppression of IL-12p70, while TNF- $\alpha$  neutralizing antibodies had no effect on IL-12p70 production. Blockade of IL-10 in cultured PBMC from children with malaria increased IL-12p70 production. Hemozoin increased gene expression of suppressor of cytokine signaling (SOCS)-3, however, silencing of SOCS-3 expression using siRNA did not alter hemozoin-induced suppression of IL-12p40 transcripts.

The ratio of IL-10/IL-12 production was inversely related to malaria severity and dose-dependently associated with hemozoin. Taken together, these results suggest that ingestion of hemozoin by monocytes inhibits IL-12 gene products via up-regulation of IL-10 gene expression.

### **3.1.2. Introduction**

Malaria continues to represent a global health threat and is estimated to cause 1.5 to 2.7 million deaths annually (World Health Organization 1997). The majority of these deaths occur in children less than 5 years of age, due to their lack of naturally acquired immunity to malaria (Breman *et. al.* 2001). In areas of holoendemic *Plasmodium falciparum* transmission the primary clinical manifestations of severe malaria in young children are anemia, hyperparasitemia, and respiratory distress, and the mortality rate can be as high as 25-30% (Breman *et. al.* 2001). Furthermore, the prevalence of malaria in children 1 to 4 years of age residing in these areas is 83%, with malarial anemia (MA) being the most common clinical manifestation of malaria and CM occurring rarely (Bloland *et. al.* 1999).

The innate immune response to malaria is characterized by the production of interleukin (IL)-12 from mononuclear cells (Crutcher *et. al.* 1995). IL-12 is a pro-inflammatory cytokine important for the early activation of a type I immune response through enhanced differentiation of CD4<sup>+</sup> T cells into T<sub>H</sub>1 cells, and the subsequent production of interferon (IFN)- $\gamma$  (Crutcher *et. al.* 1995, Stevenson *et. al.* 1995). In circulating blood, monocytes are the primary producers of bioactive IL-12p70 (D'Andrea *et. al.* 1992), a heterodimer of IL-12p35 and IL-12p40 subunits encoded by separate genes (Kobayashi *et. al.* 1989). In cultured monocyte-derived macrophages,



IL-12p70 production is negatively regulated by pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$  (Ma *et. al.* 2000), and anti-inflammatory cytokines, such as IL-10 (Aste-Amezaga *et. al.* 1998), at the level of p40 gene transcription.

The ability to produce IL-12 appears to be protective in animal models of malaria. An early and sustained induction of IL-12 mediates resistance to severe malaria in mice infected with *P. chabaudi* (Sam *et. al.* 1999), which occurs, at least in part, through enhancement of erythropoiesis (Mohan *et. al.* 1998). Furthermore, administration of recombinant IL-12 (rIL-12) to mice infected with *P. yoelli* or rhesus macaques infected with *P. cynomolgi* results in 100% protection (Hoffman *et. al.* 1997, Sedegah *et. al.* 1994). Mice deficient for the IL-12p40 gene are unable to resolve parasitemia, and following treatment with rIL-12, the ability to clear parasitemia is restored (Su *et. al.* 2002). In murine splenic cells, suppression of IL-12 appears to be mediated by increased IL-10 production (Xu *et. al.* 2001), however, the regulatory effect of IL-10 on IL-12 production during human malaria is currently undefined.

Previous studies from our laboratories illustrated that Gabonese children with severe malaria [characterized primarily as high-density parasitemia (HDP)] have suppressed systemic production of IL-12, and elevated production of both TNF- $\alpha$  and IL-10, relative to children with mild disease (Luty *et. al.* 2000). Suppression of systemic IL-12 in individuals with severe malaria has recently been reported by others (Chaiyaroj *et. al.* 2004, Malaguarnera *et. al.* 2002, Malaguarnera *et. al.* 2002, Musumeci *et. al.* 2003). Furthermore, our studies in Gabonese children demonstrated that the ratio of IL-10/IL-12 is significantly higher in children with severe malaria compared to children with mild malaria (Perkins *et. al.* 2000), suggesting that the

relative production of these cytokines may be a pathogenic determinant in disease severity. Suppression of systemic IL-12 in children with severe malaria may be a consequence of reduced peripheral blood mononuclear cell (PBMC) IL-12 production; baseline and mitogen-stimulated IL-12 production is significantly lower in cultured PBMC from children with severe malaria relative to PBMC from children with mild disease (Luty *et. al.* 2000).

Phagocytosis of parasitic products, such as hemozoin (malarial pigment), influences cytokine and effector molecule production in cultured human PBMC (Biswas *et. al.* 2001, Keller *et. al.* 2004, Keller *et. al.* 2004, Mordmuller *et. al.* 1998, Pichyangkul *et. al.* 1994, Sherry *et. al.* 1995). Hemozoin is an insoluble coordination polymer of heme subunits formed during plasmodia-specific hemoglobin catabolism to avoid the toxic effects of heme (Slater 1992). Blood mononuclear cells rapidly phagocytose free hemozoin, which is released into the blood stream upon rupture of parasitized red blood cells (RBC) (Schwarzer *et. al.* 1998). Studies by Mordmuller *et al.* illustrate that monocytes stimulated with high concentrations of hemozoin have elevated TNF- $\alpha$  and IL-10 production (Mordmuller *et. al.* 1998). Suppression of systemic IL-12 in Gabonese children with malaria is also significantly associated with the number of hemozoin-containing circulating neutrophils and monocytes (Luty *et. al.* 2000).

Previous studies have examined several transcription factors, such as c-Rel, NF-kappaB, IFN regulatory factor-1, and ets-2, which are not involved in IL-10- and/or TNF- $\alpha$ -mediated suppression of IL-12p40 gene expression (Ma *et. al.* 2000). More recently, a family of Jak/Stat suppressors, collectively known as suppressors of cytokine signaling (SOCS), has been identified which can suppress LPS- or IFN- $\gamma$ -induced gene transcription, such as IL-12p40, through

blockade of the Jak/Stat signaling cascade [for review see (Larsen *et. al.* 2002)]. Furthermore, it has been shown that IL-10 inhibits activation of STAT1, which may occur through induction of SOCS-3 (Ito *et. al.* 1999), however, the association of SOCS-3 with suppression of IL-12p70 production has not been examined.

Since at the time of these experiments specific inhibitors of SOCS-3 were not available, we utilized small interfering RNA (siRNA) technology to silence SOCS-3 gene expression. siRNA relies on the binding of a complementary sequence of nucleotides to the transcript of interest, which targets the double stranded mRNA for degradation by the RNA-induced silencing complex (RISC) (Hammond *et. al.* 2001). This relies on 21-23mers which do not trigger the antiviral response in mammalian cells (Elbashir *et. al.* 2001, Elbashir *et. al.* 2001).

We hypothesized that hemozoin ingestion by circulating mononuclear phagocytes is a contributing factor of IL-12 suppression in malaria, which may be due to excessive IL-10 and/or TNF- $\alpha$  production. To examine this potential mechanism, plasma levels of IL-12p70, IL-10, and TNF- $\alpha$  were determined in aparasitemic children and children with low- or high-density parasitemia. Soluble IL-12p70, IL-10, and TNF- $\alpha$  production and the temporal profile of IL-12p35, IL-12p40, IL-10, and TNF- $\alpha$  mRNA induction was determined in PBMC stimulated with physiologic concentrations of hemozoin. We further characterized the regulatory effects of IL-10 and TNF- $\alpha$  on IL-12p70 production in cultured PBMC from healthy, malaria-naïve US donors stimulated with hemozoin, and from children with acute malaria, by blocking endogenous IL-10 or TNF- $\alpha$  with neutralizing antibodies. Silencing of suppressor of cytokine signaling (SOCS)-3 with small interfering (si)RNA on IL-12p70 production was also investigated.

### 3.1.3. Subjects and Methods

**3.1.3.1. Study Participants:** Children (n = 128, age 0 to 3 years) were recruited from a hospital-based longitudinal study at the Siaya District Hospital in Siaya District, western Kenya as part of our ongoing activities examining the genetic basis of severe malarial anemia (manuscript submitted). In this holoendemic area of malaria transmission, residents receive approximately 100-300 infective mosquito bites per year (Bloland *et. al.* 1999). The primary clinical manifestations of severe childhood malaria are severe anemia and hyperparasitemia (Bloland *et. al.* 1999). Children with cerebral malaria, a rare event in this high transmission setting (Bloland *et. al.* 1999), were excluded from the study. Heel/finger-prick blood (~100  $\mu$ L) was obtained to determine parasitemia status and Hb concentration. Peripheral blood smears were prepared and stained with Giemsa reagent and examined under oil immersion for malaria parasites. Asexual malaria parasites were counted against 300 leukocytes and parasite densities estimated assuming a count of 8,000 white blood cells per microliter of blood. Hb levels were measured using a HemoCue® system (HemoCue AB, Angelholm, Sweden). Children with malaria were given antimalarials and the appropriate supportive therapy as required. All blood samples were obtained prior to treatment with antimalarials. Based upon the presence and density of parasitemia, children were categorized into three groups; 1) healthy controls (HC, no detectable peripheral parasitemia and hemoglobin [(Hb)  $\geq$  11.0 g/dL], low-density parasitemia (LDP, parasitemia < 10,000 parasites/ $\mu$ L and any Hb concentration), high-density parasitemia (HDP, parasitemia  $\geq$  10,000 parasites/ $\mu$ L and any Hb concentration). Informed consent was obtained from the parents/guardians of participating children.

Healthy, malaria-naïve adult donors (n = 21) were recruited from the University of Pittsburgh, U.S.A. The study was approved by the ethics committees of the University of Pittsburgh Institutional Review Board and the Kenya Medical Ethical Board.

### **3.1.3.2. Isolation and Culture of Peripheral Blood Mononuclear Cells and CD14+ and**

**CD14- Cells:** For in vitro experiments in cells from U.S. adults (n = 21) or children with malaria (n = 12), venous blood (40 mL and < 3 mL, respectively) was drawn into EDTA-containing vials. PBMC were prepared using ficoll/Hypaque as described earlier (Weinberg *et al.* 1981), and, for experiments in cells from U.S. donors, CD14+ cells were isolated from PBMC fractions using CD14+ magnetic beads (Miltenyi Biotech, Auburn, CA) as per the manufacturer's protocol. PBMC and monocytes were plated at  $1 \times 10^6$  cells per mL in Dulbecco's modified Eagles medium (DMEM) containing 10% pooled human serum (heat inactivated at 56°C for 30 min). Cultures were stimulated with media alone (controls) or LPS [100 ng/mL (Alexis Corp., San Diego, CA)] and IFN- $\gamma$  [200 U/mL (BD Pharmingen, San Diego, CA)]. Cultured PBMC from healthy U.S. adults were also stimulated with LPS and IFN- $\gamma$  in the presence of hemozoin (10, 1.0, and 0.1  $\mu\text{g/mL}$ ) or  $\beta$ -hematin (10  $\mu\text{g/mL}$ ). The effect of endogenous IL-10 and TNF- $\alpha$  on IL-12p70 production was determined by the addition of neutralizing antibodies for IL-10 [0.1, 0.5, and 1.0  $\mu\text{g/mL}$  (R&D Systems, Minneapolis, MN)] and TNF- $\alpha$  [1.0, 10, and 100 ng/mL (R&D Systems, Minneapolis, MN)] or exogenous IL-10 [0.2, 2.0, and 20 ng/mL (Endogen, Woburn, MA)] and TNF- $\alpha$  [0.2, 2.0, and 20 ng/mL (BD Pharmingen, Bedford, MA)]. Concentrations of hemozoin used in the present studies were comparable to those in children with severe (10  $\mu\text{g/mL}$  hemozoin) and mild (1.0  $\mu\text{g/mL}$

hemozoin) malaria based on our previously calculated results (Keller *et. al.* 2004). Hemozoin and  $\beta$ -hematin preparations were extensively sonicated prior to addition to the cultures.

**3.1.3.3. Hemozoin and  $\beta$ -hematin Preparation:** Crude hemozoin was isolated as previously described (Keller *et. al.* 2004). Briefly, *in vitro* cultures of *P. falciparum*-infected red blood cells were cultured to a parasitemia of 3-5% containing predominately late trophozoites and early schizonts. Cultured red blood cells (RBC) were isolated and spun at 2,000 rpm for 10 min, and the resulting pellet was resuspended in 40 mL of 0.01 M PBS (pH 7.2) with 2 mL saponin for 10 min. The solution was then spun at 14,000 rpm for 15 min, and the pelleted material was washed in PBS until the resulting pellet was dark red and free from the white-colored RBC cellular components (4-7 times). The final pellet was dried, weighed, and resuspended in filter-sterilized H<sub>2</sub>O at a final concentration of 1.0 mg/mL. The final solution was extensively sonicated to disperse the hemozoin.

$\beta$ -hematin was formed in a 4.5 M acidic acetate solution at pH 4.5 by the method of Egan *et al.* (Egan *et. al.* 2001). Briefly, hemin chloride (Sigma, St. Louis, MO) was added to a 0.1 M solution of NaOH followed by the addition of HCl at 60°C. A solution of acetate was then added, and the mixture was incubated for 150 min at 60°C without stirring. The  $\beta$ -hematin was then spun at 14,000 rpm in a microcentrifuge, washed 3X with filter-sterilized H<sub>2</sub>O, and dried at 60°C under vacuum. The final pellet was weighed and then resuspended at 1.0 mg/mL in filter-sterilized H<sub>2</sub>O. The resuspended  $\beta$ -hematin was sonicated extensively to disperse the preparation. Under these preparation conditions,  $\beta$ -hematin has an identical infrared spectroscopy pattern as detergent-purified hemozoin, and  $\beta$ -hematin crystals appear

morphologically similar to natural hemozoin crystals as evidenced by scanning electron microscopy (Egan *et. al.* 2001). Endotoxin levels were found to be less than 0.01 EU/mL using the Limulus Amebocyte Lysate test (BioWhittaker, Walkersville, MD).

**3.1.3.4. IL-12p70, IL-10, and TNF- $\alpha$  Determination:** Plasma and supernatant concentrations of IL-12p70, IL-10, and TNF- $\alpha$  were determined by quantitative sandwich ELISA (BD Pharmingen, San Diego, CA), with a sensitivity of detection  $\geq 7.8$  pg/mL for each of the cytokines. 96-well plates were coated with an anti-cytokine specific monoclonal antibody followed by blocking for 1 hr and subsequent washing. Samples were incubated at room temperature for 2 hrs, washed, and incubated with a biotin-conjugated cytokine-specific detection antibody and a horseradish peroxidase-streptavidin conjugate. Plates were washed and incubated with TMB Substrate Solution (Pharmingen, San Diego, CA) for 30 min with protection from light. Absorbance was determined at 450 nm.

**3.1.3.5. Quantitative real time RT-PCR:** Total RNA was isolated from PBMC by the GITC method (Chomczynski *et. al.* 1987). Total RNA (1  $\mu$ g) was reverse transcribed into cDNA, and cytokine gene expression was analyzed by quantitative real time RT-PCR on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). cDNA (100 ng) was amplified in duplicate with specific primer/probe sets for IL-12p35, IL-12p40, IL-10, TNF- $\alpha$ , and SOCS-3 [Accession Numbers NM\_008351, NM\_002187, NM\_000572, NM\_000594, and NM\_003955, respectively (Applied Biosystems, Foster City, CA)]. To control for non-specific background fluorescence, no template controls were included in triplicate. An endogenous control gene,  $\beta$ -actin [Accession Number NM\_001101 (Applied Biosystems, Foster City, CA)],

was used as a reference gene to normalize cDNA loading between samples. Data were compared using the  $-\Delta\Delta C_T$  method as previously described (Keller *et. al.* 2004).

**3.1.3.6. Preparation and Nucleofection of siRNA:** GAPDH siRNA was prepared in vitro using the Silencer siRNA Construction Kit following the manufacturer's instructions (Ambion, Austin TX). SOCS-3 siRNA (target sequence: 3'-AAG ACC CAG TCT GGG ACC AAG AA-5') was purchased from Qiagen Inc. (Valencia, CA). PBMC were nucleofected using the Human T Cell Nucleofector Kit according to the manufacturer's specifications (Amaxa Biosystems, Gaithersburg, MD). PBMC were suspended in 100  $\mu$ L of nucleofection reagent and nucleofected with 5  $\mu$ g of SOCS-3 or GAPDH siRNA on the Nucleofector I (Amaxa Biosystems, Gaithersburg, MD). Nucleofected cells were then transferred to pre-warmed DMEM containing 10% pooled human serum. Cultures were stimulated with LPS and IFN- $\gamma$ , or LPS and IFN- $\gamma$  in the presence of a high dose (10  $\mu$ g/mL) of hemozoin.

**3.1.3.7. Statistical Analyses:** Supernatant concentrations of IL-12p70, IL-10, and TNF- $\alpha$  (pg/mL) and plasma concentrations of IL-12p70 (pg/mL) and IL-10 (pg/mL) were measured in triplicate at several different dilutions. IL-12p35, IL-12p40, IL-10, TNF- $\alpha$ , and SOCS-3 mRNA expression was measured in duplicate. Pairwise comparisons between conditions were performed by the Mann-Whitney U test. Multiple group comparisons were performed by the Kruskal-Wallis test. Chi-square analysis was used to determine differences between proportions. All tests were deemed significant at the  $P \leq 0.05$  level (all  $P$  values corrected for multiple comparisons when appropriate).



### 3.1.4. Results

**3.1.4.1. Participant Characteristics:** Children were enrolled as part of a longitudinal cohort study and categorized according to density of parasitemia. We have found that parasite density does not correlate with the degree of anemia in our current study population (Ong'echa *et. al.* 2005), and as such, these studies examine malaria disease severity based on hyperparasitemia. The clinical and laboratory characteristics of the study participants are shown in Table 2. There was no significant difference in age (mos) or gender between the groups (Table 2). Children in the LDP and HDP groups had significantly elevated admission temperatures ( $^{\circ}\text{C}$ ) and significantly lower Hb concentrations (g/dL) than the HC group ( $P < 0.01$ , and  $P < 0.001$ , respectively), however, these variables did not differ between the LDP and HDP groups (Table 2). Parasite density was significantly higher in the HDP group relative to the LDP group ( $P < 0.001$ , Table 2).

**3.1.4.2. Plasma Levels of IL-12p70, IL-10, and TNF- $\alpha$  in Children with Malaria:** We have previously observed suppression of IL-12p70 and increased IL-10 and TNF- $\alpha$  in plasma from children with severe malaria, where the primary determinant of severe disease was HDP (Luty *et. al.* 2000, Perkins *et. al.* 2000). Therefore, we examined the relationship between disease severity (i.e., HDP) and cytokine levels in plasma from children with malaria. Plasma levels of IL-12p70 were significantly lower in the HDP group relative to HC group ( $P < 0.01$ ), and non-significantly lower than the LDP group (Figure 4A). Plasma IL-10 levels were significantly higher in the LDP and HDP groups relative to the HC group ( $P < 0.01$  and  $P < 0.01$ , respectively, Figure 1B). Furthermore, IL-10 was significantly elevated in the HDP group

relative to LDP ( $P < 0.05$ , Figure 1B). TNF- $\alpha$  was significantly elevated in the LDP and HDP groups relative to HC ( $P < 0.01$  and  $P < 0.01$ , respectively, Figure 4C), and significantly increased in the HDP group relative to LDP ( $P < 0.05$ , Figure 4C).

**3.1.4.3. Effect of Hemozoin on IL-12p70, IL-10, and TNF- $\alpha$  Production:** Since children with HDP had decreased IL-12p70 and increased IL-10 and TNF- $\alpha$ , we hypothesized that parasitic products, such as hemozoin, may be responsible for dysregulation of cytokine production. To examine the effect of hemozoin on bioactive IL-12p70, IL-10, and TNF- $\alpha$ , PBMC or CD14<sup>+</sup> monocytes were cultured from healthy, malaria-naïve adults and stimulated with media alone (control), LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), or LPS and IFN- $\gamma$  in the presence of physiologically relevant concentrations of hemozoin (10, 1.0, and 0.1  $\mu\text{g/mL}$ ) or  $\beta$ -hematin (10  $\mu\text{g/mL}$ ). Since PBMC from healthy individuals cultured under baseline conditions produce negligible amounts of IL-12, cell cultures were stimulated with LPS and IFN- $\gamma$  to promote IL-12 synthesis. Stimulation of PBMC or monocytes with LPS and IFN- $\gamma$  significantly elevated IL-12p70 production at 48 hrs in culture ( $P < 0.01$  and  $P < 0.01$ , respectively), and the addition of a high dose (10  $\mu\text{g/mL}$ ) of hemozoin or  $\beta$ -hematin significantly decreased LPS- and IFN- $\gamma$ -promoted IL-12p70 production ( $P < 0.05$  and  $P < 0.05$ , respectively, Figures 5A and B). IL-12p70 was not detectable in culture supernatants from hemozoin- or  $\beta$ -hematin-treated PBMC in the absence of LPS and IFN- $\gamma$  stimulation (data not shown). LPS and IFN- $\gamma$  stimulation significantly elevated PBMC- and monocyte-derived IL-10 production ( $P < 0.01$  and  $P < 0.01$ , respectively), and a high dose of hemozoin or  $\beta$ -hematin significantly augmented LPS- and IFN- $\gamma$ -induced IL-10 ( $P < 0.05$  and  $P < 0.05$ , respectively, Figures 5C and D). Stimulation of PBMC

and monocytes significantly augmented TNF- $\alpha$  production ( $P < 0.05$  and  $P < 0.05$ , respectively), and addition of a high dose of hemozoin or  $\beta$ -hematin significantly increased TNF- $\alpha$  in cultured PBMC and monocytes ( $P < 0.05$  and  $P < 0.05$ , respectively, Figures 5E and F). IL-12p70 and IL-10 were not detectable in cultured CD14<sup>-</sup> cells (Figures 5B and D), and CD14<sup>-</sup> cell TNF- $\alpha$  production was not altered in any culture condition examined (Figure 5E). Results presented here illustrate that physiological concentrations of hemozoin and  $\beta$ -hematin suppress PBMC- and monocyte-derived IL-12p70 production, and augment IL-10 and TNF- $\alpha$ .

#### **3.1.4.4. Effect of Hemozoin on the Time-dependent Production of IL-12p35, IL-12p40, IL-**

**10, and TNF- $\alpha$  Transcripts:** Regulation of cytokine production during an inflammatory event occurs primarily at the level of *de novo* transcription, therefore, the effect of hemozoin on the induction and temporal profile of PBMC mRNA expression for IL-12p35, IL-12p40, IL-10 and TNF- $\alpha$  was examined using real time RT-PCR. PBMC were stimulated with media alone (controls), LPS and IFN- $\gamma$ , or LPS and IFN- $\gamma$  in the presence of hemozoin (10  $\mu$ g/mL). Stimulation of PBMC with LPS and IFN- $\gamma$  significantly augmented IL-12p35 mRNA expression at all time points examined ( $P < 0.05$ , Figure 6A), and the addition of hemozoin did not significantly alter IL-12p35 mRNA production at any time point examined (Figure 6A). Stimulated PBMC produced significantly higher levels of IL-12p40 mRNA at 8 and 24 hrs ( $P < 0.01$ ), and hemozoin significantly suppressed IL-12p40 mRNA at these time points ( $P < 0.01$ , Figure 6B). LPS- and IFN- $\gamma$  significantly augmented IL-10 mRNA at 8, 24, and 48 hrs ( $P < 0.01$ ), and hemozoin significantly enhanced IL-10 transcript expression at 24 hrs ( $P < 0.01$ , Figure 6C). TNF- $\alpha$  mRNA expression was significantly enhanced in LPS- and IFN- $\gamma$ -stimulated PBMC at 2, 4, and 8 hrs ( $P < 0.01$ ), and hemozoin significantly elevated TNF- $\alpha$  mRNA

expression at these time points ( $P < 0.01$ , Figure 6D). Taken together, results presented here illustrate that dysregulation of soluble IL-12p70, IL-10, and TNF- $\alpha$  production by hemozoin is a consequence of suppressed IL-12p40 mRNA expression and increased IL-10 and TNF- $\alpha$  mRNA expression.

**3.1.4.5. Effect of IL-10 and TNF- $\alpha$  on Hemozoin-induced IL-12p70 Suppression:** Since exogenous IL-10 and TNF- $\alpha$  suppress IL-12 production in monocyte-derived macrophages (Aste-Amezaga *et. al.* 1998, Ma *et. al.* 2000), we postulated that hemozoin-induced increases in endogenous IL-10 and/or TNF- $\alpha$  may suppress IL-12p70 production in cultured PBMC. To test this, neutralizing antibodies for IL-10 (0.1, 0.5, and 1.0  $\mu\text{g/mL}$ ) and TNF- $\alpha$  (1.0, 10, and 100 ng/mL), or exogenous IL-10 (0.2, 2.0, and 20 ng/mL) and TNF- $\alpha$  (0.2, 2.0, and 20 ng/mL), were added to LPS- and IFN- $\gamma$ -stimulated PBMC in the presence of a high dose (10  $\mu\text{g/mL}$ ) of hemozoin, and IL-12p70 production was measured in culture supernatants at 48 hrs. Stimulation of PBMC with LPS and IFN- $\gamma$  significantly increased IL-12p70 production ( $P < 0.05$ ), which was significantly decreased by hemozoin ( $P < 0.05$ , Figure 7A). In both LPS- and IFN- $\gamma$ -stimulated cells, and stimulated cells treated with hemozoin, IL-12p70 production was significantly increased upon the addition of an intermediate (0.5  $\mu\text{g/mL}$ ) and a high (1.0  $\mu\text{g/mL}$ ) dose of IL-10 neutralizing antibody ( $P < 0.05$  and  $P < 0.05$ , respectively), and these levels did not significantly differ from each other (Figure 7A). Supernatant IL-10 levels were dose-dependently lower in the presence of increasing doses of IL-10 neutralizing antibody in stimulated cells and stimulated cells treated with hemozoin (Figure 7B). The addition of exogenous IL-10 dose-dependently suppressed IL-12p70 production in LPS- and IFN- $\gamma$ -stimulated PBMC in the presence and absence of hemozoin (Figure 7C).

The addition of all examined doses of TNF- $\alpha$  neutralizing antibody to LPS- and IFN- $\gamma$ -stimulated PBMC, and stimulated cells treated with hemozoin, did not significantly alter IL-12p70 production compared to stimulated conditions, and stimulated cells treated with hemozoin, in the absence of antibody, respectively (Figure 7D). Addition of hemozoin to LPS- and IFN- $\gamma$ -stimulated PBMC significantly suppressed IL-12p70 production in the presence of all examined doses of TNF- $\alpha$  neutralizing antibody ( $P < 0.05$  for all conditions, Figure 7D). Supernatant TNF- $\alpha$  levels were dose-dependently decreased by increasing doses of TNF- $\alpha$  neutralizing antibody in both stimulated cells and stimulated cells in the presence of hemozoin (Figure 7E). Addition of hemozoin to LPS- and IFN- $\gamma$ -stimulated PBMC in the presence of all examined doses of exogenous TNF- $\alpha$  significantly suppressed IL-12p70 production ( $P < 0.05$  for all conditions, Figure 7F). Taken together, these results illustrate that IL-12p70 production in hemozoin-treated PBMC is restored by neutralizing endogenously produced IL-10, but not by blocking endogenous TNF- $\alpha$ .

#### **3.1.4.6. Effect of IL-10 on IL-12p70 in Cultured PBMC from Children with Malaria:**

Since hemozoin-induced IL-10 production mediated the suppression of IL-12p70 in cultured PBMC from healthy, malaria-naïve donors, we wished to determine if neutralizing IL-10 in cultured PBMC from children with malaria would also cause increased IL-12p70 production. To examine this potential mechanism, PBMC were cultured from children with malaria and stimulated with media alone (controls), LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), or LPS and IFN- $\gamma$  in the presence of IL-10 neutralizing antibodies (0.5  $\mu$ g/mL) or exogenous IL-10 (2.0 ng/mL). LPS and IFN- $\gamma$  significantly increased IL-12p70 production ( $P < 0.05$ , Figure 8).

Addition of IL-10 antibodies caused a significant increase in IL-12p70 over stimulated conditions ( $P < 0.05$ ), and exogenous IL-10 significantly decreased IL-12p70 production ( $P < 0.05$ , Figure 8). Thus, neutralizing endogenously produced IL-10 in cultured PBMC from children with malaria augments IL-12p70 production.

**3.1.4.7. Effect of Hemozoin on SOCS-3 Transcripts:** Previous studies have shown that IL-10 induces SOCS-3 (Ito *et. al.* 1999), which is known to suppress the induction of inflammatory genes in response to LPS (Berlato *et. al.* 2002) or IFN- $\gamma$  (Ito *et. al.* 1999), via blockade of the JAK-STAT signaling cascade [for review see (Larsen *et. al.* 2002)]. To determine if hemozoin upregulates SOCS-3 gene expression, real time RT-PCR was used to examine the temporal profile of SOCS-3 message in PBMC stimulated with media alone (controls), LPS and IFN- $\gamma$ , or LPS and IFN- $\gamma$  in the presence of hemozoin (10  $\mu\text{g/mL}$ ). LPS and IFN- $\gamma$  stimulation significantly induced SOCS-3 mRNA at 8 and 24 hrs ( $P < 0.05$  and  $P < 0.05$ , respectively), and the addition of hemozoin significantly augmented SOCS-3 message at these time points ( $P < 0.05$  and  $P < 0.05$ , respectively, Figure 9).

**3.1.4.8. Effect of SOCS-3 siRNA on Hemozoin-induced Suppression of IL-12 Transcripts:** Since hemozoin induced SOCS-3 transcripts, we postulated that suppression of SOCS-3 may restore IL-12p40 transcripts in hemozoin-treated PBMC. Using siRNA technology, we determined the effect of silencing SOCS-3 expression on hemozoin-induced suppression of IL-12p40 transcripts at 24 hrs in culture, the time when SOCS-3 was induced by hemozoin. PBMC were nucleofected with siRNA specific for SOCS-3 or a control siRNA specific for GAPDH. Nucleofected cells and nucleofection control cells (i.e., PBMC

nucleofected in the absence of siRNA) were stimulated with media alone (controls), LPS and IFN- $\gamma$ , or LPS and IFN- $\gamma$  in the presence of hemozoin (10  $\mu\text{g}/\text{mL}$ ). PBMC were harvested at 24 hrs for determination of SOCS-3, GAPDH, and IL-12p40 transcripts using real time RT-PCR. Stimulation of PBMC with LPS and IFN- $\gamma$  significantly augmented SOCS-3 transcripts ( $P < 0.05$ ), and the addition of hemozoin further increased SOCS-3 expression ( $P < 0.05$ , Figure 10A). SOCS-3 siRNA significantly decreased SOCS-3 transcripts in both LPS and IFN- $\gamma$ -stimulated cells, and stimulated cells treated with hemozoin ( $P < 0.05$  and  $P < 0.05$ , respectively, Figure 5A). GAPDH siRNA did not significantly alter SOCS-3 transcripts under any condition examined (Figure 10A).

LPS and IFN- $\gamma$ , or LPS and IFN- $\gamma$  in the presence of hemozoin, did not significantly alter GAPDH transcripts, and nucleofection of PBMC with SOCS-3 siRNA did not significantly alter GAPDH gene expression under any condition examined (Figure 10B). GAPDH transcripts were significantly lower in both stimulated PBMC and hemozoin-treated PBMC nucleofected with GAPDH siRNA ( $P < 0.05$  and  $P < 0.05$ , respectively, Figure 10B).

LPS and IFN- $\gamma$  significantly augmented IL-12p40 gene expression ( $P < 0.05$ ), and hemozoin significantly decreased IL-12p40 transcript levels ( $P < 0.05$ , Figure 11C). IL-12p40 gene expression was not significantly altered by SOCS-3 or GAPDH siRNA in LPS- and IFN- $\gamma$ -stimulated PBMC, or stimulated PBMC treated with hemozoin (Figure 11C). Data presented here illustrate that hemozoin-induced suppression of IL-12p40 transcripts is not mediated through increased SOCS-3 gene expression.

### 3.1.5. Discussion

High IL-12 production in malaria appears to promote a protective response, at least in part, through activation of IFN- $\gamma$  which initiates the immune cascade (Crutcher *et. al.* 1995, Stevenson *et. al.* 1995). Decreased production of IL-12 in murine models of malaria has been associated with severe disease and higher mortality (Sam *et. al.* 1999), and reduced erythropoiesis (Mohan *et. al.* 1998). In human malaria, plasma levels of IL-12 are lower in patients with severe malaria relative to patients with mild malaria (Chaiyaroj *et. al.* 2004, Luty *et. al.* 2000, Malaguarnera *et. al.* 2002, Malaguarnera *et. al.* 2002, Musumeci *et. al.* 2003, Perkins *et. al.* 2000), which as reported here appears to be due, at least in part, to increased levels of parasitemia. In our previous study, suppression of IL-12 was associated with an increased percentage of neutrophils and monocytes containing hemozoin (Luty *et. al.* 2000). This is further illustrated by the fact that cultured PBMC from children with severe malaria, who had higher density parasitemias than age-matched children with mild malaria, produced lower levels of baseline- and mitogen-induced IL-12 (Luty *et. al.* 2000), suggesting that a phagocytosed parasitic product(s) encountered *in vivo* may be responsible for suppression of IL-12.

Since monocytes are one of the major cellular sources of systemic IL-12p40, and thus of circulating IL-12p70 (D'Andrea *et. al.* 1992), we hypothesized that ingestion of hemozoin by blood mononuclear cells was responsible for suppression of IL-12p70. Here we show that ingestion of physiologically relevant concentrations of crude hemozoin significantly reduced LPS- and IFN- $\gamma$ -promoted PBMC production of bioactive IL-12p70 at the level of p40 transcription. Furthermore, hemozoin suppressed IL-12p70 production in freshly isolated CD14<sup>+</sup> monocytes, while cultured CD14<sup>-</sup> cells (mainly CD4 and CD8 T cells and B cells) failed



to produce detectable amounts of IL-12p70 in response to LPS and IFN- $\gamma$ . Previous studies have shown that ingestion of detergent purified hemozoin by activated dendritic cells (DCs) upregulates IL-12 production (Coban *et. al.* 2002), however, our unpublished observations have shown that hemozoin inhibits maturation and cytokine production in cultured monocyte-derived DC (Keller *et. al.*, unpublished observations). Also, it is important to note that mature DCs comprise approximately 0.7% of blood mononuclear cells in children with severe malaria (Urban *et. al.* 2001), and would likely not contribute to the overall circulating IL-12p70 production in children with severe disease. Furthermore, recent studies have shown that DC maturation is inhibited by crude lysates of hemozoin (Skorokhod *et. al.* 2004), suggesting negative implications of hemozoin on DCs during human malaria.

Regulation of IL-12 production appears to be important for the immunopathogenesis of other protozoan infections. The *Leishmania major* cell surface molecule lipophosphoglycan (LPG) selectively inhibits IL-12p40 transcription in activated murine macrophages independent of NF- $\kappa$ B signaling, but does not alter production of other pro-inflammatory mediators, such as TNF- $\alpha$  (Piedrafita *et. al.* 1999). In murine models of *Toxoplasma gondii*, early mortality of IL-10-deficient mice is delayed by neutralizing endogenous IL-12 (Neyer *et. al.* 1997). Viral infections, such as measles and HIV, are also associated with suppressed IL-12 production (Chehimi *et. al.* 1994, Chougnet *et. al.* 1996), and in the case of measles virus, can actively inhibit IL-12 production in mononuclear cells (Karp *et. al.* 1996). Therefore, hemozoin-induced suppression of IL-12p70 may serve to protect *Plasmodium* parasites from immune clearance by down-regulating Type 1-mediated responses.

In agreement with previous studies (Biswas *et. al.* 2001, Mordmuller *et. al.* 1998, Pichyangkul *et. al.* 1994, Sherry *et. al.* 1995), results presented here show that ingestion of hemozoin or  $\beta$ -hematin by cultured PBMC augments TNF- $\alpha$  and IL-10 production. Hemozoin-induced increases in IL-10 and TNF- $\alpha$  production appear to be regulated at the level of transcription. Furthermore, the present studies show that hemozoin augments LPS- and IFN- $\gamma$ -induced TNF- $\alpha$  and IL-10 production in isolated CD14<sup>+</sup> monocytes, while the CD14<sup>-</sup> fraction of cultured PBMC do not produce these cytokines in response to LPS and IFN- $\gamma$  or hemozoin stimulation. Although CD3<sup>+</sup> T-cells have been shown to influence the early immune response to *P. falciparum*-infected RBC (Scragg *et. al.* 1999), the response to phagocytosis of free hemozoin appears to be independent of direct T-cell interactions with monocyte/macrophages. Therefore, it appears that phagocytosis of hemozoin by cultured human monocytes suppresses IL-12p70, and augments TNF- $\alpha$  and IL-10 production.

IL-12 synthesis is inhibited by pro- and anti-inflammatory cytokines, such as TNF- $\alpha$  and IL-10, respectively (Aste-Amezaga *et. al.* 1998, Ma *et. al.* 2000). Here we show for the first time in human mononuclear cells that hemozoin-induced over-expression of IL-10, but not TNF- $\alpha$ , is responsible for suppression of IL-12p70. Previous studies in cultured murine splenic cells from mice infected with malaria showed that suppression of IL-12 was due to increased IL-10 production (Xu *et. al.* 2001), which, as suggested by the present studies, may have been due to ingestion of hemozoin by these cells. The negative regulatory action of IL-10 on IL-12 production is also evident in chronic diseases, such as systemic lupus erythematosus (SLE), where suppressed IL-12 production in PBMC from patients with SLE is restored by blocking endogenous IL-10 (Liu *et. al.* 1998).

Although we found that overproduction of IL-10 is responsible for suppression of IL-12p70, other factors may still be involved in dysregulation of IL-12 during human malaria. Cytokines such as TGF- $\beta$ 1 [for review see (Wahl 1994)], and effector molecules such as PGE<sub>2</sub> [for review see (van der Pouw Kraan *et. al.* 1995)], are known to down-regulate the pro-inflammatory response, however, our previous studies show that both molecules are suppressed in children with severe malaria (Perkins *et. al.* 2001, Perkins *et. al.* 2000), suggesting that increased production of these anti-inflammatory cytokines is not responsible for suppression of IL-12 in childhood malaria. Furthermore, our previous *in vitro* studies show that hemozoin significantly suppressed LPS- and IFN- $\gamma$ -promoted PGE<sub>2</sub> production in PBMC from healthy donors (Keller *et. al.* 2004). Therefore, the cellular mechanism of IL-12 suppression in malaria is most likely not due to increased TGF- $\beta$ 1 or PGE<sub>2</sub> production. Taken together, these results illustrate that hemozoin-induced IL-10 overproduction from human PBMC suppresses IL-12p70 production.

