ROLE OF CHEMOKINE-CHEMOKINE RECEPTORS IN THE PATHOGENESIS OF SEVERE *PLASMODIUM FALCIPARUM* MALARIA IN CHILDREN: IMPLICATIONS FOR MALARIA-HIV INTERACTION

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ABSTRACT

Molecular determinants of malaria pathogenesis are largely undefined. Chemokines and chemokine receptors, regulate immune responses, may thus determine malaria severity. Further, by regulating HIV pathogenesis, they may constitute a crucial link in malaria-HIV interaction. Understanding biologic mechanisms underlying malaria-HIV interaction has important public health utility in designing rational therapeutic and preventive strategies. Malaria could potentially modulate HIV-1 infection through alteration in expression of CD4 and chemokine receptors, required for cellular entry. This study has determined circulating levels and transcriptional profiles of β-chemokines (MIP-1α, MIP-1β, and RANTES) in ex vivo peripheral blood mononuclear cells (PBMCs) of children with varying degrees of malaria severity. Additional *in vitro* experiments assessed the effects of stimulation of PBMCs with crude hemozoin (Hz) or synthetic hemozoin (sHz) on CD4, β-chemokine and chemokine receptor (CCR5 and CXCR4) protein expression and transcript formation. Plasma MIP-1α and MIP-1β levels were significantly elevated in mild and severe malaria, while RANTES levels decreased with increasing disease severity. β-chemokine gene expression closely matched circulating β-chemokine profile, illustrating that PBMCs are a primary source for β-chemokine production during malaria. Healthy children with a history of severe malaria had lower baseline RANTES production than children with a history of mild malaria, suggesting inherent differences in

RANTES production. *In vitro* experiments in PBMCs from healthy malaria-naïve donors showed that Hz and sHz promote a similar pattern of β-chemokine protein secretion and transcript expression. FACS analysis showed that Hz and sHz induced similar patterns of cellular surface expression of CD4, CCR5 and CXCR4 on PBMCs. Hz or sHz-exerted differential effects on $CD14^+$ and $CD3^+$ subsets, and this modulatory effect part to transcriptional regulation based on gene expression profiles obtained for respective antigens. Additional studies showed that HIV-1 replicates differently in monoctye-derived macrophages (MDMs) stimulated with either Hz or sHz. sHz enhanced HIV-1 replication while Hz had an inhibitory effect. Results presented here demonstrate a distinct profile of β-chemokine expression in children with severe malaria, which is promoted by *P. falciparum* derived hemozoin. Further, Hz modulates expression of surface antigens required for HIV-1 entry, defining a possible mechanism for HIV-malaria interaction.

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CHAPTER 1

1.1. General Introduction

Malaria is a major cause of morbidity and mortality in tropical countries (1). Children less than 5 years of age, pregnant women and nonimmune people (e.g. travelers) are at the highest risk of severe disease (1). For example, the annual death toll from malaria is estimated to be one million children in Africa; 90% of annual global malaria mortality (1). Young children are especially susceptible to malaria due to their non-immune status (2). Malaria in pregnancy results in anemia, low birth weight, preterm delivery and high neonatal mortality (3). The etiologic agents for malaria in human are four species of protozoal parasites: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium falciparum*, all transmitted by female *Anopheline* mosquitoes. Of these parasites, *P. falciparum* and to a lesser extent *P. vivax* are the major cause of morbidity and mortality in malaria (4).

The life cycle of *Plasmodium spp* involves distinct stages in the mosquito vector and the human host (5). Sporozoite forms of the parasite are injected subcutaneously by mosquito vector during a blood meal, and the parasites rapidly migrate to the liver. Within the liver, after a period of dormancy (how long), the sporozoites mature in the liver hepatocytes and become merozoites which are released into the circulation resulting in infection of red blood cells (RBCs). Rupture of parasitized RBCs (pRBCs) results in the release of merozoites and expansion of the population into neighboring RBCs. The sexual phase of the parasites in RBCs results in male and female gametocytes that are ingested by the mosquito vector during a blood meal. The release of merozoites from ruptured RBCs corresponds with the paroxysms of fever and other clinical manifestations of infection.

From a therapeutic perspective, malaria presents enormous challenge. There is widespread increase in resistance to first line therapies, including chloroquine and sulphadoxine/pyramethamine (6). This coupled with the fact that there is yet no vaccine currently available (7) makes malaria a major health and socioeconomic challenge. The use of insecticide-impregnated bed nets is widely being implemented as a prevention strategy against malaria in areas of high transmission (8, 9).

Another consideration in malaria pathogenesis is the current recognition of the potential interaction existing between malaria and viral, bacterial and other parasitic infections. HIV infection and the resultant acquired immune deficiency syndrome (AIDS) represent a major co-infection particularly in the developing countries where there is geographical overlap in the prevalence of malaria and HIV (10). Approximately 40 million people are currently living with HIV/AIDS, and majority of these cases are the Sub-saharan Africa (11).

HIV belongs to lentivirus subfamily of retroviruses, characterized by possession of the enzyme reverse transcriptase that enables replication of the single-stranded viral RNA genome though a double-stranded DNA intermediate (12). Synthesized viral DNA integrates within the host cellular DNA as proviral sequence achieved by a virally encoded integrase enzyme, followed by replication using host cellular components (12). Cellular tropism for viral particles is defined principally by expression of CD4, and chemokine receptors (e.g. CCR5 and CXCR4) on the surface of host cells that serve as the primary receptor and co-receptor, respectively, for entry of the virus into the cells (13). An immunologic hallmark of HIV infection is the progressive depletion of $CD4^+$ T helper cells, resulting in immune deficiency state that predisposes an individual to opportunistic infections, and ultimately death (14).

In adult population, the sexual route represents the main mode of transmission of the virus, while in pediatric populations over 90% of infections are acquired through mother-to-child transmission. Currently there is no therapeutic regime that can "cure" HIV infection. Highly active antiretroviral therapy (HAART) is regarded as the standard of care (15) but is limited by side-effects, emergence of resistant strains, and lack of availability for much of the developing world (15). An effective vaccine against HIV remains elusive (16), illustrating that an improved understanding the basic biology of the virus and possible interactions with other infections remains critical for efforts aimed at designing effective therapies and vaccine strategies against HIV.

CHAPTER 2

2.1 Chemokines as Mediators of Malaria Pathogenesis

2.1.1 Determinants of Malaria Disease Severity

Malaria is one of the greatest influences of infectious origin on morbidity and mortality in sub-Saharan Africa (1). In young children who are largely non- or semi-immune to *Plasmodium falciparum* malaria, severe disease primarily manifests as cerebral malaria (CM) and/or severe malarial anemia (SMA) (17). In areas of hyperendemicity, such as Lambaréné, Gabon, severe malaria generally affects children less than 5 years of age due to the lack of naturally acquired immunity (2). The primary clinical manifestations of severe malaria in this area are SMA and high-density parasitemia with only rare occurrences of CM.

Although the molecular determinants of mild versus severe malaria are largely undefined, current evidence suggests a potential role for the relative balance between pro- and anti-inflammatory cytokines (18-22). Elevated levels of pro-inflammatory cytokines during the acute phase of infection appear to limit disease progression, while a bias towards an anti-inflammatory response appears to promote increased pathogenesis (21, 23-25). Additional soluble inflammatory mediators which may be important in malaria pathogenesis are chemokines, a superfamily of small structurally related proteins (8-17 kDa) that regulate innate and adaptive immunity (26).

2.1.2. Chemokine Nomenclature and Biological Function

Chemokines are categorized into four classes based on the number and spacing of N-terminal cysteine residues (27). These classes can be more broadly defined as CXC- (or α-) chemokines [e.g. stromal cell derived factor (SDF)-1] and the CC- (or β-) chemokines [e.g. macrophage

inflammatory protein (MIP-1α), MIP-1β, and Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES)] (27). The actions of chemokines are mediated by a family of 7-transmembrane G protein-coupled receptors (26). Binding of chemokines to their cognate receptors results in an intracellular signaling cascade that culminates in diverse biological functions, such as chemotactic recruitment of inflammatory cells, leukocyte activation, angiogenesis, hematopoiesis, and antimicrobial effects (26).