Increased production of bioactive IL-12p70 is dependent on *de novo* induction of IL-12p40 gene expression (Kobayashi *et. al.* 1989), therefore, we postulated that hemozoin-induced IL-10 overproduction was suppressing IL-12 through dysregulation of a transcription factor(s). Previous studies have identified several transcription factors, such as c-Rel, NF- $\kappa$ B, IFN regulatory factor-1, and ets-2, which are not involved in suppression of IL-12p40 (Ma *et. al.* 2000). More recently, a family of Jak/Stat suppressors, collectively known as SOCS, has been identified which can suppress LPS- or IFN- $\gamma$ -induced genes through blockade of the Jak/Stat signaling cascade [for review see (Larsen *et. al.* 2002)]. Furthermore, it has been shown that IL-10 inhibits activation of STAT1, which may occur through induction of SOCS-3 (Ito *et. al.*

1999). Using real time RT-PCR, we show here that hemozoin augments levels of SOCS-3 transcripts, however, silencing of SOCS-3 expression using siRNA illustrated that hemozoin-induced expression of SOCS-3 was not responsible for suppression of IL-12p40 transcripts. Although SOCS-3 was not responsible for suppression of IL-12p40 transcripts, it is likely that alterations in signaling cascades, such as the JAK/STAT pathway, by over-expression of IL-10 may be responsible for suppression of IL-12p70 in hemozoin-treated PBMC.

In the present studies, we propose a model of IL-12 suppression in human malaria in which ingestion of hemozoin, as a consequence of elevated parasitemia, by circulating monocytes induces over-expression of IL-10 which in turn inhibits *de novo* IL-12p40 transcription. Although other factors likely play a role in suppression of IL-12 during human malaria, results presented here illustrate that hemozoin-induced suppression of IL-12 can be corrected by blocking endogenous levels of IL-10. Although current research is focused on designing/implementing vaccines towards malaria, the present studies suggest that suppression of IL-12 by hemozoin may hinder the efficacy of novel vaccines.

**Table 2. Clinical and laboratory characteristics of study participants.**

	HC	LDP	HDP	<i>P</i>
No. of participants	28	35	65	
Age, <i>mos.</i>	11.86 (1.39)	12.90 (1.17)	11.60 (0.76)	0.62 <sup>a</sup>
Sex, <i>no.</i> (%)				
Male	12 (43)	19 (54)	27 (42)	0.45 <sup>b</sup>
Female	16 (57)	16 (46)	38 (58)	
Admission temperature, °C	36.84 (0.17)	37.81 (0.19)	37.58 (0.13)	< 0.01 <sup>a</sup>
Hemoglobin, <i>g/dL</i>	11.53 (0.17)	7.45 (0.38)	7.57 (0.28)	< 0.001 <sup>a</sup>
Parasitemia, <i>parasites/μL</i>	0	4,636 (498)	41,121 (4,012)	< 0.001 <sup>c</sup>

Children (n = 128) were recruited as part of a hospital-based longitudinal study and categorized according to parasitemia status; healthy controls (HC, absence of peripheral parasitemia and Hb  $\geq$  11.0 g/dL), low-density parasitemia (LDP, parasitemia < 10,000 parasites/ $\mu$ L and any Hb concentration), high-density parasitemia (HDP, parasitemia  $\geq$  10,000 parasites/ $\mu$ L and any Hb concentration). Heel/finger-prick blood (~100  $\mu$ L) was obtained to determine parasitemia status and Hb concentration. Peripheral blood smears were prepared and stained with Giemsa reagent and examined under oil immersion for malaria parasites. Asexual malaria parasites were counted against 300 leukocytes and parasite densities estimated assuming a count of 8,000 white blood cells per microliter of blood. Hb levels were measured using a HemoCue® system (HemoCue AB, Angelholm, Sweden). Data are presented as the mean (SEM) unless otherwise noted.

<sup>a</sup>Statistical significance determined by Kruskal-Wallis test.

<sup>b</sup>Statistical significance determined by Chi-square analysis.

<sup>c</sup>Statistical significance determined by Mann-Whitney U test.

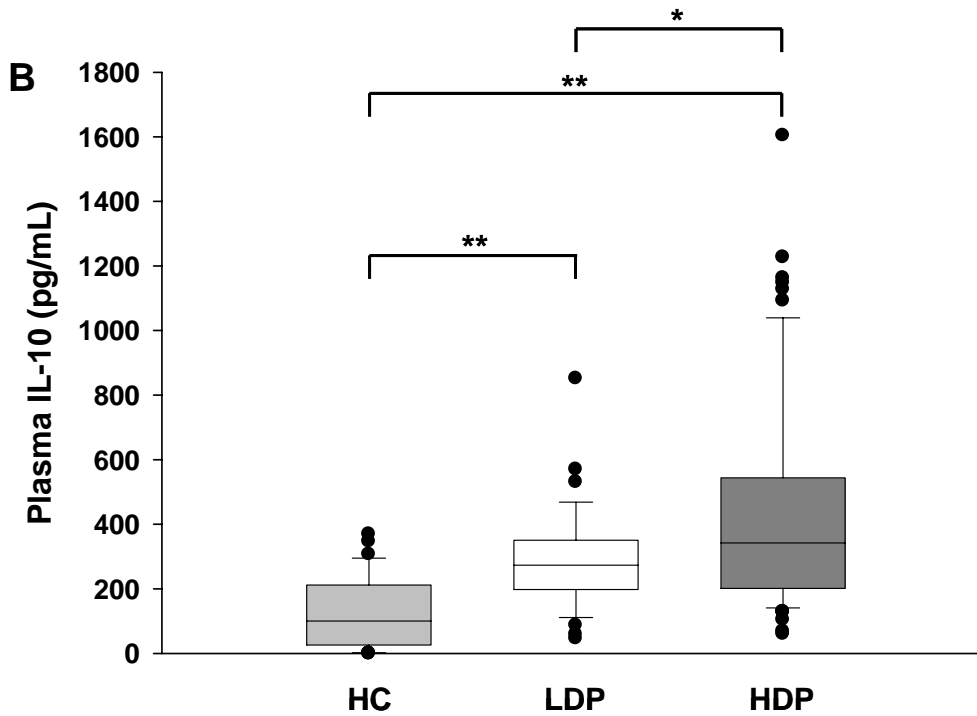
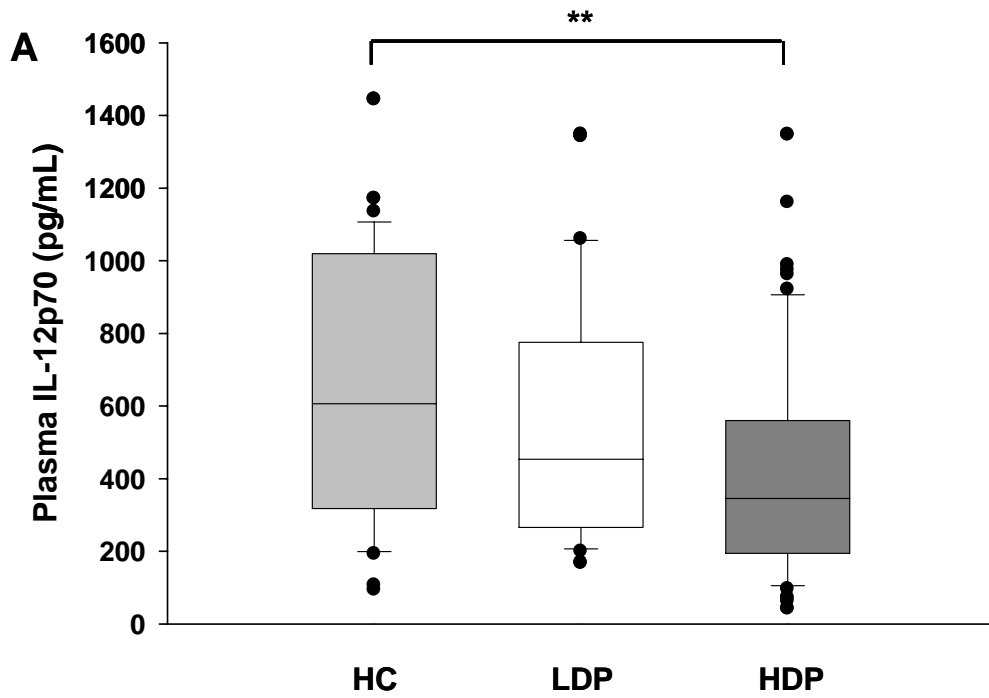


Figure 4. IL-12p70, IL-10, and TNF- $\alpha$  levels in plasma from children with varying degrees of malaria severity.

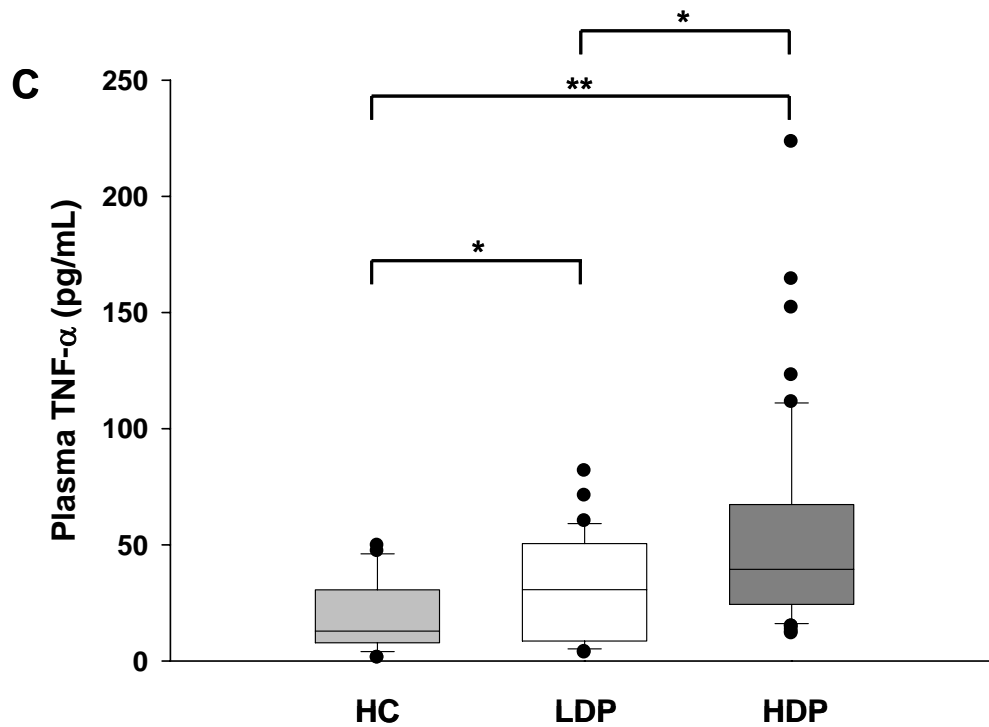


Figure 4. *IL-12p70, IL-10, and TNF- $\alpha$  levels in plasma from children with varying degrees of malaria severity.* Plasma was obtained from malaria-exposed, healthy children (n = 28), children with low-density parasitemia (LDP, n = 35), or children with high-density parasitemia (HDP, n = 65) for (A) *IL-12p70*, (B) *IL-10*, and (C) *TNF- $\alpha$*  determination. The box represents the interquartile range, the line represents the median, the whiskers represent 95% CI, and the symbols are outliers. Statistical significance was determined by the Mann-Whitney U test, \* $P < 0.05$ , \*\* $P < 0.01$ , (all  $P$  values corrected for multiple comparisons).

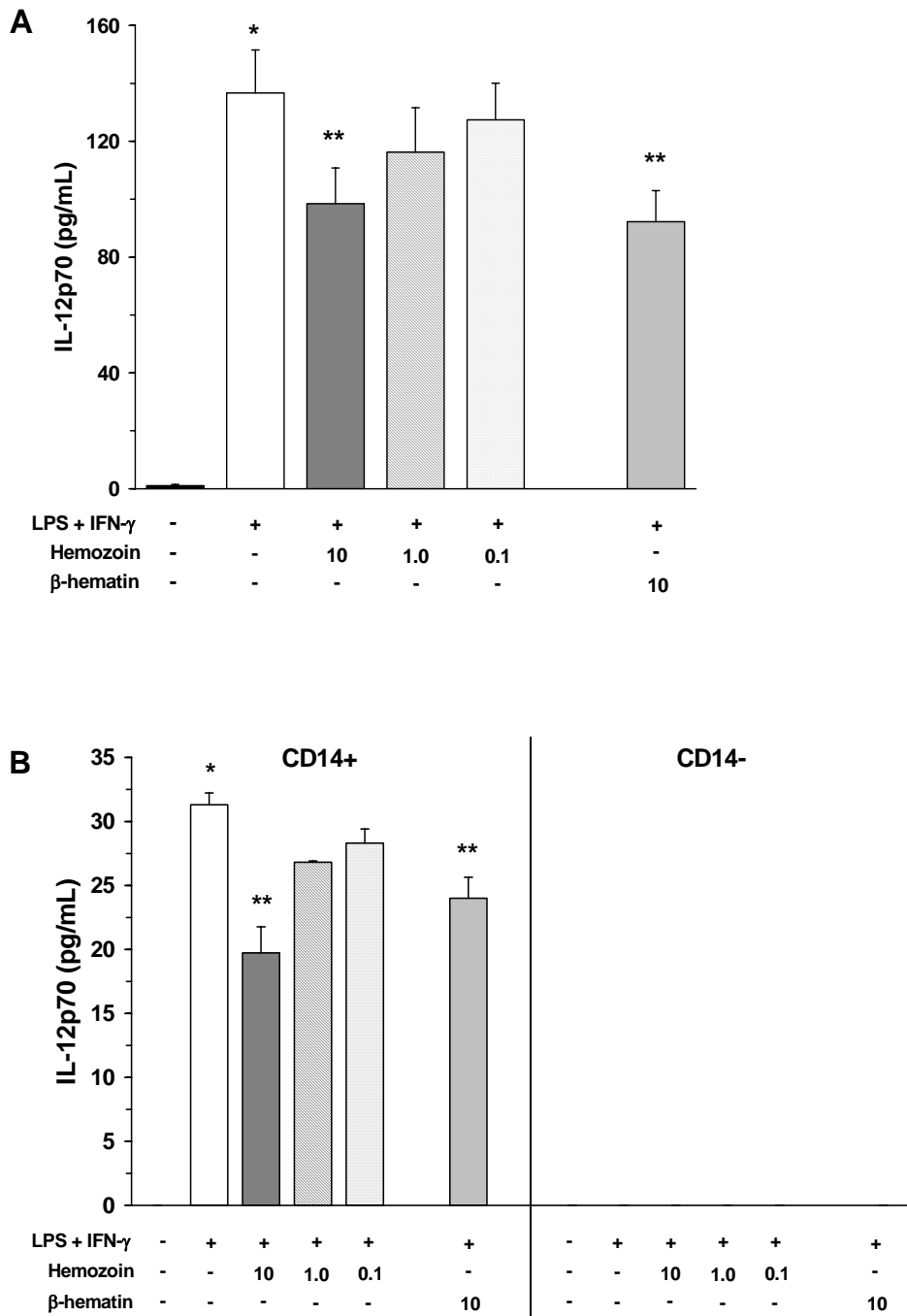


Figure 5. Effect of hemozoin on IL-12p70, IL-10, and TNF- $\alpha$  production in cultured PBMC and monocytes.



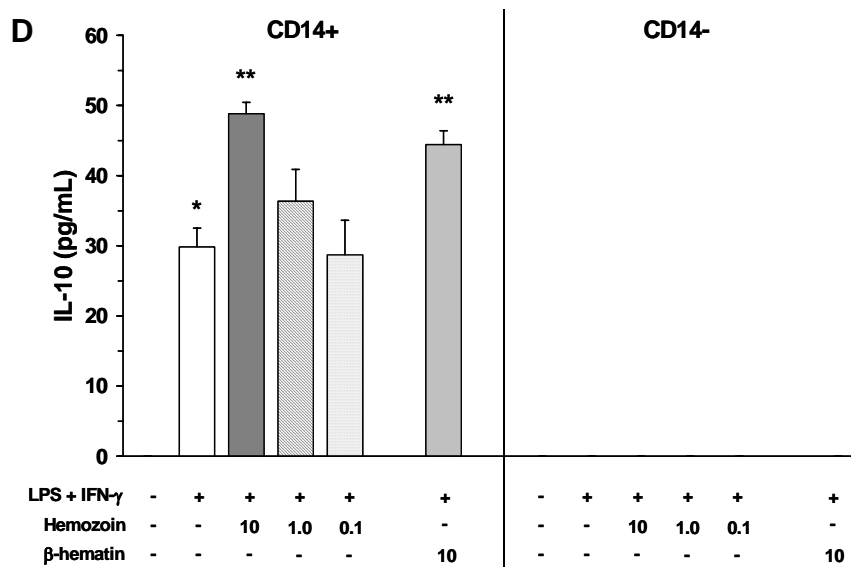
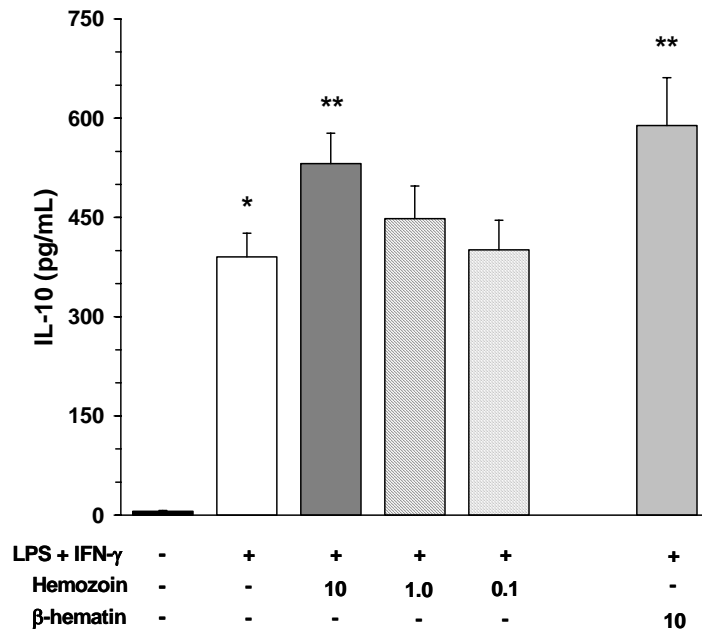


Figure 5. Effect of hemozoin on IL-12p70, IL-10, and TNF- $\alpha$  production in cultured PBMC and monocytes.

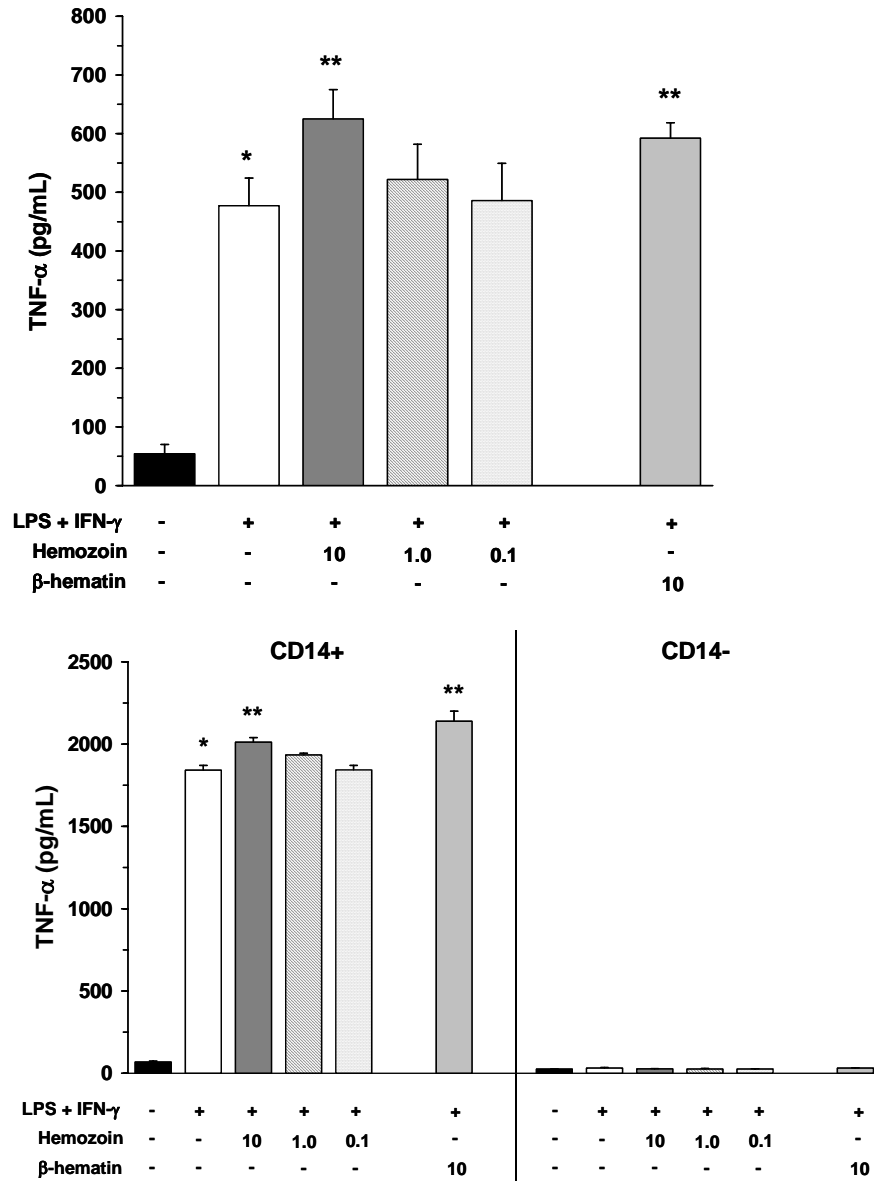


Figure 5. Effect of hemozoin on IL-12p70, IL-10, and TNF- $\alpha$  production in cultured PBMC and monocytes. (A, C, and E) PBMC ( $1 \times 10^6$  cells/mL,  $n = 15$ ) or (B, D, and F) CD14<sup>+</sup> and CD14<sup>-</sup> cells ( $1 \times 10^6$  cells/mL,  $n = 3$ ) from healthy, malaria-naïve donors were stimulated with media alone, LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), or LPS and IFN- $\gamma$  in the presence of varying concentrations of hemozoin (10, 1.0, and 0.1  $\mu$ g/mL) or  $\beta$ -hematin (10  $\mu$ g/mL). Supernatants were removed at 48 hrs for (A and B) IL-12p70, (C and D) IL-10, and (E and F) TNF- $\alpha$  determination. Values are the mean  $\pm$  SEM. Statistical significance was determined by Mann-Whitney U test, \* $P < 0.05$  compared to unstimulated conditions, \*\* $P < 0.05$  compared to LPS and IFN- $\gamma$  stimulation.

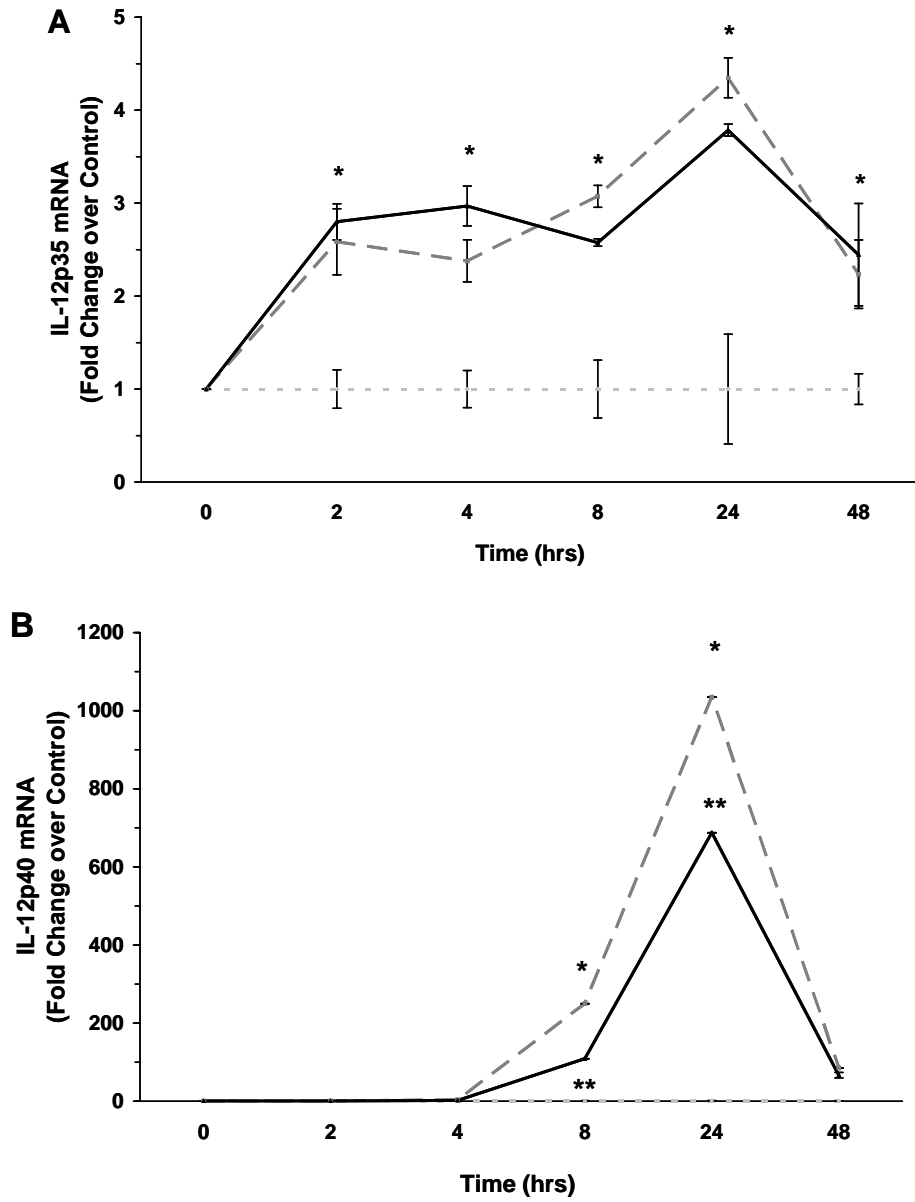


Figure 6. Temporal effect of hemozoin on IL-12p35, IL-12p40, IL-10, and TNF- $\alpha$  transcripts.

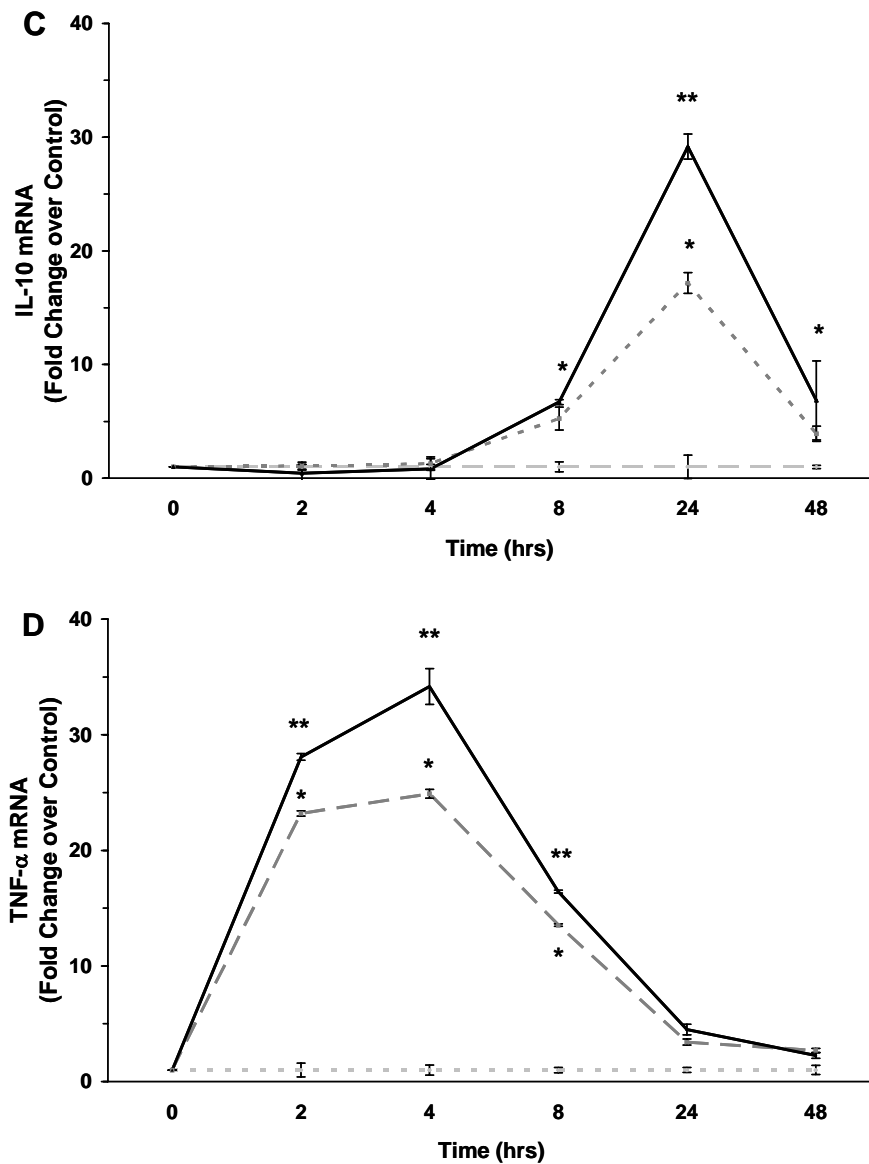


Figure 6. Temporal effect of hemozoin on IL-12p35, IL-12p40, IL-10, and TNF- $\alpha$  transcripts. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone (dashed black line), LPS (100 ng/mL) and IFN- $\gamma$  [200 U/mL, (dashed gray line)], or LPS and IFN- $\gamma$  in the presence of hemozoin [10  $\mu$ g/mL, (solid black line)]. Cells were collected at 2, 4, 8, 24, and 48 hrs for (A) IL-12p35, (B) IL-12p40, (C) IL-10, and (D) TNF- $\alpha$  mRNA determination by real time RT-PCR. Values are the mean  $\pm$  SEM,  $n = 3$ . Statistical significance was determined by Mann-Whitney U test. \* $P < 0.01$  compared to unstimulated conditions, \*\* $P < 0.01$  compared to LPS and IFN- $\gamma$  stimulation.

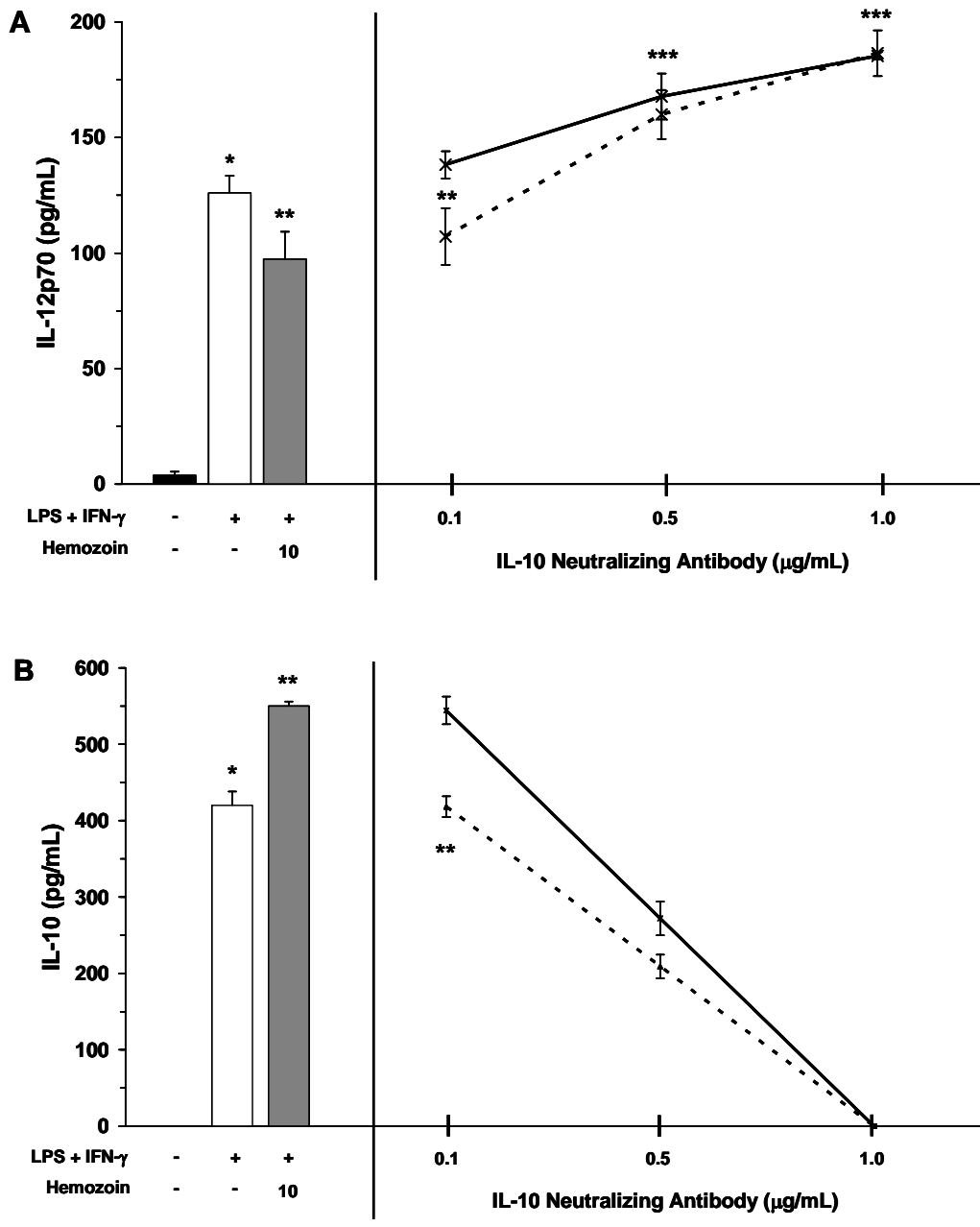


Figure 7. Effect of IL-10 and TNF- $\alpha$  on hemozoin-induced IL-12p70 suppression.

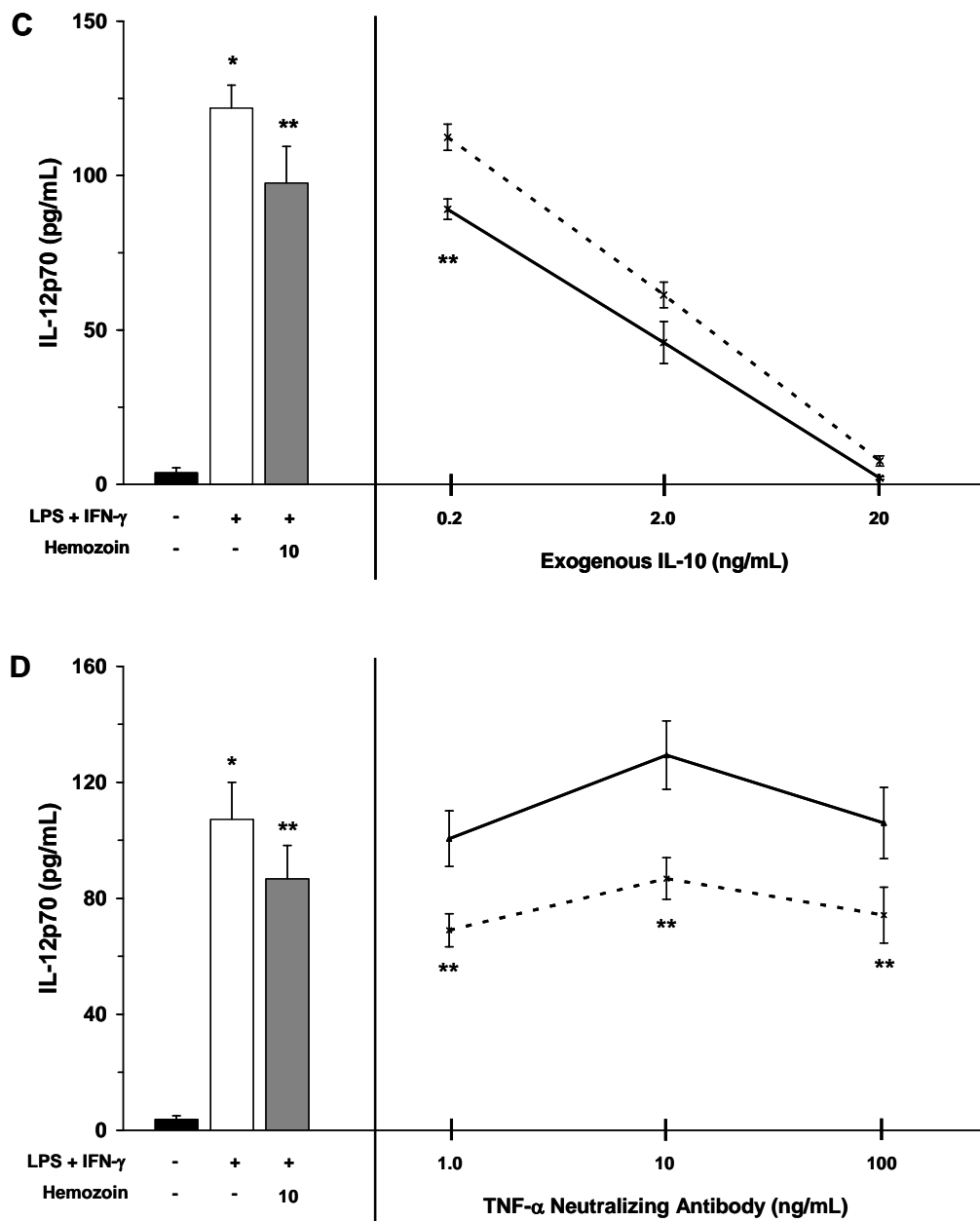


Figure 7. Effect of IL-10 and TNF- $\alpha$  on hemozoin-induced IL-12p70 suppression.

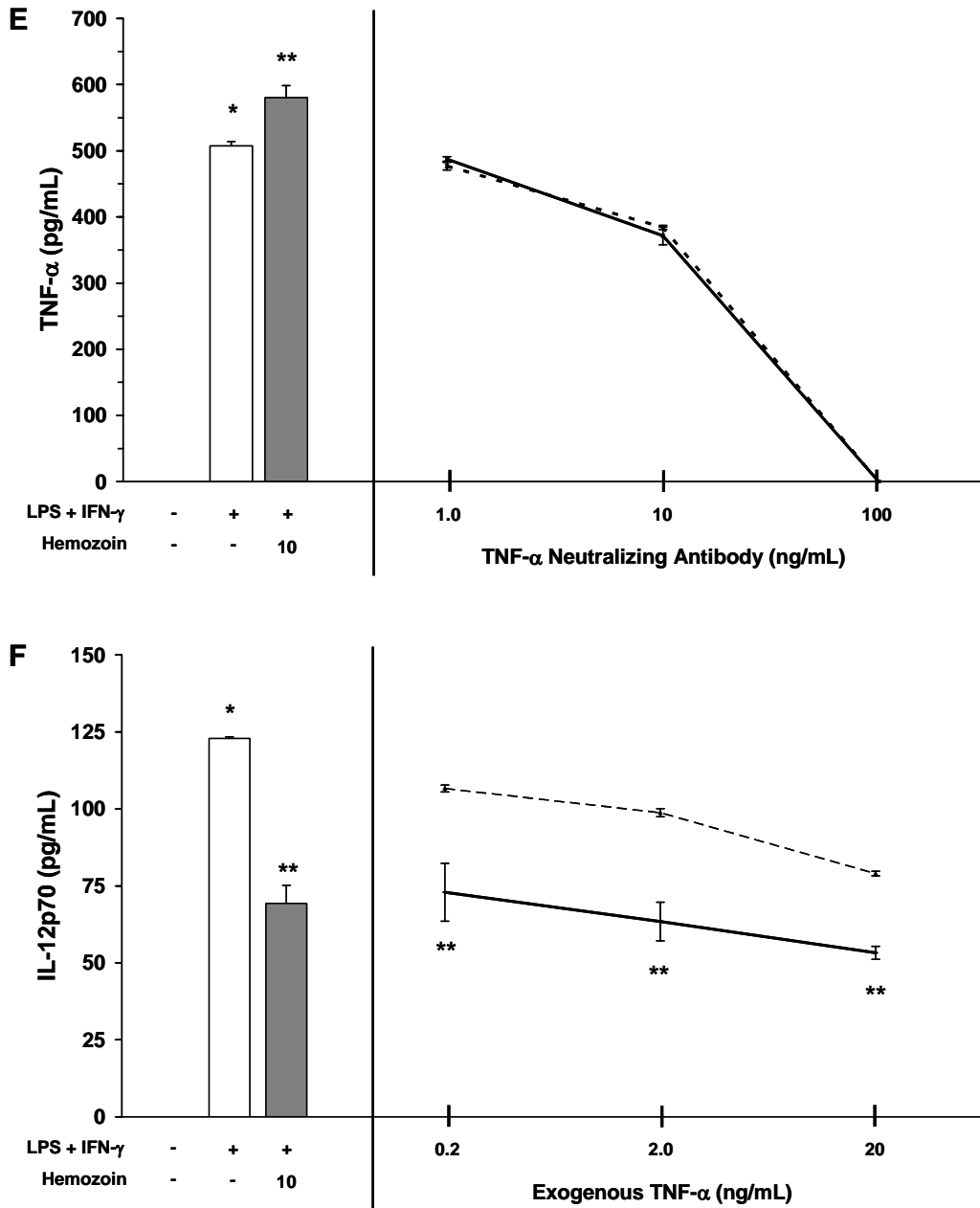
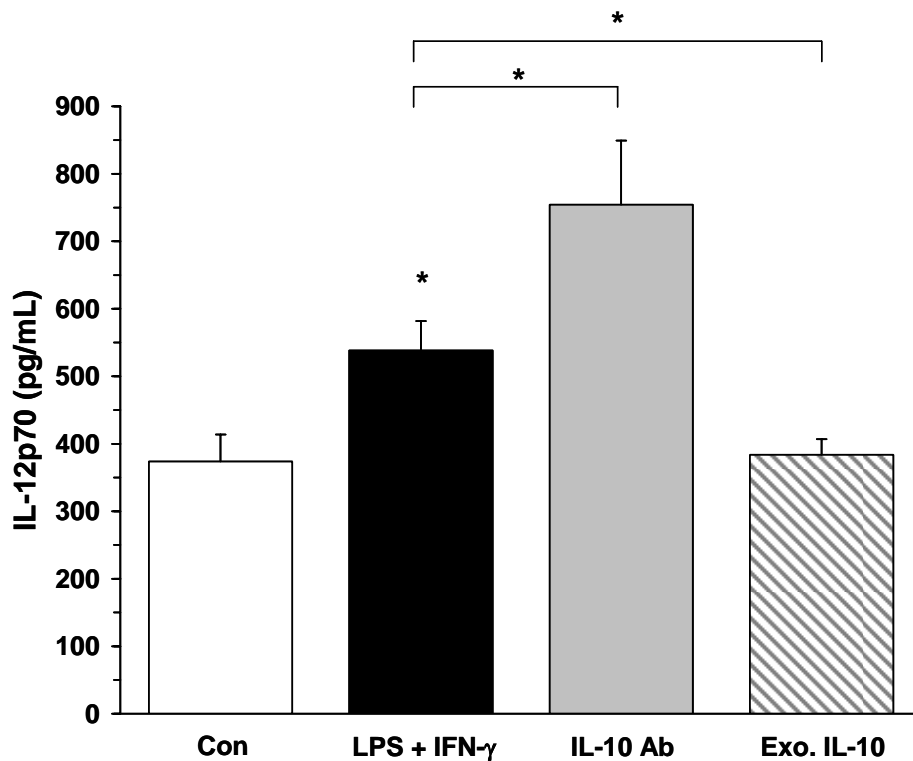


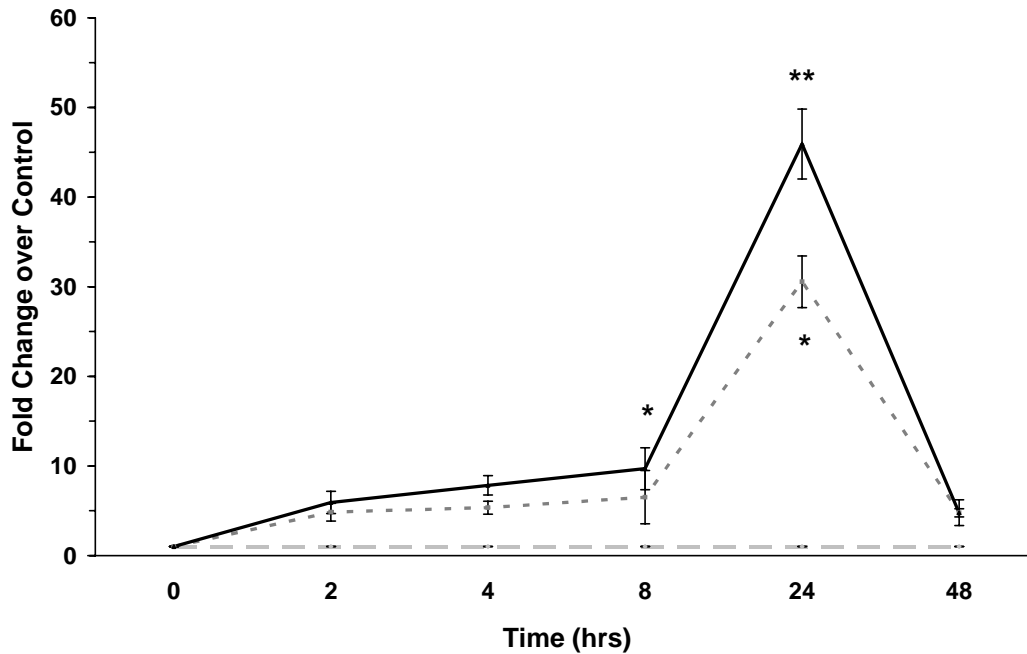
Figure 7. Effect of IL-10 and TNF- $\alpha$  on hemozoin-induced IL-12p70 suppression. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), or LPS and IFN- $\gamma$  in the presence of hemozoin (10  $\mu$ g/mL). IL-10 neutralizing antibodies (0.1, 0.5, and 1.0  $\mu$ g/mL) were added to LPS- and IFN- $\gamma$ -stimulated PBMC (dashed black line) and stimulated PBMC in the presence of hemozoin (solid black line), and supernatant concentrations of (A) IL-12p70 and (B) IL-10 were measured at 48 hrs. (C) Exogenous IL-10 (0.2, 2.0, and 20 ng/mL) was added to LPS- and IFN- $\gamma$ -stimulated PBMC and stimulated PBMC treated with

hemozoin, and supernatant IL-12p70 was measured at 48 hrs. TNF- $\alpha$  neutralizing antibodies (1.0, 10, and 100 ng/mL) were added to LPS- and IFN- $\gamma$ -stimulated PBMC and stimulated PBMC treated with hemozoin, and supernatant (D) IL-12p70 and (E) TNF- $\alpha$  was measured at 48 hrs. (F) Exogenous TNF- $\alpha$  (0.2, 2.0, and 20 ng/mL) was presented to LPS- and IFN- $\gamma$ -stimulated PBMC and stimulated PBMC treated with hemozoin, and supernatant IL-12p70 was measured at 48 hrs. Values are the mean  $\pm$  SEM, n = 3. Statistical significance was determined by Mann-Whitney U test. \* $P$  < 0.01 compared to unstimulated conditions, \*\* $P$  < 0.01 compared to LPS and IFN- $\gamma$  stimulation. \*\*\* $P$  < 0.01 compared to LP- and IFN- $\gamma$ - or hemozoin-stimulated conditions in the absence of antibody.





*Figure 8. Effect of IL-10 on IL-12p70 in cultured PBMC from children with malaria.* PBMC ( $1 \times 10^6$  cells/mL) were obtained from children with acute SMA ( $n = 12$ ) and cultured in the presence of media alone (controls), LPS (100 ng/mL) and IFN- $\gamma$ (200 U/mL), or LPS and IFN- $\gamma$  in the presence of IL-10 neutralizing antibodies (0.5  $\mu$ g/mL) or exogenous IL-10 (2.0 ng/mL). IL-12p70 production was determined in plasma at 48 hrs. Statistical significance determined by the Mann-Whitney U test, \* $P < 0.05$  (corrected for multiple comparisons).



*Figure 9. Temporal effect of hemozoin on SOCS-3 transcripts.* PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone (dashed black line), LPS (100 ng/mL) and IFN- $\gamma$  [200 U/mL, (dashed gray line)], or LPS and IFN- $\gamma$  in the presence of hemozoin [10  $\mu$ g/mL, (solid black line)]. Cells were collected at 2, 4, 8, 24, and 48 hrs for SOCS-3 mRNA determination by real time RT-PCR. Values are the mean  $\pm$  SEM, n = 3. Statistical significance was determined by Mann-Whitney U test. \* $P < 0.01$  compared to unstimulated conditions, \*\* $P < 0.01$  compared to LPS and IFN- $\gamma$  stimulation.

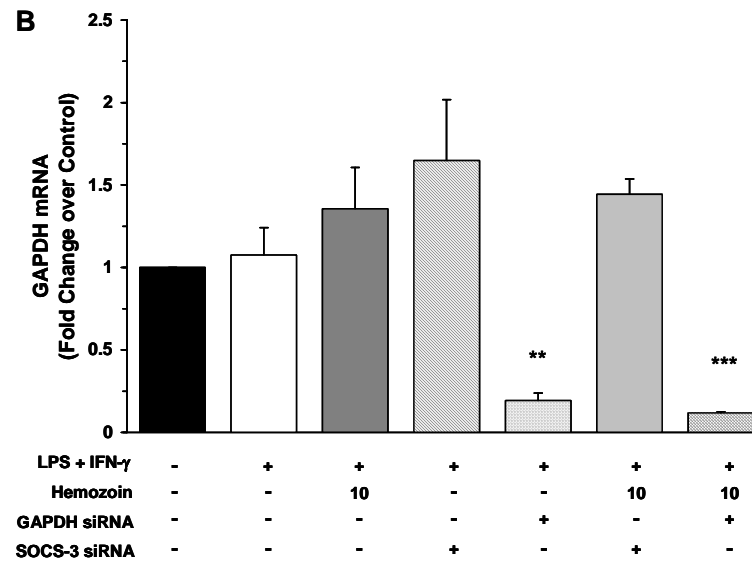
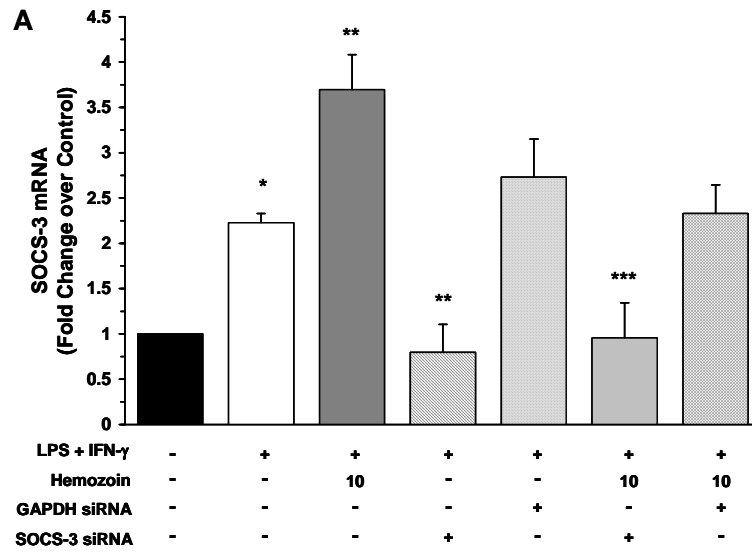


Figure 10. Effect of SOCS-3 siRNA on hemozoin-induced suppression of IL-12 transcripts

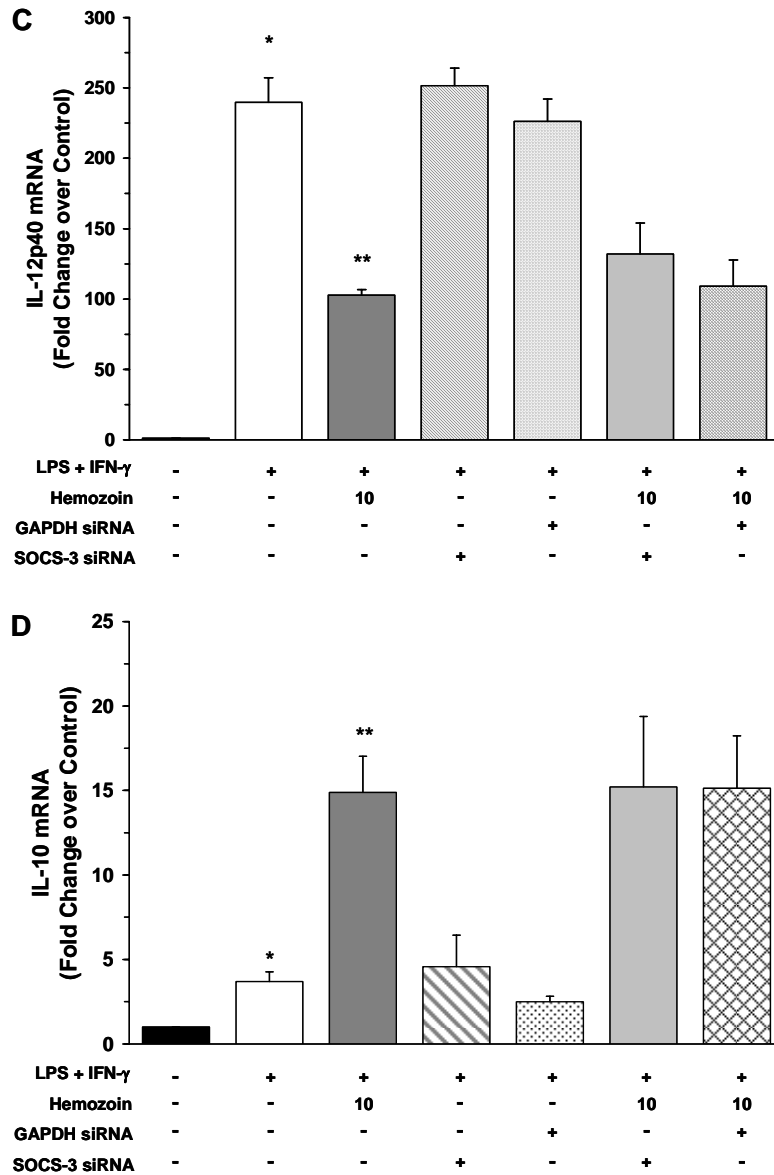


Figure 10. Effect of SOCS-3 siRNA on hemozoin-induced suppression of IL-12 transcripts PBMC ( $1 \times 10^6$  cells/mL) were nucleofected with siRNA for SOCS-3 or GAPDH (control siRNA) and stimulated with media alone, LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), or LPS and IFN- $\gamma$  in the presence of hemozoin [10  $\mu$ g/mL]. Cells were collected at 48 hrs for (A) SOCS-3, (B) GAPDH, (C) IL-12p40, and (D) IL-10 mRNA determination by real time RT-PCR. Values are the mean  $\pm$  SEM, n = 3. Statistical significance was determined by the Mann-Whitney U test. \* $P$  < 0.05 compared to unstimulated conditions, \*\* $P$  < 0.05 compared to LPS and IFN- $\gamma$  stimulated conditions, \*\*\* $P$  < 0.05 compared to stimulated conditions in the presence of hemozoin.

#### 4. CHAPTER 4: SPECIFIC AIM 2

**Specific Aim 2: To determine if hemozoin decreases COX-2 gene expression and the subsequent production of PGE<sub>2</sub> in cultured human PBMC.**

*Hypothesis 1:* Hemozoin suppresses COX-2-derived PGE<sub>2</sub> production in cultured human PBMC through suppression of *de novo* COX-2 transcripts and protein, and/or inhibition of COX-2 enzyme activity.

Previous studies in Gabonese children with malaria illustrated that COX-2 gene products, and subsequent PGE<sub>2</sub> production, were significantly lower in children with severe disease (Perkins *et. al.* 2001), however, the mechanism of COX-2-derived PGE<sub>2</sub> suppression in malaria remains unknown. To investigate Hypothesis 1, PGE<sub>2</sub> production was examined in cultured PBMC from healthy, malaria-exposed children and children with mild MA, and cultured PBMC from healthy, malaria-naïve U.S. adults stimulated with hemozoin or  $\beta$ -hematin. Further experiments in hemozoin-stimulated PBMC examined COX-2 protein and transcripts, and the effects of IL-10 on hemozoin-induced suppression of PGE<sub>2</sub> production. The effect of hemozoin on COX-2 enzymatic activity was examined using a COX inhibitor screening assay.

The first part of Hypothesis 1 of Specific Aim 2 has been addressed in the following manuscript which has been published in the journal *Molecular Medicine*. The second part of Hypothesis 1 examining the effect of hemozoin on inhibition of COX-2 enzymatic activity has been addressed following presentation of the manuscript.

**4.1. HYPOTHESIS 1: PRESENTATION OF MANUSCRIPT: REDUCED PERIPHERAL PGE<sub>2</sub> BIOSYNTHESIS IN *PLASMODIUM FALCIPARUM* MALARIA OCCURS THROUGH HEMOZOIN-INDUCED SUPPRESSION OF BLOOD MONONUCLEAR CELL COX-2 GENE EXPRESSION VIA AN IL-10-INDEPENDENT MECHANISM.**

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#### 4.1.1. Abstract

Molecular immunologic determinants of disease severity during *Plasmodium falciparum* malaria are largely undetermined. Our recent investigations showed that peripheral blood mononuclear cell (PBMC) COX-2 gene expression and plasma PGE<sub>2</sub> production are suppressed in children with falciparum malaria relative to healthy, malaria-exposed children with partial immunity. Furthermore, decreased COX-2/PGE<sub>2</sub> levels were significantly associated with increased plasma IL-10, an anti-inflammatory cytokine that inhibits the expression of COX-2 gene products. To determine the mechanism(s) responsible for COX-2-derived PGE<sub>2</sub> suppression, PBMC were cultured from children with falciparum malaria. PGE<sub>2</sub> production was suppressed under baseline and COX-2-promoting [stimulation with lipopolysaccharide (LPS) and interferon (IFN)- $\gamma$ ] conditions over prolonged periods, suggesting that an *in vivo*-derived product(s) was responsible for reduced PGE<sub>2</sub> biosynthesis. Hemozoin (malarial pigment) was investigated as a source of COX-2/PGE<sub>2</sub> suppression in PBMC from healthy, malaria-naïve adults. In addition, synthetically prepared hemozoin,  $\beta$ -hematin, was used to investigate the effects of the core iron component of hemozoin, ferriprotoporphyrin (FP)-IX. Physiologic concentrations of hemozoin or  $\beta$ -hematin suppressed LPS- and IFN- $\gamma$ -induced COX-2 mRNA in a time- and dose-dependent manner resulting in decreased COX-2 protein and PGE<sub>2</sub> production. Suppression of COX-2/PGE<sub>2</sub> by hemozoin was not due to decreased cell viability as evidenced by examination of mitochondrial bioactivity. These data illustrate that ingestion of FPIX by blood mononuclear cells is responsible for suppression of COX-2/PGE<sub>2</sub>. Although hemozoin induced over-production of IL-10, neutralizing IL-10 antibodies failed to restore PGE<sub>2</sub> production. Thus, acquisition of hemozoin by blood mononuclear cells is responsible for suppression of PGE<sub>2</sub> in malaria through

inhibition of *de novo* COX-2 transcripts via molecular mechanisms independent of increased IL-10 production.

#### **4.1.2. Introduction**

Although the underlying pathophysiologic determinants of severe malaria are only partially defined, cytokines and effector molecules are important immunologic determinants for regulating disease susceptibility. Our recent investigations have focused on defining the role of prostaglandins (PGs) as immunomodulatory factors in malaria. Eicosanoids, such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) regulate macrophage function, vascular permeability, extracellular adhesion molecules, fever, and cytokine production (Vane *et. al.* 1998). PGE<sub>2</sub> formation is initiated by the actions of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) which releases arachidonic acid (AA) from the membrane phospholipid bilayer in response to inflammatory stimuli (Murakami *et. al.* 1997). Free AA is rapidly metabolized to PGH<sub>2</sub> via the enzymatic activity of the cyclooxygenase (COX) enzymes, of which there are two distinct isoforms encoded by separate genes: COX-1 and COX-2 (Appleton *et. al.* 1996, Vane *et. al.* 1998). COX-1 is constitutively expressed in most tissues and generates PGs for physiologic homeostasis, while COX-2 is an inducible gene that generates high levels of PGs during an inflammatory event (Seibert *et. al.* 1994). Following formation of PGH<sub>2</sub> by COX, PGE<sub>2</sub> synthase converts PGH<sub>2</sub> to PGE<sub>2</sub> (Smith *et. al.* 1991).

Our previous studies showed that plasma levels of PGE<sub>2</sub> were significantly reduced in children with malaria, and were significantly associated with increased plasma interleukin (IL)-10 levels (Perkins *et. al.* 2001). Furthermore, peripheral blood mononuclear cell (PBMC) COX-2 gene products were inversely related to disease severity (Perkins *et. al.* 2001). We have also



shown that children with cerebral malaria (CM) have suppressed systemic levels of PGE<sub>2</sub> (Perkins *et. al.* 2005). Although suppression of COX-2/PGE<sub>2</sub> in our previous studies was associated with enhanced severity of falciparum malaria, the mechanism(s) for decreased PGE<sub>2</sub> synthesis remain undefined.

During the erythrocytic stage of malaria, *Plasmodia* digest host hemoglobin, releasing heme in the process. To avoid the toxic effects of heme, enzymes within the parasitophorous vacuole catalyze the polymerization of heme into a non-toxic insoluble compound, hemozoin (malarial pigment) [for review see (Francis *et. al.* 1997)]. Hemozoin is composed predominately of protein and a smaller fraction of carbohydrates, lipids, and nucleic acids which are attached to the polymerized iron core structure, ferriprotoporphyrin (FP)-IX (Goldie *et. al.* 1990). Upon rupture of infected erythrocytes, hemozoin is released into the blood stream and phagocytosed by circulating monocytes and tissue macrophages (Schwarzer *et. al.* 1998). Hemozoin is also acquired by monocytes/macrophages through phagocytosis of parasitized erythrocytes (Arese *et. al.* 1997). Experiments in cultured blood mononuclear cells illustrate that hemozoin augments the release of both pro-inflammatory cytokines, such as TNF- $\alpha$  (Pichyangkul *et. al.* 1994), and anti-inflammatory cytokines such as IL-10 (Mordmuller *et. al.* 1998). This appears important in the current context since TNF- $\alpha$  induces high levels of sustained COX-2 gene expression (Perkins *et. al.* 1997), while IL-10 decreases COX-2 gene expression through de-stabilization of COX-2 message (Niuro *et. al.* 1995). Additional affects of hemozoin on blood mononuclear cells include; inhibition of oxidative burst and protein kinase C activity (Schwarzer *et. al.* 1993), reduction of microbicidal and anti-tumor capabilities (Fiori *et. al.* 1993), impaired expression of

MHC class II and CD54 expression (Schwarzer *et. al.* 1998), and an inability of monocytes to undergo repeated phagocytosis (Schwarzer *et. al.* 1992).

In the present investigations, PGE<sub>2</sub> production was examined in cultured PBMC from children with falciparum malaria under baseline conditions and following treatment with COX-2/PGE<sub>2</sub> inducing stimuli: LPS and IFN- $\gamma$ . In addition, COX-2 mRNA and protein, and PGE<sub>2</sub> formation were examined in cultured PBMC from malaria-naïve healthy donors stimulated with LPS and IFN- $\gamma$  in the presence of physiologically relevant doses [10, 1.0, and 0.1  $\mu$ g/mL, (Keller *et. al.* 2004)] of hemozoin or  $\beta$ -hematin (synthetic malarial pigment). The effect of hemozoin on IL-10 production and the ability of IL-10 to down-regulate PGE<sub>2</sub> biosynthesis were also investigated.

#### **4.1.3. Subjects and Methods**

**4.1.3.1. Study Participants:** Children (n = 30, age 2 to 7 years) were recruited from a longitudinal prospective study at the Albert Schweitzer Hospital in Lambaréné, Gabon in the Province of Moyen Ogooue. In this hyperendemic area of malaria transmission, the primary clinical manifestations of severe childhood malaria is severe anemia and/or hyperparasitemia, with cerebral malaria rarely occurring (Kun *et. al.* 1998). Classification of malaria was defined according to our previously published methods (Perkins *et. al.* 2001, Perkins *et. al.* 2000). Only those children with mild malaria [defined by parasitemias < 100,000 parasites/ $\mu$ L and the absence of any signs or symptoms of severe malaria (Kun *et. al.* 1998, Perkins *et. al.* 2001)] were included in the present study due to the limited amount of blood available from severe cases. Healthy, malaria-exposed subjects were defined as participants with a previous episode(s) of

malaria and the absence of a positive thick blood film for malaria, or any other illnesses, within the last 4 weeks. All blood samples were obtained prior to treatment with antimalarials. Informed consent was obtained from the parents of participating children.

Healthy, malaria-naïve adult donors (n = 21) were recruited from the University of Pittsburgh, U.S.A. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital, Duke University Medical Center, and the University of Pittsburgh Investigational Review Boards.

**4.1.3.2. Isolation and Culture of Peripheral Blood Mononuclear Cells:** For in vitro experiments in Gabonese children (n = 30) and U.S. adults (n = 21), venous blood (3 and 40 mL, respectively) was drawn into EDTA-containing vials. PBMC were prepared using ficoll/Hypaque as described earlier (Weinberg *et. al.* 1981), and plated at  $1 \times 10^6$  cells per mL in Dulbecco's modified Eagles medium (DMEM) containing 10% pooled human serum (heat inactivated at 56°C for 30 min). Cells were incubated with media alone (controls) or concomitant stimulation with LPS [100 ng/mL (Alexis Corp., San Diego, CA)] and IFN- $\gamma$  [200 U/mL (BD Pharmingen, San Diego, CA)]. For experiments in healthy adults, cultures were incubated with varying concentrations of hemozoin (10, 1.0, and 0.1  $\mu\text{g/mL}$ ) or  $\beta$ -hematin (10, 1.0, and 0.1  $\mu\text{g/mL}$ ) in the presence and absence of LPS and IFN- $\gamma$  stimulation. For neutralization of endogenous IL-10, cultures were stimulated with IL-10 neutralizing antibodies (1.0, 0.5, and 0.1  $\mu\text{g/mL}$ ).

**4.1.3.3. Hemozoin and  $\beta$ -hematin Preparation:** Crude hemozoin was isolated from in vitro cultures of *P. falciparum*-infected red blood cells (strain Pf-D6) according to our previously

published methods (Keller *et. al.* 2004). Briefly, cultured red blood cells (RBC) were treated with 2 mL saponin for 10 min and the solution was washed in PBS and spun at 14,000 rpm for 15 min (4-7 times). The final pellet was dried, weighed, and resuspended in filter-sterilized H<sub>2</sub>O at a concentration of 1.0 mg/mL. As a control, a parasite-free RBC lysate was prepared from uninfected RBC following the protocol outlined above for the hemozoin preparation.

$\beta$ -hematin was formed in a 4.5 M acidic acetate solution at pH 4.5 by the method of Egan *et al.* (Egan *et. al.* 2001). Briefly, hemin chloride (Sigma, St. Louis, MO) was added to a 0.1 M solution of NaOH followed by addition of HCl and acetate. The mixture was incubated for 150 min at 60°C without stirring, spun at 14,000 rpm, washed 3X with filter-sterilized H<sub>2</sub>O, and dried at 60°C under vacuum. The final pellet was weighed and resuspended at 1.0 mg/mL in filter-sterilized H<sub>2</sub>O. Resuspended  $\beta$ -hematin was sonicated extensively to disperse the preparation. Under these preparation conditions,  $\beta$ -hematin has an identical infrared spectroscopy pattern as detergent-purified hemozoin, and  $\beta$ -hematin crystals appear morphologically similar to natural hemozoin crystals as evidenced by scanning electron microscopy (Egan *et. al.* 2001).

**4.1.3.4. PGE<sub>2</sub> and IL-10 Measurements:** Concentrations of PGE<sub>2</sub> and IL-10 were measured in the supernatants of cultured PBMC using a quantitative indirect sandwich enzyme immunoassay (Cayman Chem., Ann Arbor, MI) and quantitative sandwich ELISA (Pharmingen, San Diego, CA), respectively, according to our previously defined methods (Perkins *et. al.* 2003). Sensitivity of detection for PGE<sub>2</sub> and IL-10 was  $\geq 7.8$  pg/mL.

**4.1.3.5. MTT Assay:** Cell viability was determined by the MTT assay, which measures active mitochondrial conversion of MTT salt into formazan crystals. Briefly, 20  $\mu$ L of MTT salt

[5 mg/mL in 1X PBS, (Sigma, St. Louis, MO)] were added to cultured cells 5 hrs prior to termination of cultures. At the end of culture, supernatants were aspirated and 200  $\mu$ L of DMSO were added to dissolve the formazan crystals. Plates were incubated for 5 min at room temperature. Absorbance was determined at 550 nm.

**4.1.3.6. Immunoblot Analyses:** Cellular extracts were prepared according to our previously defined methods (Perkins *et. al.* 1997). Protein samples (30  $\mu$ g) were fractionated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and COX-2 antigen was measured by our previously defined methods (Perkins *et. al.* 2001). Extracts of mouse macrophage cells (RAW 264.7), stimulated with lipopolysaccharide (*E. coli* LPS 0127B8, 1.0  $\mu$ g/mL) and IFN- $\gamma$  (10 ng/mL) were used as a positive control. Densitometry readings for COX-2 protein expression were obtained using commercially available software (ScanImage, Scion Corporation).

**4.1.3.7. Quantitative real time RT-PCR:** Total RNA was isolated from PBMC by the GITC method (Chomczynski *et. al.* 1987). Total RNA (1  $\mu$ g) was reverse transcribed into cDNA, and COX-2 gene expression was analyzed by quantitative real time RT-PCR on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). cDNA (100 ng) was amplified in duplicate with COX-2 specific primers and probe as follows: forward, 5'-CCT TCC TCC TGT GCC TGA TG-3'; reverse, 5'-ACA ATC TCA TTT GAA TCA GGA AGC T-3'; probe (FAM labeled), 5'-TGC CCG ACT CCC TTG GGT GTC A-3' [sequence information obtained from (Sales *et. al.* 2001)]. The COX-2 cDNA was amplified for 40 cycles following the parameters specified by the manufacturer (Applied Biosystems, Foster City, CA). To control

for non-specific background fluorescence, no template controls were included in triplicate. An endogenous control gene,  $\beta$ -actin [Accession Number NM\_001101 (Applied Biosystems, Foster City, CA)], was used as a reference gene to normalize cDNA loading between samples. Data was compared using the  $-\Delta\Delta C_T$  method as previously described (Keller *et. al.* 2004).

**4.1.3.8. Statistical Analyses:** Supernatant concentrations of PGE<sub>2</sub> (pg/mL), and IL-10 (pg/mL) were measured in triplicate and at several different dilutions. COX-2 mRNA expression was measured in duplicate. Pairwise comparisons between conditions, including pairwise tests of dose-dependent effects, were performed by the Mann-Whitney U test. Multiple group comparisons for dose-dependent effects were performed using the Kruskal-Wallis test (statistical significance set at  $P \leq 0.05$ ).

#### **4.1.4. Results**

**4.1.4.1. PGE<sub>2</sub> Production in Cultured PBMC from Children with Malaria:** To determine if mononuclear phagocyte production of PGE<sub>2</sub> is altered in children with falciparum malaria, PGE<sub>2</sub> synthesis was determined in culture supernatants of PBMC from healthy, malaria-exposed children (control, n = 20) and children with malaria (malaria, n = 10). To minimize the influence of the *in vivo* environment, PBMC were cultured for 120 hrs. PGE<sub>2</sub> synthesis was examined under baseline conditions (media alone) and in the presence of COX-2-promoting stimuli (LPS and IFN- $\gamma$ ). Under baseline conditions, PGE<sub>2</sub> production was lower in the malaria group compared to the control group ( $P < 0.05$ , Figure 11). Stimulation of PBMC augmented PGE<sub>2</sub> production in the control group ( $P < 0.01$ ), but failed to significantly elevate PGE<sub>2</sub> formation in the malaria group (Figure 11). Production of PGE<sub>2</sub> in stimulated PBMC was lower in the

malaria group relative to the control group ( $P < 0.05$ , Figure 10). The percent of hemozoin containing monocytes in the cultured cells from children with malaria was 5.6%. In addition, the relative percent of monocytes and lymphocytes in the two groups of children were equivalent (control: 7.1% monocytes and 54.3%, lymphocytes, and malaria group: 7.9% monocytes and 51.0% lymphocytes). The relative proportion of monocytes and lymphocytes remained comparable when determined in a subset of the healthy controls ( $n = 10$ ) and children with malaria ( $n = 10$ ) following ficoll/Hypaque separation. These results suggest that monocyte-derived PGE<sub>2</sub> synthesis is significantly suppressed in children with malaria and is refractory to COX-2 promoting stimuli.

**4.1.4.2. Effect of Hemozoin on PGE<sub>2</sub> Production:** Since PBMC from children with malaria had significantly reduced baseline and stimulated PGE<sub>2</sub> synthesis over a 120 hr period, we postulated that an in vivo parasite-driven event induced PGE<sub>2</sub> suppression. As such, a crude hemozoin preparation, representing that most closely observed during a natural infection, was added to cultured PBMC. Since these experiments required larger volumes of blood than were available in anemic children, PBMC were cultured from healthy, malaria-naive adult US donors, followed by stimulation with media alone, LPS and IFN- $\gamma$ , and LPS and IFN- $\gamma$  in the presence of physiologic concentrations of hemozoin [10, 1.0, and 0.1  $\mu\text{g}/\text{mL}$ , (Keller *et. al.* 2004)]. LPS- and IFN- $\gamma$ -stimulation increased PGE<sub>2</sub> levels over baseline conditions at 24 hrs ( $P < 0.05$ ), with hemozoin having no effect on PGE<sub>2</sub> production (Figure 12). However, at 48 hrs, and at all subsequent time points examined, a high and intermediate concentration of hemozoin reduced PGE<sub>2</sub> production ( $P < 0.05$ , Figure 12). Treatment of cultures with a RBC lysate did not alter PGE<sub>2</sub> production at any of the time points examined (Figure 12).

Since suppression of PGE<sub>2</sub> by hemozoin could result from hemozoin-induced cell death, the temporal profile of cell viability was examined using the MTT assay. LPS and IFN- $\gamma$  stimulation decreased cell viability at 72, 96, and 120 hrs ( $P < 0.05$ ), with the addition of hemozoin having no effect on cell viability (Figure 13). Thus hemozoin significantly suppresses PGE<sub>2</sub> through mechanisms independent of altering cell viability.

Because PGE<sub>2</sub> production varied in different individuals, we investigated the effect of hemozoin on a larger cohort of malaria-naïve healthy adults ( $n = 18$ ) at 48 and 72 hrs, the time at which the greatest reductions in PGE<sub>2</sub> were observed (see Figure 12). Cultures were also stimulated with  $\beta$ -hematin to determine the effects of the core malarial pigment compound, FPIX, on PGE<sub>2</sub> synthesis in the absence of host- and parasite-derived products. PBMC were stimulated with LPS and IFN- $\gamma$  in the presence of hemozoin or  $\beta$ -hematin, and data were expressed as percent change in PGE<sub>2</sub> production in hemozoin-treated cells relative to cells stimulated with LPS and IFN- $\gamma$ . The addition of both hemozoin and  $\beta$ -hematin to PBMC caused a dose-dependent decrease in PGE<sub>2</sub> production (Figure 14), suggesting that FPIX is responsible for suppression of PBMC-derived PGE<sub>2</sub> production.

**4.1.4.3. Effect of Hemozoin and  $\beta$ -hematin on COX-2 Gene Products:** Because inflammatory-derived PGE<sub>2</sub> is dependent on de novo COX-2 mRNA and protein, we hypothesized that hemozoin suppresses PGE<sub>2</sub> through blockade of COX-2 gene expression. As such, COX-2 protein expression was determined by immunoblot analysis, using a COX-2-specific monoclonal antibody, in stimulated cells in the presence and absence of hemozoin or  $\beta$ -hematin. Stimulation of cells increased COX-2 protein levels at 48 hrs (Figures 15A - D) with



the addition of hemozoin or  $\beta$ -hematin causing a dose-dependent reduction in COX-2 protein (Figures 15A - 4D).

To establish if hemozoin caused a decrease in COX-2 transcripts, COX-2 message was examined using quantitative real time RT-PCR. At 48 hrs in culture, LPS- and IFN- $\gamma$ -stimulated PBMC produced higher levels of COX-2 mRNA ( $P < 0.01$ , Figure 16). The addition of a high and intermediate concentration of hemozoin or  $\beta$ -hematin reduced COX-2 transcript formation ( $P < 0.01$ , Figure 16).

To determine the kinetics by which COX-2 transcripts are suppressed by hemozoin, the time-dependent induction of COX-2 mRNA was examined. LPS- and IFN- $\gamma$ -stimulation increased COX-2 mRNA by 8 hrs, which peaked at 24 hrs ( $P < 0.01$ , Figure 17). The addition of hemozoin decreased COX-2 transcript formation at 24 and 48 hrs ( $P < 0.01$ , Figure 17). Taken together, results presented here demonstrate that decreased PGE<sub>2</sub> production occurs in hemozoin-treated cells through reductions in COX-2 transcripts and protein.

**4.1.4.4. Effect of Endogenous IL-10 on Hemozoin-induced PGE<sub>2</sub> Suppression:** Anti-inflammatory cytokines, such as IL-10, suppress de novo COX-2 transcription in monocytes (Niuro *et. al.* 1995). Moreover, we have previously shown that decreased PGE<sub>2</sub> production in children with falciparum malaria was significantly associated with increased plasma IL-10 production (Perkins *et. al.* 2001). We, therefore, investigated if hemozoin-induced suppression of COX-2/PGE<sub>2</sub> was a consequence of increased IL-10 production. IL-10 was determined in PBMC stimulated with media alone, LPS and IFN- $\gamma$ , and LPS and IFN- $\gamma$  in the presence of

hemozoin. At 48 hrs, stimulation increased IL-10 production ( $P < 0.05$ ), which was further augmented by the addition of hemozoin ( $P < 0.05$ , Figure 18A).