In addition to their role in regulating the immune response during viral and bacterial infections, chemokines also appear to mediate the host-immune response during protozoan infections (28). For example, recent studies have shown that *P. falciparum* during pregnancy is characterized by elevations in MIP-1α, monocyte chemoattractant protein (MCP)-1, I-309, and interleukin (IL)-8 transcripts which are associated with increased monocyte density in the placental intervillous space (29, 30). Additional studies in women with malaria during pregnancy show that MIP-1α and MIP-1β are elevated in cultured intervillous leukocytes (31). Serum concentrations of MIP-1α and interleukin (IL)-8 are also increased in adults with severe and complicated falciparum malaria (32).

2.1.3. Hemozoin (Hz) Synthesis and Function

Recent studies in our laboratory found that malarial pigment (hemozoin, Hz) is one of the parasitic products responsible for regulating the production of effector molecules, such as nitric oxide (NO) and prostaglandin-E2 (PGE2) from peripheral blood mononuclear cells (PBMCs) during acute falciparum malaria in children (33, 34). Hz is a coordinated aggregation polymer of heme produced by parasites within red blood cells (RBCs) during the digestion of host hemoglobin [for review see (35)]. Following lysis of parasitized RBC, Hz is rapidly

phagocytosed by neutrophils, monocytes, and tissue macrophages. Naturally acquired Hz is composed of host- and parasite-derived lipids, proteins, and nucleic acids which are associated with the polymerized iron core structure, ferriprotoporphorin (FP)-IX (36). Upon removal of proteins and lipids from Hz, the FP-IX core remains, which is structurally identical to synthetic pigment, β-hematin (sHz) (37). Our laboratory, as well as others, have shown that Hz and sHz promote similar patterns of pro- and anti-inflammatory cytokines, effector molecules, and chemokines, such as IL-1β, TNF-α, IL-10, NO, PGE2, MIP-1α, and MIP-1β from cultured human PBMCs as those observed in children with malaria (33, 34, 38, 39). Hz is a key molecule that mediates malaria-associated immunosuppression (40), and its phagocytosis by monocytes alters expression of MHC class II, CD54 and CD11c which may result in impairment of antigen presentation (41). Recent evidence in murine model of malaria indicates that in vivo administration of hemozoin rapidly up regulates chemokines and chemokine receptor expression, and this effect is associated with increased cellular infiltration (42).

Although chemokines are important for bridging innate and adaptive immune responses and regulating hematopoietic maturation (43, 44), the profile of chemokine expression and their potential role in regulating disease severity in children with *P. falciparum* malaria has not been defined. As such, we determined circulating protein levels and transcript profiles of β-chemokines (MIP-1α, MIP-1β and RANTES) and an α-chemokine (SDF-1) in plasma and PBMCs, respectively, in children with varying degrees of *P. falciparum* malaria. Additional studies were conducted in cultured PBMCs to determine if Hz and sHz induced a similar profile of chemokine transcripts and protein as that found in children with malaria.

2.2 Malaria and HIV Interaction

2.2.1 Epidemiological Evidence

It is becoming increasingly apparent that subtle interactions exist between infectious pathogens, and that a full understanding of any given infection process can only be achieved if potential specific interactions with other infectious agents are elucidated. Human immunodeficiency virus (HIV) infection and *Plasmodium falciparum*-induced malaria are both associated with high morbidity and mortality, particularly in the developing countries (11, 45). With regards to global prevalence, considerable geographical overlap between malaria and HIV-1 infection suggests possible interaction between these infections (10). Approximately 40 million people are living with HIV/AIDS, with over 70% in Sub-saharan Africa (11). Malaria accounts for approximately 1-3 million deaths annually, with over 90% of the 300-500 annually reported infections occurring in Sub-saharan Africa (2, 46). Understanding interaction between HIV and malaria, thus, has enormous public health importance, particularly in the context of designing effective intervention strategies in populations where HIV and malaria are prevalent.

Early epidemiological studies showed lack of association between HIV and malaria (47-49). These studies were, however, limited in design and technical capability to assess immune compromise accurately by the use of CD4 counts and HIV-1 viral loads. Recent evidence, however, supports potential reciprocal interaction between HIV-1 and malaria. Advanced HIV immunosuppression is associated with higher density parasitemia and more clinical illness in adults (50-53). These findings have further been supported by additional observational studies in cohorts of pregnant women, indicating that HIV infection results in increased prevalence of placental malaria (54-56). The underlying mechanism-based explanation for increased frequency of placental malaria in HIV-coinfected women is presently unknown with conflicting evidence

regarding the role of the humoral immune response (57, 58), and limited data on cellular immune response in these women (59). Interestingly, studies in pediatric populations are inconclusive on the effect of HIV infection on the clinical course of malaria (60, 61). Conversely, malaria has been shown to impact on the clinical course of HIV infection. Acute *P. falciparum* infection is associated with increased HIV-1 viral loads in non-pregnant (62) and pregnant (63-65) adult populations. However, in spite of demonstrable increases in placental viral loads in women with malaria, the effect of this process on mother-to-child transmission is not clearly defined (64, 66, 67).

2.2.3. Potential Mechanism (s) of Interaction

Despite these numerous epidemiological studies supporting evidence for malaria-HIV interactions, there is limited data on potential biological mechanisms that define the interaction. Previous studies by Xiao and colleagues (68) showed that increased cellular activation, mediated by TNF-α, and induced by malarial antigen stimulation resulted in enhanced HIV-1 replication in human mononuclear cells. Cellular activation as a mechanism for malaria-induced HIV replication has further been supported by data generated in vitro model (69), transgenic mice model (70) and clinical settings (71). The latter study showed that parasite clearance achieved with antimalarial therapy results in decreased in HIV-1 particles derived from macrophages as a consequence of diminution in systemic immune activation.

An additional mechanism through which malaria could influence HIV pathogenesis is by modulation of receptors required viral entry and *in vivo* dissemination. HIV-1 entry into susceptible cells is mediated by interactions with CD4 and chemokine receptors which serve as primary and co-receptors, respectively (72). Chemokine and chemokine receptors belong to a

superfamily of proteins, whose principal role involves leukocyte chemotaxis (73). HIV-1 interacts with CCR5 and CXCR4 via gp120, specifically its V3 loop, which determines cellular tropism (74, 75). On the basis of chemokine receptor utilization, HIV-1 strains have been categorized as R5, X4 or R5-X4, corresponding to macrophage tropic, T cell tropic, or dual tropic, respectively (13). Evolution of X4 viruses is associated with progression to clinical end-stage disease (76-78). Investigating the effects of malaria on chemokine and chemokine receptor expression is therefore critical in understanding the initial establishment of HIV-1 infection of susceptible cells, and the clinical progression of disease through alterations in the proportion of *in vivo* cellular targets. A recent study has shown that soluble malarial antigens regulate expression of CCR5 and CXCR4 on human $CD4^+$ mononuclear cells (79).

To determine potential mechanisms by which malaria affects HIV-1 infection, this study utilized an *in vitro* model based on stimulation of human peripheral blood mononuclear cells (PBMCs) with crude plasmodium falciparum-derived pigment, hemozoin (Hz), or its synthetic form (sHz). The present study has analyzed surface expression of CD4, CCR5 and CXCR4, and quantitated the respective transcripts by real time RT-PCR. In addition, susceptibility of monocyte-derived macrophages (MDMs) stimulated with either Hz or sHz was determined following infection with M-tropic HIV-1.

2.3 Purpose

These studies were conducted to define the profile of β-chemokines in children with acute P. falciparum infection, and to determine association between β-Chemokine profile and malaria severity. Additional experiments were designed to define molecular basis of interaction between malaria and HIV. Surface expression and transcript formation of antigens required for HIV-1

entry were analyzed following stimulation of PBMCs with Hz or sHz to determine if this results in altered infectivity of human mononuclear cells with HIV-1.