Since addition of hemozoin to PBMC increased IL-10 production, and suppressed COX-2-derived PGE<sub>2</sub> formation, we determined if neutralizing antibodies to IL-10 could restore hemozoin-induced reductions in PGE<sub>2</sub>. As shown in Figure 18B, addition of IL-10 neutralizing antibodies failed to restore PGE<sub>2</sub> production. Measurement of IL-10 confirmed that endogenously produced IL-10 was dose-dependently reduced in culture supernatants with increasing concentrations of IL-10 neutralizing antibodies (Figure 18C). Determination of IL-10 transcripts by real time RT-PCR showed that the addition of IL-10 neutralizing antibodies had no effect on IL-10 transcript levels (data not shown). In addition, the addition of isotype-matched control antibodies had no effect on IL-10 levels (data not shown). Thus, suppression of COX-2-derived PGE<sub>2</sub> occurs through an IL-10-independent mechanism.

#### **4.1.5. Discussion**

Our previous studies demonstrated that plasma bicyclo-PGE<sub>2</sub> and *ex vivo* blood mononuclear cell COX-2 levels are suppressed in children with malaria (Perkins *et. al.* 2001). In the present study, we extend those findings by showing that cultured PBMC from children with malaria have reduced baseline and stimulated PGE<sub>2</sub> production. Since cells were cultured for 120 hrs to avoid influence of host-derived *in vivo* regulatory factors, we postulated that suppression of PGE<sub>2</sub> was due to a phagocytosed parasitic product(s). Because hemozoin can persist in mononuclear cells for prolonged periods (Day *et. al.* 1996), the effect of hemozoin on PGE<sub>2</sub> was examined. A crude hemozoin isolate was used since this preparation mimics the hemozoin moiety acquired during a natural infection. Consistent with studies illustrating that phagocytosis of hemozoin by

monocytic cells occurs within 24 hrs with the biologic effects occurring over prolonged periods (Olliaro *et. al.* 2000, Schwarzer *et. al.* 2001), hemozoin significantly decreased PGE<sub>2</sub> synthesis in PBMC from 48 hrs onward.

The molecular mechanism responsible for hemozoin-induced PGE<sub>2</sub> suppression does not occur through reduced cell viability, since the addition of hemozoin to stimulated PBMC did not alter mitochondrial bioactivity (i.e., MTT assay) at the time points selected for the mechanism-based studies (48 and 72 hrs). It is important to note that after 72 hrs in culture, hemozoin reduced cell viability, which was further reduced by LPS and IFN- $\gamma$  treatment. Thus, it was difficult to interpret experimental results in which cells were pretreated with hemozoin followed by stimulation. Although results presented here clearly illustrate that hemozoin suppresses COX-2-derived PGE<sub>2</sub> biosynthesis, it appears that the natural acquisition of hemozoin in the cytokine-activated *in vivo* milieu differs slightly from that offered by the *in vitro* environment. This rationale is supported by the fact that hemozoin reduced PGE<sub>2</sub> biosynthesis by 15 to 53% in cells from healthy donors versus the 70% reduction observed in children with malaria. The apparent discrepancy between *in vitro* hemozoin-induced reductions in PGE<sub>2</sub> and naturally acquired hemozoin in mononuclear cells from children with malaria may be due to additional host- and/or parasite-derived factors independent of hemozoin. We are currently investigating the effects of additional host immune factors and parasitic products that may alter PGE<sub>2</sub> synthesis during acute malaria.

While we consistently observed PGE<sub>2</sub> suppression in PBMC stimulated with hemozoin, there was substantial variation in PGE<sub>2</sub> production in different individuals. To address this

variation, data were expressed as percent reduction in PGE<sub>2</sub> relative to stimulated conditions in a larger number of individuals (n = 18) at time-points when the cell viability was greatest (*i.e.*, 48 and 72 hrs). Expression of data in this manner “normalized” individual variation and demonstrated a dose-dependent suppressive effect of hemozoin on PGE<sub>2</sub> biosynthesis. These experiments further demonstrated that β-hematin dose-dependently reduced PGE<sub>2</sub> production. Since β-hematin is a synthetically prepared compound of FPIX, which lacks host- and parasite-derived proteins and lipids, and is structurally identical to purified (detergent-treated) hemozoin (Pagola *et. al.* 2000, Slater *et. al.* 1991), it appears that FPIX is responsible for suppression of PGE<sub>2</sub>.

Our previous results showed that *ex vivo* PBMC COX-2 protein and mRNA were decreased in children with malaria (Perkins *et. al.* 2001). This, along with our previous data showing that *de novo* induction of COX-2 message is required for high levels of sustained PGE<sub>2</sub> production (Perkins *et. al.* 1997), prompted us to investigate hemozoin-induced changes in COX-2 expression. Hemozoin and β-hematin dose-dependently suppressed COX-2 mRNA and protein, indicating that PGE<sub>2</sub> suppression is due to reduced levels of COX-2 gene products. Results from kinetic experiments illustrated that hemozoin inhibits *de novo* COX-2 message at 8 and 24 hrs. Thus, reductions in COX-2 protein and PGE<sub>2</sub> formation at 48 hrs is consistent with decreased COX-2 transcripts at earlier time points.

Previous studies in individuals with CM showed increased COX-2 protein in Durck’s granulomas within the central nervous system (Deininger *et. al.* 2000). However, our results in plasma and PBMC from children with malaria (Perkins *et. al.* 2001), and here in hemozoin-treated PBMC, illustrate that COX-2/PGE<sub>2</sub> is suppressed in circulating blood mononuclear cells

and plasma. Our recent results in Tanzanian children with CM also show that peripheral PGE<sub>2</sub> levels are decreased during acute disease, with the most profound reductions in PGE<sub>2</sub> being associated with increased disease sequelae (Perkins *et. al.* 2005). We have further shown that placental monocytes from women with malaria during pregnancy have decreased PGE<sub>2</sub> formation; there was a dose-dependent reduction in PGE<sub>2</sub> with increasing amounts of naturally acquired hemozoin (Perkins *et. al.* 2003). Recent studies in a murine model of CM also showed that COX-2 transcripts were significantly increased within the brain, but non-significantly decreased in PBMC (Ball *et. al.* 2004). Furthermore, infection of mice with *P. berghei* K173, a parasite strain causing anemia in the absence of CM, significantly decreased peripheral COX-2 transcripts (Ball *et. al.* 2004). Thus, during acute malaria, COX-2/PGE<sub>2</sub> regulation within the brain differs from COX-2/PGE<sub>2</sub> regulation in the local tissue compartments and in circulating blood monocytes.

Our previous studies showed that decreased plasma PGE<sub>2</sub> levels were significantly associated with increased plasma IL-10 concentrations (Perkins *et. al.* 2001). This is consistent with data illustrating that IL-10 decreases PGE<sub>2</sub> through suppression and/or destabilization of COX-2 transcripts (Niuro *et. al.* 1995). As such, we determined if hemozoin-induced PGE<sub>2</sub> suppression occurred through increased IL-10 production. Hemozoin augmented LPS- and IFN- $\gamma$ -induced IL-10 production, however, hemozoin-induced IL-10 production does not appear responsible for PGE<sub>2</sub> suppression since IL-10 neutralizing antibodies failed to restore PGE<sub>2</sub>. Thus, the molecular mechanism(s) by which hemozoin suppresses COX-2/PGE<sub>2</sub> remains unclear. Previous studies show that activation of the protein kinase C (PKC) signaling pathway is essential for COX-2 induction in human intestinal myofibroblasts (Mifflin *et. al.* 2002), and

that inhibition of PKC attenuates IL-1 $\beta$  induction of COX-2/PGE<sub>2</sub> in human pulmonary epithelial cells (Lin *et. al.* 2000). This appears important for results presented here since previous studies found that hemozoin suppresses PKC activity in cultured monocytes (Schwarzer *et. al.* 1993). We are currently determining if reduced levels of COX-2 in malaria occur through inhibition of PKC signal transduction pathways.

Although decreased systemic PGE<sub>2</sub> levels are associated with increasing disease severity, it is unclear how suppressed peripheral PGE<sub>2</sub> promotes enhanced malaria pathogenesis. Potential pathogenic effects may occur through suppression of erythropoiesis since previous studies demonstrate that PGE<sub>2</sub> enhances burst forming unit erythroid formation (Dupuis *et. al.* 1998), suggesting that suppression of systemic PGE<sub>2</sub> by hemozoin may reduce formation of new RBC. Additional mechanisms by which decreased systemic PGE<sub>2</sub> could promote increased pathogenesis may occur through over-expression of the pro-inflammatory response. For example, PGE<sub>2</sub> is important for resolution of the late-phase immune response through promotion of T<sub>H</sub>2-derived cytokine production [for review see (Phipps *et. al.* 1991)]. PGE<sub>2</sub> also regulates the pro-inflammatory response by suppressing *de novo* TNF- $\alpha$  transcription (Kunkel *et. al.* 1988), and by modulating the secretion of soluble TNF- $\alpha$  receptors (Choi *et. al.* 1996). PGE<sub>2</sub> may also alter malaria pathogenesis through modulation of lymphocytic activity. Previous studies in cultured human mononuclear cells from individuals with acute falciparum malaria showed that the non-selective COX inhibitor, indomethacin, reversed malarial antigen-induced suppression of lymphoproliferation (Riley *et. al.* 1989). However, levels of COX-2/PGE<sub>2</sub> were not determined in these studies (Riley *et. al.* 1989). Subsequent studies showed that exogenous addition of PGE<sub>2</sub> failed to suppress T-cell proliferation (Alves *et. al.* 1992). The apparent

discrepancy in these results may be related to the fact that indomethacin alters a number of cellular functions in a COX-independent fashion [for review see (Teveder *et. al.* 2001)]. We are currently investigating the effects of COX-2/PGE<sub>2</sub> suppression on malaria pathogenesis in children with severe malarial anemia.

#### **4.1.6. Acknowledgements**

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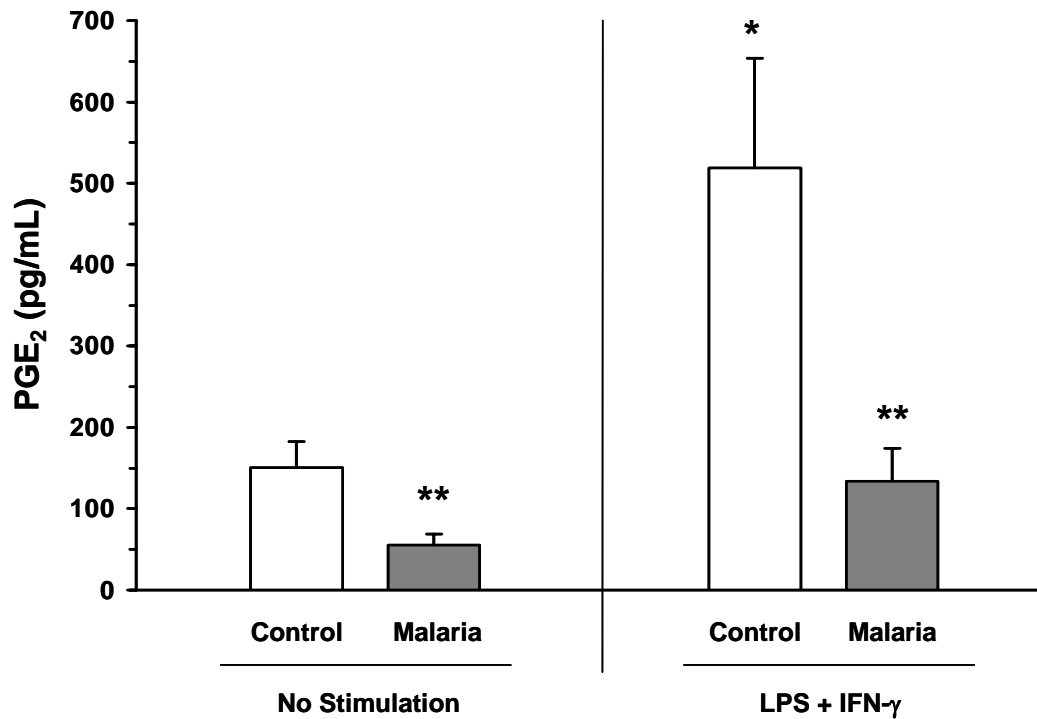


Figure 11. PGE<sub>2</sub> production in cultured PBMC from children with malaria. PBMC ( $1 \times 10^6$  cells/mL) from healthy malaria-exposed children (Control,  $n = 20$ ) and children with malaria (Malaria,  $n = 10$ ) were cultured in the presence and absence of LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL). Supernatants were collected at 120 hrs for PGE<sub>2</sub> determination. Values are the mean  $\pm$  SEM. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.01$  compared to unstimulated conditions, \*\* $P < 0.05$  compared to control.



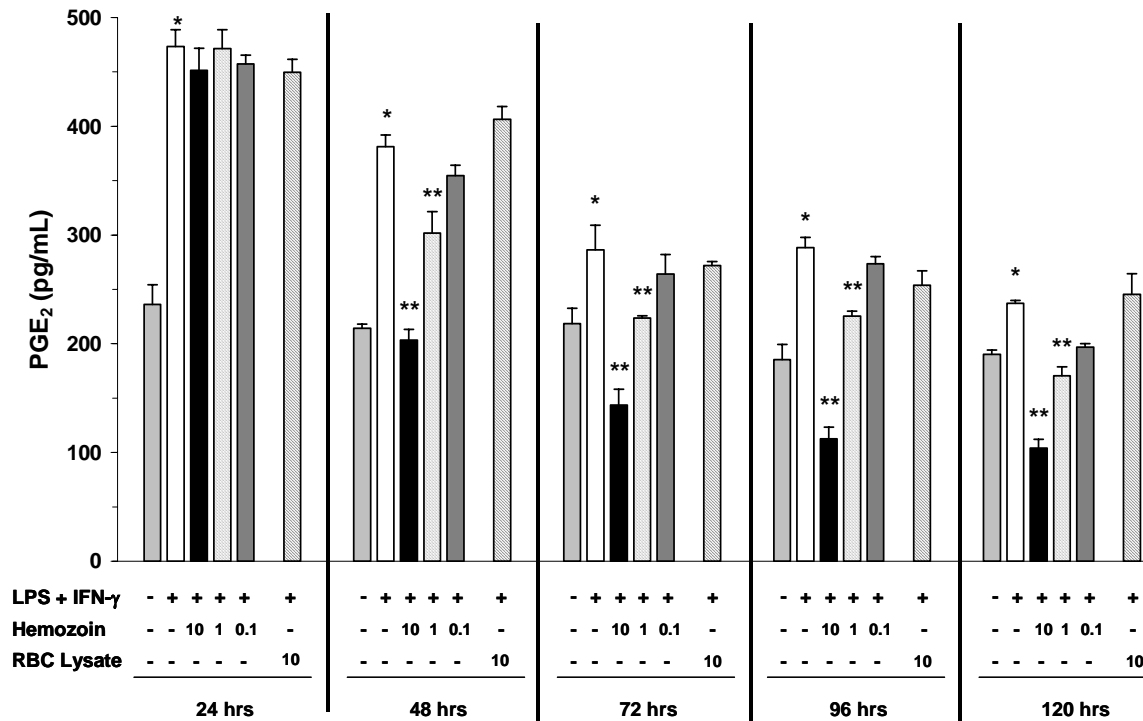


Figure 12. Temporal effect of hemozoin on PGE<sub>2</sub> production. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), and LPS and IFN- $\gamma$  in the presence of hemozoin (10, 1.0, and 0.1  $\mu$ g/mL) and an RBC lysate (10  $\mu$ g/mL). Supernatants were removed at 24, 48, 72, 96, and 120 hrs for PGE<sub>2</sub> determination. Values are the mean  $\pm$  SEM and are representative of three independent experiments. Statistical significance determined by the Mann-Whitney U test. \*P < 0.05 compared to control (media alone), \*\*P < 0.01 compared to stimulated conditions.

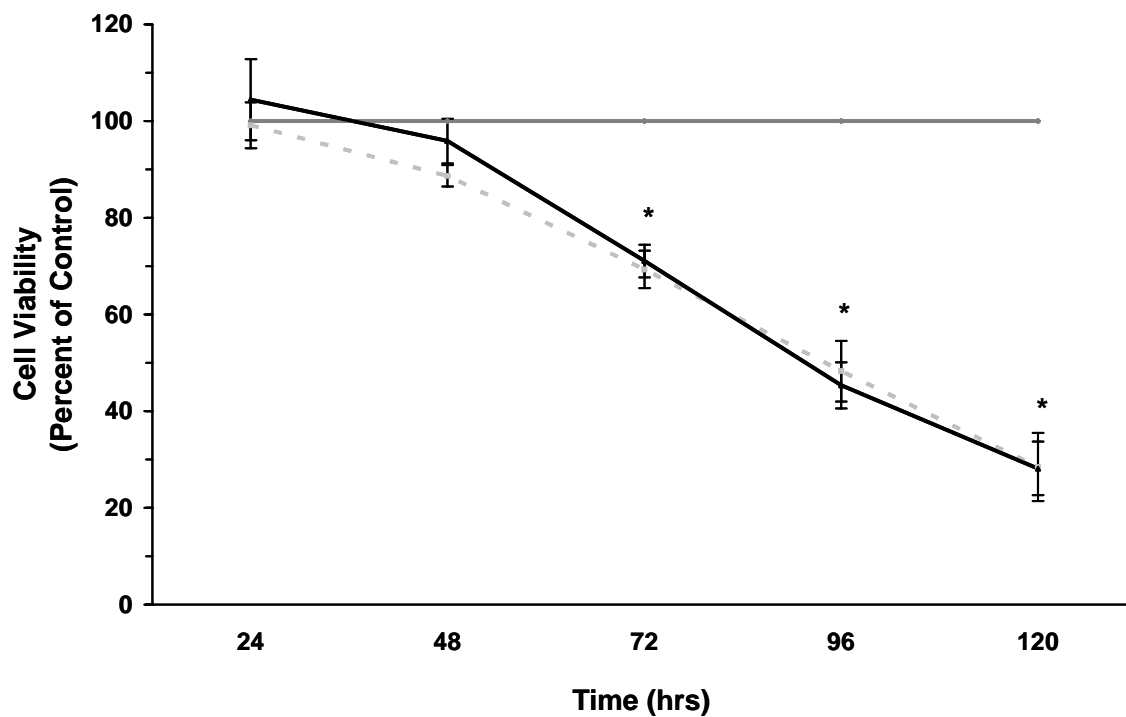


Figure 13. Temporal effect of hemozoin on cell viability. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone (solid gray line), LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL) (dashed gray line), and LPS and IFN- $\gamma$  in the presence of hemozoin [10  $\mu$ g/mL, (solid black line)]. Cell bioactivity was determined by the MTT assay at 24, 48, 72, 96, and 120 hrs. Values are the mean  $\pm$  SEM,  $n = 3$ . Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.05$ .

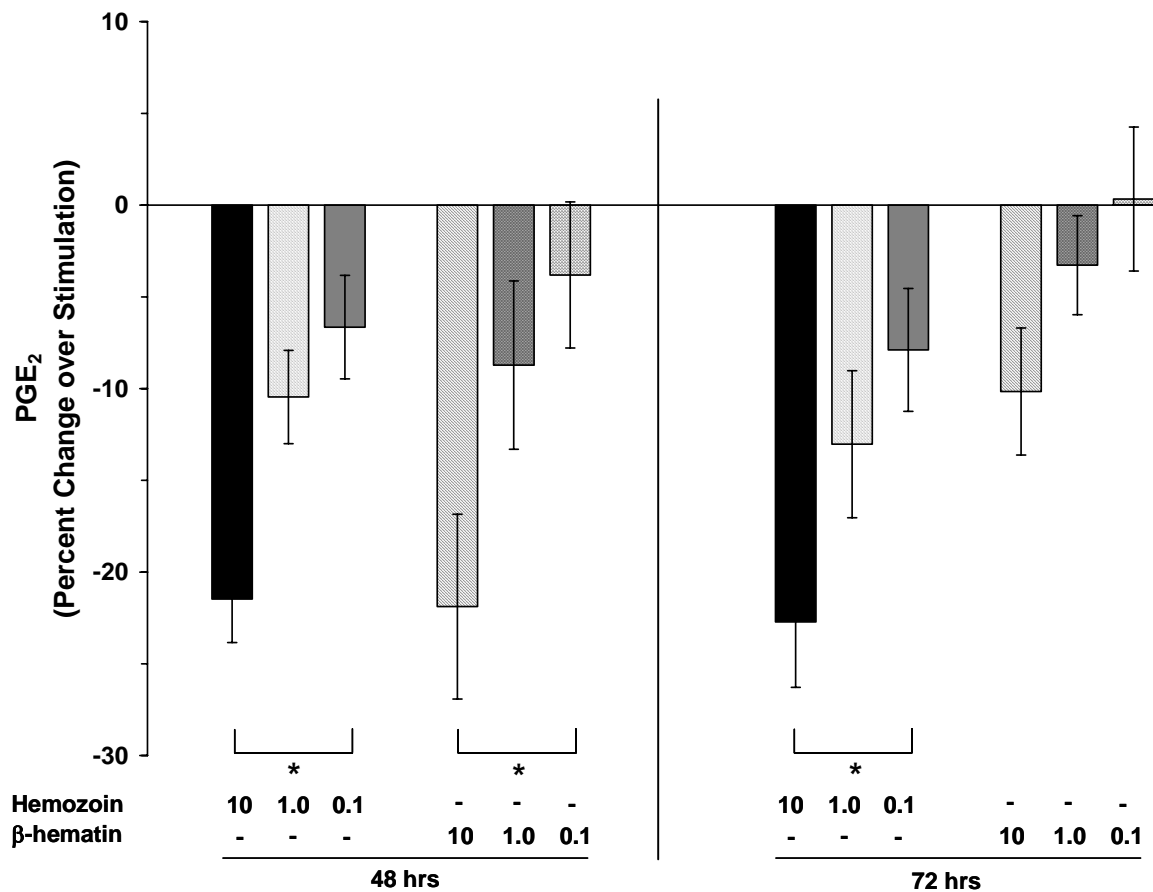


Figure 14. Effect of hemozoin and  $\beta$ -hematin on  $PGE_2$  production. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL) and LPS and IFN- $\gamma$  in the presence of hemozoin (10, 1.0, and 0.1  $\mu$ g/mL) or  $\beta$ -hematin (10, 1.0, and 0.1  $\mu$ g/mL). Supernatants were removed at 48 and 72 hrs for  $PGE_2$  determination. Values are the mean  $\pm$  SEM, n=18. Data are expressed as percent change over LPS and IFN- $\gamma$  stimulation. Statistical significance was determined by the Kruskal-Wallis test, \* $P < 0.05$ .

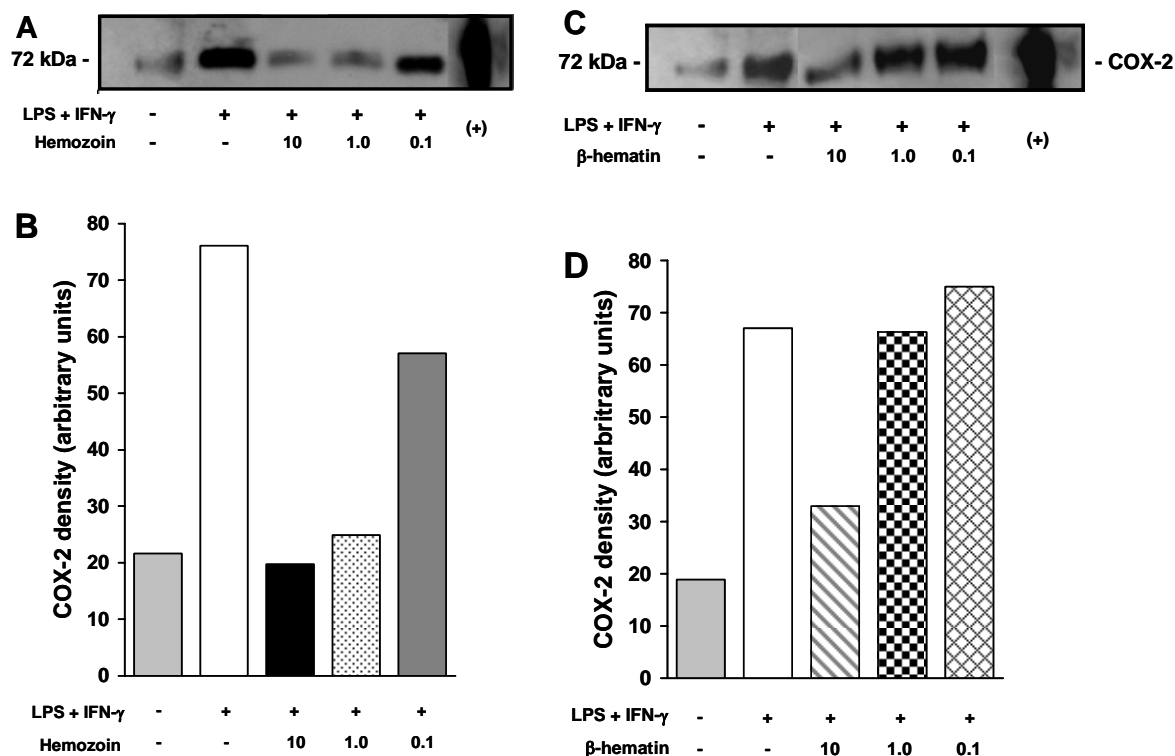


Figure 15. Effect of hemozoin and  $\beta$ -hematin on COX-2 protein expression. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), and LPS and IFN- $\gamma$  in the presence of hemozoin (10, 1.0, and 0.1  $\mu$ g/mL) or  $\beta$ -hematin (10, 1.0, and 0.1  $\mu$ g/mL). Cells were harvested at 48 hrs for COX-2 protein determination by immunoblot analysis. Shown above is a representative immunoblot of four independent experiments in different individuals for cells treated with hemozoin (A) or  $\beta$ -hematin (C). As a positive control (+), extracts of mouse macrophage cells (RAW 264.7), stimulated with lipopolysaccharide (*E. coli* LPS 0127B8, 1.0  $\mu$ g/mL) and IFN- $\gamma$  (10 ng/mL) were included. COX-2 antigen expression was quantified using commercially available software (ScnImage, Scion Corporation) in cells treated with (B) hemozoin or (D)  $\beta$ -hematin and expressed as relative arbitrary units.

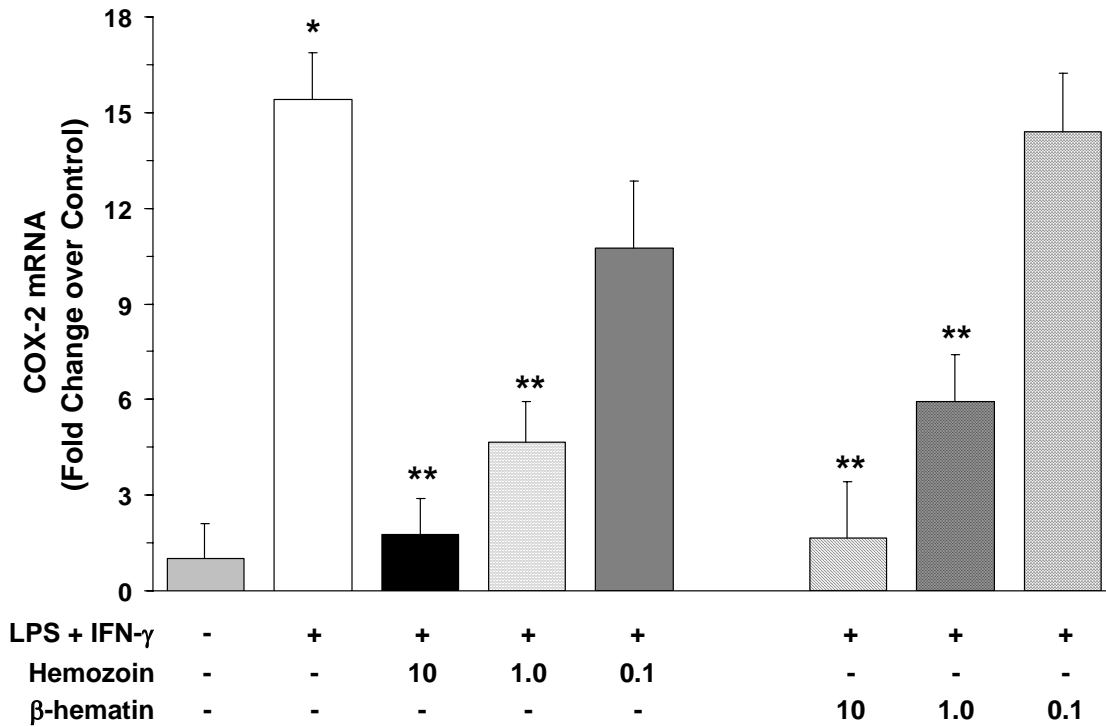


Figure 16. Effect of hemozoin on COX-2 mRNA expression. COX-2 mRNA was quantified for four individuals by Real time RT-PCR. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), and LPS and IFN- $\gamma$  in the presence of hemozoin (10, 1.0, and 0.1  $\mu$ g/mL) or  $\beta$ -hematin (10, 1.0, and 0.1  $\mu$ g/mL). Cells were collected at 48 hrs for COX-2 mRNA determination. Values are the mean  $\pm$  SEM, n=4. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.01$  compared to control (media alone), \*\* $P < 0.01$  compared to stimulated conditions.

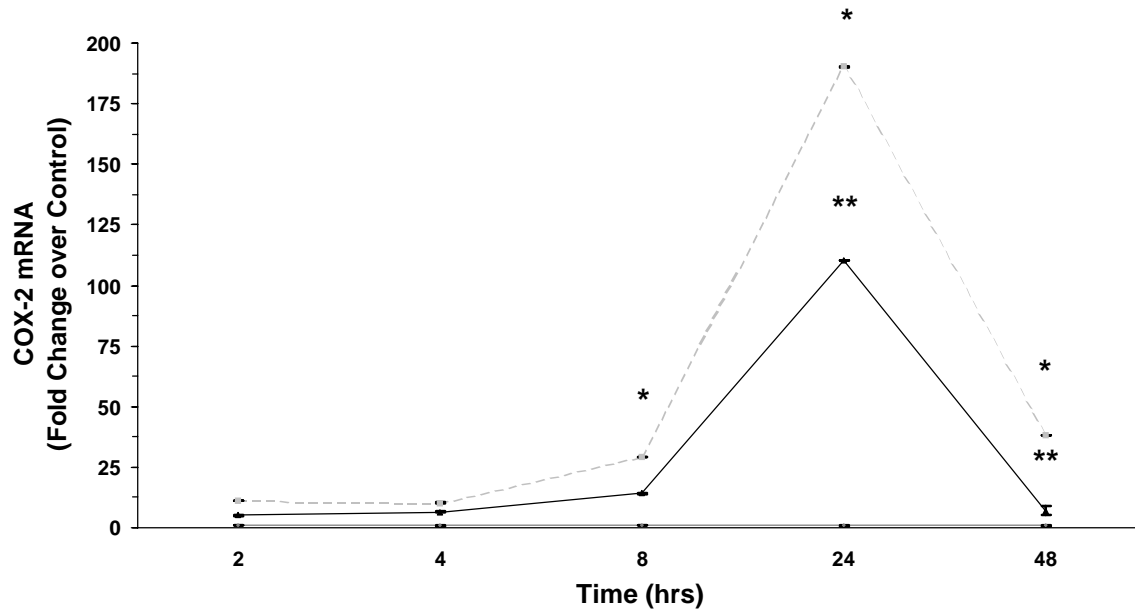


Figure 17. Temporal effect of hemozoin on COX-2 transcripts. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone (solid gray line), LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL) (dashed gray line), and LPS and IFN- $\gamma$  in the presence of hemozoin [10  $\mu$ g/mL, (solid black line)]. Cells were collected at 2, 4, 8, 24, and 48 hrs for COX-2 mRNA determination. Values are the mean  $\pm$  SEM and are representative of three independent experiments. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.01$  compared to control (media alone), \*\* $P < 0.01$  compared to stimulated conditions.

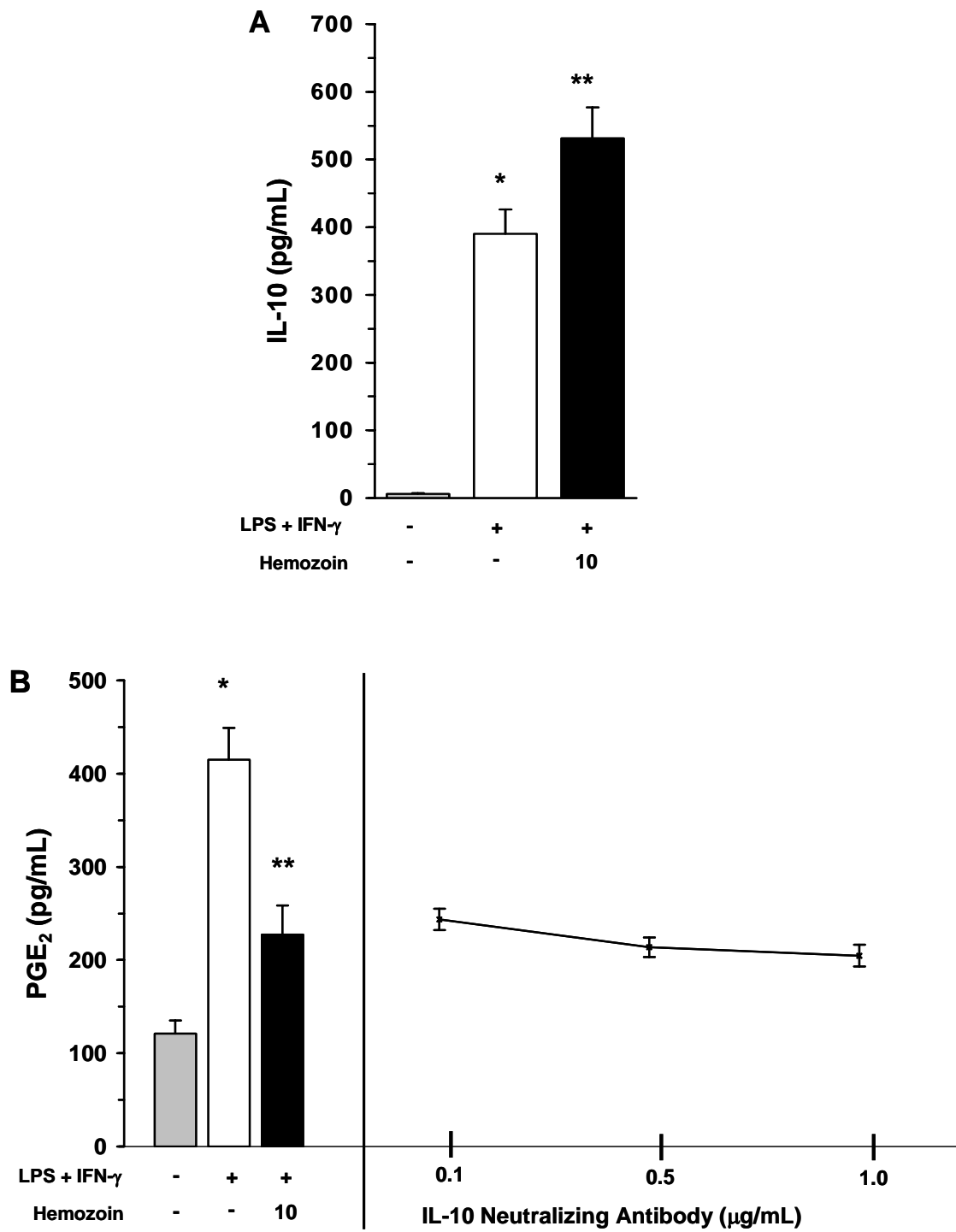


Figure 18. Effect of endogenous IL-10 on hemozoin-induced PGE<sub>2</sub> suppression.

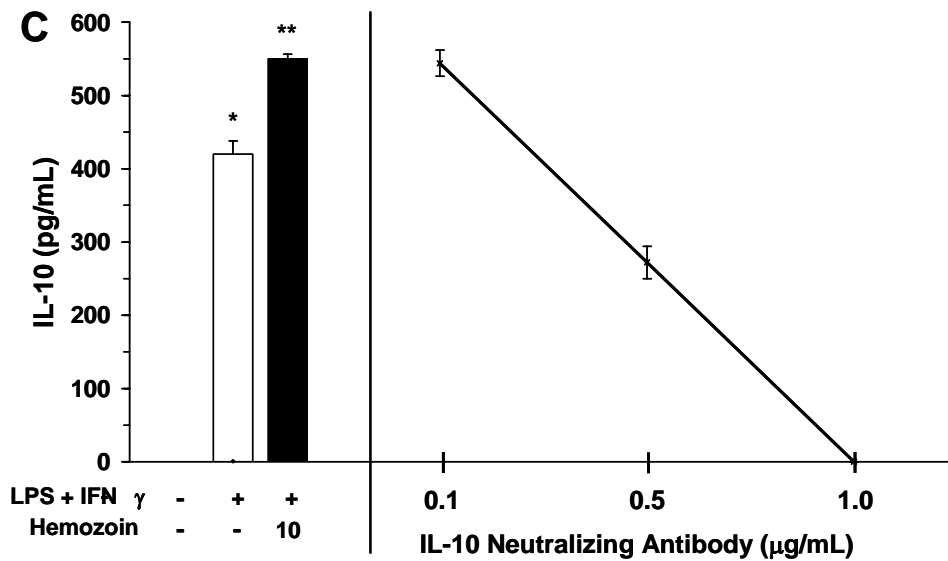


Figure 18. Effect of endogenous IL-10 on hemozoin-induced PGE<sub>2</sub> suppression. PBMC (1x10<sup>6</sup> cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN-γ (200 U/mL), and LPS and IFN-γ in the presence of hemozoin [10 μg/mL, (solid black line)]. (A) Supernatants were obtained at 48 hrs for IL-10 determination. (B) Cultures were incubated in the presence of varying concentrations of an IL-10 neutralizing antibody (1.0, 0.5, and 0.1 μg/mL) and supernatants were obtained at 48 hrs for PGE<sub>2</sub> determination. Values are the mean ± SEM, n=10 and 5, respectively. Statistical significance was determined by the Mann-Whitney U test. \**P* < 0.01 compared to control (media alone), \*\**P* < 0.05 compared to stimulated conditions.



## 4.2. EFFECT OF HEMOZOIN ON COX-2 ENZYMATIC ACTIVITY

### 4.2.1. Introduction

Regulation of COX-2-dependent PGE<sub>2</sub> production occurs at several levels, including suppression and/or destabilization of *de novo* COX-2-transcripts and/or through inhibition of COX-2 enzymatic activity. Data presented above illustrate that hemozoin suppresses PGE<sub>2</sub> production through reductions in *de novo* COX-2 mRNA and protein expression, however, inhibition of COX-2 enzyme activity by hemozoin has not been investigated.