CHAPTER 3

3.1 Materials and Methods

3.1.1 Study Participants

Children (n=43, 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 manifestation of severe childhood malaria is severe anemia and/or hyperparasitemia, with cerebral malaria rarely occurring (18, 80). Classification of malaria was defined according to our previously published methods (81) with inclusion criteria for severe cases characterized by parasitemias greater than 250,000 parasites/μL and/or the presence of severe anemia (i.e. hemoglobin ≤ 5 g/dL) (81). Matched mild malaria cases were defined by parasitemias less than 100,000 parasites/μL and the absence of any signs or symptoms of severe malaria. Healthy, malaria-exposed subjects were defined as those participants with a previous episode(s) of either mild or severe 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 and/or antipyretics. Routine clinical evaluations and laboratory measures were used 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.

For in vitro experiments to determine the effect of Hz or sHz on production of β-chemokines, healthy malaria-naïve adult volunteers (n=5) were recruited at the University of Pittsburgh, US. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital, Duke University Medical Center Investigational Review Board, and the University of Pittsburgh Investigational Review Board.

3.1.2. Isolation and Culture of Peripheral Blood Mononuclear Cells (PBMCs)

Venous blood (50 mL) was drawn from healthy adult US donors into EDTA-containing vials. Plasma was separated and PBMCs were prepared using Ficoll/Hypaque according to previous methods (82). PBMCs collected from the interface were subsequently washed twice with Dulbecco's Modified Eagles Medium (DMEM) [Invitrogen Corporation, Carlsbad, CA] supplemented with 10mM HEPES, 10mM Penicillin/Streptomycin).

To assess the effects of Hz or sHz on the production of chemokines, PBMCs were plated at a density of 1 X 10^6 cells/mL in DMEM supplemented with 10mM HEPES, 10mM Penicillin/Streptomycin, and 10% pooled human serum (heat inactivated at 56^{o}C for 30 min). Cells were incubated with media alone (control) or physiologic amounts of Hz or sHz representing a concentration comparable to that during severe malaria (i.e., 10 μg/mL) (33). Cells were kept at 37^{o}C in a humid atmosphere of 5% CO_2. Supernatants and cell pellets were collected at 4, 24, 48 and 120 hr.

3.1.3. Preparation of Crude Hemozoin and Synthetic Hemozoin

Protocols for preparation of crude Hz and sHz have been described previously (34). In brief, Hz was derived from *in vitro* cell cultures of *P. falciparum*-infected red blood cells (strain Pf-D6) harvested when the parasitemia level was 3 to 5%, and late trophozoites and early schizonts were the predominant forms. Infected RBCs were spun at 2,000 rpm for 10 min, and re-suspended in 40 mL of 0.01 M phosphate-buffered saline (pH 7.2) with 2 mL of 1% saponin solution for 10 min. Cell lysates were then subjected to ultracentrifugation (14,000 rpm for 15 min) and resultant cell pellets were washed 3 to 5 times to remove lipid membranes. Following the final

wash step, the pellet was dried overnight, weighed, and re-suspended in filter-sterilized H_2O at a concentration of 1.0 mg/mL.

sHz was prepared in a 4.5 M acidic acetate solution (pH 4.5) as previously described (37). In brief, hemin chloride (Sigma, St. Lous, MO) was added to a 0.1 M solution of NaOH followed by addition of HCl at 60^{o}C. sHz was crystallized by addition of an acidic solution of acetate and the mixture was incubated at 60^{o}C for 150 min. The solution was then centrifuged and washed thrice with distilled (dH_2O), and dried at 60^{o}C under vacuum. The final pellet was weighed and re-suspended in filter-sterilized H_2O at a concentration of 1.0 mg/mL.

3.1.4. Chemokine ELISAs

Quantitative ELISA was performed with commercially available assays to determine MIP-1α, MIP-1β, and RANTES (Biosource International, Camarillo, CA) and SDF-1 (DuoSet: R&D Minneapolis, MN) levels in plasma or culture supernatants. The standard deviation (SD) of replicate samples was less than 10% of the mean in all experiments. Sample concentrations of each chemokine were quantified from standard curves generated with recombinant chemokines and analyzed with Softmax Software (Molecular Devices Corporation, Sunnyvale, CA). The lower limit of detection for the chemokines analyzed was 15 pg/ml.

3.1.5. Quantitative Real Time RT-PCR

Total RNA was extracted from PBMCs by the GITC method (83). Following isopropanol precipitation, RNA was pelleted at 13,000 rpm for 15 min and resuspended in RNase-free water to a final concentration of 1.0 μg/μL. Extracted RNA (1.0 μg) was reverse transcribed using random primers to generate cDNA. One-tenth (2.0 μl) of the resulting cDNA was then used as a

template for Taqman real time PCR amplification on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Each PCR reaction was set up in duplicate with 2.0 μl of cDNA template in a total volume of 25 μl comprised of Taqman®Universal Master Mix, and specific primer/probe sets for chemokine transcripts: MIP-1α (Hs00234142_mL), MIP-1β (Hs00605740_mL), RANTES (Hs00174575_mL), SDF-1 (Hs00171022_mL), CCR5 (Hs00152917_ml) and CD4 (Hs00181217_ml) (Applied Biosystems, Foster City, CA). Transcripts for CXCR4 were analyzed using previously described primer-probe sets (84). Amplification of β-actin {Accession Number NM_001101 (Applied Biosystems, Foster City, CA)} served as an endogenous control to normalize loading of cDNA samples. For experiments in ex vivo PBMC from Gabonese children, data were compared by subtracting the β-actin cycle threshold (C_T) value from the experimental gene C_T for each sample, and expressed as fold-change relative to β-actin levels ($2^{-\Delta C_T}$). For experiments in cultured PBMCs, data were compared using the $^{-\Delta\Delta C_T}$ method as previously described (33).

3.1.6. Flow Cytometry Analyses

Expression of surface antigens on PBMC determined by a three-color FACS analysis with the antibody panels shown in Table 2. All the monoclonal antibodies were purchased from BD Biosciences–Pharmingen, (San Diego CA). Cells were incubated with the mAb for 30 min at 4^{o}C, washed twice, and then fixed in 1% paraformaldehyde solution and analyzed on a Coulter EPICS XL Flow Cytometer (Coulter Corporation). Matched isotype antibody controls; Flourescein Isothyanate (FITC)-labeled mouse IgG1 (FITC) (clone: MOPC-21), Phycoerythrin (PE)-labeled mouse IgG2b (Clone: 27-35) and Phycoerythrin-Cytochrome 5 (PE-Cy5)-labeled mouse IgG2a (clone: G155-178) were used to control for non-specific binding. Acquired data

were analyzed with Summit® Software version 3.1 (DakoCytomation, Fort Collins, CO) to determine expression of CD4, CCR5 and CXCR4 on CD14+ and CD3+ cell subsets. Data were presented as percent expression of CD4, CCR5 or CXCR4 on CD14+ monocytes or CD3+ lymphocyte populations, and plotted as relative expression (mean ± s.e.m) between cells stimulated with either Hz or sHz and unstimulated control cells.