Inhibitors of COX-1 and COX-2 enzymatic activity have been placed into three classes; class I which act as competitive substrate inhibitors, class II which elicit conformational changes in the protein structure of the enzyme, and class III which irreversibly acetylate the enzyme (Smith *et. al.* 1996). Inhibition of COX-2 enzymatic activity by specific COX-2 class II inhibitors, such as NS-398, occurs by reversible binding of the inhibitor to COX-2, followed by the formation of a COX-2-inhibitor complex which slowly dissociates over time (Copeland *et. al.* 1994). To determine if hemozoin interferes with COX-2 enzymatic activity, hemozoin and  $\beta$ -hematin were added to an *in vitro* COX-2 Inhibitor Screening Assay (Cayman Chem. Ann Arbor, MI). Furthermore, it has been proposed that hemozoin may bind to free heme (Schwarzer *et. al.* 1999), a necessary co-factor for COX-2 activity. Hemozoin binding to free heme may therefore

decrease COX-2 enzymatic activity and the subsequent production of PGE<sub>2</sub> through removal of a critical co-factor required for optimal enzymatic efficiency. As such, we examined the ability of hemozoin and β-hematin to bind free heme using an *in vitro* heme binding assay.

#### **4.2.2. Methods**

**4.2.2.1. COX-2 Inhibitor Screening Assay:** To determine if hemozoin interferes with COX-2 enzymatic activity, the COX-2 Inhibitor Screening Assay (Cayman Chem. Ann Arbor, MI) was used following the manufacturer's instructions. Briefly, COX-2 recombinant human enzyme was incubated in the presence or absence of hemozoin (10, 1.0, 0.1 μg/mL), β-hematin (10, 1.0, 0.1 μg/mL), or a COX-2-specific inhibitor, NS-398 (1 x 10<sup>-5</sup> M, positive control), for 10 min at 37°C. Exogenous arachidonic acid was then added to the reactions for 2 min, and the reaction was terminated by the addition of 1M HCl. The resultant PGH<sub>2</sub> was then non-enzymatically converted to PGF<sub>2α</sub> by the addition of stannous chloride. PGF<sub>2α</sub> was determined by EIA, with a sensitivity of detection ≥ 15.6 pg/mL. Data were expressed as percent of COX-2 enzyme activity (i.e., PGF<sub>2α</sub> formation) relative to control (no inhibitor) conditions. Statistical significance was determined by the Mann-Whitney U test at the *P* < 0.05 level.

**4.2.2.2. Heme Binding Assay:** To determine if hemozoin and β-hematin bind to heme, an *in vitro* heme-binding assay was conducted. Briefly, 100 μL volumes of varying concentrations of an exogenous heme solution (100, 50, 25, 12.5, 6.25, 3.13, 1.66, and 0.78 μg/mL) were incubated in the absence or presence of a high concentration (10 μg/mL) of hemozoin or β-hematin for 48 hrs at 37°C and 5% CO<sub>2</sub>. The plates were then centrifuged for 10 min at 3,000

rpm to pellet the hemozoin and  $\beta$ -hematin, and 20  $\mu$ L volumes of each sample were transferred to a 96-well plate, followed by the addition of 180  $\mu$ L of heme quantitation reagent (250  $\mu$ L of 2.5% Triton-X100 dissolved in methanol into 9.75 mL ethanol). Absorbance was obtained at 405 nm on a plate reader. Data were extrapolated against a freshly prepared standard curve of heme solution (serial dilutions of 100  $\mu$ g/mL of heme) and expressed as heme units ( $\mu$ g/mL). Statistical significance was determined by the Mann-Whitney U test at the  $P < 0.05$  level.

### **4.2.3. Results**

**4.2.3.1. Effect of Hemozoin and  $\beta$ -hematin on COX-2 Enzymatic Activity:** COX-2 enzyme activity was determined in the absence or presence of hemozoin (10, 1.0, 0.1  $\mu$ g/mL),  $\beta$ -hematin (10, 1.0, 0.1  $\mu$ g/mL), or a COX-2 specific inhibitor, NS-398 ( $1 \times 10^{-5}$  M). Hemozoin and  $\beta$ -hematin dose-dependently suppressed COX-2 enzyme activity ( $P < 0.05$  and  $P < 0.05$ , respectively, Figure 19). The positive control, NS-398, significantly inhibited COX-2 enzyme activity ( $P < 0.01$ , Figure 19).

**4.2.3.2. Ability of Hemozoin and  $\beta$ -hematin to Bind Free Heme:** The heme binding assay revealed that, relative to control (free heme incubated alone for 48 hrs), the addition of hemozoin or  $\beta$ -hematin significantly reduced the amount of heme detected at high doses (i.e., 100, 50, 25, and 12.5  $\mu$ g/mL,  $P < 0.05$  for all tests, Figure 20). The addition of NS-398 ( $1 \times 10^{-5}$  M) did not significantly alter free heme concentrations relative to control. Taken together, results presented here suggest that hemozoin inhibits COX-2-dependent enzymatic conversion of arachidonic acid to PGH<sub>2</sub>, at least in part, through reductions in free heme.

#### 4.2.4. Discussion

Although hemozoin suppressed *de novo* COX-2 transcripts and subsequent PGE<sub>2</sub> formation in the presence of inflammatory mediators (see Figures 12 - 17), it was important to determine if hemozoin inhibited PGE<sub>2</sub> formation through a mechanism such as enzymatic inhibition that was independent of down-regulation of *de novo* COX-2 transcripts. It has been proposed that hemozoin may act as a “sink” for free heme (Schwarzer *et. al.* 1999), which is a necessary co-factor for COX-2 activity. Results presented here demonstrate that incubation of COX-2 lysates with hemozoin and  $\beta$ -hematin dose-dependently inhibited COX-2 enzyme activity, suggesting that the core iron component of hemozoin, FP-IX, can interfere with the enzymatic activity of COX-2. However, high concentrations (10  $\mu$ g/mL) of hemozoin and  $\beta$ -hematin did not inhibit COX-2 enzymatic activity to the level of suppression observed by the specific COX-2 inhibitor, NS-398. These results are not unexpected since the mechanism of inhibition by NS-398 is direct binding to COX-2 versus removal of a critical co-factor (i.e., heme) through binding to hemozoin. Experimental results presented here support the notion that hemozoin binds to free heme in solution, thereby sequestering free heme that is required for COX-2 enzymatic function. Taken together, these data suggest that hemozoin-induced suppression of PGE<sub>2</sub> occurs at several levels; 1) reduction of *de novo* COX-2 transcripts and 2) inhibition of COX-2 enzymatic activity.

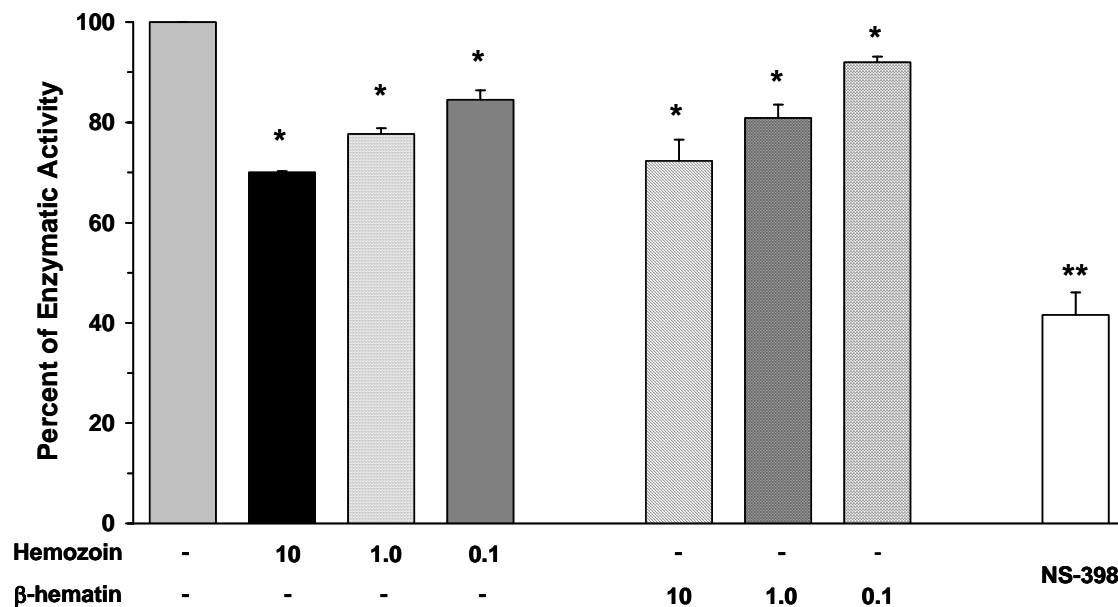


Figure 19. Effect of hemozoin and  $\beta$ -hematin on COX-2 enzymatic activity. COX-2 enzymatic activity was determined *in vitro* in the presence of hemozoin (10, 1.0, 0.1  $\mu\text{g}/\text{mL}$ ) and  $\beta$ -hematin (10, 1.0, 0.1  $\mu\text{g}/\text{mL}$ ), and a COX-2-specific inhibitor, NS-398 ( $1 \times 10^{-5}$  M, positive control). Values are the mean  $\pm$  SEM,  $n = 3/\text{condition}$ , and are expressed as percent of enzymatic activity relative to control. Statistical significance determined by the Mann-Whitney U test, \* $P < 0.05$ , \*\* $P < 0.01$ .

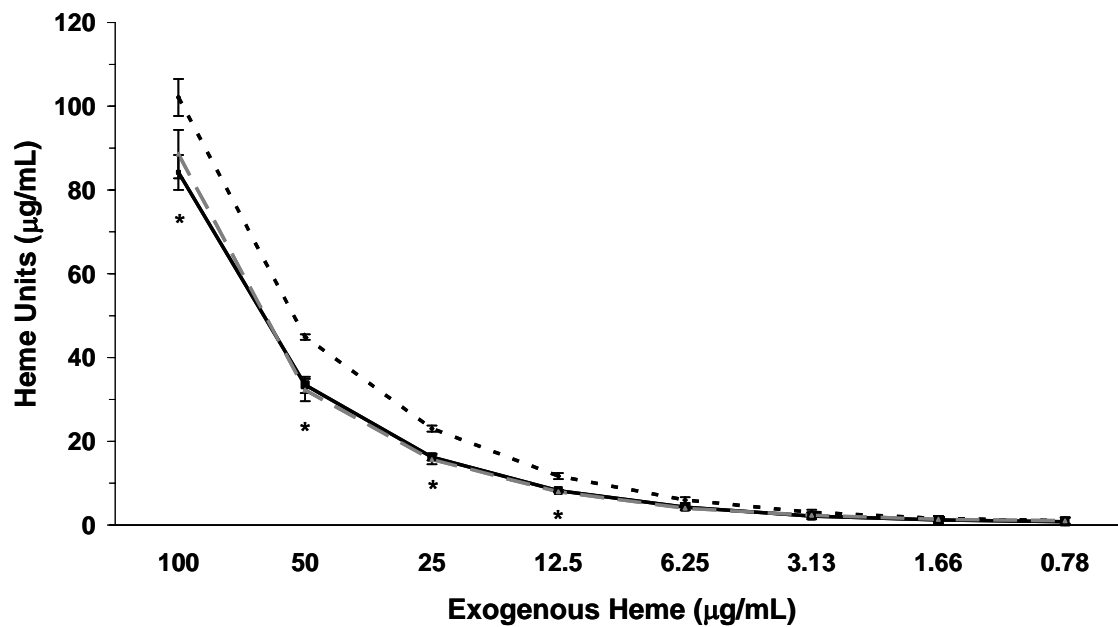


Figure 20. Effect of hemozoin and  $\beta$ -hematin on free heme. Varying concentrations of exogenous heme (100, 50, 25, 12.5, 6.25, 3.13, 1.66, and 0.78  $\mu\text{g/mL}$ ) were incubated for 48 hrs in the absence (control, dashed black line) or presence of a high concentration (10  $\mu\text{g/mL}$ ) of hemozoin (solid black line) or  $\beta$ -hematin (dashed gray line). Values are the mean  $\pm$  SEM,  $n = 3$ , and are expressed as heme units ( $\mu\text{g/mL}$ ). Statistical significance was determined by the Mann-Whitney U test,  $*P < 0.05$ .

**4.3. SPECIFIC AIM 2, HYPOTHESES 2 AND 3: PRESENTATION OF  
MANUSCRIPT ENTITLED: *HEMOZOIN-INDUCED SUPPRESSION OF COX-2-  
DERIVED PGE<sub>2</sub> ALLOWS OVER-PRODUCTION OF TNF- $\alpha$ : ASSOCIATION WITH THE  
PATHOGENESIS OF CHILDHOOD MALARIAL ANEMIA.***

Hypothesis 2 and 3 of Specific Aim 2 are examined in the following manuscript that has been submitted for publication in the *Journal of Infectious Diseases*.

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#### **4.3.1. Abstract**

Establishment of immune correlates based on molecular mechanisms involved in malaria pathogenesis will be useful in the development and testing of malaria vaccine candidates. We have previously shown that cyclooxygenase (COX)-2 and bicyclo-prostaglandin E<sub>2</sub> (bicyclo-PGE<sub>2</sub>) are suppressed in children with malarial anemia (MA). Furthermore, hemozoin (malarial pigment) suppressed monocyte/macrophage COX-2/PGE<sub>2</sub>, which allowed over-expression of tumor necrosis factor (TNF)- $\alpha$ . Because elevated TNF- $\alpha$  and reduced PGE<sub>2</sub> are associated with decreased erythropoiesis, we determined if bicyclo-PGE<sub>2</sub> relative to TNF- $\alpha$  (bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio) was associated with childhood MA. Relative to healthy subjects, the bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio was significantly lower in children with MA ( $P < 0.01$ ), and significantly associated with hemoglobin levels ( $r = 0.653$ ,  $P < 0.01$ ). In addition, the PGE<sub>2</sub>/TNF- $\alpha$  ratios examined in PBMC treated with physiologic concentrations of hemozoin were dose-dependently decreased. Thus, the PGE<sub>2</sub>/TNF- $\alpha$  ratio is an important immunologic correlate of MA, and ingestion of hemozoin by monocyte/macrophages appears responsible for decreased levels of PGE<sub>2</sub> relative to TNF- $\alpha$ .

#### **4.3.2. Introduction**

Malarial anemia (MA) is a multifaceted clinical manifestation of malaria with varying etiologies including intravascular and extravascular hemolysis of infected and non-infected RBCs, and suppression of erythropoiesis (Hillman *et al.* 1996). Depending on previous exposure to malaria (number of episodes), malaria transmission rates within a given geographic location (holoendemic versus hyperendemic), and the age at which the first MA episode occurs, the underlying causes of decreased Hb concentrations (anemia) are likely different. In areas with



high transmission rates of *Plasmodium falciparum*, mortality rates can be as high as 25-30% in children less than 5 years of age (Breman *et. al.* 2001). The high mortality rate in this age group is primarily due to MA (Breman *et. al.* 2001). Although the molecular immunologic basis that determines whether a child will develop mild versus severe forms of MA remains undefined, soluble mediators of immunity such as, cytokines and effector molecules appear to play an important role in the development of naturally acquired immunity.

Acute *P. falciparum* malaria is characterized by high systemic levels of tumor necrosis factor (TNF)- $\alpha$  (Perkins *et. al.* 2000), a pro-inflammatory cytokine which is both protective by restricting parasitemia (Kremsner *et. al.* 1995) and pathogenic by promoting anemia (Clark *et. al.* 1988). Our previous studies showed that plasma levels of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>, determined as the stable end-product: bicyclo-PGE<sub>2</sub>) and peripheral blood mononuclear cells (PBMC) cyclooxygenase-2 (COX-2) gene products are significantly reduced in acute childhood falciparum malaria (Perkins *et. al.* 2001). Eicosinoids such as PGE<sub>2</sub> are formed when phospholipase A<sub>2</sub> (PLA<sub>2</sub>) cleaves arachidonic acid (AA) from the phospholipid bilayer releasing free AA which is rapidly metabolized to PGH<sub>2</sub> via the enzymatic activity of the COX enzymes, COX-1 and COX-2, encoded by separate genes (Appleton *et. al.* 1996, Murakami *et. al.* 1997, Vane *et. al.* 1998). COX-1 is constitutively expressed in most tissues and generates prostaglandins (PGs) for physiologic homeostasis, and COX-2 is an inducible early response gene that generates high levels of PGs during inflammation (Seibert *et. al.* 1994). We have previously shown that TNF- $\alpha$  promotes stabilization of COX-2 transcripts and protein which generates high levels of sustained prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) biosynthesis (Perkins *et. al.* 1997). Once formed, PGE<sub>2</sub> can limit the biologic activity of TNF- $\alpha$  by inhibiting TNF- $\alpha$  transcription

and enhancing the release of soluble TNF- $\alpha$  receptors (Choi *et. al.* 1996, Kunkel *et. al.* 1988). Consistent with the fact that PGE<sub>2</sub> down-regulates TNF- $\alpha$ , abrogation of PGE<sub>2</sub> synthesis by indomethacin, a non-specific COX-1 and COX-2 inhibitor, promotes over-production of TNF- $\alpha$  from monocytes (Hart *et. al.* 1989).

We have previously shown that mononuclear phagocyte ingestion of malarial pigment (examined as both a crude hemozoin preparation and as synthetically-derived  $\beta$ -hematin) suppress COX-2 transcripts and the subsequent formation of PGE<sub>2</sub> (Keller *et. al.* 2004). Hemozoin is formed during the erythrocytic stage of malaria when the parasite catalyzes heme, which is released upon digestion of host hemoglobin, into an insoluble polymer that is no longer toxic to the parasite (Slater 1992). Hemozoin is acquired by circulating monocytes and neutrophils and tissue macrophages through phagocytosis of both parasitized erythrocytes and free hemozoin released upon erythrocytic lysis (Arese *et. al.* 1997, Schwarzer *et. al.* 1998). Our previous results showed that increased numbers of pigment containing monocytes and neutrophils in circulation are associated increased TNF- $\alpha$  and interleukin (IL)-10, and decreased IL-12 acute-phase cytokine levels (Luty *et. al.* 2000). Thus, phagocytosis of hemozoin by circulating monocytes and neutrophils appears to induce immunologic dysregulation during acute malaria by promoting over-production of TNF- $\alpha$ , IL-10, and suppression of PGE<sub>2</sub> and IL-12. Although the relative expression of TNF- $\alpha$ , IL-10, and IL-12 have been investigated and shown to be associated with malaria disease severity (Kurtzhals *et. al.* 1998, Othoro *et. al.* 1999, Perkins *et. al.* 2000), biologically significant relationships between TNF- $\alpha$  and PGE<sub>2</sub> have not previously been examined in malaria.

The present studies, therefore, investigated the relationship between COX-2/PGE<sub>2</sub> and TNF- $\alpha$  in cultured PBMC from healthy, malaria-naïve donors in the presence of physiologically relevant concentrations (10, 1.0, and 0.1  $\mu\text{g/mL}$ ) of hemozoin or  $\beta$ -hematin (Keller *et. al.* 2004). In addition, since erythropoietic maturation is enhanced by PGE<sub>2</sub> and suppressed by TNF- $\alpha$  (Dupuis *et. al.* 1998, Skobin *et. al.* 2000), and PGE<sub>2</sub> inhibits the production of TNF- $\alpha$  (Kunkel *et. al.* 1988), we determined if plasma levels of bicyclo-PGE<sub>2</sub> relative to TNF- $\alpha$  (the bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio) were associated with MA [defined by hemoglobin (Hb) concentrations] in children with falciparum malaria.

### **4.3.3. Subjects and Methods**

**4.3.3.1. Study Participants:** Children (n = 41, age 2 to 7 years) were recruited from a longitudinal prospective study at the Albert Schweitzer Hospital in Lambaréné, Gabon in the Province of Moyen Ogooue. In this area of hyperendemic malaria transmission, the primary clinical manifestations of severe childhood malaria are severe anemia and/or hyperparasitemia, with cerebral malaria occurring rarely (Kun *et. al.* 1998). Classification of malaria was defined according to World Health Organization guidelines (World Health Organization 1990). Children with mild MA were defined as those study participants with a Hb level between 9.0 and 10.9 g/dL in the presence of any density parasitemia, and moderate MA cases were defined as those study participants with a Hb level between 5.1 and 8.9 g/dL in the presence of any density parasitemia. Healthy, malaria-exposed subjects were defined as those participants with the absence of a positive thick blood film for malaria, or any other illnesses, within the last 4 weeks. All blood samples were obtained prior to treatment with antimalarials and/or antipyretics. We used routine clinical evaluations and laboratory measures to evaluate the patients. Children with

malaria were given antimalarials and the appropriate supportive therapy as required. Informed consent was obtained from the parents of participating children.

Healthy, malaria-naïve adult donors (n = 21) were recruited from the University of Pittsburgh, U.S.A. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital, Duke University Medical Center Investigational Review Board, the University of Tübingen, and the University of Pittsburgh Institutional Review Board.

**4.3.3.2. Isolation and Culture of Peripheral Blood Mononuclear Cells:** For in vitro experiments in U.S. adults (n = 21), venous blood (40 mL) was drawn into EDTA-containing vials. PBMC were prepared using ficoll/Hypaque as described earlier (Weinberg *et. al.* 1981), and plated at  $1 \times 10^6$  cells per mL in Dulbecco's modified Eagles medium (DMEM) containing 10% pooled human serum (heat inactivated at 56°C for 30 min; complete medium). Cells were incubated with media alone (controls) or with varying concentrations of hemozoin (10, 1.0, and 0.1 µg/mL) or β-hematin (10, 1.0, and 0.1 µg/mL). For determining the effect of exogenous PGE<sub>2</sub> on cytokine production, cultures were stimulated with HPLC-grade PGE<sub>2</sub> [( $1 \times 10^{-5}$  M to  $1 \times 10^{-9}$  M) Cayman Chem., Ann Arbor, MI]. For experiments with COX-2-specific inhibitors, PBMC in the absence and presence of β-hematin were cultured with varying concentrations of DuP-697 ( $1 \times 10^{-10}$  –  $1 \times 10^{-6}$  M) or indomethacin ( $1 \times 10^{-8}$  –  $1 \times 10^{-4}$  M).

**4.3.3.3. Preparation of Hemozoin and β-hematin:** A crude preparation of hemozoin was isolated as described earlier (Keller *et. al.* 2004). Briefly, *P. falciparum*-infected red blood cells (pRBC) (strain Pf-D6, 3-5% parasitemia, predominately late trophozoites and early schizonts)

were isolated from in vitro cultures and lysed with saponin. Samples were spun at 14,000 rpm for 15 min, and the pelleted material was washed in PBS until free from the white-colored red blood cell (RBC) cellular components. The final pellet was dried, weighed, and resuspended in filter-sterilized H<sub>2</sub>O at a final concentration of 1.0 mg/mL. The final solution was extensively sonicated to disperse the hemozoin.

$\beta$ -hematin was formed in a 4.5 M acidic acetate solution at pH 4.5 by the method of Egan *et al.* (Egan *et al.* 2001). Briefly, hemin chloride (Sigma, St. Louis, MO) was added to a 0.1 M solution of NaOH followed by the addition of HCl at 60°C. A solution of acetate was then added, and the mixture was incubated for 150 min at 60°C without stirring. The  $\beta$ -hematin was then spun at 14,000 rpm in a microcentrifuge, washed 3X with filter-sterilized H<sub>2</sub>O, and dried at 60°C under vacuum. The final pellet was weighed and resuspended at 1.0 mg/mL in filter-sterilized H<sub>2</sub>O. The resuspended  $\beta$ -hematin was sonicated extensively to disperse the preparation. Under these preparation conditions,  $\beta$ -hematin has an identical infrared spectroscopy pattern as detergent-purified hemozoin, and  $\beta$ -hematin crystals appear morphologically similar to natural hemozoin crystals as evidenced by scanning electron microscopy (Egan *et al.* 2001).

**4.3.3.4. Measurement of Prostaglandins and TNF- $\alpha$ :** To determine plasma PGE<sub>2</sub> concentrations in children with malaria, we measured the stable end-metabolite of PGE<sub>2</sub>, bicyclo-PGE<sub>2</sub> by competitive EIA (Cayman, Ann Arbor, MI) according to our previously described methods (Perkins *et al.* 2001). Since PGE<sub>2</sub> is rapidly metabolized in biologic solutions, the most accurate means of measuring PGE<sub>2</sub> in plasma is by converting PGE<sub>2</sub> and the intermediary metabolites to the stable end product bicyclo-PGE<sub>2</sub>. Briefly, plasma was

precipitated in ethanol at 4°C and then purified by solid phase extraction with DSC-18 (octadecyl bonded silica, Suppelco, Bellefonte, PA). PGs were eluted with ethyl acetate and dried under vacuum centrifugation. Samples were derivatized overnight at 37°C followed by re-suspension in buffer. Bicyclo-PGE<sub>2</sub> was then determined by quantitative sandwich enzyme immunoassay (EIA) technique (Cayman Chemical Co., Ann Arbor, MI).

PGE<sub>2</sub> was measured in the supernatants of cultured PBMC using a quantitative indirect sandwich EIA (Cayman Chem., Ann Arbor, MI) with a sensitivity of detection  $\geq 7.8$  pg/mL. Briefly, samples were incubated overnight in goat anti-mouse IgG-coated plates in the presence of a mouse IgG anti-PGE<sub>2</sub> monoclonal antibody and a PGE<sub>2</sub>-acetylcholinesterase tracer. After washing, the plates were incubated at room temperature with Ellman's reagent, and absorbance readings were obtained at 550 nm.

Concentrations of TNF- $\alpha$  in plasma from children and supernatants from cultured PBMC were determined by quantitative sandwich ELISA (sensitivity of detection  $\geq 4.4$  pg/mL), following the manufacturers specifications (Pharmingen, San Diego, CA).

**4.3.3.5. Immunoblot Analyses:** Cellular extracts were prepared according to our previously defined methods (Perkins *et. al.* 1997). Protein samples (30  $\mu$ g) were fractionated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and COX-2 antigen was measured by our previously defined methods (Perkins *et. al.* 2001). Extracts of mouse macrophage cells (RAW 264.7), stimulated with lipopolysaccharide (E. coli LPS 0127B8, 1.0  $\mu$ g/mL) and IFN- $\gamma$  (10 ng/mL) were used as a positive control. Densitometry readings for COX-

2 protein expression were obtained using commercially available software (ScanImage, Scion Corporation).

**4.3.3.6. Quantitative real time RT-PCR:** Total RNA was isolated from PBMC by the GITC method (Chomczynski *et. al.* 1987). Total RNA (1  $\mu$ g) was reverse transcribed into cDNA, and COX-2 and TNF- $\alpha$  gene expression was analyzed by quantitative real time RT-PCR on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). cDNA (100 ng) was amplified in duplicate with COX-2 specific primers and probe as follows: forward, 5'-CCT TCC TCC TGT GCC TGA TG-3'; reverse, 5'-ACA ATC TCA TTT GAA TCA GGA AGC T-3'; probe (FAM labeled), 5'-TGC CCG ACT CCC TTG GGT GTC A-3' [sequence information obtained from (Sales *et. al.* 2001)], or with specific primer/probe sets for TNF- $\alpha$  [Accession Number NM\_000594 (Applied Biosystems, Foster City, CA)]. The cDNA was amplified for 40 cycles following the parameters specified by the manufacturer (Applied Biosystems, Foster City, CA). To control for non-specific background fluorescence, no template controls were included in triplicate. An endogenous control gene,  $\beta$ -actin [Accession Number NM\_001101 (Applied Biosystems, Foster City, CA)], was used as a reference gene to normalize cDNA loading between samples. Data was compared using the  $-\Delta\Delta C_T$  method as previously described (Keller *et. al.* 2004).

**4.3.3.7. Statistical Analyses:** Plasma concentrations of bicyclo-PGE<sub>2</sub> (pg/mL) and TNF- $\alpha$  (pg/mL) and supernatant concentrations of PGE<sub>2</sub> (pg/mL) and TNF- $\alpha$  (pg/mL) were measured in duplicate at several different dilutions. Pairwise comparisons between conditions were performed by the Mann-Whitney U test. Multiple group comparisons were performed using the

Kruskal-Wallis test (statistical significance set at  $P \leq 0.05$ ). Linear regression was used to determine the association between the bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio and hemoglobin (Hb) levels with statistical significance set at  $P \leq 0.05$ .

#### **4.3.4. Results**

**4.3.4.1. Effect of Hemozoin and  $\beta$ -hematin on COX-2 Protein Expression:** Our previously published results showed that hemozoin or  $\beta$ -hematin suppress LPS- and IFN- $\gamma$ -induced COX-2 mRNA and protein, and PGE<sub>2</sub> production (Keller *et. al.* 2004). Although COX-2-derived PGE<sub>2</sub> production is enhanced by cellular activation (e.g., LPS and IFN- $\gamma$  stimulation), our previous results show that baseline COX-2 gene expression is detectable in cultured human PBMC (Keller *et. al.* 2004) and is highly expressed in *ex vivo* PBMC from healthy malaria-exposed children (Perkins *et. al.* 2001). In the current experiments, we sought to determine if hemozoin or  $\beta$ -hematin reduced COX-2-derived PGE<sub>2</sub> production in mononuclear cells that were not primed with COX-2 inducing stimuli. To accomplish this, the effects of hemozoin or  $\beta$ -hematin on COX-2 protein expression were examined at 48 hrs, a time in which hemozoin is successfully phagocytosed by monocytes (Olliaro *et. al.* 2000, Schwarzer *et. al.* 2001), and does not alter cell viability (Keller *et. al.* 2004). Stimulation of PBMC with hemozoin or  $\beta$ -hematin (10  $\mu$ g/mL) reduced baseline COX-2 protein expression (Figures 21A and B), demonstrating that baseline levels of COX-2 protein in PBMC are reduced following phagocytosis of hemozoin and  $\beta$ -hematin.



**4.3.4.2. Effect of Hemozoin and  $\beta$ -hematin on COX-2 and TNF- $\alpha$  Transcripts:** To determine if hemozoin- and  $\beta$ -hematin-promoted down-regulation of baseline COX-2 protein resulted from a suppression of COX-2 mRNA formation, PBMC were stimulated with media alone or a high concentration (10  $\mu\text{g}/\text{mL}$ ) of hemozoin or  $\beta$ -hematin for 48 hrs. As shown in Figure 22A, the addition of hemozoin or  $\beta$ -hematin to cultured PBMC suppressed COX-2 transcripts ( $P < 0.05$  and  $P < 0.05$ , respectively).

To examine if TNF- $\alpha$  transcripts were induced by hemozoin or  $\beta$ -hematin, PBMC were stimulated with media alone or a high concentration (10  $\mu\text{g}/\text{mL}$ ) of hemozoin or  $\beta$ -hematin for 48 hrs. Addition of hemozoin or  $\beta$ -hematin to cultured PBMC induced TNF- $\alpha$  transcripts ( $P < 0.05$  and  $P < 0.05$ , respectively, Figure 22B). Taken together, these results illustrate that hemozoin and  $\beta$ -hematin can reduce COX-2 transcripts and induce TNF- $\alpha$  mRNA formation in the absence of mitogenic stimulation.

**4.3.4.3. Effect of Hemozoin and  $\beta$ -hematin on Baseline PGE<sub>2</sub> and TNF- $\alpha$  Production:** To determine if hemozoin- and  $\beta$ -hematin-promoted down-regulation of baseline COX-2 transcripts and protein resulted in suppression of PGE<sub>2</sub> biosynthesis, PBMC were stimulated with media alone or varying physiologically relevant concentrations of hemozoin (10, 1.0, and 0.1  $\mu\text{g}/\text{mL}$ ) or  $\beta$ -hematin (10, 1.0, and 0.1  $\mu\text{g}/\text{mL}$ ,) for 48 hrs. As shown in Figure 23A, the addition of hemozoin or  $\beta$ -hematin to cultured PBMC dose-dependently suppressed PGE<sub>2</sub> production ( $P < 0.05$ ), illustrating that hemozoin and  $\beta$ -hematin can reduce spontaneous PBMC PGE<sub>2</sub> production in the absence of mitogenic stimulation.

Previous studies indicate that hemozoin and  $\beta$ -hematin increase TNF- $\alpha$  production in human PBMC (Biswas *et. al.* 2001, Mordmuller *et. al.* 1998, Pichyangkul *et. al.* 1994, Sherry *et. al.* 1995). Since PGE<sub>2</sub> is known to inhibit TNF- $\alpha$  transcription (Choi *et. al.* 1996, Kunkel *et. al.* 1988), we determined if TNF- $\alpha$  levels were elevated under experimental conditions that suppressed PGE<sub>2</sub> formation. TNF- $\alpha$  synthesis was determined in culture supernatants of PBMC stimulated with media alone or varying concentrations of hemozoin (10, 1.0, and 0.1  $\mu$ g/mL) or  $\beta$ -hematin (10, 1.0, and 0.1  $\mu$ g/mL) for 48 hrs. Addition of hemozoin and  $\beta$ -hematin to PBMC dose-dependently increased baseline TNF- $\alpha$  production ( $P < 0.01$ , Figure 23B). Thus, these results show that hemozoin and  $\beta$ -hematin significantly elevate TNF- $\alpha$  production under the same experimental conditions that suppress COX-2-derived PGE<sub>2</sub> production.

Although the inverse pattern of PGE<sub>2</sub> and TNF- $\alpha$  production suggested a potential biologic relationship, to further explore the association between PGE<sub>2</sub> and TNF- $\alpha$ , both factors were measured in identical culture supernatants from hemozoin and  $\beta$ -hematin-treated PBMC and expressed as the PGE<sub>2</sub>/TNF- $\alpha$  ratio. The PGE<sub>2</sub>/TNF- $\alpha$  ratio decreased with increasing concentrations of hemozoin ( $P < 0.01$ , Figure 23C), demonstrating that hemozoin induces an immunologic profile characterized by low levels of PGE<sub>2</sub> and elevated production of TNF- $\alpha$ .

#### **4.3.4.4. Effect of Exogenous PGE<sub>2</sub> Addition on Hemozoin- and $\beta$ -hematin-induced TNF-**

**$\alpha$  Production:** Since TNF- $\alpha$  production in monocytes is decreased by PGE<sub>2</sub> (Choi *et. al.* 1996, Kunkel *et. al.* 1988), and hemozoin induced low levels of PGE<sub>2</sub> relative to TNF- $\alpha$  in our experimental paradigm, we determined if the exogenous addition of PGE<sub>2</sub> could reduce TNF- $\alpha$

production in hemozoin- and  $\beta$ -hematin-treated PBMC. To test this hypothesis, exogenous PGE<sub>2</sub> ( $1 \times 10^{-9}$  to  $1 \times 10^{-5}$  M) was added to PBMC treated with a high dose (10  $\mu$ g/mL) of hemozoin or  $\beta$ -hematin, and TNF- $\alpha$  production was determined at 48 hrs. Addition of hemozoin or  $\beta$ -hematin increased TNF- $\alpha$  production ( $P < 0.05$ ), which was dose-dependently suppressed by the addition of exogenous PGE<sub>2</sub> ( $P < 0.05$ , Figure 24). Results presented here demonstrate that hemozoin- and  $\beta$ -hematin-induced TNF- $\alpha$  production is reduced by physiologically relevant concentrations of PGE<sub>2</sub>, illustrating that suppression of COX-2-derived PGE<sub>2</sub> by hemozoin and  $\beta$ -hematin promotes high levels of TNF- $\alpha$  synthesis.

**4.3.4.5. Effect of COX-2 Inhibitors on  $\beta$ -hematin-induced TNF- $\alpha$  Production:** Since  $\beta$ -hematin-induced suppression of PGE<sub>2</sub> caused over-expression of TNF- $\alpha$ , we wished to determine if further reductions in PGE<sub>2</sub> by blockade of COX-2 enzymatic activity in the presence of  $\beta$ -hematin would further augment TNF- $\alpha$  production. To test this hypothesis, PBMC were stimulated with media alone or a high concentration (10  $\mu$ g/mL) of  $\beta$ -hematin in the absence or presence of COX-2-specific inhibitors, DuP-697 ( $1 \times 10^{-10}$  -  $1 \times 10^{-6}$  M) or indomethacin ( $1 \times 10^{-8}$  -  $1 \times 10^{-4}$  M). Supernatants were collected at 48 hrs for TNF- $\alpha$  determination. Addition of high concentrations of DuP-697 ( $1 \times 10^{-7}$  and  $1 \times 10^{-6}$  M) and indomethacin ( $1 \times 10^{-4}$  M) significantly increased TNF- $\alpha$  production in unstimulated PBMC ( $P < 0.05$ ,  $P < 0.05$ , and  $P < 0.05$ , respectively, Figures 25A and C).  $\beta$ -hematin significantly increased TNF- $\alpha$  production ( $P < 0.05$  and  $P < 0.05$ , respectively), and addition of DuP-697 or indomethacin to  $\beta$ -hematin-stimulated cultures dose-dependently augmented TNF- $\alpha$  production, with high concentrations of

DuP-697 ( $1 \times 10^{-8}$  -  $1 \times 10^{-6}$  M) and indomethacin ( $1 \times 10^{-5}$  and  $1 \times 10^{-4}$  M) significantly increasing TNF- $\alpha$  levels ( $P < 0.05$  for all tests, Figures 25A and C).

To further examine the interaction between reduced PGE<sub>2</sub> biosynthesis and increased TNF- $\alpha$  production, the IC<sub>50</sub> concentration for PGE<sub>2</sub> was calculated for DuP-697 and indomethacin for our *in vitro* system, and the corresponding TNF- $\alpha$  concentration was then determined. To accomplish this, PGE<sub>2</sub> levels were determined under the same experimental conditions when TNF- $\alpha$  was augmented.  $\beta$ -hematin significantly suppressed PGE<sub>2</sub> production ( $P < 0.05$  and  $P < 0.05$ , respectively Figures 25B and D), and the addition of DuP-697 or indomethacin to unstimulated or  $\beta$ -hematin-stimulated cells dose-dependently suppressed PGE<sub>2</sub> ( $P < 0.05$  for all tests, Figures 25B and D).

Using the dose-response curve generated for TNF- $\alpha$  and PGE<sub>2</sub> (Table 3), the IC<sub>50</sub> was calculated for each of the COX-2-specific inhibitors (Table 4). In our *in vitro* assays, the IC<sub>50</sub> concentrations for DuP-697 and indomethacin were  $7.50 \times 10^{-9}$  and  $2.73 \times 10^{-7}$  M, respectively (Table 3). The corresponding TNF- $\alpha$  concentrations at the PGE<sub>2</sub> IC<sub>50</sub> concentrations were 166.33 and 112.11 pg/mL for DuP-697 and indomethacin, respectively (Table 4). In our *in vitro* assays using COX-2-specific inhibitors, when one half of the PGE<sub>2</sub> production was suppressed, DuP-697 caused a greater than 100% induction in TNF- $\alpha$  levels over  $\beta$ -hematin alone, while indomethacin induced a 50% increase in TNF- $\alpha$  (Table 4). These results illustrate that  $\beta$ -hematin-induced TNF- $\alpha$  production, as a consequence of reduced PGE<sub>2</sub> biosynthesis, can be further increased by inhibition of COX-2, suggesting that administration of compounds which target COX-2 during malaria may lead to a further suppression in PGE<sub>2</sub> formation.

**4.3.4.6. Clinical and Immunologic Parameters in Children with MA:** To determine if the in vitro findings reflected physiologically relevant changes in PGE<sub>2</sub> and TNF- $\alpha$  during acute malaria, the relationship between PGE<sub>2</sub> and TNF- $\alpha$  was examined in healthy, malaria-exposed children (n = 19), and children with mild (n = 10) and moderate MA (n = 12). Characteristics of the study participants are summarized in Table 4. There were no cases of severe MA (i.e., Hb < 5.0 g/dL) available for the current analyses. As shown in Table 5, age (years) between the different groups was not significantly different. Children with moderate MA had higher admission temperature ( $P < 0.01$ ), geometric mean parasitemia (parasites/ $\mu$ L,  $P < 0.05$ ), and lower Hb concentrations (g/dL,  $P < 0.001$ ) than children with mild MA (Table 5). Plasma bicyclo-PGE<sub>2</sub> (pg/mL) and TNF- $\alpha$  (pg/mL) levels were also different between the groups ( $P < 0.01$  and  $P < 0.001$ , respectively, Table 5).

**4.3.4.7. Association of the Plasma Bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  Ratio with MA Disease Severity:** Although measuring cytokines and effector molecules individually and comparing their levels with disease pathogenesis is useful, expressing the cytokines and effector molecules in biologically relevant proportions (ratios) may better reflect immunologic correlates of disease. Since PGE<sub>2</sub> is known to inhibit TNF- $\alpha$  production from mononuclear cells (Kunkel *et. al.* 1988), and the PGE<sub>2</sub>/TNF- $\alpha$  ratio was significantly reduced with increasing concentrations of hemozoin, we determined if plasma levels of bicyclo-PGE<sub>2</sub> relative to TNF- $\alpha$  (bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio) differed according to MA status. The plasma bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio decreased with increasing disease severity, with the lowest ratio present in children with moderate MA ( $P < 0.01$ , Figure 26). The plasma bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio also differed

between the mild MA and moderate MA groups ( $P < 0.01$ , Figure 26). These results show that increasing severity of MA is defined by a proportional decrease in circulating levels of bicyclo-PGE<sub>2</sub> relative to TNF- $\alpha$ .

#### **4.3.4.8. Association of the Plasma Bicyclo-PGE<sub>2</sub>/TNF- $\alpha$ Ratio with Hemoglobin**

**Concentrations:** In vitro experiments with CD34+ stem cells show that elevated levels of TNF- $\alpha$  suppress erythropoiesis (Skobin *et. al.* 2000), while the addition of PGE<sub>2</sub> enhances erythropoiesis (Dupuis *et. al.* 1998). We, therefore, hypothesized that the relative expression of bicyclo-PGE<sub>2</sub> to TNF- $\alpha$  may be an important immunologic marker/mediator for MA. As shown in Figure 27, the bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio was significantly correlated with Hb concentrations in children with MA ( $r = 0.62$ ,  $P < 0.01$ ). These results illustrate that reduced Hb concentrations are present in an inflammatory milieu characterized by low levels of circulating PGE<sub>2</sub> and high levels of TNF- $\alpha$ .

#### **4.3.5. Discussion**

Development of a protective versus pathogenic response to malaria is largely determined by host-mediated release of soluble factors that govern anti-parasitic activity. Although the role of inflammatory-derived PGE<sub>2</sub> in regulating host immunity to parasitic infections has been examined for a number of tropical pathogens [for review see (Daugochies *et. al.* 2000)], the functions of PGE<sub>2</sub> in malarial immunity remains largely undefined. Our previous studies in Gabonese children showed that plasma bicyclo-PGE<sub>2</sub> levels and PBMC COX-2 gene expression are suppressed during acute malaria, with the lowest levels of COX-2/PGE<sub>2</sub> associated with the most severe disease (Perkins *et. al.* 2001). Additional studies in our laboratory demonstrated that

phagocytosis of hemozoin by stimulated human blood mononuclear cells is responsible, at least in part, for decreased COX-2-derived PGE<sub>2</sub> biosynthesis during malaria (Keller *et. al.* 2004). The molecular basis of reduced COX-2/PGE<sub>2</sub> during malaria appears to be due to the ability of hemozoin to inhibit COX enzymatic activity via binding of host-derived heme, a requisite co-factor for maximal COX activity, which results in decreased COX-2 transcript formation (see Figures 19 and 20). The effect of reduced COX-2-derived PGE<sub>2</sub> on additional soluble mediators (i.e., TNF- $\alpha$ ) that regulate innate immunity to malaria, however, was previously undefined. Results presented here in cultured, non-stimulated PBMC from healthy, malaria-naïve donors show that suppression of COX-2/PGE<sub>2</sub> production by hemozoin and  $\beta$ -hematin promotes over-production of TNF- $\alpha$ . These *in vitro* findings were also investigated in children with *P. falciparum*-induced MA which revealed that the relative production of bicyclo-PGE<sub>2</sub> to TNF- $\alpha$  (expressed as the bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio) is significantly associated with anemia.

Results presented here suggest that phagocytosis of hemozoin by blood mononuclear cells is, at least in part, responsible for suppression of PGE<sub>2</sub> and over-production of TNF- $\alpha$  during malaria. In agreement with previous reports (Biswas *et. al.* 2001, Mordmuller *et. al.* 1998, Pichyangkul *et. al.* 1994, Sherry *et. al.* 1995), results presented here show that ingestion of hemozoin and  $\beta$ -hematin by human blood mononuclear cells increases TNF- $\alpha$  production. TNF- $\alpha$  is a pleiotropic cytokine that can serve as a protective factor, by limiting malaria parasitemia (Kremsner *et. al.* 1995), and if over-expressed, a pathogenic molecule that can promote MA (Clark *et. al.* 1988). It, therefore, appears that strict regulation of TNF- $\alpha$  synthesis during a malaria infection is essential. Our previous studies showed that TNF- $\alpha$  promotes high levels of PGE<sub>2</sub> production through induction of sustained COX-2 mRNA and protein expression (Perkins

*et. al.* 1997). Although TNF- $\alpha$  increases COX-2 gene expression, reciprocal interactions also occur in which PGE<sub>2</sub> directly limits the actions of TNF- $\alpha$  by: 1) suppressing *de novo* TNF- $\alpha$  transcription (Kunkel *et. al.* 1988), and 2) regulating the secretion of soluble TNF- $\alpha$  receptors (Choi *et. al.* 1996). Results presented here showing that exogenous PGE<sub>2</sub> dose-dependently decreases TNF- $\alpha$  production in hemozoin-treated cells provides direct evidence that decreased PG production allows over-production of TNF- $\alpha$ .

The relationship between suppression of PGE<sub>2</sub> and over-production of TNF- $\alpha$  in response to hemozoin appear biologically significant for the development of MA, since PGE<sub>2</sub> enhances burst forming unit erythroid (BFU-E) formation (Dupuis *et. al.* 1998), while TNF- $\alpha$  inhibits differentiation of CD34+ hematopoietic precursors into BFU-E (Skobin *et. al.* 2000). The *in vivo* significance of the interactions between PGE<sub>2</sub> and TNF- $\alpha$  are supported in the present study by the fact that reduced bicyclo-PGE<sub>2</sub> and increased TNF- $\alpha$  in plasma are associated with decreasing Hb concentrations. The effect of a low bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio on the development of anemia may involve several mechanisms. Since PGE<sub>2</sub> can inhibit the release of TNF- $\alpha$  from monocytes (Kunkel *et. al.* 1988), we propose that the low monocyte-derived PGE<sub>2</sub> production results in the inability of PGE<sub>2</sub> to feedback and decrease TNF- $\alpha$  production. This would result in high levels of TNF- $\alpha$  that may then induce intravascular and extravascular hemolysis of pRBC and non-pRBCs by the reticuloendothelial system, particularly those RBCs towards the end of their 120-day life span (Hillman *et. al.* 1996). Once hemolysis of RBCs occurs during acute malaria, it is then necessary to increase erythropoiesis to restore the lost RBC population. Thus, we propose a dual mechanism in which low levels of PGE<sub>2</sub> relative to TNF- $\alpha$  contribute to increased hemolysis and decreased production of RBCs.



While the role of PGs in MA remains only partially defined, it appears that COX-2/PGE<sub>2</sub> may be regulated differently in the central nervous system than in the periphery. Previous studies showed that COX-2 protein levels are increased within Durck's granulomas in patients with cerebral malaria (CM) (Deininger *et. al.* 2000). Recent studies also illustrated that COX-2 transcripts were significantly increased within the brain, but non-significantly decreased in PBMC of mice with CM (Ball *et. al.* 2004). These experiments further demonstrated that infection with a parasite strain that causes anemia in the absence of cerebral complications (*P. berghei* K173) significantly decreased COX-2 transcripts in the periphery (Ball *et. al.* 2004). Our recent studies in Tanzanian children showed that systemic levels of PGE<sub>2</sub> (measured as urinary bicyclo-PGE<sub>2</sub>/creatinine) are suppressed during CM (Perkins *et. al.* 2005). We have also demonstrated that naturally acquired hemozoin is associated with decreased PGE<sub>2</sub> production from cultured placental blood mononuclear cells from women with falciparum malaria during pregnancy (Perkins *et. al.* 2003). Results presented here extend the previous findings by illustrating that decreased levels of inflammatory-derived PGs during malaria can influence additional mediators such as TNF- $\alpha$  that are known to be important in malaria pathogenesis. Additional biologic interactions between PGE<sub>2</sub> and TNF- $\alpha$  are important for regulation of the febrile response. This appears particularly relevant for malaria since the universal hallmark of the disease is fever. Results in our previous studies showed that the lowest levels of COX-2/PGE<sub>2</sub> in circulation are observed in children with the highest fevers (Perkins *et. al.* 2001), an event that relies upon systemically derived pro-inflammatory cytokine (e.g., TNF- $\alpha$ ) induction of COX-2/PGE<sub>2</sub> production within the preoptic area of the anterior hypothalamus (POAH) (Katsuura *et. al.* 1990). Based on our previous results, and data presented here, it appears that hemozoin-induced suppression of COX-2/PGE<sub>2</sub> in circulating mononuclear cells may allow up-

regulation of TNF- $\alpha$  in the periphery, which may then increase COX-2/PGE<sub>2</sub> in the POAH for generation of the febrile response. Thus, low levels of COX-2/PGE<sub>2</sub> in the periphery may result in pro-inflammatory-induced up-regulation of COX-2/PGE<sub>2</sub> in the brain, an event that is supported by the findings to date (Ball *et. al.* 2004, Deininger *et. al.* 2000, Xiao *et. al.* 1999).

In conclusion, we show that the relative production of PGE<sub>2</sub> to TNF- $\alpha$  is significantly lower in children with malaria and that phagocytosis of hemozoin by PBMC may be responsible for the suppression of this ratio. Furthermore, the PGE<sub>2</sub>/TNF- $\alpha$  ratio significantly correlates with Hb concentrations, indicating that this ratio may be an important immunologic marker and potential mediator of MA. Although we propose that changes in PGE<sub>2</sub> and TNF- $\alpha$  production contribute to the development of MA, and that the PGE<sub>2</sub>/TNF- $\alpha$  ratio represents a valid immunologic correlate of disease, it is important to note that MA is a condition influenced by many complex genetic and immunologic factors.

#### **4.3.6. Acknowledgments**

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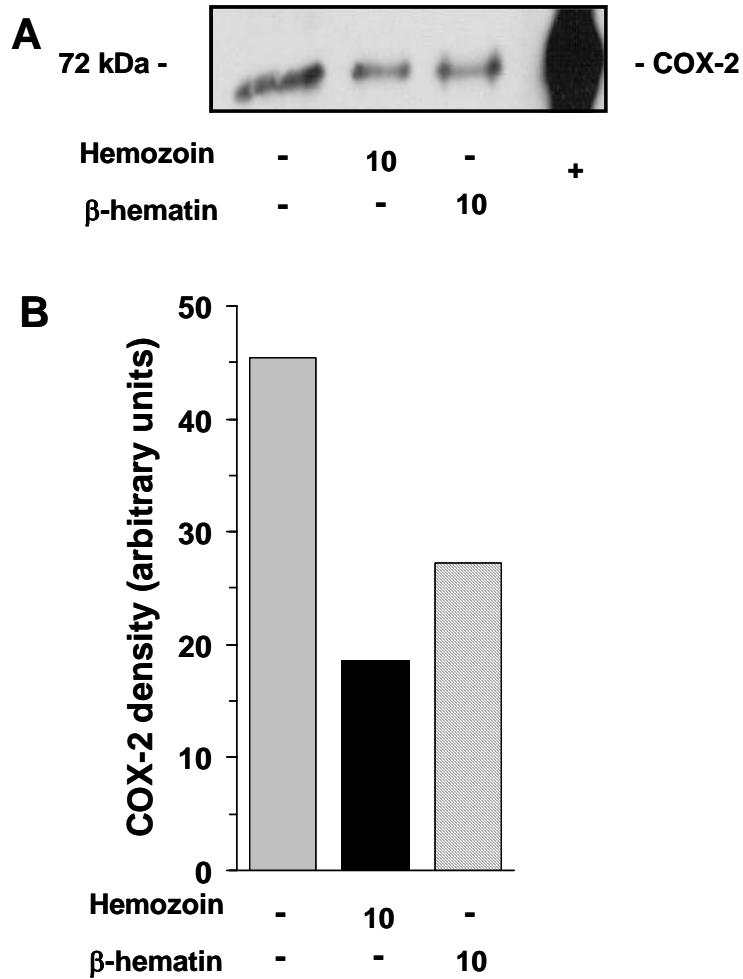


Figure 21. Effect of hemozoin and  $\beta$ -hematin on baseline COX-2 protein expression. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone or a high concentration (10  $\mu$ g/mL) of hemozoin or  $\beta$ -hematin. Cells were harvested at 48 hrs for COX-2 protein determination by immunoblot analysis. (A) Shown above is a representative immunoblot of four independent experiments in different individuals. As a positive control (+), extracts of mouse macrophage cells (RAW 264.7), stimulated with lipopolysaccharide (*E. coli* LPS 0127B8, 1.0  $\mu$ g/mL) and IFN- $\gamma$  (10 ng/mL) were included. (B) COX-2 antigen expression was quantified using commercially available software (ScnImage, Scion Corporation).

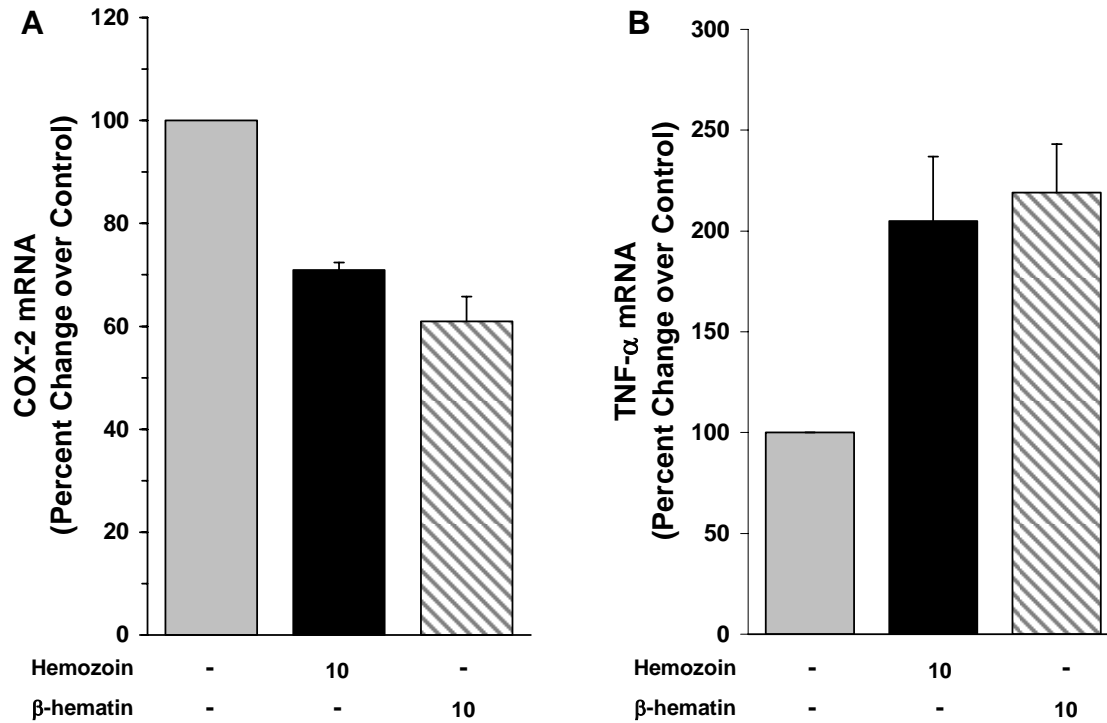


Figure 22. Effect of hemozoin and  $\beta$ -hematin on baseline COX-2 and TNF- $\alpha$  transcripts. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone or a high concentration (10  $\mu$ g/mL) of hemozoin or  $\beta$ -hematin. Cells were harvested at 48 hrs for (A) COX-2 and (B) TNF- $\alpha$  mRNA determination by Taqman real time RT-PCR. Data were expressed as percent change of unstimulated conditions. Values are the mean  $\pm$  SEM, n=3. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.05$  compared to control (media alone), \*\* $P < 0.01$  compared to control (media alone).

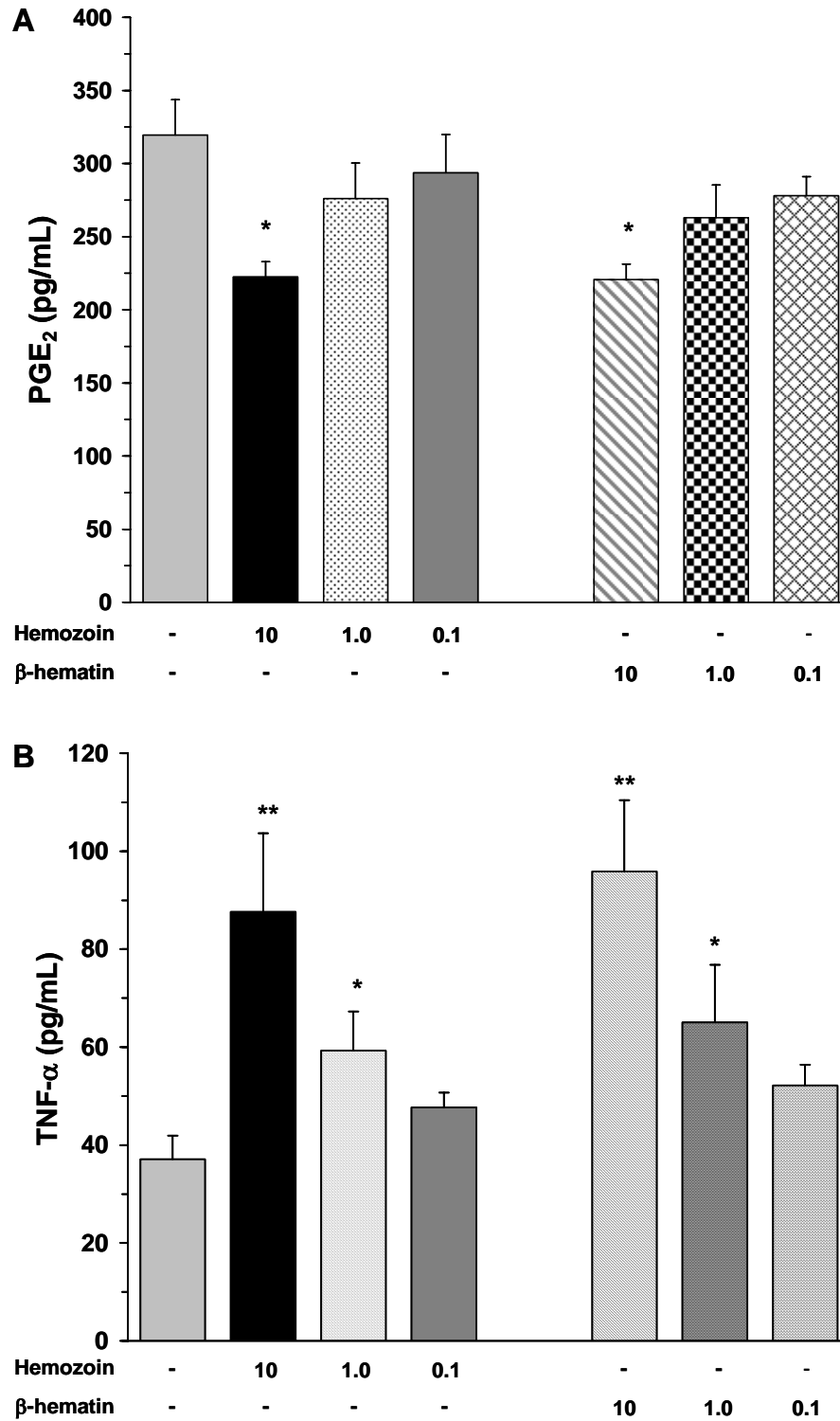


Figure 23. Effect of hemozoin and  $\beta$ -hematin on baseline PGE<sub>2</sub> and TNF- $\alpha$  production.

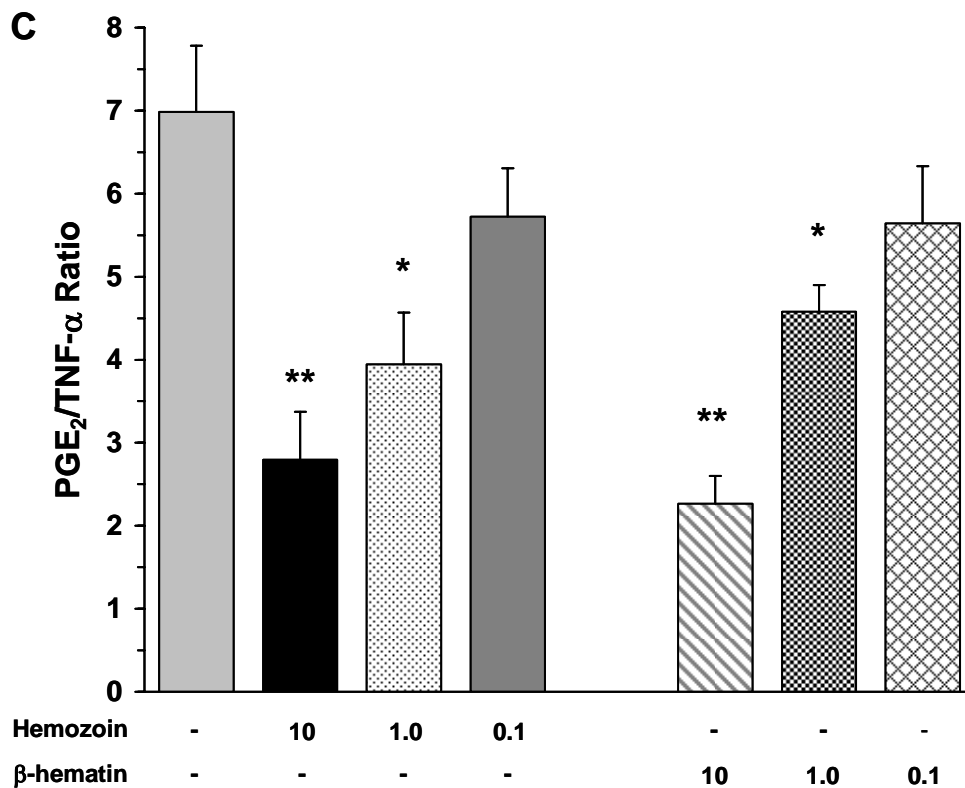


Figure 23. Effect of hemozoin and  $\beta$ -hematin on baseline PGE<sub>2</sub> and TNF- $\alpha$  production. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone or varying concentrations of hemozoin (10, 1.0, and 0.1  $\mu$ g/mL) or  $\beta$ -hematin (10, 1.0, and 0.1  $\mu$ g/mL). Supernatants were removed at 48 hrs for (A) PGE<sub>2</sub> and (B) TNF- $\alpha$  determination by EIA and ELISA, respectively. (C) Data was expressed as the ratio of PGE<sub>2</sub>/TNF- $\alpha$ . Values are the mean  $\pm$  SEM, n=10. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.05$  compared to control (media alone), \*\* $P < 0.01$  compared to control (media alone).

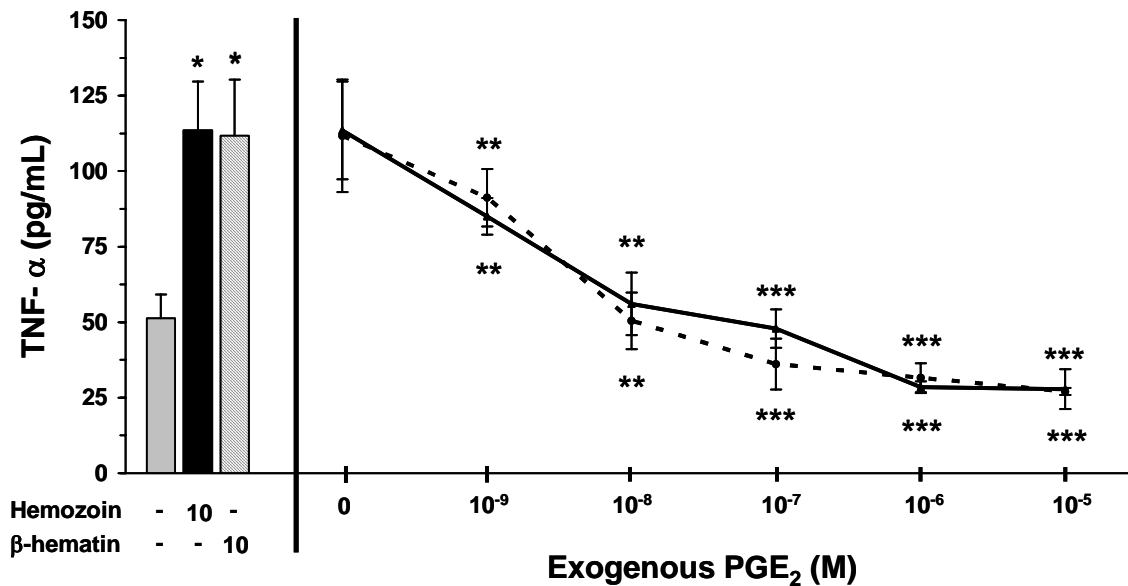


Figure 24. Effect of exogenous PGE<sub>2</sub> addition on hemozoin- and  $\beta$ -hematin-induced TNF- $\alpha$  production. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone or a high concentration (10  $\mu$ g/mL) of hemozoin or  $\beta$ -hematin. Cells were treated with increasing concentrations of PGE<sub>2</sub> ( $1 \times 10^{-9}$ - $1 \times 10^{-5}$ M) in the presence of a high concentration (10  $\mu$ g/mL) of hemozoin (solid black line) or  $\beta$ -hematin (dashed black line) and supernatants were collected at 48 hrs for TNF- $\alpha$  determination. Values are mean  $\pm$  SEM and are representative of three independent experiments. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.01$  compared to control (media alone), \*\* $P < 0.05$  compared to no exogenous PGE<sub>2</sub>, \*\*\* $P < 0.01$  compared to no exogenous PGE<sub>2</sub>.

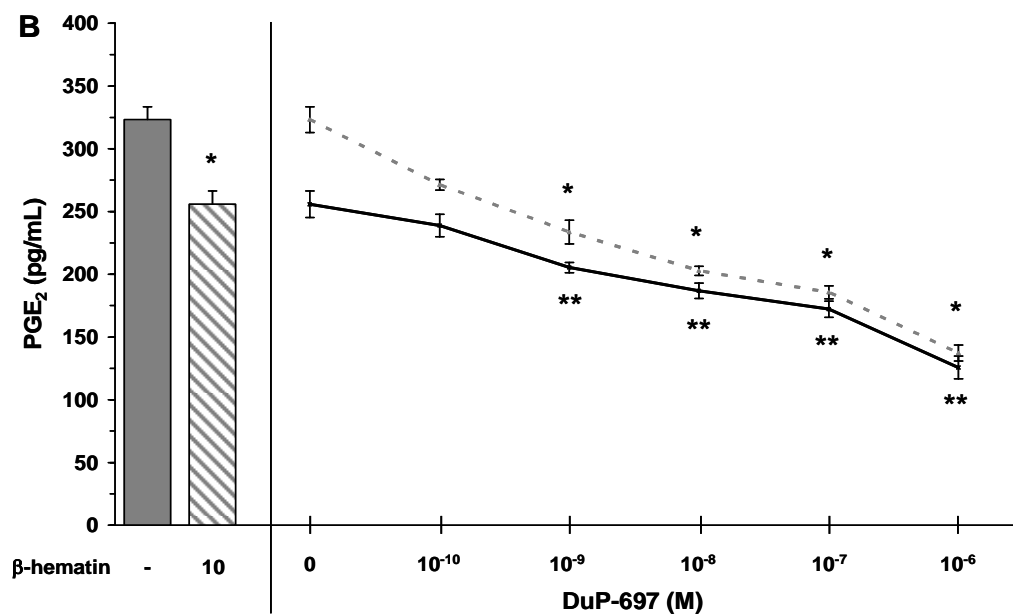
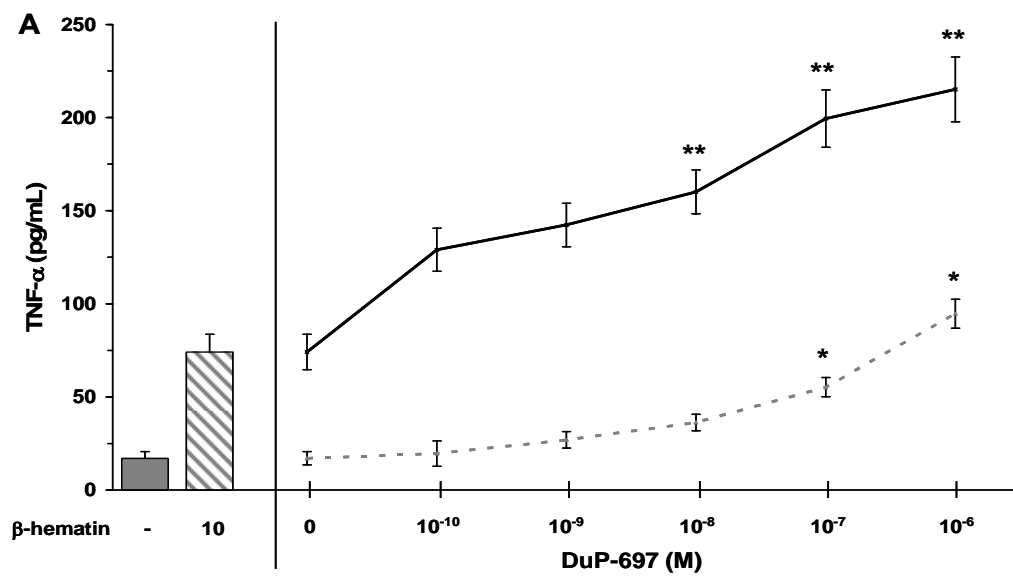


Figure 25. Effect of COX-2 inhibitors on  $\beta$ -hematin-induced TNF- $\alpha$  production and PGE<sub>2</sub> suppression.



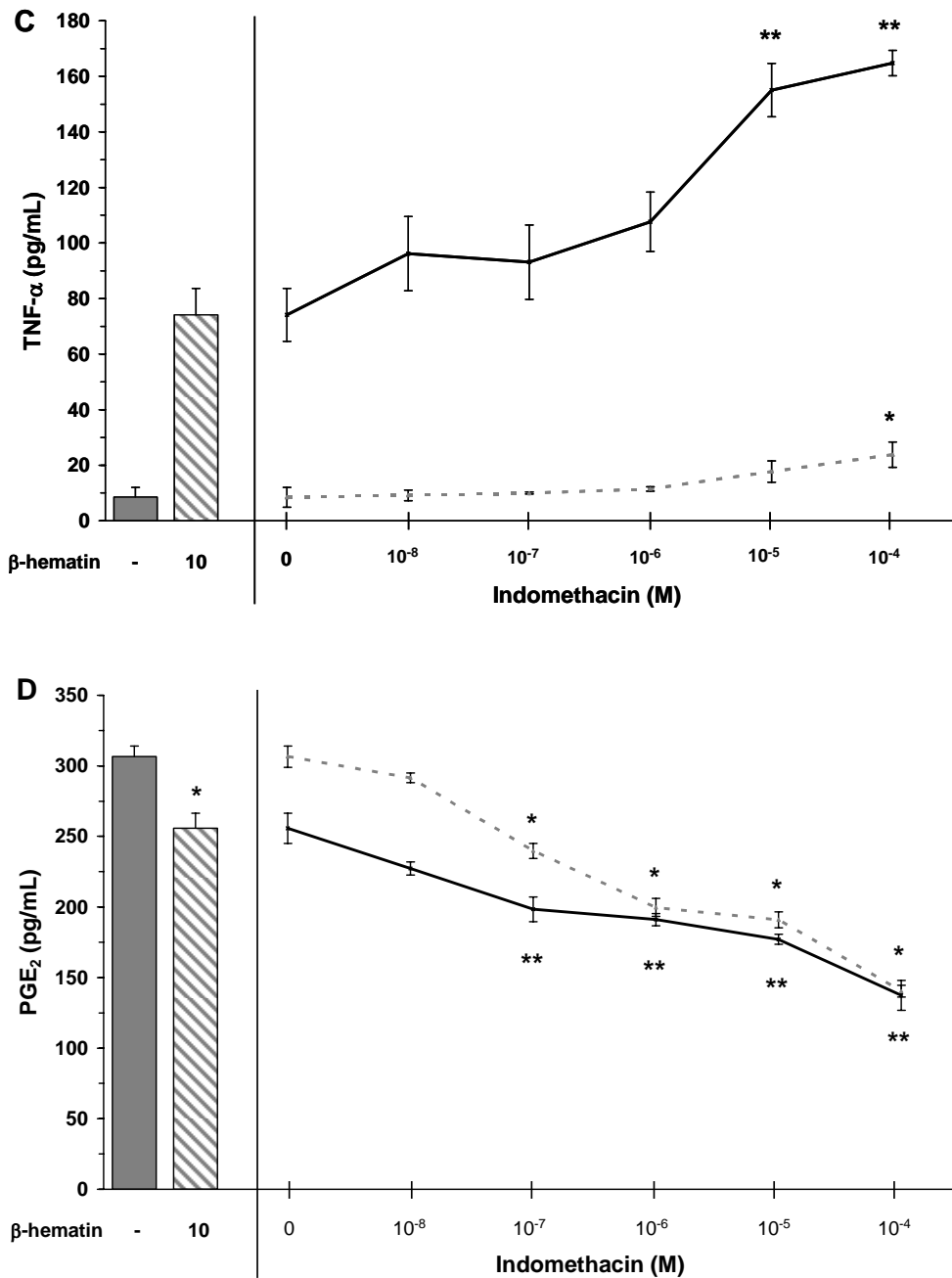


Figure 25. Effect of COX-2 inhibitors on  $\beta$ -hematin-induced TNF- $\alpha$  production and PGE<sub>2</sub> suppression. PBMC ( $1 \times 10^6$  cells/mL) were stimulated with media alone or a high concentration (10  $\mu$ g/mL) of  $\beta$ -hematin. Cells were treated with increasing concentrations of (A and B) DuP-697 ( $1 \times 10^{-10}$  -  $1 \times 10^{-6}$  M) or (C and D) indomethacin ( $1 \times 10^{-8}$  -  $1 \times 10^{-4}$  M) in the presence of media alone (dashed gray line) or (10  $\mu$ g/mL) of  $\beta$ -hematin (dashed black line) and supernatants were

collected at 48 hrs for (A and C) TNF- $\alpha$  and (B and D) PGE<sub>2</sub> determination. Values are mean  $\pm$  SEM and are representative of 3 independent experiments. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.01$  compared to control (media alone), \*\* $P < 0.05$  compared to no exogenous PGE<sub>2</sub>, \*\*\* $P < 0.01$  compared to no exogenous PGE<sub>2</sub>.

Table 3. Effect of COX-2-specific inhibitors on PGE<sub>2</sub> and TNF- $\alpha$  production.

	DuP-697		Indomethacin	
	PGE <sub>2</sub> <sup>a</sup>	TNF- $\alpha$ <sup>a</sup>	PGE <sub>2</sub>	TNF- $\alpha$
Control	323.22	17.07	306.56	16.55
$\beta$ -hematin	252.32	79.22	257.78	74.13
Conc. (M)				
1x10 <sup>-10</sup>	238.84	129.07	ND <sup>b</sup>	ND <sup>b</sup>
1x10 <sup>-9</sup>	205.39	142.31	ND <sup>b</sup>	ND <sup>b</sup>
1x10 <sup>-8</sup>	186.83	160.05	227.26	96.17
1x10 <sup>-7</sup>	172.17	199.39	198.33	93.11
1x10 <sup>-6</sup>	125.74	215.16	191.10	107.63
1x10 <sup>-5</sup>	ND <sup>b</sup>	ND <sup>b</sup>	177.11	155.06
1x10 <sup>-4</sup>	ND <sup>b</sup>	ND <sup>b</sup>	137.37	164.78

<sup>a</sup>Supernatant concentrations of PGE<sub>2</sub> (pg/mL) and TNF- $\alpha$  (pg/mL) in  $\beta$ -hematin-stimulated PBMC, values are representative of 3 independent experiments.

<sup>b</sup>ND = not determined for the indicated concentration.

Table 4. PGE<sub>2</sub> IC<sub>50</sub> Concentrations for COX-2-specific inhibitors and corresponding TNF- $\alpha$  concentrations.

	DuP-697	Indomethacin
PGE <sub>2</sub> IC <sub>50</sub> Conc. (M) <sup>a</sup>	7.50 x 10 <sup>-9</sup>	2.73 x 10 <sup>-7</sup>
TNF- $\alpha$ (pg/mL) <sup>b</sup>	166.33	112.11
Increase over $\beta$ -hematin (%)	109.96	51.23

<sup>a</sup>PGE<sub>2</sub> IC<sub>50</sub> values were determined by subtracting the concentration of PGE<sub>2</sub> obtained with the highest dose of COX-2 inhibitor from the PGE<sub>2</sub> value obtained at the lowest concentration. Dose-response curves for DuP-697 and indomethacin were obtained by plotting the PGE<sub>2</sub> levels (pg/mL) in Table 3 against the concentration of COX-2 inhibitor. The PGE<sub>2</sub> IC<sub>50</sub> concentration for each COX-2 inhibitor was then determined against the dose-response curve.

<sup>b</sup>TNF- $\alpha$  concentrations at the PGE<sub>2</sub> IC<sub>50</sub> concentration were determined by subtracting the concentration of TNF- $\alpha$  obtained with the lowest dose of COX-2 inhibitor from the TNF- $\alpha$  value obtained at the highest concentration. Dose-response curves for DuP-697 and indomethacin were obtained by plotting the TNF- $\alpha$  levels (pg/mL) in Table 3 against the concentration of COX-2 inhibitor. The TNF- $\alpha$  concentration for the corresponding PGE<sub>2</sub> IC<sub>50</sub> concentration for each COX-2 inhibitor was then determined against the dose-response curve.

Table 5. Clinical, parasitologic, laboratory, and immunologic parameters in study participants.