3.1.7. HIV-1 Infection of Monoctye-derived Macrophages (MDMs)

To determine differences in susceptibility of MDMs stimulated with Hz or sHz to HIV-1 infection, human monocytes were isolated from buffy coats of HIV-seronegative healthy adult blood donors (University of Pittsburgh, USA) by Ficoll/Hypaque density gradient centrifugation and positive isolation with CD14 MicroBeads (Milteny Biotec Inc, Auburn, CA). $CD14^+$ enriched monocytes were plated at a density of 1 X 10^6 cells/mL in DMEM supplemented with 10mM HEPES, 10mM Penicillin/Streptomycin, and 10% pooled human serum (heat inactivated at 56^{o}C for 30 min). Cells were incubated with media alone (control) or physiologic amounts of Hz or sHz as described above for PBMCs. Cells were then cultured for 7 days in media containing 10ng/mL granulocyte-macrophage-colony stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN), and then exposed to M-tropic HIV-1_{BAL} (AIDS Research and Reference, MD) (MOI = 0.1) for 2 hr. To determine whether the effect of Hz or sHz was through soluble factors released upon cellular stimulation with malarial pigment versus a direct effect of malarial pigment, culture supernatants (conditioned medium) were harvested from unstimulated control cells, and Hz-or sHz-stimulated cells and used in parallel infections at 1:1 dilution with fresh medium. Unbound virus was washed three times, and cells were kept at 37^{o}C in a humid atmosphere of 5% CO_2 for an additional 5 days. Supernatants were harvested from the cultures

at days 0, 3 and 5 post-infection and analyzed for p24 using commercial ELISA (NEN™ Life Science Products, Boston, MA) with a detection limit of 26 pg/mL.

3.1.8. Statistical Analyses

Mann-Whitney U tests (statistical significance set at $p < 0.05$) were used to analyze for differences in concentration of chemokines in plasma and chemokine gene expression profiles between groups, and Pearson correlations were conducted to examine the linear associations among the variables of interest. For *in vitro* experiments with human PBMCs, chemokine concentrations in culture supernatants and gene expression were analyzed for statistical significance between Hz- or sHz-stimulated cells and unstimulated control cells using Mann-Whitney U tests ($p < 0.05$). Similarly, surface expression of CD4, CCR5 and CXCR4 on monocytes and lymphocyte subpopulations and gene expression were analyzed for statistical significance between Hz- or sHz-stimulated cells and unstimulated control cells using Mann-Whitney U tests ($p < 0.05$).

CHAPTER 4

4.0. Results

4.1. β-chemokine profile in children with malaria

4.1.1. Circulating levels of chemokines in children with malaria

To quantify circulating levels of β-chemokines, plasma samples were obtained from children with varying degrees of malaria disease; healthy controls (HC, n = 23), mild malaria (MM, n = 10) and severe malaria (SM, n = 10), and analyzed by ELISA. MIP-1α was largely undetectable in the plasma of the HC group, but was elevated in children with MM ($p < 0.01$) and SM ($p < 0.01$, Fig. 1A). Similarly, levels of MIP-1β were higher in children with MM ($p < 0.01$) and SM ($p < 0.01$) compared to HC (Fig. 1B). Although the SM group had higher levels of MIP-1α and MIP-1β than the MM group, levels were not significantly different between the groups. In contrast to MIP-1α and MIP-1β, circulating levels of RANTES were lower in children with SM compared to HC ($p < 0.01$, Fig. 1C). Although median RANTES levels were lower in plasma of children with MM relative to HC, the difference was not statistically significant, but were, however, significantly higher than in children with SM ($p < 0.05$). Additional experiments revealed that plasma SDF-1 was not detectable in any of the children in the present study (data not shown)

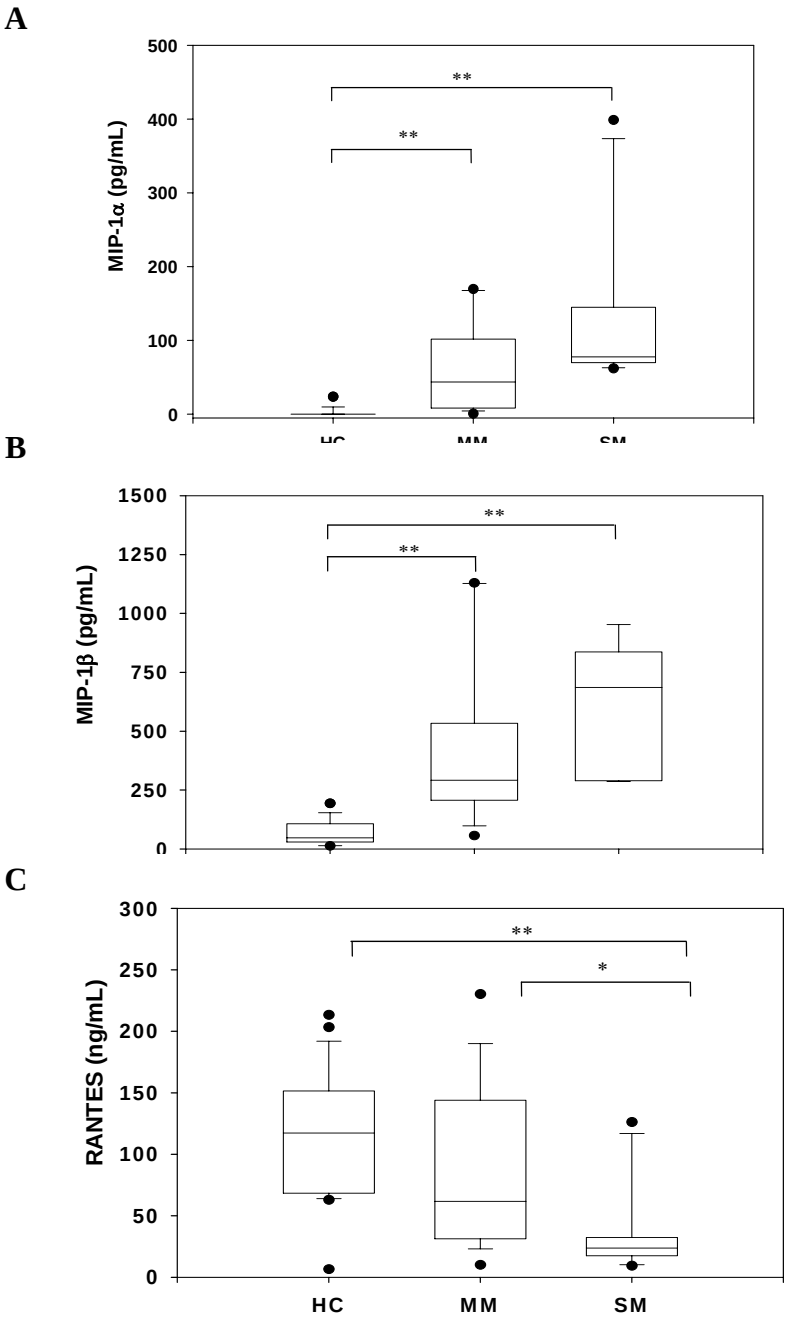

Figure 1. Plasma Levels of Chemokines in Children with Malaria.
β-chemokines (MIP-1α, MIP-1β and RANTES) and α-chemokine (SDF-1) levels were determined in plasma of healthy children (HC, n=23), those with mild malaria (MM, n=10), and severe malaria (SM, n=10) with commercial ELISA. Each box plot represents the range (25th -75th percentiles) chemokine concentration for each category, with the median value at the intersect. MIP-1α (A) and MIP-1-β (B) were significantly elevated in plasma of children with MM and SM compared to HC ($p < 0.001$). Children with SM had significantly lower plasma levels of RANTES than HC ($p < 0.01$) (C). Levels of RANTES in children with MM, though lower compared to HC were not significantly different ($p > 0.05$) but were significantly higher than in children with SM ($p < 0.05$).

4.1.2 β-Chemokine Transcript Profiles

To determine the potential cellular source(s) of circulating chemokines, gene expression profiles for MIP-1α , MIP-1β, and RANTES were determined by Taqman real time RT-PCR in ex vivo PBMCs isolated from children with varying degrees of malaria. Relative to HC, MIP-1α expression was significantly increased in children with either MM ($p < 0.05$) or SM ($p < 0.05$, Fig. 2A). Similarly, MIP-1β gene expression was significantly elevated in children with MM and SM compared to HC ($p < 0.05$, Fig. 2B). In contrast, RANTES was decreased in children with SM ($p < 0.05$), and slightly lower in children with MM ($p > 0.05$, Fig. 2C). SDF-1 transcripts were not detectable in any of the specimens analyzed (data not presented).