Characteristic	Healthy Children	Mild MA <sup>a</sup>	Moderate MA <sup>b</sup>	<i>P</i>
No. of subjects	19	10	12	
Age (years)	4.7 ± 0.5	4.9 ± 0.5	4.6 ± 0.5	NS
Admission temperature (°C)	37.2 ± 0.2	38.7 ± 0.4	39.1 ± 0.3	<0.01 <sup>c</sup>
Hyperparasitemia				
≥250,000 parasites/μL, <i>n</i>	0	2	5	
Parasitemia (/μL)	0	124,708 ± 40,452	257,895 ± 61,548	<0.05 <sup>d</sup>
Geomean parasitemia (/μL)	0	69,829	181,103	
Hemoglobin (g/dL)	ND	9.9 ± 0.2	6.4 ± 0.2	< 0.001 <sup>d</sup>
Bicyclo-PGE <sub>2</sub> (pg/mL)	29.0 ± 3.2	19.7 ± 2.5	12.0 ± 2.4	< 0.01 <sup>c</sup>
TNF-α (pg/mL)	85.5 ± 13.8	173.2 ± 55.3	252.9 ± 41.6	< 0.001 <sup>c</sup>

NOTE. Data represent the mean ± SEM unless otherwise noted. None of the subjects had cerebral malaria or severe MA. ND, not determined. NS, not significant.

<sup>a</sup>Mild MA cases were defined as Hb between 9.0 and 10.9 g/dL with any density parasitemia.

<sup>b</sup>Moderate MA cases were defined as Hb between 5.1 and 8.9 g/dL with any density parasitemia.

<sup>c</sup>Statistical significance determined by Kruskal-Wallis test.

<sup>d</sup>Statistical significance determined by Mann-Whitney U test.

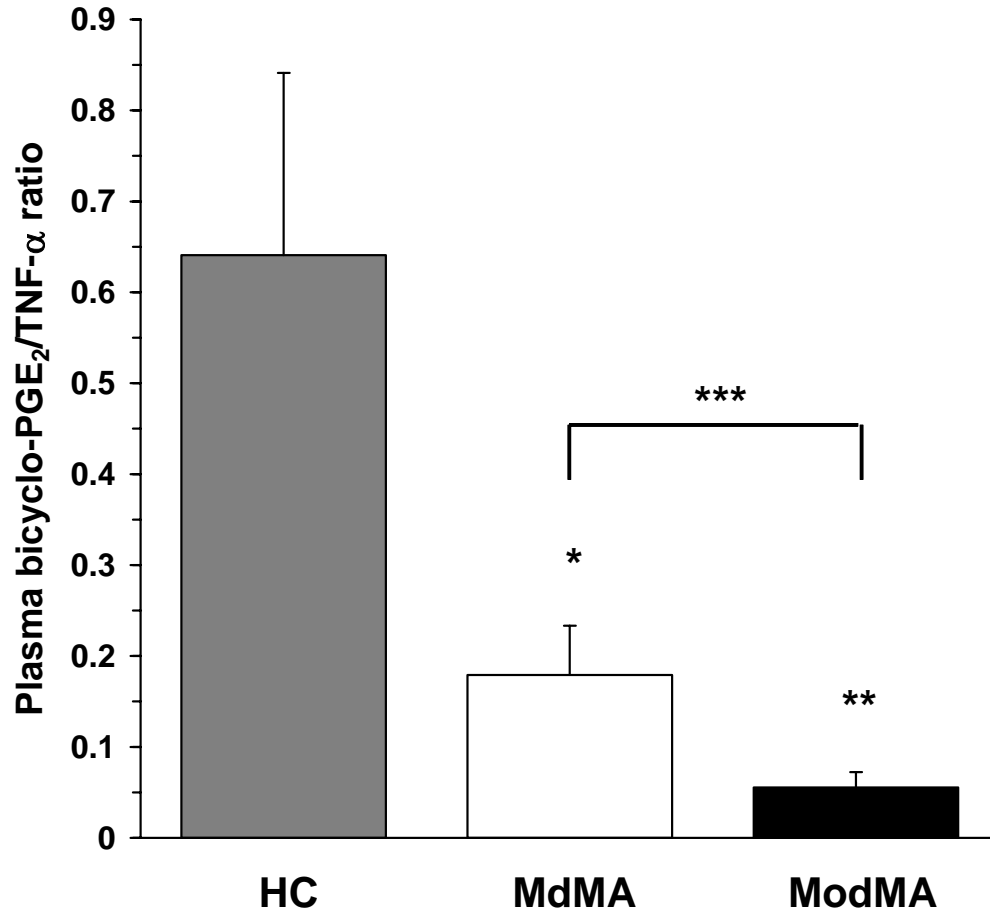


Figure 26. Association of the plasma bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio with disease severity. Plasma was obtained from healthy, malaria-exposed children (HC, n = 19), children with mild MA (MdMA, n = 10), and children with moderate MA (ModMA, n = 12) and bicyclo-PGE<sub>2</sub> and TNF- $\alpha$  levels were determined by EIA and ELISA, respectively. Data are expressed as the ratio of bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  production. Values are the mean  $\pm$  SEM for each group. Statistical significance was determined by the Mann-Whitney U test. \* $P$  < 0.05 compared to HC, \*\* $P$  < 0.01 compared to HC, \*\*\* $P$  < 0.05.

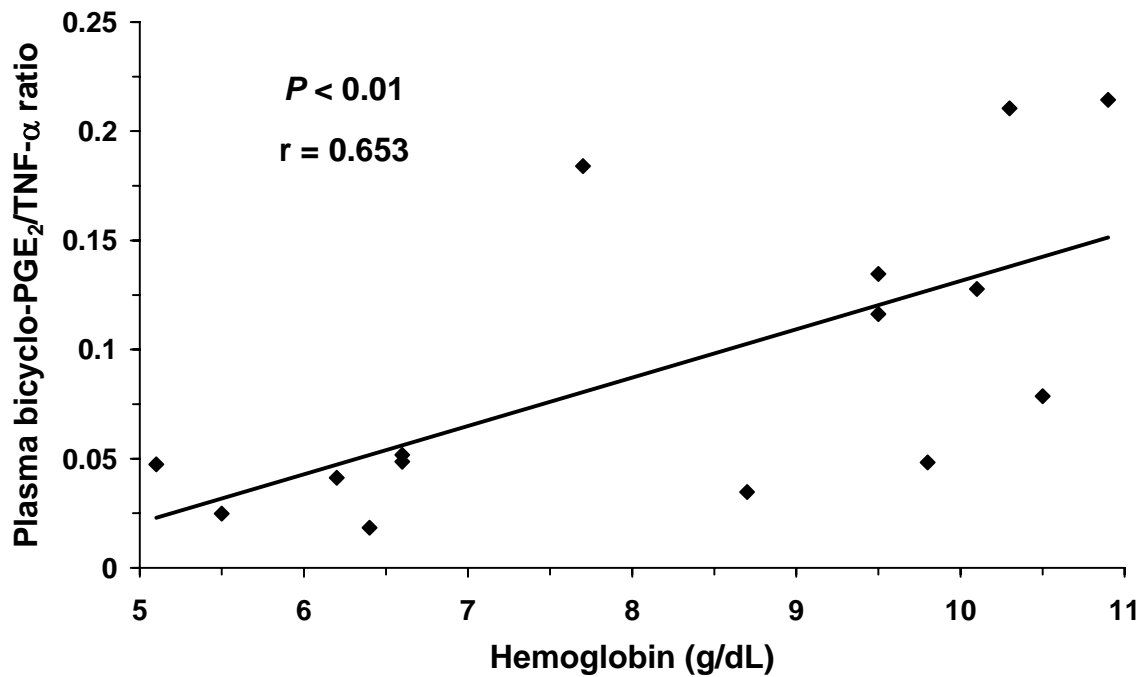


Figure 27. Association of the plasma bicyclo-PGE<sub>2</sub>/TNF-α ratio with hemoglobin concentrations. Plasma was obtained from children with mild and moderate MA (n = 22) and bicyclo-PGE<sub>2</sub> and TNF-α levels were determined by EIA and ELISA, respectively. Hb concentrations (g/dL) were measured with a Hemocue®. Regression analysis was used to examine the relationship between plasma bicyclo-PGE<sub>2</sub>/TNF-α ratios and Hb levels.

#### **4.4. SPECIFIC AIM 2, HYPOTHESIS 4: POLYMORPHISMS IN THE PROMOTER OF COX-2 CONDITION THE CLINICAL OUTCOMES IN CHILDREN WITH MA.**

##### **4.4.1. Introduction**

Malarial anemia (MA) is a consequence of many underlying etiologies, however, previous studies have identified single nucleotide polymorphisms (SNPs) in key immunoregulatory genes that are associated with susceptibility to or protection against severe disease. In particular, SNPs in the promoter regions of inducible immune response genes can regulate the production of effector molecules during an inflammatory event, and thereby mediate disease severity during blood-stage malaria. Three SNPs in the promoter region of the TNF- $\alpha$  gene (G-238A, G-308A, and G-376A) have been separately associated with increased severe malaria when the A allele is present (McAlpine *et. al.* 1992, McGuire *et. al.* 1994, McGuire *et. al.* 1999). Previous studies conducted in Gabonese children with malaria illustrated that a SNP in the NOS2 promoter (NOS2 G-954C, or NOS2<sup>Lamberéné</sup>) provided protection from severe malaria (Kun *et. al.* 2001, Kun *et. al.* 1998). Although the role of SNPs in the promoters of TNF- $\alpha$  and NOS2 have been examined in the context of malaria, there have been no studies to date examining the role of SNPs in the COX-2 gene with malaria disease severity.

To investigate the role of SNPs in the promoter region of COX-2, we sequenced the COX-2 promoter region for genetic variability in DNA isolated from children (n = 16) with varying degrees of malaria severity [defined as protected (absence of SMA or HDP during the first year of life) or susceptible (presence of SMA or HDP during the first year of life)]. Of the



six novel polymorphisms identified in the COX-2 promoter region, a C to T transition at position -512 was analyzed in the present studies. Furthermore, the -512T allele in the COX-2 promoter ablates a nuclear factor (NF)-E site and a potential glucocorticoid response (GR) binding site (Table 6), suggesting that the T allele may decrease COX-2 gene expression and the subsequent production of PGE<sub>2</sub>.

#### **4.4.2. Subjects and Methods**

**4.4.2.1. Study Participants:** Genomic DNA samples were obtained from the Centers for Disease Control and Prevention (CDC). Children were enrolled as part of the Asembo Bay Cohort Project (ABCP) from 1992 to 1996 and followed every 2 weeks for the presence and severity of *P. falciparum* malaria (Bloland *et. al.* 1999). We received the samples in a “blinded fashion” such that the clinical variables of each participant were unknown during the time of genotyping. Furthermore, only data on the length of time each child had high-density parasitemia (HDP,  $\geq 10,000$  parasites/ $\mu$ L), malarial anemia (MA, Hb < 8.0 g/dL and any density parasitemia), and severe MA (SMA, Hb < 5.0 g/dL and any density parasitemia) were made available on a subset of the children (i.e., 394 out of 877). All subjects were given standard treatment according to Kenya Ministry of Health guidelines. The study was approved by the ethics committees of the University of Pittsburgh, the Centers for Disease Control and Prevention, and the Kenya Medical Ethics Review Board.

**4.4.2.2. Genotyping:** The COX-2 C-512T polymorphism was analyzed by sequence-specific PCR followed by restriction nuclease digest with HPY188 III. The PCR reaction conditions were as follows: 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 320 uM each of dATP, dCTP, dGTP, and

dTTP, 0.625 U Taq DNA polymerase (all reagents obtained from Applied Biosystems, Foster City, CA), 200 nM forward primer, 5' - GCT TCC TCT CCA GGA ATC - 3', and 200 nM reverse primer, 5' - GAG CAG ATA TAC AGC CTA - 3'. The cycle conditions were: 94°C for 5 min, 40 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 7 min. PCR products (10 µL) were digested in 20 µL volume with 1.5 U of HPY188 III, 100X BSA, and 1X NEB buffer 4 (all reagents obtained from New England Biolabs, Beverly, MA) overnight (~18 hrs) at 37°C followed by deactivation of enzyme at 65°C for 20 min. Genotypes were then analyzed by electrophoresis using a 3% agarose gel. Enzyme digest of the 200 bp PCR product with HPY188 III specifically digests the C allele, resulting in 117 and 82 bp fragments.

**4.4.2.3. Statistical Analyses:** The distribution of CC, CT, and TT genotypes according to disease severity (i.e., HDP, MA, and SMA) was determined using Chi-square analyses. Logistic regression was used to determine the association of the COX-2 C-512T polymorphism with HDP, MA, and SMA. The CC genotype was used as the baseline since this genotype was the most prevalent in the present study population.

#### **4.4.3. Results**

This COX-2 C-512T SNP was analyzed in a birth cohort of children (n = 877) from western Kenya as part of the Asembo Bay Cohort Project (ABCP). The frequency of the polymorphism in the study population was 83.24%, 11.40%, and 5.36% for the C/C, C/T, and T/T genotypes, respectively (Table 7). The allelic distribution was 0.889 and 0.111 for the C and T alleles, respectively. To assess the association of the COX-2 -512 polymorphism with malaria disease

severity, children were grouped according to the presence or absence of HDP ( $\geq 10,000$  parasites/ $\mu\text{L}$ ), MA (Hb  $< 8.0$  g/dL and any density parasitemia), or SMA (Hb  $< 5.0$  g/dL and any density parasitemia) during the first year of follow-up. Of the 877 children genotyped, 394 had complete clinical data, and of these, 94.41% (372 of 394), 81.47% (321 of 394), and 34.26% (135 of 394) had HDP, MA, and SMA, respectively (Table 8). When categorized according to the CC, CT, and TT genotypes, 94.46% (307 of 325), 90.24% (37 of 41), and 100% (28 of 28), respectively, had HDP, 80.92% (263 of 325), 78.05% (32 of 41), and 92.86% (26 of 28) had MA, and 34.15% (111 of 325), 31.71% (13 of 41), and 39.29% (11 of 28) had SMA (Table 8). There were no significant differences in the proportion of genotypes for HDP, MA, or SMA ( $P = 0.22$ ,  $P = 0.25$ ,  $P = 0.81$ , respectively, Table 9).

#### **4.4.4. Discussion**

Polymorphisms that regulate the production of key immunoregulatory genes, such as TNF- $\alpha$  and IL-10, have been associated with malaria disease severity, suggesting that genetic variability plays a major role in the acquisition of protective immunity to malaria. Our previous studies illustrate that circulating PBMC COX-2 and PGE<sub>2</sub> are important factors associated with malaria disease severity (Keller *et. al.* 2004, Perkins *et. al.* 2005, Perkins *et. al.* 2001), therefore, identifying polymorphisms that regulate the production of COX-2 gene expression during malaria will likely yield important information.

Results presented here show that a novel polymorphism in the COX-2 promoter, COX-2 C-512T, is present in African children. Although non-significant in these studies, the CT genotype was protective against HDP relative to the CC genotype. Our previous studies showed that

increased production of COX-2 and PGE<sub>2</sub> was associated with protection against severe malaria (defined by SMA and/or HDP) in Gabonese children (Perkins *et. al.* 2001). The COX-2 -512T allele ablates a potential GR binding site, and since glucocorticoids down-regulate COX-2 transcript formation, the presence of a T allele may promote increased levels of COX-2, and subsequent PGE<sub>2</sub> production, through ablation of this negative signaling cascade. Alternatively, the loss of an NF-E binding site in the presence of the T allele may decrease the promotion of COX-2 transcript formation. Although the CT genotype protected against HDP, all children with the TT genotype had HDP, suggesting that homozygotes may not be able to limit parasitemia. Furthermore, the TT genotype appears to be associated with an increased risk of MA relative to both the CC and CT genotypes (OR = 3.06 and OR = 3.66, respectively, see Table 9). Further studies in a larger cohort with a higher number of polymorphic heterozygotic individuals will likely yield important information on the association of the COX-2 C-512T polymorphism with malaria disease severity.

Table 6. Known transcription factor binding sites associated with COX-2 -512.

<b>Polymorphism</b>	<b>-512 (C-T)</b>
Known TF Binding Site (C)	<b>NF-E</b> (ctgt <b>C</b> )
Known TF Binding Site (T)	<b>GR</b> (tgt <b>C</b> cc) <b>unknown</b> (t <b>T</b> cc)

-TF binding sites identified by Transfac database at:

<http://bimas.dcrn.nih.gov/molbio/signal/>

Table 7. Number and frequency of COX-2 -512 genotypes.

	Number of children, (%)
<b>CC</b>	730 (83.24)
<b>CT</b>	100 (11.40)
<b>TT</b>	47 (5.36)
<b>Total</b>	877 (100)

The COX-2 C-512T polymorphism was determined in genomic DNA from children (n = 877) residing in a holoendemic area of malaria transmission. Data are n (%).

Table 8. Number and percentage of COX-2 genotypes with HDP, MA, and SMA.

	<b>All Children (n = 394)</b>	<b>CC (n = 325)</b>	<b>CT (n = 41)</b>	<b>TT (n = 28)</b>	<i>p</i>
<b>HDP</b>	372	307 (94.46)	37 (90.24)	28 (100)	0.22
<b>MA</b>	321	263 (80.92)	32 (78.05)	26 (92.86)	0.25
<b>SMA</b>	135	111 (34.15)	13 (31.71)	11 (39.29)	0.81

The COX-2 C-512T polymorphism was examined in genomic DNA from children (n = 394) residing in a holoendemic area of malaria transmission. Participants were visited every 2 weeks over the first year of life and examined for the presence of HDP ( $\geq 10,000$  parasites/ $\mu$ L), MA (Hb < 8.0 g/dL), or SMA (Hb < 6.0 g/dL). Data are n (%) of children with each genotype who had HDP, MA, or SMA at any one visit. Statistical significance between the proportions of genotypes within each clinical variable was determined by Chi-square analysis.

Table 9. Association of COX-2 -512 polymorphism with malaria disease severity.

	<b>HDP (n = 372)</b>			<b>MA (n = 321)</b>			<b>SMA (n = 135)</b>		
	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>
<b>CT<sup>a</sup></b>	0.54	0.47 – 1.69	0.29	0.84	0.38 – 1.85	0.66	0.90	0.45 – 1.80	0.76
<b>TT<sup>a</sup></b>	-	-	0.99	3.06	0.71 – 13.26	0.13	1.25	0.56 – 2.76	0.58
<b>TT<sup>b</sup></b>	-	-	0.99	3.66	0.73 – 18.42	0.12	1.39	0.51 – 3.80	0.52

The COX-2 C-512T polymorphism was examined in genomic DNA from children (n = 394) residing in a holoendemic area of malaria transmission. Participants were visited every 2 weeks over the first year of life and examined for the presence of HDP ( $\geq 10,000$  parasites/ $\mu$ L), MA (Hb < 8.0 g/dL), or SMA (Hb < 6.0 g/dL). Odds Ratios (OR) and 95% confidence intervals (CI) were determined by logistic regression analyses.

<sup>a</sup> Odds ratio relative to CC.

<sup>b</sup> Odds ratio relative to CT.

## 5. CHAPTER 5, SPECIFIC AIM 3

**Specific Aim 3: To determine if children with MA have altered NO production and to determine if ingestion of malarial products (hemozoin) regulates NO production through changes in induction of *de novo* NOS2 transcripts.**

At present, it is unclear if increased levels of circulating NO are protective or pathogenic in acute malaria. Previous studies in adults and children with malaria have illustrated a protective role for elevated NO during acute malaria (Kremsner *et. al.* 1996), and in healthy children with a history of mild malaria (Perkins *et. al.* 1999). The association of NO with MA, however, has not been investigated. Both hypotheses of Specific Aim 3 have been addressed in the following manuscript which has been published in the journal *Infection and Immunity*.



**5.1. PRESENTATION OF MANUSCRIPT: *ELEVATED NITRIC OXIDE PRODUCTION IN CHILDREN WITH MALARIAL ANEMIA: HEMOZOIN-INDUCED NITRIC OXIDE SYNTHASE TYPE 2 TRANSCRIPTS AND NITRIC OXIDE IN BLOOD MONONUCLEAR CELLS.***

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### 5.1.1. Abstract

Experiments outlined here investigate the role of nitric oxide (NO) in the pathogenesis of *Plasmodium falciparum*-induced malarial anemia (MA). These results show that *ex vivo* and *in vitro* NO synthase (NOS) activity in peripheral blood mononuclear cells (PBMC) is significantly elevated in children with MA, and inversely associated with hemoglobin levels. Additional experiments in PBMC from non-malaria exposed donors demonstrate that physiologic amounts of *P. falciparum*-derived hemozoin augment NOS2 transcripts and NO production. Results here illustrate that elevated NO production in children with MA is associated with decreased hemoglobin concentrations, and that hemozoin can induce NOS2-derived NO formation in cultured blood mononuclear cells.

### 5.1.2. Manuscript

A lack of acquired immunity to *Plasmodium falciparum* malaria in young children appears to underlie the high rate of morbidity and mortality from malaria in endemic areas of sub-Saharan Africa (Breman *et. al.* 2001). In areas of high transmission, the predominate manifestations of severe malaria are hyperparasitemia and malarial anemia (MA) (Breman *et. al.* 2001). Although the molecular mechanisms responsible for effective malarial immunity remain elusive, production of nitric oxide (NO) appears to be an important marker and potential mediator of disease severity. Previous studies show that elevated plasma levels of NO metabolites [ $\text{NO}_2^-$  plus  $\text{NO}_3^-$  ( $\text{NO}_x$ )] are associated with enhanced parasite clearance time in Gabonese adults and children with malaria (Kremsner *et. al.* 1996). In addition, our previous results show that healthy, malaria-exposed Gabonese children with a history of mild malaria have significantly elevated levels of peripheral blood mononuclear cell (PBMC) NO production and nitric oxide

synthase (NOS) enzyme activity compared to their age-matched cohorts with a history of severe malaria (Perkins *et. al.* 1999). Protection against severe malaria in this population of children appears to be, at least in part, related to a polymorphism in NOS type 2 gene [NOS2, inducible NOS, iNOS]] which produces high levels of NO during an inflammatory event. For example, we have recently shown that a single nucleotide polymorphism in the promoter region of the NOS2 gene [NOS2<sup>Lamberéné</sup> (NOS2 G-954C)] is associated with increased *in vivo* and *in vitro* baseline NO production, and protection against malaria (Kun *et. al.* 1998, Kun *et. al.* 2001). Although increased NO production appears to be associated with protection against malaria in the Gabonese children we have previously investigated, elevated levels of NO can suppress erythropoiesis (Shami *et. al.* 1996), and induce apoptosis in cultured CD34+ cells (Reykdal *et. al.* 1999). Moreover, since the studies examining the functional significance of the NOS2<sup>Lamberéné</sup> polymorphism were conducted in healthy children, we extended our previous investigations by examining the association between NO production and anemia during acute *P. falciparum* malaria.

**5.1.2.1. NOS Enzyme Activity in Ex Vivo PBMC:** To determine if NO production is altered in children with MA, NOS enzyme activity was measured in ex vivo PBMC from healthy, malaria-exposed children (n = 26) and children with mild (n = 19) or severe malaria (n = 14) according to our previously described methods (Weinberg *et. al.* 1994). PBMC were selected for investigation since monocytes are a primary source of NO during blood-stage malaria. Furthermore, measurement of NOS enzyme activity was selected as the index for determining NO production since this assay, unlike measurement of plasma NO<sub>x</sub>, is not influenced by dietary intake of nitrates. Participants were recruited from a longitudinal prospective study at the Albert Schweitzer Hospital in Lambaréné, Gabon in the Province of

Moyen Ogooue. Severe malaria cases were defined according to World Health Organization guidelines [parasitemias  $> 250,000$  parasites/ $\mu\text{L}$  and/or the presence of severe anemia (i.e., hemoglobin (Hb)  $\leq 5.0$  g/dL)]. Mild malaria cases were defined by parasitemias  $< 100,000$  parasites/ $\mu\text{L}$  and the absence of any signs or symptoms of severe malaria. Routine clinical evaluations and laboratory measures were used to evaluate the subjects; all blood samples were obtained prior to treatment with antimalarials and/or antipyretics. Children with acute malaria were given antimalarials and the appropriate supportive therapy as required. Healthy children were defined as those participants with a previous episode(s) of malaria and the absence of a positive thick blood film for malaria, or any other illnesses, within the last 4 weeks. Informed consent was obtained from the parents of all participating children. The study was approved by the ethics committees of the International Foundation of the Albert Schweitzer Hospital in Lambaréné, the University of Tübingen, Duke University Medical Center Investigational Review Board, and the University of Pittsburgh Institutional Review Board.

As shown in Figure 28, relative to healthy children, NOS enzyme activity was significantly higher in *ex vivo* PBMC from children with mild ( $P < 0.01$ ) and severe ( $P < 0.01$ ) malaria. Although the severe malaria group had higher NOS enzyme activity than the mild malaria group, the difference between the two groups was not significant ( $P = 0.14$ , Figure 28). As a control, PBMC lysates were incubated with specific (L-NIL) and non-specific NOS2 inhibitors (L-NMMA) which demonstrated that the NOS enzyme activity in the assays was NOS2 specific. Taken together, these experiments are the first reports illustrating that NOS enzyme activity is significantly elevated in circulating mononuclear cells from children with acute MA.

**5.1.2.2. Association of NOS Enzyme Activity with Malarial Anemia:** To further assess the relationship between elevated NO production and MA, we examined the association between ex vivo NOS enzyme activity and hemoglobin (Hb) levels in children with mild and severe MA. There was a significant inverse correlation between NOS enzyme activity and Hb concentration ( $r = -0.57$ ,  $P < 0.05$ , Figure 29), illustrating that elevated PBMC NO production is associated with MA. Consistent with previous observations in asymptomatic, malaria-exposed children (Anstey *et. al.* 1999), the significant association between elevated NO production and decreased Hb levels in children with acute malaria shown here illustrates that increased NO production may be involved in the pathogenesis of MA. Since NO can inhibit erythropoiesis (Shami *et. al.* 1996) and induce apoptosis (Reykdal *et. al.* 1999) in hematopoietic precursors, we postulate that excessively high levels of NO during acute malaria may contribute to suppression of erythropoiesis. However, based on the current study design, it cannot be ruled out that there is a non-causal relationship between increased NO production and decreased Hb concentrations in children with MA.

**5.1.2.3. Baseline and Stimulated Levels of NOS Enzyme Activity in Cultured PBMC:** Since elevated ex vivo PBMC NOS enzyme activity could arise from stimulation by both host-derived inflammatory cytokines and parasitic products, PBMC isolated from children with and without malaria were cultured for 7 days according to our previously described methods (Weinberg *et. al.* 1981). This length of time in culture should remove the influence of the in vivo milieu on NO production. Briefly, PBMC were plated in Dulbecco's modified Eagles medium (DMEM) supplemented with 10 mM HEPES, 10 mM penicillin/streptomycin, and 10% pooled human serum (heat inactivated at 56°C for 30 min). PBMC were cultured from healthy

children (n = 26) and children with MA (n = 14, Hb ranging from 6.2 – 10.7 g/dL) under baseline conditions [media alone (controls)] and following NO-inducing stimuli [LPS, 100 ng/mL (Alexis Corp., San Diego, CA) and IFN- $\gamma$ , 200 U/mL (BD Pharmingen, San Diego, CA)]. PBMC cultures were not prepared from children with severe MA (Hb < 5.0 g/dL) since the anemia precluded drawing enough blood for our in vitro experimental design. We have previously shown that stimulation of cultured human PBMC with LPS and IFN- $\gamma$  increases NO production in culture supernatants through augmentation of NOS enzyme activity in immune activated patients with rheumatoid arthritis (Sharara *et. al.* 1997, St. Clair *et. al.* 1996). Children with malaria had significantly higher baseline ( $P < 0.05$ ) and LPS- and IFN- $\gamma$ -promoted NOS enzyme activity, relative to malaria-exposed healthy control children ( $P < 0.01$ , Figure 30). However, LPS- and IFN- $\gamma$ -stimulation failed to increase NOS enzyme activity in PBMC from healthy children. This may be a consequence of the absence of in vivo immune activation/priming of PBMC in healthy children. As noted above, specific (L-NIL) and non-specific NOS2 inhibitors (L-NMMA) were used to demonstrate that the NOS enzyme activity was NOS2 specific. These results demonstrate that cultured PBMC from children with MA have significantly elevated baseline and stimulated NO production during acute disease.

**5.1.2.4. Estimated Hemozoin Concentrations in Children with Malaria:** Since PBMC from children with MA were cultured for 7 days, and therefore no longer influenced by positive and negative in vivo NOS2 regulatory factors (e.g., cytokines), we postulated that a malarial product(s) encountered in vivo may be responsible for elevated levels of NOS enzyme activity in children with acute malaria. Based on our previous studies illustrating that the in vivo acquisition of hemozoin (malarial pigment) alters the production of cytokines and effector

molecules (Luty *et. al.* 2000, Perkins *et. al.* 2003), we investigated hemozoin as a potential mediator of augmented NO production in children with MA. Since there are presently no reports defining the concentrations of hemozoin in phagocytic cells in children with varying degrees of malaria disease severity, we estimated the hemozoin content in children with mild and severe malaria to ensure that the amount of hemozoin used for the in vitro experiments was physiologically relevant. Table 10 illustrates our methodology for calculating the concentration of hemozoin in circulating blood in children with malaria. The geometric mean parasitemia used for our calculations was based on our previous studies in Gabonese children with mild and severe malaria (Perkins *et. al.* 2000). The concentration of hemozoin per parasitized RBC (pRBC) was derived from previous studies using in vitro cultures of *P. falciparum* (Egan 2002). Estimated concentrations of hemozoin were determined by multiplying the geometric mean parasitemia in children with mild and severe malaria (41,369 and 275,005, respectively) by the concentration of isolated hemozoin per pRBC (47 fg/pRBC, Table 10). Based on these calculations, children with mild malaria would have 1.9  $\mu\text{g}$  of hemozoin per mL of circulating blood, while children with severe malaria would have 12.9  $\mu\text{g}$  of hemozoin per mL of circulating blood. Based on these calculations, the estimated physiological concentrations of hemozoin selected for the in vitro experiments were 10, 1.0, and 0.1  $\mu\text{g}/\text{mL}$ .

**5.1.2.5. Effect of Hemozoin on PBMC NO<sub>x</sub> Production:** Hemozoin is an insoluble coordinated polymer of heme subunits formed during the detoxification of heme by Plasmodia (Slater 1992). Upon rupture of pRBC, hemozoin is released into the circulation and is rapidly phagocytosed by mononuclear cells (Schwarzer *et. al.* 1998). Hemozoin exists as a coordinated heme polymer containing a conglomeration of host- and parasite-derived lipids and proteins

(Ashong *et. al.* 1989, Goldie *et. al.* 1990). Therefore, we used a crude isolate of hemozoin that was not subjected to proteinase K treatment and/or acetone washing, which more closely mimics the moiety acquired during a natural infection. Crude hemozoin was isolated from *in vitro* cultures of *P. falciparum*-infected RBC (strain Pf-D6) when the parasitemia was 3-5% and late trophozoites and early schizonts were the predominate forms. RBC were spun at 2,000 rpm for 10 min, and the resulting pellet was resuspended in 40 mL of 0.01 M PBS (pH 7.2) with 2 mL saponin for 10 min. The solution was then spun at 14,000 rpm for 15 min, and the pelleted material was washed in PBS until the resulting pellet was dark red and free from the white-colored RBC cellular components (4-7 times). The final pellet was dried, weighed, and resuspended in filter-sterilized H<sub>2</sub>O at a final concentration of 1.0 mg/mL, and the final solution was extensively sonicated to disperse the hemozoin. Since larger volumes of blood were required for the *in vitro* assays, experiments with hemozoin were conducted in cultured PBMC from healthy, non-malaria-exposed U.S. adults. Cultured PBMC were stimulated with media alone, LPS and IFN- $\gamma$ , or LPS and IFN- $\gamma$  in the presence of hemozoin (10, 1.0, and 0.1  $\mu$ g/mL) for 48 hrs. Stimulation of cells with hemozoin was performed in the presence of LPS and IFN- $\gamma$  since cultured human blood mononuclear cells typically require priming/activation to produce NO (Weinberg 1998). Production of NO was determined by measuring NO<sub>x</sub> in culture supernatants according to our previously described methods (Perkins *et. al.* 1999). It was also confirmed that hemozoin does not interfere with the Griess reaction. LPS- and IFN- $\gamma$ -stimulation non-significantly increased NO<sub>x</sub> (Figure 31). The addition of a high dose (10  $\mu$ g/mL) of hemozoin to LPS- and IFN- $\gamma$ -stimulated PBMC significantly augmented NO<sub>x</sub> ( $P < 0.01$ ), while the intermediate and low doses of hemozoin (1.0, and 0.1  $\mu$ g/mL, respectively) failed to significantly elevate NO<sub>x</sub> (Figure 31). Moreover, additional experiments revealed that



hemozoin alone, in the absence of stimulation, failed to significantly increase NO<sub>x</sub> (data not shown). Results presented here illustrate that a crude preparation of hemozoin increases NO production in human PBMC.

**5.1.2.6. Effect of Hemozoin on NOS2 Transcripts:** To determine if hemozoin increased NO<sub>x</sub> by induction of NOS2 mRNA, we cultured PBMC from healthy, malaria-naïve U.S. adults with media alone, LPS and IFN- $\gamma$ , or LPS and IFN- $\gamma$  in the presence of hemozoin (10, 1.0, and 0.1  $\mu\text{g}/\text{mL}$ ) for 48 hr followed by measurement of NOS2 mRNA by real time RT-PCR. To accomplish this, total RNA was isolated from cultured PBMC by the GITC method (Chomczynski *et. al.* 1987), and reverse transcribed into cDNA. NOS2 gene expression was analyzed by quantitative real time RT-PCR on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) with NOS2-specific primers and probe [Assay ID Hs00167248\_m1 (Applied Biosystems, Foster City, CA)] following the parameters specified by the manufacturer. To control for non-specific background fluorescence, no template controls were included in triplicate. An endogenous control gene,  $\beta$ -actin [Assay ID 4326315E (Applied Biosystems, Foster City, CA)], was used as a reference gene to normalize cDNA loading between samples. Stimulation of PBMC with LPS and IFN- $\gamma$  non-significantly elevated NOS2 transcripts at 48 hrs (Figure 32). Addition of both high (10  $\mu\text{g}/\text{mL}$ ) and intermediate (1.0  $\mu\text{g}/\text{mL}$ ) amounts of hemozoin significantly augmented LPS- and IFN- $\gamma$ -induced NOS2 transcripts ( $P < 0.05$ , Figure 32). Previous studies have reported difficulties in detecting NOS2 transcripts in human mononuclear cells [for review see (Weinberg 1998)], however, using sensitive quantitative methods such as real time RT-PCR, we were able to detect NOS2 message. Although NOS2 transcripts were not highly abundant in cultured PBMC, the addition of

hemozoin significantly increased de novo NOS2 message, demonstrating that hemozoin-induced NO production can occur through increased NOS2 transcription.

Results presented here show that NOS enzyme activity is elevated in children with mild and severe MA, and that high levels of NOS enzyme activity are associated with anemia. The role of NO in malaria pathogenesis remains controversial since studies in subjects with cerebral malaria have shown that NO is associated with protection (Anstey *et. al.* 1996, Kremsner *et. al.* 1996, Perkins *et. al.* 1999, Rockett *et. al.* 1991), while others have found either no effect or adverse consequences of elevated NO production (Agbenyega *et. al.* 1997, Al-Yaman *et. al.* 1997, Al-Yaman *et. al.* 1996, Maneerat *et. al.* 2000, Taylor *et. al.* 1998). However, this is the first report that has directly examined the association of NO production with acute MA. Although polymorphisms in the NOS2 promoter [NOS2<sup>Lamberéné</sup> (NOS2 G-954C) and C-1173T] have been associated with protection against malaria (Hobbs *et. al.* 2002, Kun *et. al.* 2001, Kun *et. al.* 1998) there were not enough children in our sample population with these particular polymorphisms to provide meaningful statistically valid comparisons (data not shown).

As shown here, cultured PBMC from children with MA have higher baseline and stimulated NOS enzyme activity relative to healthy, malaria-exposed children. Furthermore, a crude isolate of *P. falciparum*-derived hemozoin enhances NOS2 transcripts and NO production in cultured human PBMC, suggesting that ingestion of hemozoin may account for increased NOS activity in children with acute malaria. These results are in agreement with a recent report showing that a purified preparation of hemozoin and a synthetic preparation of hemozoin ( $\beta$ -hematin) increased IFN- $\gamma$ -induced NOS2 transcripts and NO production in a murine macrophage cell line (Jaramillo *et. al.* 2003). Although concentrations of hemozoin used in those studies

were 2.5 to 5 times higher than the estimated concentrations for children with mild and severe malaria presented here (Table 10), the present studies demonstrate that ingestion of physiologically relevant concentrations of hemozoin (10 and 1.0  $\mu\text{g}/\text{mL}$ ) significantly enhance LPS- and IFN- $\gamma$ -promoted NOS2 transcripts and NO production in human PBMC. These results are in contrast to several studies in cultured murine peritoneal macrophages in which *P. vinckei*-derived hemozoin reduced NO production (Prada *et. al.* 1996), and  $\beta$ -hematin decreased LPS-induced NO and TNF- $\alpha$  production (Taramelli *et. al.* 2000). The apparent discrepancy between those results and results presented here may be a consequence of the murine origin of macrophages and/or the concentration of  $\beta$ -hematin, which was 10-fold higher than concentrations used in the present study. Moreover, since regulation of the human NOS2 gene is substantially different than that of the murine NOS2 gene (de Vera *et. al.* 1996), hemozoin-induced activation and regulation of NOS2 and subsequent NO production may be different in human and murine systems.

Based on our previous results (Kun *et. al.* 2001, Perkins *et. al.* 1999) and results presented here, we propose that elevated baseline levels of NO in healthy children, and increased levels of NO during the early phases of the immune response to acute malaria, protect against the development of severe disease. However, if parasite growth is not effectively limited during the early phases of the immune response, sustained overproduction of NO may lead to the development of SMA. We are currently testing this hypothesis in a hospital-based study in Kenyan children with SMA.