4.1.3. Association of ß-chemokine Expression with Prior Disease Severity

Since the history of prior malaria exposure was available for children in the HC group, data were further stratified into those children with previous mild malaria (PMM, n = 13) and those with previous severe malaria (PSM, n = 10). These groups were analyzed for circulating levels of RANTES and transcript levels in ex vivo PBMCs. As shown in Fig. 3A, the PMM group had significantly higher plasma RANTES levels than the PSM group ($p < 0.05$). RANTES transcripts were significantly lower in children with PMM than those with PSM ($p < 0.01$ Fig. 3B). Levels of MIP-1α and MIP-1β protein and transcripts were not significantly different between the PMM and PSM groups (data not shown).

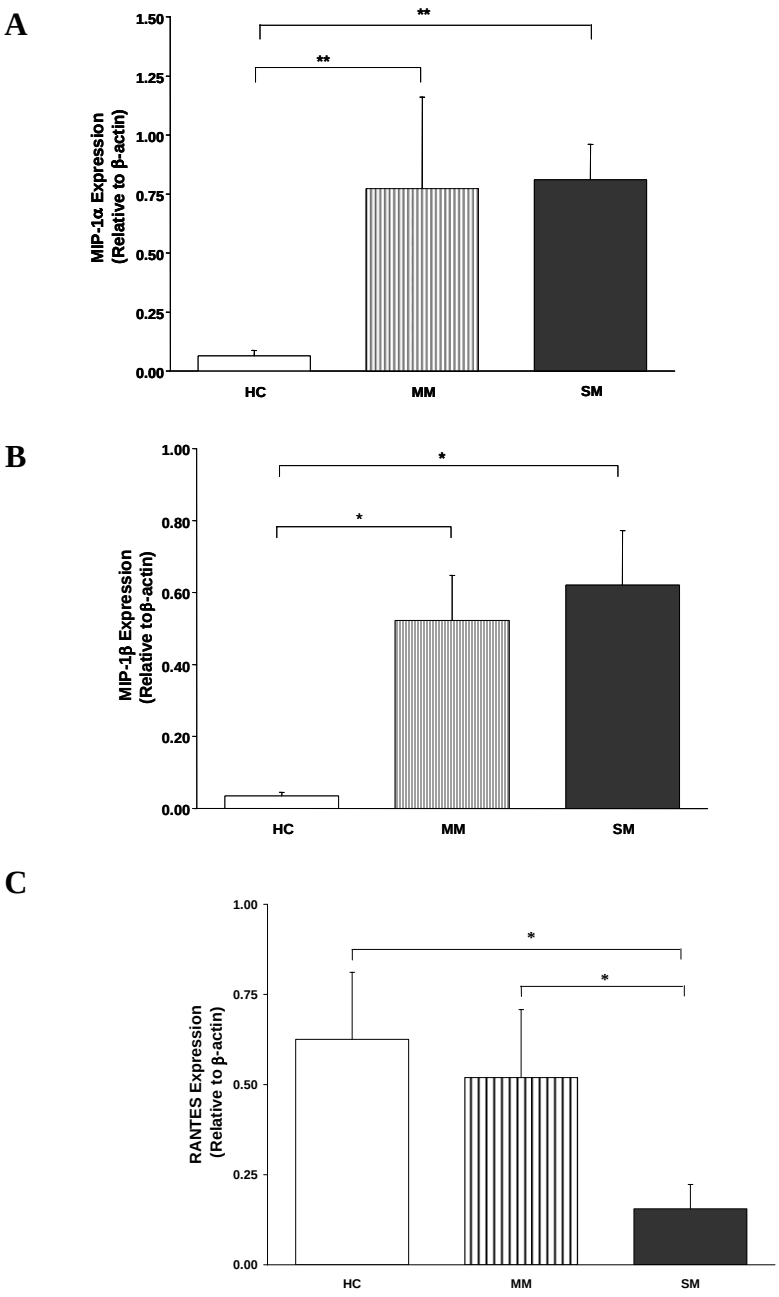

Figure 2. Transcriptional Profiles of Chemokines in Children with Malaria.
MIP-1α, MIP-1β, RANTES and SDF-1 mRNA expression was determined by real time RT-PCR on cDNA isolated from PBMC of healthy children (HC, n=23), those with mild malaria (MM, n=10) and severe malaria (SM, n=10). Gene expression for each chemokine is presented relative to expression of endogenous β-actin. MIP-1α (A) and MIP-1β mRNA levels (B) were significantly higher in children with MM and SM compared to HC ($p < 0.05$). RANTES mRNA levels were significantly lower in children with SM compared to HC (C) ($p < 0.05$). Similarly, among malaria cases, children with SM had significantly lower expression of RANTES mRNA compared to patients with MM ($p < 0.05$). SDF-1 transcripts were not detectable in any of the specimens analyzed.

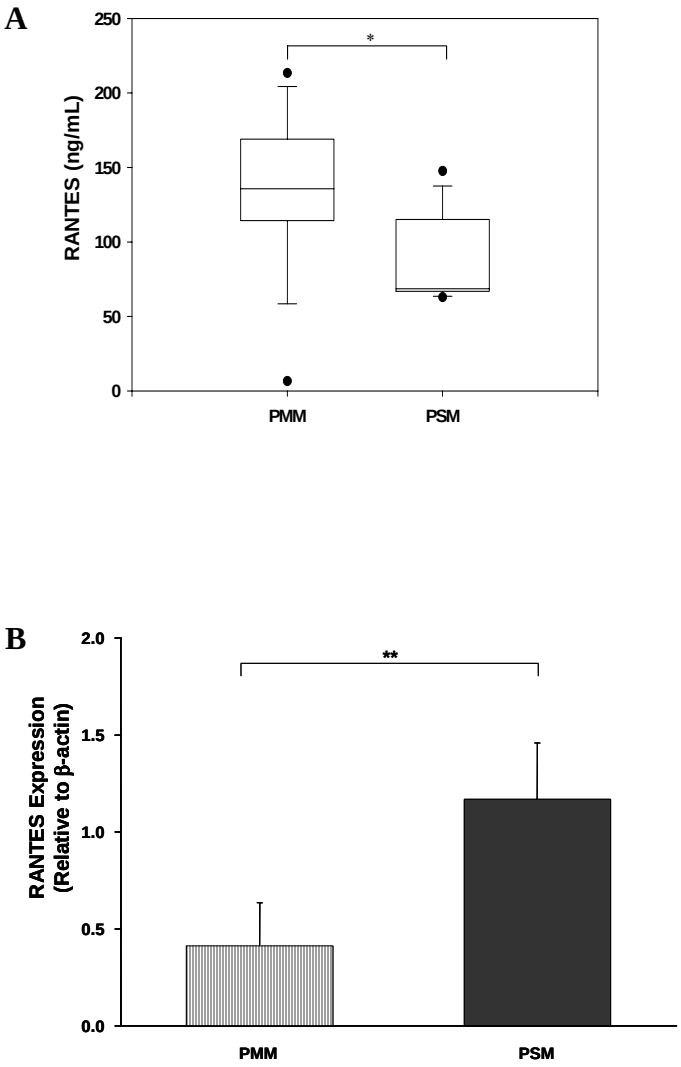

Figure 3. Circulating RANTES and ex vivo PBMC mRNA Levels in Healthy Children with Prior Malaria Exposure.

RANTES levels in healthy children (HC) with a history of either mild malaria (PMM, n =14) or severe malaria (PSM, n = 9) were determined in plasma by commercial sandwich antibody ELISA, and gene expression profiles were determined by real time RT-PCR on RNA isolated from PBMC (expressed relative to endogenous β-actin). The PSM group had significantly lower plasma RANTES levels than the PMM group ($p < 0.05$) (A). In contrast, RANTES mRNA levels were significantly higher in PSM compared to PMM cases ($p < 0.01$) (B). MIP-1α and MIP-1β plasma levels and gene transcripts were not significantly different in either PMM or PSM children (data not presented). ** indicates statistical significance; $p < 0.01$, and * indicates statistical significance; $p < 0.05$ compared to controls.