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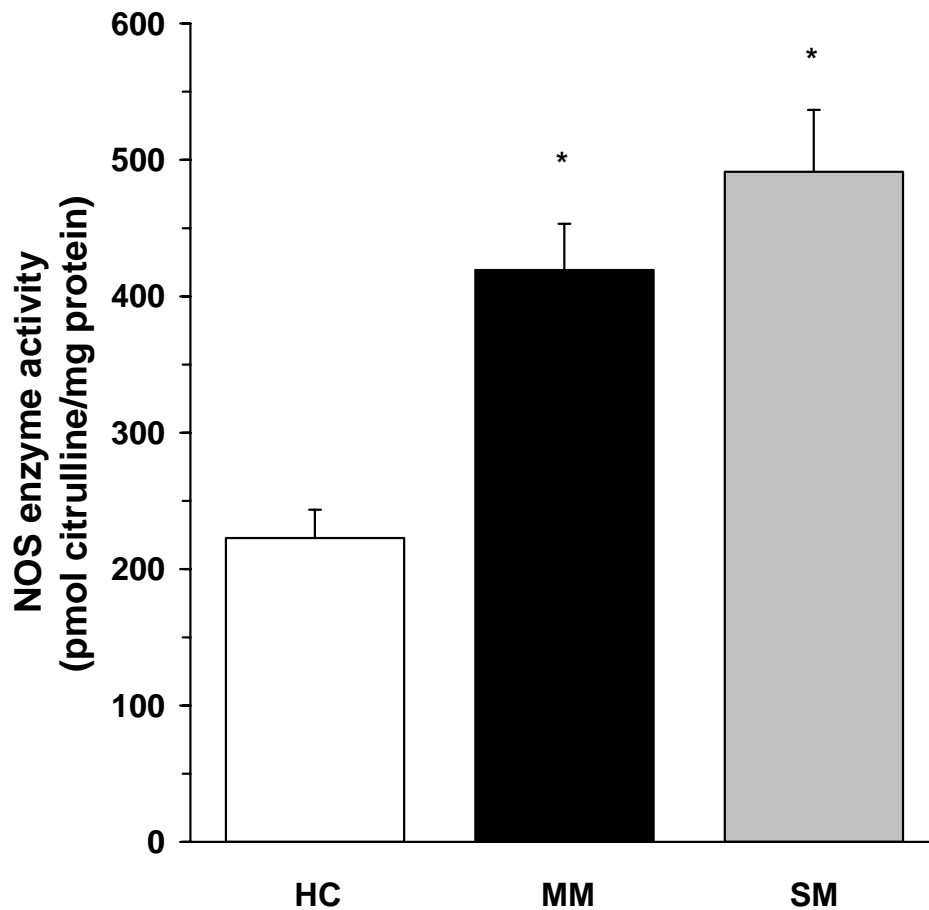


Figure 28. NOS enzyme activity in *ex vivo* (non-cultured) PBMC. PBMC were collected from healthy Gabonese children (HC, n = 26) and children with mild malaria (MM, n = 19) or severe malaria (SM, n = 14). Cell lysates were prepared and *ex vivo* NOS enzyme activity (pmol citrulline/mg protein) was determined by measuring the conversion of  $^{14}\text{C}$ -L-arginine to  $^{14}\text{C}$ -L-citrulline. The graph shows the mean  $\pm$  SEM for each of the groups. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.01$  compared to HC.

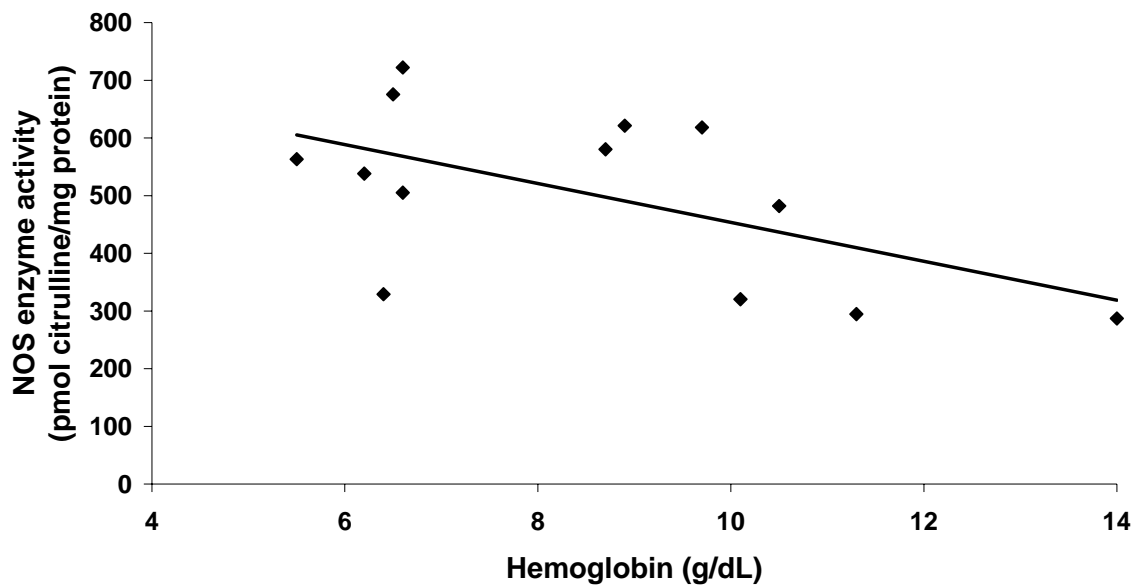
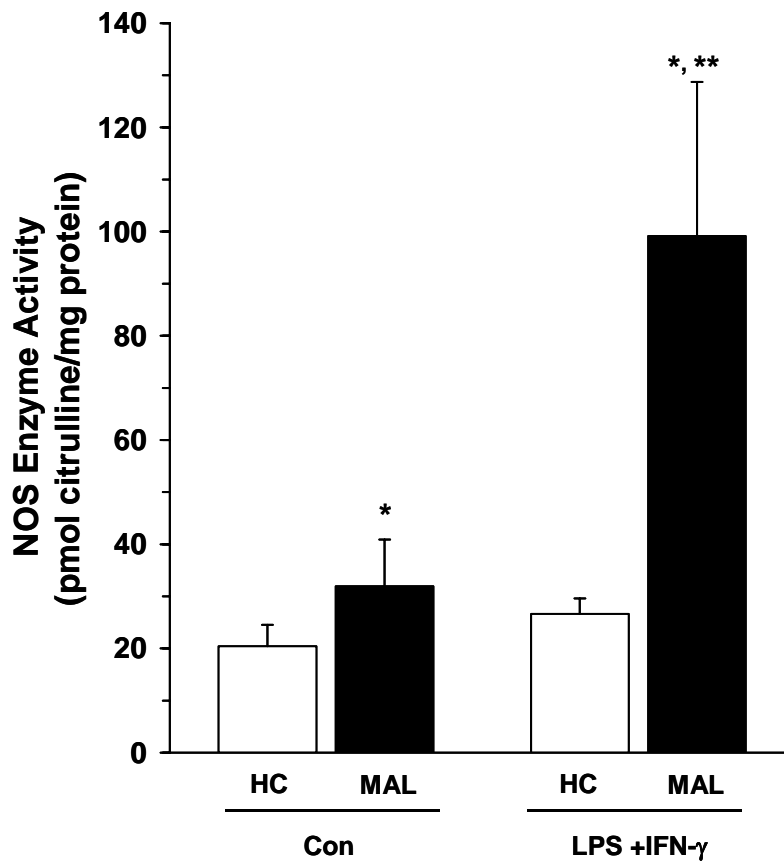


Figure 29. Association of NOS enzyme activity with hemoglobin. PBMC were collected from children (n = 13) with malaria and cell lysates were prepared for *ex vivo* NOS enzyme activity (pmol citrulline/mg protein) determination. Hemoglobin was measured using a Hemocue<sup>TM</sup>. Statistical significance was determined by linear regression.



*Figure 30. NOS enzyme activity in cultured PBMC.* PBMC ( $1 \times 10^6$  cells/mL) were collected from healthy Gabonese children (HC,  $n = 26$ ) and children with malaria (MAL,  $n = 14$ ). Cultures were prepared and incubated for 7 days with media alone (Con) or LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL). Cell lysates were prepared from cultured PBMC and NOS enzyme activity (pmol citrulline/mg protein) was determined. The graph shows the mean  $\pm$  SEM for each of the groups. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.05$  compared to HC, \*\* $P < 0.01$  compared to unstimulated MAL.

Table 10. Estimated hemozoin concentrations in children with malaria.

	<b>Mild malaria</b>	<b>Severe malaria</b>
Geometric mean parasitemia / $\mu\text{L}$ <sup>a</sup>	41,369	275,005
Hemozoin concentration per pRBC <sup>b</sup>	47 fg/pRBC	47 fg/pRBC
Estimated hemozoin concentration in children with malaria <sup>c</sup>	1.9 $\mu\text{g/mL}$	12.9 $\mu\text{g/mL}$

<sup>a</sup> From children with mild (n = 17) and severe (n = 12) malaria (Perkins *et. al.* 2000).

<sup>b</sup> From cultured parasites (Egan 2002).

<sup>c</sup> Estimated concentrations of hemozoin in children with malaria were determined by multiplying the concentration of hemozoin (fg/pRBC) obtained from cultured parasites by the geometric mean parasitemia (/ $\mu\text{L}$ ) in children with mild and severe malaria.



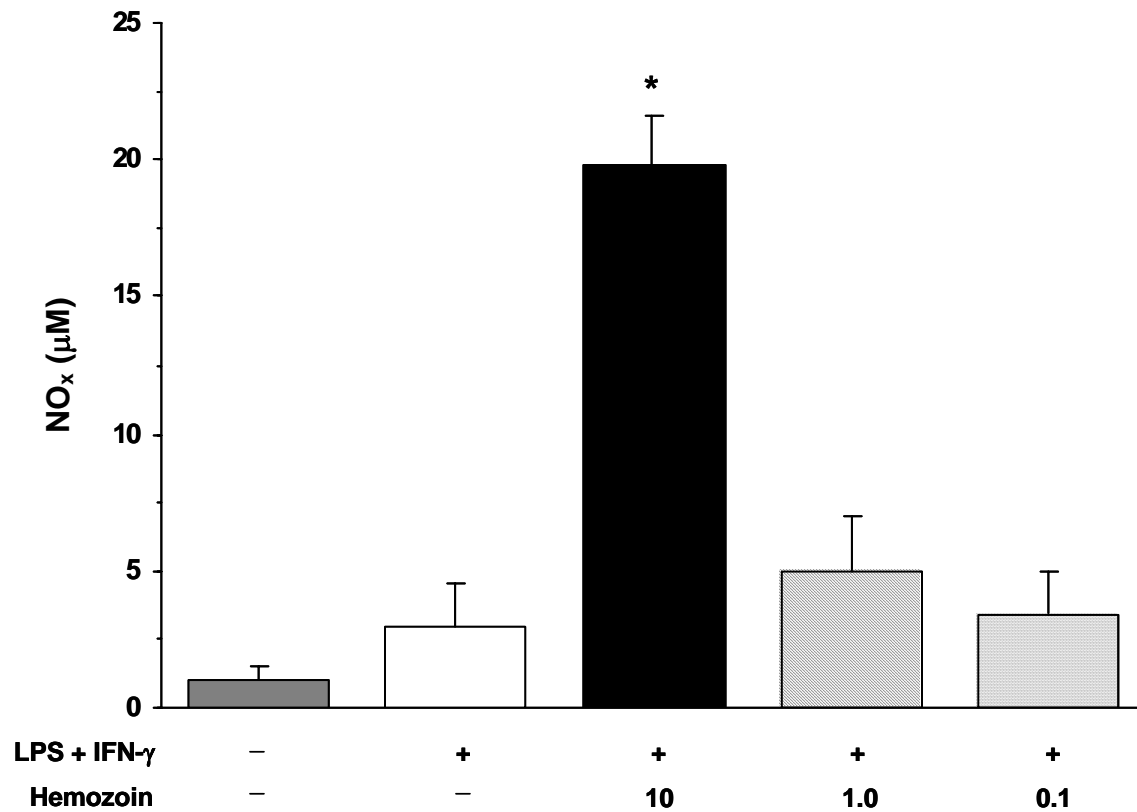


Figure 31. Effect of hemozoin on NO<sub>x</sub> production in cultured PBMC. PBMC ( $1 \times 10^6$  cells/mL) from healthy, malaria-naïve U.S. donors were stimulated with media alone, LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), or LPS and IFN- $\gamma$  in the presence of hemozoin (10, 1.0, and 0.1  $\mu\text{g/mL}$ ). NO<sub>x</sub> was determined in culture supernatants at 48 hrs via the Griess reaction. Values are the mean  $\pm$  SEM, n = 10. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.05$  compared to stimulated conditions.

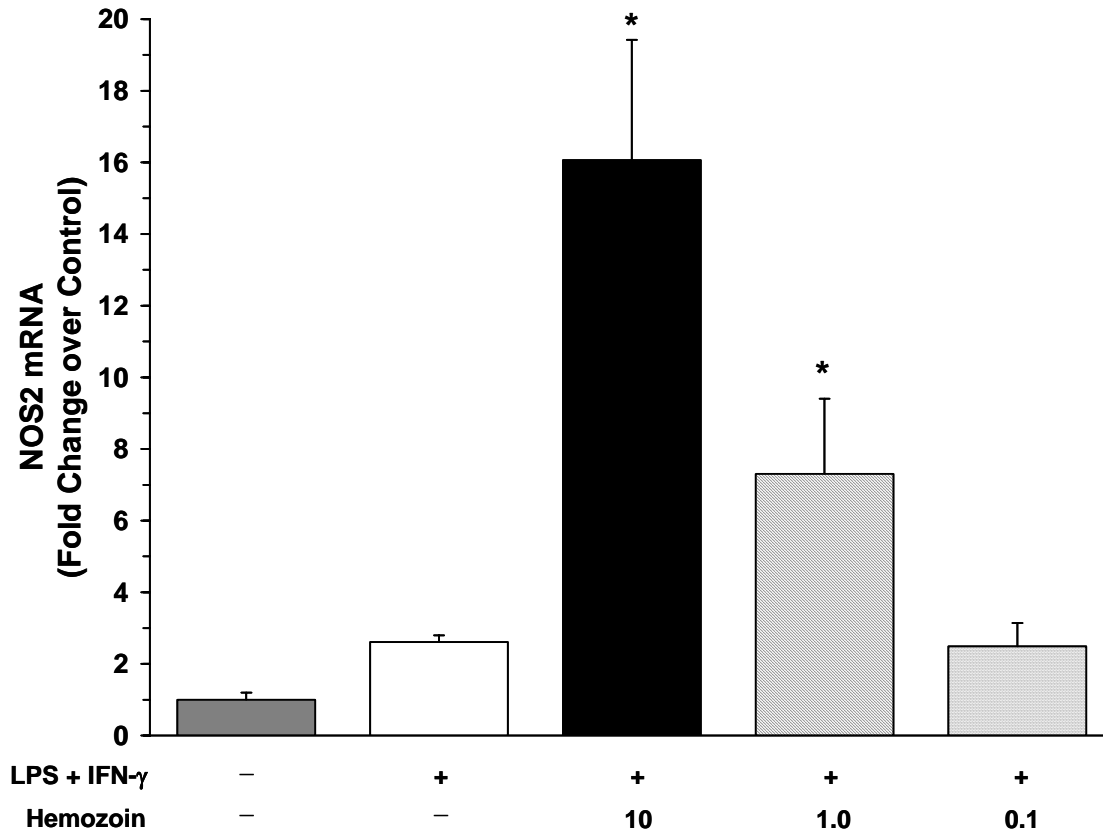


Figure 32. Effect of hemozoin on NOS2 mRNA expression. NOS2 mRNA was quantified for three individuals by Real time RT-PCR. PBMC ( $1 \times 10^6$  cells/mL) were cultured with media alone, LPS (100 ng/mL) and IFN- $\gamma$  (200 U/mL), or LPS and IFN- $\gamma$  in the presence of hemozoin (10, 1.0, and 0.1  $\mu$ g/mL). Cells were collected at 48 hrs for NOS2 mRNA determination. Values are the mean  $\pm$  SEM, n = 3. Statistical significance was determined by the Mann-Whitney U test. \* $P < 0.05$  compared to stimulated conditions.

## 6. DISCUSSION

Protection against severe malaria is regulated, at least in part, by the coordinated production of pro-inflammatory cytokines, which help to limit parasitemia, and anti-inflammatory cytokines that down-regulate the pro-inflammatory response. While a controlled pro-inflammatory response is associated with an increased parasite clearance (Kremsner *et. al.* 1995), an overly robust pro-inflammatory response is associated with increased severity of malaria (Luty *et. al.* 2000, Perkins *et. al.* 2000). Since anti-inflammatory cytokines, such as IL-10, reduce pro-inflammatory cytokine production, the relative production of pro- and anti-inflammatory cytokines in the inflammatory milieu ultimately determines clinical outcomes to severe malaria.

Increased production of IL-12p70 during the erythrocytic stage of malaria offers protection against severe disease, at least in part, through initiating a type 1 immune response (Stevenson *et. al.* 2004). The importance of IL-12 in severe malaria is further evidenced by the sterile protection of both non-human primates (Hoffman *et. al.* 1997) and mice (Sedegah *et. al.* 1994) against lethal malaria following administration of recombinant IL-12p70 prior to parasite inoculation. Furthermore, previous studies, as well as data presented here, show that IL-12p70 levels in plasma are reduced in children with severe malaria (Chaiyaroj *et. al.* 2004, Luty *et. al.* 2000, Malaguarnera *et. al.* 2002, Malaguarnera *et. al.* 2002, Musumeci *et. al.* 2003, Perkins *et. al.* 2000), illustrating that increased production of IL-12p70 during acute *P. falciparum* malaria

is associated with protection against severe disease. Although a previous study showed that IL-12p70 levels are not decreased during acute malaria (Lyke *et. al.* 2004), this study was performed in a population with a mixed phenotype of disease that included patients with CM, SMA, HDP, and hypoglycemia. Findings presented here demonstrate that reductions in IL-12p70 during acute SMA may be responsible for an inability to initiate an adequate malaria-specific immune response during blood stage malaria.

Another pro-inflammatory cytokine that has been extensively studied in malaria is TNF- $\alpha$ . Results presented here, as well as previous studies (Luty *et. al.* 2000, Perkins *et. al.* 2000), show that elevated TNF- $\alpha$  production during acute malaria is associated with severe disease. However, increased plasma levels of TNF- $\alpha$  have been correlated with a decreased parasite clearance time (Kremsner *et. al.* 1995). These findings suggest that there is an optimal range of TNF- $\alpha$  production that protects against severe malaria in which elevated TNF- $\alpha$  production during acute malaria is beneficial, while over-production of TNF- $\alpha$  leads to enhanced pathogenesis.

Anti-inflammatory cytokines, such as IL-10, negatively regulate both IL-12 and TNF- $\alpha$  production by decreasing *de novo* transcript formation (Aste-Amezaga *et. al.* 1998, Fiorentino *et. al.* 1991). Our previous studies have shown that IL-10 increases with increasing disease severity (Luty *et. al.* 2000, Perkins *et. al.* 2000), in agreement with results presented here in children with acute HDP. As mentioned above, despite conflicting observations of plasma cytokine levels in children with malaria from different geographic locations, the relative production of pro- and anti-inflammatory cytokines is consistent across populations with differing malaria endemicities

and is a good index for determining the association between cytokine dysregulation and disease severity. Consistent with these observations (Perkins *et. al.* 2000, Rhee *et. al.* 2001), results presented here illustrate that the ratio of IL-10/IL-12p70 is highest in children with severe malaria, suggesting that a more robust anti-inflammatory response, relative to a pro-inflammatory response, is associated with increased pathogenesis.

One important function of cytokines, such as TNF- $\alpha$ , during an inflammatory response is to induce the production of effector molecules, such as COX-2-derived PGE<sub>2</sub> and NOS2-dependent NO that regulate immunity (Perkins *et. al.* 1997, Perkins *et. al.* 1998). Previous studies illustrated that COX-2 transcripts and protein, and subsequent PGE<sub>2</sub> production, were reduced in Gabonese children with severe malaria (Perkins *et. al.* 2001). Suppression of PGE<sub>2</sub> during acute malaria has also been shown to occur during placental malaria (Perkins *et. al.* 2003) and CM (Perkins *et. al.* 2005). Results presented here extend these findings by illustrating that PBMC are the cellular source of suppressed COX-2-derived PGE<sub>2</sub> and increased NOS enzyme activity and NO production during acute *P. falciparum* malaria. Thus, during severe malaria, the pattern of cytokine production results in a reduction of PGE<sub>2</sub> and increased NO formation. This appears important since PGE<sub>2</sub> augments BFU-E proliferation (Dupuis *et. al.* 1998) and increased NO suppresses BFU-E formation (Shami *et. al.* 1996), suggesting that the relative production of effector molecules during acute childhood malaria may lead to decreases in erythropoiesis.

Although our results have shown that IL-12p70 and PGE<sub>2</sub> are decreased during severe malaria (Luty *et. al.* 2000, Perkins *et. al.* 2001, Perkins *et. al.* 2000), and IL-10, TNF- $\alpha$ , and NO are increased (Keller *et. al.* 2004, Luty *et. al.* 2000, Perkins *et. al.* 2000), it was unclear at the

time of these studies if dysregulation in cytokine and effector molecule production during severe malaria was a consequence of the host-immune response, or if a parasite antigen(s) was responsible for the observed pattern of immune production.

Ingestion of hemozoin by cultured human PBMC has been shown to induce pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$  (Biswas *et. al.* 2001, Mordmuller *et. al.* 1998, Pichyangkul *et. al.* 1994, Sherry *et. al.* 1995), and chemokines, such as MIP-1 $\alpha$  and MIP-1 $\beta$  (Sherry *et. al.* 1995). Results presented here support these findings for TNF- $\alpha$ , and further illustrate that hemozoin augments IL-10 and NO production, and decreases IL-12p70 and PGE<sub>2</sub> formation. As mentioned above, cytokine production in children with severe malaria is characterized by increased TNF- $\alpha$ , IL-10, and NO production, and decreased synthesis of IL-12p70 and PGE<sub>2</sub>. Furthermore, our recent observations illustrate that circulating MIP-1 $\alpha$  and MIP-1 $\beta$  levels are reduced in Gabonese children with severe malaria relative to children with mild disease (Ochiel *et. al.* 2005). Thus, it appears that immune dysregulation during severe childhood malaria is, at least partially, due to ingestion of hemozoin by PBMC.

One mechanism by which hemozoin may increase malaria disease severity is through suppression of IL-12p70, which is thought to initiate the immune response to malaria, at least in part, through induction of IFN- $\gamma$  from CD4<sup>+</sup> T cells [for review see (Stevenson *et. al.* 2004)]. Recent studies illustrate that hemozoin suppresses IFN- $\gamma$  production in cultured human PBMC (Deshpande *et. al.* 2004), which may be a consequence of reduced IL-12p70 production reported here and elsewhere (Deshpande *et. al.* 2004). However, it is highly interesting that pro-inflammatory molecules, such as TNF- $\alpha$  and NO, are increased in response to hemozoin

phagocytosis (Biswas *et. al.* 2001, Keller *et. al.* 2004, Mordmuller *et. al.* 1998, Pichyangkul *et. al.* 1994, Sherry *et. al.* 1995), since IL-12p70-induction of IFN- $\gamma$  appears to play a major role in increasing gene expression of these molecules during blood stage malaria [for review see (Stevenson *et. al.* 2004)].

Hemozoin may promote severe malaria through suppression of erythropoiesis. In response to acute anemia elicited by intravascular and extravascular hemolysis of both parasitized RBC (pRBC) and non-pRBC (Hillman *et. al.* 1996), increased production of new RBC is essential for recovery. Erythropoiesis is regulated by growth factors, such as hormones and cytokines [for review see (Means 2004)], and the inflammatory pattern during SMA most likely leads to suppression of the erythropoietic response [for review see (McDevitt *et. al.* 2004)]. This is further evidenced by the suppression of BFU-E formation by high levels of TNF- $\alpha$  (Skobin *et. al.* 2000), IL-10 (Oehler *et. al.* 1999), and NO (Shami *et. al.* 1996) and by reductions in IL-12p70 (Mohan *et. al.* 1998). Furthermore, exogenous PGE<sub>2</sub> enhances BFU-E formation and proliferation in cultured CD34<sup>+</sup> cells (Dupuis *et. al.* 1998), indicating the importance of this lipid mediator in promoting RBC formation. The low bicyclo-PGE<sub>2</sub>/TNF- $\alpha$  ratio reported here, and the high ratio of IL-10/IL-12p70 (Perkins *et. al.* 2000), in children with severe malaria suggests that the circulating cytokine milieu may promote suppression of erythropoiesis due to dysregulation in the immune response promoted by phagocytosis of hemozoin. The fact that hemozoin is present in large amounts in tissue macrophages from children with prolonged exposure to *P. falciparum*, and is present in high quantities in bone marrow from malaria-infected adults (Abdallah 1990), further suggests that local production of inflammatory mediators in bone marrow stromal cells may further suppress erythropoiesis.

Table 11. Effect of hemozoin ingestion on cytokine and effector molecule production in cultured mononuclear cells.

Cytokine	Cell Type	Effect of Hz	REF
IFN- $\gamma$	Human	Decrease	(Deshpande <i>et. al.</i> 2004)
IL-1 $\beta$	Human	Increase	(Pichyangkul <i>et. al.</i> 1994)
	Human	Increase	(Biswas <i>et. al.</i> 2001)
	Human	Increase	(Deshpande <i>et. al.</i> 2004)
	Murine	No effect	(Taramelli <i>et. al.</i> 1995)
IL-2	Human	Decrease	(Deshpande <i>et. al.</i> 2004)
IL-6	Murine	Decrease	(Prada <i>et. al.</i> 1995)
IL-10	Human	Increase	(Mordmuller <i>et. al.</i> 1998)
	Human	Increase	(Keller <i>et. al.</i> 2004)
	Human	Increase	(Deshpande <i>et. al.</i> 2004)
IL-12p70	Human	Increase	(Mordmuller <i>et. al.</i> 1998)
	Human	Decrease	(see Figure 5A)
	Human	Decrease	(Deshpande <i>et. al.</i> 2004)
MIF	Murine	Increase	(Martiney <i>et. al.</i> 2000)
MIP-1 $\alpha$	Human	Increase	(Sherry <i>et. al.</i> 1995)
	Murine	Increase	(Jaramillo <i>et. al.</i> 2005)
	Human	Increase	(Ochiel <i>et. al.</i> 2005)
MIP-1 $\beta$	Human	Increase	(Sherry <i>et. al.</i> 1995)
	Murine	Increase	(Jaramillo <i>et. al.</i> 2005)
	Human	Increase	(Ochiel <i>et. al.</i> 2005)
MIP-2	Murine	Increase	(Jaramillo <i>et. al.</i> 2005)
MCP-1	Murine	Increase	(Jaramillo <i>et. al.</i> 2005)
NO	Murine	Decrease	(Taramelli <i>et. al.</i> 1995)
	Murine	Decrease	(Prada <i>et. al.</i> 1996)
	Murine	Increase	(Jaramillo <i>et. al.</i> 2003)
	Human	Increase	(Keller <i>et. al.</i> 2004)
Oxidative burst	Human	Decrease	(Schwarzer <i>et. al.</i> 1993)
PGE <sub>2</sub>	Human	Decrease	(Keller <i>et. al.</i> 2004)
TNF- $\alpha$	Murine	Increase	(Prada <i>et. al.</i> 1995)
	Murine	Decrease	(Taramelli <i>et. al.</i> 1995)
	Human	Increase	(Sherry <i>et. al.</i> 1995)
	Human	Increase	(Mordmuller <i>et. al.</i> 1998)
	Human	Increase	(Pichyangkul <i>et. al.</i> 1994)
	Human	Increase	(Biswas <i>et. al.</i> 2001)
	Human	Increase	(Deshpande <i>et. al.</i> 2004)
	Human	Increase	(see Figure 5E)



Hemozoin elicits a different pattern of cytokine and effector molecule production in cultured human and murine mononuclear cells. Table 11 depicts the effects of hemozoin on cultured circulating mononuclear cells in both human and murine models. Induction of IL-1 $\beta$  has been shown in human PBMC treated with hemozoin (Biswas *et. al.* 2001, Deshpande *et. al.* 2004, Pichyangkul *et. al.* 1994), however, IL-1 $\beta$  is not altered in murine cells (Taramelli *et. al.* 1995). Results presented here show that hemozoin induces NO production in cultured human PBMC (Keller *et. al.* 2004), while conflicting results have been reported on the effect of hemozoin on NO production in murine cells (Jaramillo *et. al.* 2003, Prada *et. al.* 1996, Taramelli *et. al.* 1995). Furthermore, a number of studies have shown that hemozoin augments TNF- $\alpha$  production in cultured human mononuclear cells (Biswas *et. al.* 2001, Mordmuller *et. al.* 1998, Pichyangkul *et. al.* 1994, Sherry *et. al.* 1995), despite contradicting reports in murine systems (Prada *et. al.* 1995, Taramelli *et. al.* 1995). These discrepancies could arise from the concentrations of hemozoin used in the different in vitro models. For example, using the estimated concentration of hemozoin that would be found in children with severe malaria (i.e., 10  $\mu$ g/mL) (Keller *et. al.* 2004), we have shown here that hemozoin augments IL-10 and TNF- $\alpha$ , and suppresses PGE<sub>2</sub> formation (Keller *et. al.* 2004). Recent observations in pregnant women with malaria have shown that cultured intervillous blood mononuclear cells (IVBMC) that have acquired hemozoin in vivo produce less IL-10, TNF- $\alpha$ , and PGE<sub>2</sub> (Perkins *et. al.* 2003). This suppression was correlated with the percentage of pigmented IVBMC (Perkins *et. al.* 2003), suggesting that higher concentrations, or a buildup of hemozoin deposition, causes a different pattern of inflammatory production. Alternatively, the varying results in murine studies could be due to treatment of crude hemozoin with chemicals, such as acetone or proteinase K that would remove host- and parasite-derived lipids and protein, respectively, which could dramatically

impact on the immune response. In our in vitro model system, isolated hemozoin is not subjected to purification, thus the moiety used in these studies most likely resembles the pigment that would be acquired during a natural malaria infection. Results obtained in murine systems using high concentrations of highly purified hemozoin may therefore not accurately reflect the immune profile that is elicited by in vivo acquisition of hemozoin during childhood malaria.

Although not directly investigated here, other parasite-derived antigens, such as glycosylphosphatidylinositol (GPI), have been shown to influence cytokine and effector molecule production in cultured PBMC. Initial studies revealed that purified and concentrated GPI augmented TNF- $\alpha$ , IL-1, and NO production in cultured murine macrophages (Schofield *et. al.* 1993, Tachado *et. al.* 1996). Recent studies have also shown increased IL-12 and IL-6 in response to malaria-specific GPI (Zhu *et. al.* 2005), which is dependent on NF- $\kappa$ B-mediated signaling through Toll-like receptor (TLR) 2 and TLR4 (Krishnegowda *et. al.* 2005). Despite a similar pattern of inflammatory production in response to GPI, the concentrations of GPI used in these experiments are much higher than concentrations seen in normal in vivo infections. However, GPI antibodies have been noted in malaria-immune adults (Naik *et. al.* 2000), suggesting that malaria-specific GPI function as important signaling molecules during blood-stage malaria.

The immune response elicited by PBMC ingestion of hemozoin is likely dependent on the phagocytic pathway by which hemozoin is acquired. Previous studies illustrated that detergent-treated hemozoin and  $\beta$ -hematin were present in large clusters in the absence of cellular membranes (Olliaro *et. al.* 2000), and in these studies, was found to occur independently

of coated pit formation in the absence of a specific receptor (Olliaro *et. al.* 2000). Confocal microscopy studies examining ingestion of hemozoin by PBMC showed contrasting results in which serum-opsonized hemozoin was associated with lysosomal formation (Schwarzer *et. al.* 2001), thus suggesting that phagocytosis of hemozoin is receptor-mediated. Alternatively, opsonization of hemozoin could cause phagocytosis through scavenger receptors, which would account for the discrepancy between these studies. Recent studies in mice injected with *P. falciparum*-derived hemozoin identified TLR9 as a putative recognition receptor for phagocytosis of hemozoin (Coban *et. al.* 2005). This is highly interesting as TLR9 is the pattern recognition receptor for CpG methylated DNA of bacterial origin (Krieg 2002), and in the previous studies, highly purified hemozoin and  $\beta$ -hematin were used to illustrate TLR9-dependent phagocytosis, however, both of these compounds do not contain DNA (Coban *et. al.* 2005). Removal of host- and parasite-derived components, such as proteins, lipids, and nucleic acids, from crude hemozoin that would be obtained during a natural infection, yields  $\beta$ -hematin, which was also used in these studies. Further experiments using non-treated hemozoin that would contain host- and parasite-derived components, such as proteins, lipids, and nucleic acids, in cultured human mononuclear cells will likely yield important information on the mechanism of ingestion of free hemozoin.

While phagocytosis of circulating hemozoin may occur through receptor recognition, such as TLR9, phagocytosis of RBC membrane-associated hemozoin, or phagocytosis of intact pRBC that contain large amounts of hemozoin (Celada *et. al.* 1983), most likely occurs through the scavenger receptor, CD36 [for review see (Serghides *et. al.* 2003)]. CD36 is a class B surface scavenger receptor that is involved in phagocytosis of pRBC by human monocytes

(McGilvray *et. al.* 2000). Ingestion of pRBC through CD36 is not associated with increased TNF- $\alpha$  production (McGilvray *et. al.* 2000), in contrast to results presented here for ingestion of free hemozoin and  $\beta$ -hematin by cultured human PBMC. Thus, although phagocytosis of free hemozoin elicits dysregulation of mononuclear cytokine and effector molecule production, it remains to be determined if acquiring hemozoin through CD36-mediated uptake of intact pRBC leads to altered inflammatory production.

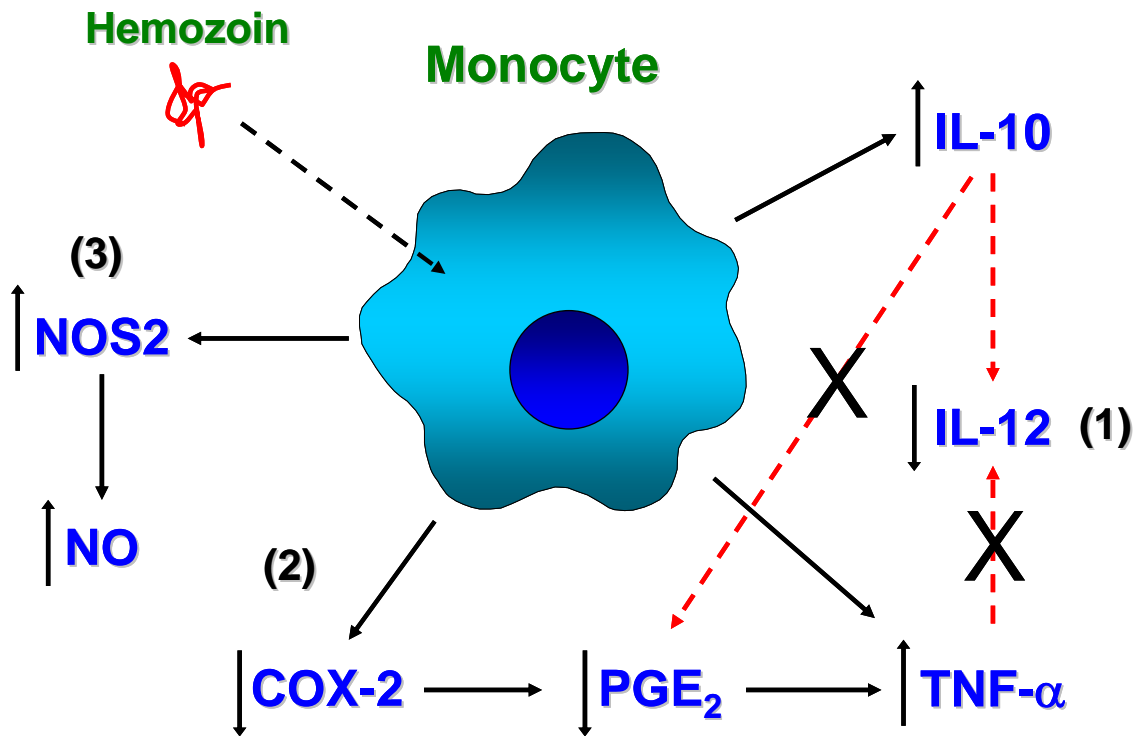
The specific signaling cascades that are activated upon ingestion of hemozoin may lead to further understanding of hemozoin-induced dysregulation of inflammatory mediator production. The first series of experiments examining the effect of hemozoin on cellular signaling illustrated that protein kinase C (PKC) activation was reduced in hemozoin-containing cells, which caused reduction in oxidative burst (Schwarzer *et. al.* 1993). Several recent observations in a cultured murine macrophage cell line, B10R, suggest that ingestion of  $\beta$ -hematin elicits activation of the extracellular signal-related kinase (ERK) 1/2 and NF- $\kappa$ b signaling pathways, which is responsible for induction of NOS2 and upregulation of chemokine expression (Jaramillo *et. al.* 2005, Jaramillo *et. al.* 2003). These studies support previous observations in our laboratories illustrating that the  $\beta$ -chemokines macrophage inflammatory protein (MIP)-1 $\alpha$  and MIP-1 $\beta$  are upregulated in severe malaria, which is regulated at least partially by PBMC ingestion of hemozoin (Ochiel *et. al.* 2005). However, experiments in cultured human monocyte-derived dendritic cells (DC) found that NF- $\kappa$ b was not induced in response to phagocytosis of hemozoin, but that DC activation was mediated through increased expression of peroxisome proliferator-activator receptor (PPAR)- $\gamma$  (Skorokhod *et. al.* 2004). The discrepancy between these results illustrates the differences between human and murine models

of malaria, in which different signaling cascades regulate the production of inflammatory mediators. Further studies in cultured human PBMC examining signaling cascades that are altered in response to phagocytosis of hemozoin may help to reveal the mechanism(s) by which hemozoin augments both NOS2-dependent NO production and chemokines levels.

In conclusion, data presented as part of this dissertation have illustrated that ingestion of hemozoin by cultured human mononuclear cells results in a similar pattern of cytokine and effector molecule production to that observed in plasma and cultured PBMC from children with severe malaria. These studies illustrate that hemozoin induces over-production of IL-10 and TNF- $\alpha$ , and that hemozoin-induced IL-10 production suppresses *de novo* IL-12p70 production (Figure 33). Furthermore, independently of IL-10 over-production, hemozoin suppresses COX-2 transcripts and protein, and the subsequent production of PGE<sub>2</sub>, which causes over-production of TNF- $\alpha$  (Figure 33). Lastly, hemozoin induced NOS2 transcripts and NO production in cultured human mononuclear cells (Figure 33). Taken together, these results show that hemozoin is capable of eliciting a pattern of inflammatory production consistent with that observed in children with severe malaria, suggesting that blockade of hemozoin formation during blood stage malaria protects against severe disease by reducing circulating monocyte-induced cytokine dysregulation.

The majority of childhood morbidity and mortality in sub-Saharan Africa is due to infection with *P. falciparum*, thus treatment and/or prevention of malaria is of great public health importance. For successful malaria vaccine designs, the pattern of soluble immune mediator production that defines protection against severe malaria must be taken into account. Therefore,

understanding the immune response that is associated with protection against severe malaria will greatly impact on the design and testing of malaria-specific vaccines. Although the acquisition of protective malarial immunity is not currently well defined, the present work has identified specific cytokines and effector molecules whose production is dysregulated during severe childhood malaria, which furthers the understanding of a beneficial immune pattern during blood stage malaria to aid in malaria-specific vaccine strategies.



*Figure 33. Effect of hemozoin on immune dysregulation.* (1) Ingestion of hemozoin by human PBMC elicits elevated production of the anti-inflammatory cytokine, IL-10, and the pro-inflammatory cytokine, TNF- $\alpha$ . Elevated IL-10, but not TNF- $\alpha$ , is responsible for decreased IL-12 production. (2) Hemozoin suppresses COX-2 transcript and protein formation, which leads to decreased PGE<sub>2</sub> production, which is not due to increased IL-10. Hemozoin-induced suppression of PGE<sub>2</sub> causes over-production of TNF- $\alpha$ . (3) Hemozoin induces NOS2 expression and NO production in cultured human PBMC.

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