4.1.4. Association of β-chemokines with Hemoglobin Concentrations and Parasite Density

To examine the linear associations between the chemokines and clinical parameters (i.e., anemia and parasitemia), bivariate Pearson correlations were conducted. These correlations were performed on the mild and severe malaria groups only (n = 29) due to the fact that hemoglobin measurements were not obtained from the healthy control group. Before conducting the analyses, all relevant variables were examined for departures from normality. Since MIP-1α and parasitemia were moderately positively skewed, these variables were normalized by square-root transformations prior to performing the analyses. Although there was a significant negative correlation between parasitemia and hemoglobin (r = -.402, p = .047), none of the other correlations were statistically significant (Table 1). As a follow-up to the correlation analyses, we conducted two linear multiple regression analyses on the mild and severe malaria groups, one each for the dependent variables of hemoglobin and parasitemia. The purpose of these analyses was to examine the unique predictive ability of each of the three chemokines (i.e., MIP-1α, MIP-1β and RANTES) after controlling for the remaining two chemokines and age. Both of the regression equations were statistically nonsignificant and, likewise, all of the standardized partial regression coefficients (β weights) were nonsignificant, indicating that none of the chemokines uniquely predicted anemia and parasitemia.

Table 1. Correlation between β-chemokines, hemoglobin, and parasitemia.

	r^a	p	r^b	p
Age	0.105	NS	-0.297	NS
MIP-1α	0.148	NS	0.033	NS
MIP-1β	-0.316	NS	0.230	NS
RANTES	0.062	NS	-0.309	NS

Table shows Pearson correlation coefficients (mild and severe malaria groups only). r^a = Hemoglobin, r^b = Parasitemia. NS = Not significant

4.1.5. Effect of Hz and sHz on PBMC Chemokine Production

Previous studies reported that parasite-derived products, such as Hz, can augment production of MIP-1α and MIP-1β from cultured human PBMCs (38). Results presented here show that β-chemokine levels were higher in children with severe malaria, and we have previously shown that children in this population with severe malaria have significantly higher circulating levels of Hz-containing monocytes and neutrophils than children with mild malaria (85). Therefore, to determine the effects of malaria pigment on β-chemokine production, PBMCs were isolated from healthy, malaria-naïve adult donors (n = 5) followed by culture with media alone (controls), Hz (10 μg/mL), or sHz (10 μg/mL). β-chemokine concentrations were determined in culture supernatants at 4, 24, 48 and 120 hr. MIP-1α and MIP-1β levels were undetectable in control conditions. However, addition of Hz or sHz increased MIP-1α and MIP-1β production by 24 hr, and this was sustained throughout the time-course ($p < 0.01$ for both chemokines at 24 and 48 hr, Fig. 4). Baseline RANTES production significantly decreased at 48 hr in control cells ($p < 0.05$, Fig. 4C). SDF-1 was undetectable in culture supernatants from stimulated and unstimulated cells (data not shown).

To determine if alterations in β-chemokine production by Hz and sHz were regulated at the transcriptional level, gene expression profiles for MIP-1α, MIP-1β, RANTES, and SDF-1 were determined by real time RT-PCR. Relative to non-stimulated control PBMCs, MIP-1α and MIP-1β expression was significantly increased by Hz and sHz at 4, 24, and 48 hr ($p < 0.05$, Fig. 5). In contrast, RANTES expression was not altered by either Hz or sHz at 4 hr (Fig. 5C). After 24 hr in culture, PBMCs stimulated with sHz had comparatively higher expression of RANTES relative to controls ($p < 0.05$, Fig. 5C). RANTES gene expression in PBMCs stimulated with Hz for 48 hr was not significantly altered, while cells stimulated with sHz had suppression of RANTES mRNA expression ($p < 0.05$, Fig. 5C). SDF-1 transcripts were undetectable in culture supernatants from stimulated and unstimulated cells at all time points (data not shown).

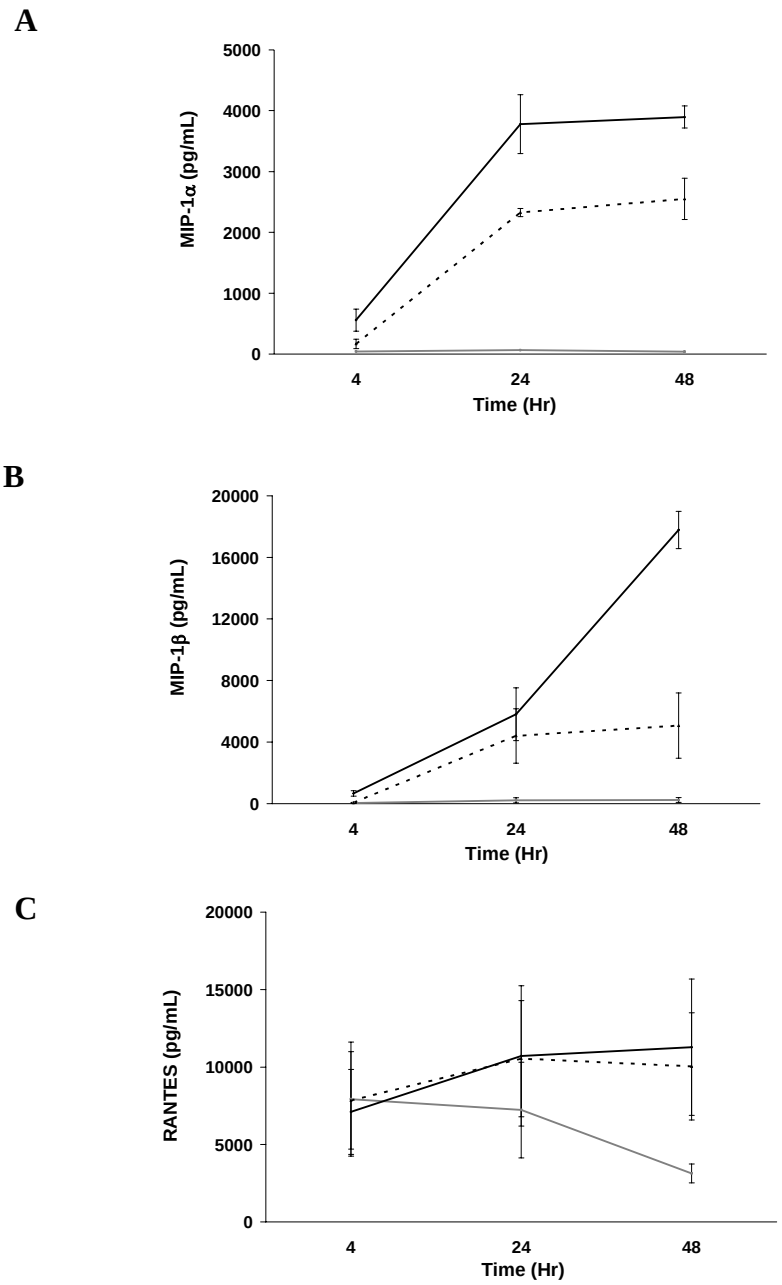

Figure 4. Effects of P. falciparum-derived and Synthetic Hemozoin on Production of Chemokines by PBMCs.

To determine in vitro production of chemokines by cultured human mononuclear cells, PBMCs (2 X 10^6/well) isolated from healthy adult volunteers (n=5) were cultured with either Hz (10 μg/mL) or sHz (10 μg/mL) for 4, 24 and 48 hr. Cell culture supernatants harvested at each time point were analyzed by commercial sandwich antibody ELISA for MIP-1α (A), MIP-1β (B) and RANTES (C). SDF-1 was undetectable in supernatants analyzed.— (unstimulated controls), — (Hz) and - - (sHz)** indicates statistical significance; $p < 0.01$, and * indicates statistical significance; $p < 0.05$ compared to non-stimulated controls.

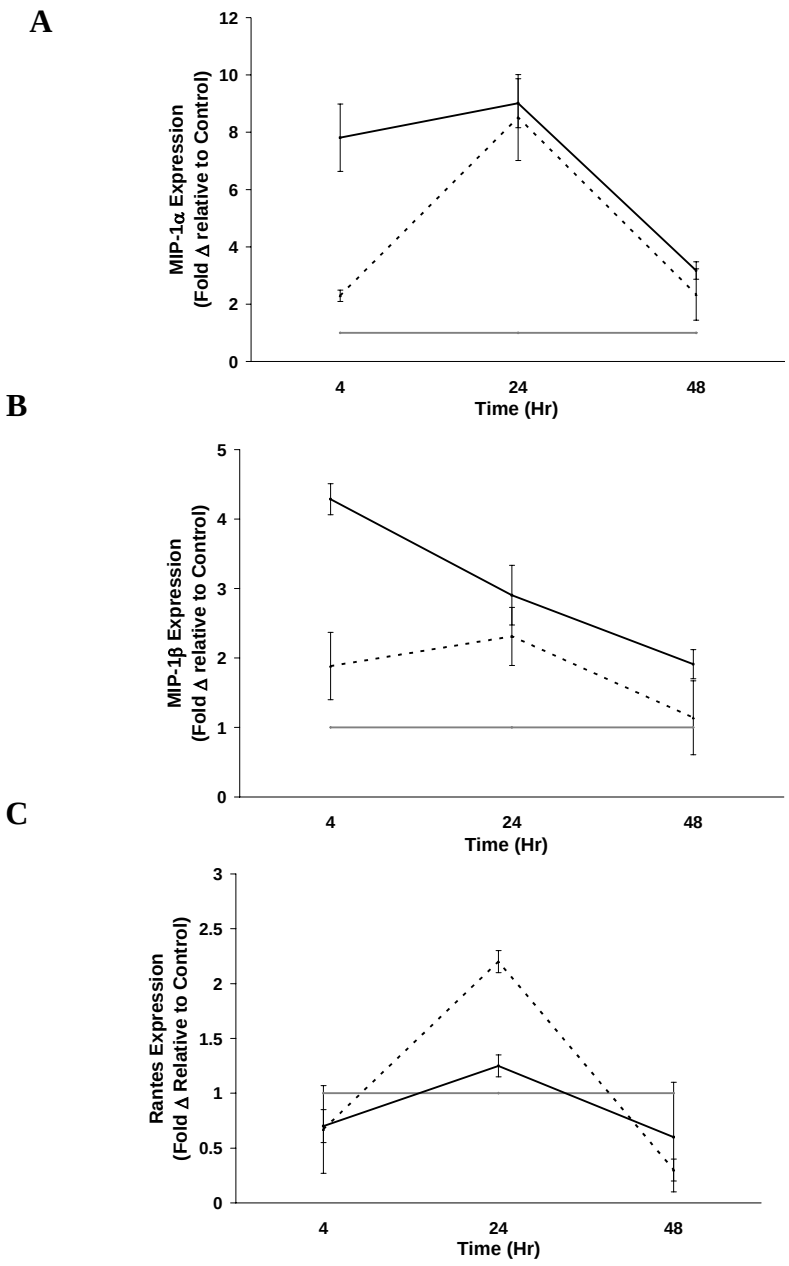

Figure 5. Effects of P. falciparum-derived and Synthetic Hemozoin on Chemokine Gene Expression by PBMCs.

To determine temporal profile of expression of chemokine genes, PBMCs (2 X 10^6/well) isolated from healthy adult volunteers (n= 5) were cultured with either Hz (10 μg/mL) or sHz (10 μg/mL) for 4, 24 and 48 hr. Unstimulated cells served as negative controls. Gene expression profile for MIP-1α (A), MIP-1β (B) and RANTES (C) RNA was determined by real time RT-PCR using specific primer/probe sets on cDNA generated from the PBMC RNA. Data are presented as fold change in gene expression in stimulated cells relative to unstimulated controls.— (unstimulated controls), — (Hz) and - - (sHz). Transcripts for SDF-1 were not detectable. ** indicates statistical significance; $p <$ 0.01, and * indicates statistical significance; $p < 0.05$ compared to non-stimulated controls.

4.2. Modulation of CD4, and Chemokine Receptors Expression on Human Mononuclear Cells by Hemozoin

4.2.1. Surface Expression of CD4, CCR5, and CXCR4 on PBMCs

To determine the pattern of surface expression of CD4, CCR5 and CXCR4 on PBMCs stimulated with either Hz or sHz, three-color FACS analysis was performed and the percent expression of these antigens was analyzed on $CD14^+$ monoctye (Figure 6) and $CD3^+$ lymphocyte (Figure 7) subsets separated based on lineage marker-specific gating. Results were presented as relative expression compared to unstimulated controls. Hz stimulation significantly up-regulated the expression of CD4 on monocytes at 4 and 48 hr in culture ($p < 0.05$) (Figure 6A). CD4 expression was also significantly up-regulated on monocytes at 48 hr with sHz stimulation. Conversely, by 120 hr of culture, Hz and sHz significantly down-regulated CD4 expression on monocytes. CCR5 and CXCR4 were significantly down-regulated by 24 hr in Hz or sHz stimulated cells ($p < 0.01$) (Figure 6B and 6C, respectively). However, these molecules were up-regulated on monocytes by 120 hr, with significant effects observed on sHz-stimulated cells ($p < 0.05$). There were no significant differences in the expression of CD4 on $CD3^+$ lymphocytes under control conditions, or following treatment with Hz and sHz (Figure 7A). In contrast, Hz and sHz up-regulated the expression of CXCR4 on $CD3^+$ cells at 4 and 24 hr (Figure 7B). CCR5 expression was undetectable (<1%) in lymphocytes (data not presented).

Table 2. Monoclonal Antibodies And Three-Color Flow Cytometry Panels

Antibody Panels		
FITC	**PE**	**PE-Cy5 (PCP)**
CD4 (RPA-T4)	CD14 (MϕP9)	CCR5 (2D7)
CD4 (RPA-T4)	CD14(MϕP9)	CXCR4 (12G5)
CD4 (RPA-T4)	CD3 (HIT3a)	CCR5 (2D7)
CD4 (RPA-T4)	CD3 (HIT3a)	CXCR4 (12G5)
Ms IgG1 (MOPC-21)	Ms IgG2b (27-35)	Ms IgG2a (G155-178)

Table shows the various monoclonal antibodies used for analysis of surface antigen expression in a three-color FACS scheme. Isotype matched mouse (Ms) mAb were used to control for non-specific binding. The mAb clones are indicated in parenthesis.

4.2.2. CD4, CCR5 and CXCR4 Transcript Profiles

To determine if the observed modulation of surface expression of these receptors was due to transcriptional changes induced by Hz or sHz, real time RT-PCR was performed to quantitate mRNA for CD4, CCR5 and CXCR4 in control, Hz or sHz stimulated PBMCs (Figure 8). CD4 expression was significantly up-regulated by both Hz and sHz at 4 hr of culture, and was significantly down-regulated at 48 and 120 hr (Figure 8A). CCR5 expression was significantly up-regulated by sHz at 24, 48 and 120 hr ($p<0.05$), and by Hz at 48 and 120 hr ($p < 0.05$) (Figure 8B). CXCR4 expression was significantly up-regulated by Hz at 24 and 120 hr ($p<0.05$) (Figure 8C).

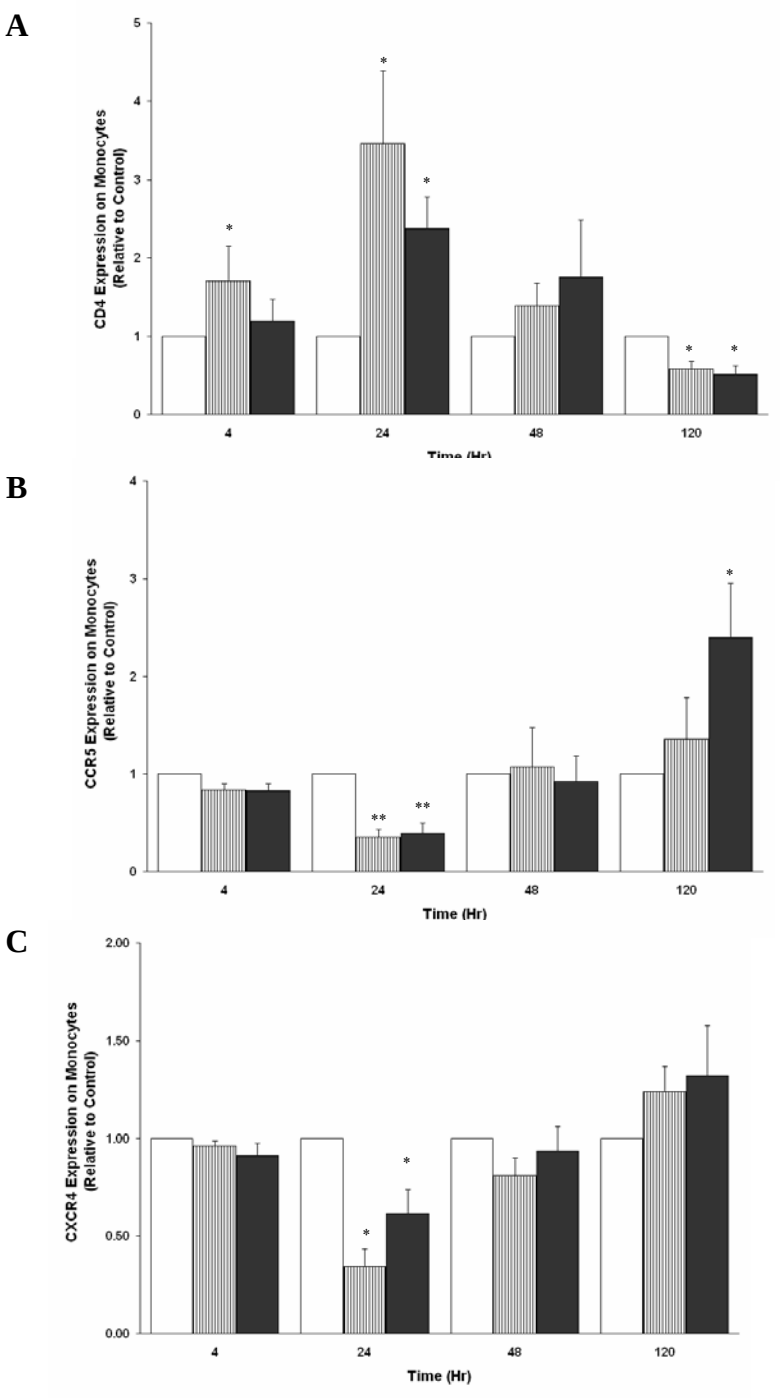

Figure 6. Analysis of Surface Expression of CD4, CCR5 and CXCR4 on Monocytes.
Pattern of surface expression of CD4, CCR5 and CXCR4 on $CD14^+$ monocyte subsets of PBMCs. PBMCs from healthy adult donors (n=5) were cultured for 4, 24 48, and 120 hr with Hz (10μg/ml) or sHz (10μg/ml). Non-stimulated cells served as control. Cells were subsequently stained for surface markers using three-color FACS scheme. Expression on monocyte subsets were delineated by lineage-specific gating set on $CD14^+$ cells. Data are presented as percent expression of CD4 (A), CCR5 (B) or CXCR4 (C) on $CD14^+$ monocytes and plotted as relative expression between cells stimulated with either Hz or sHz and unstimulated control cells. ** indicates statistical significance; $p < 0.01$, and * indicates statistical significance; $p < 0.05$ compared to non-stimulated controls.

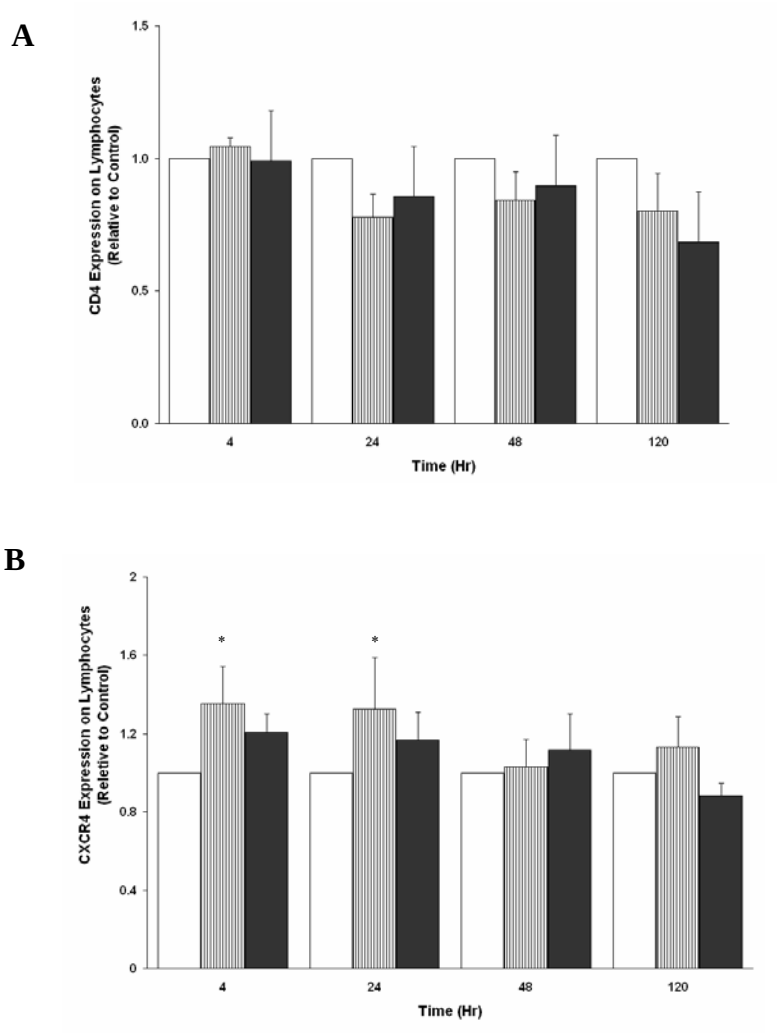

Figure 7. Analysis of Surface Expression of CD4, CCR5 and CXCR4 on Lymphocytes.
Pattern of expression of CD4 and CXCR4 on $CD3^+$ lymphocyte subsets of PBMCs. PBMCs from healthy adult donors (n=5) were cultured for 4, 24, 48, and 120 hr with Hz (10μg/ml) or sHz (10μg/ml). Expression on lymphocytes was delineated by lineage-specific gating set on $CD3^+$ cells. Data are presented as percent expression of CD4 (A), or CXCR4 (B) on $CD3^+$ monocytes and plotted as relative expression between cells stimulated with either Hz or sHz and unstimulated control cells. ** indicates statistical significance; $p < 0.01$, and * indicates statistical significance; $p < 0.05$ compared to non-stimulated controls.

4.2.3. HIV-1 Infectivity of MDMs

To determine whether Hz- or sHz-induced changes of CD4, CCR5 and CXCR4 influences susceptibility to infection with HIV-1, MDMs were infected with M-tropic HIV-1 following stimulation with either Hz or sHz for seven days. Infection was performed in the presence and absence of conditioned medium obtained from culture supernatants to determine if there were soluble factors induced by malarial pigment and/or direct effects of malarial pigment on viral replication. In a representative experiment shown in Table 2, sHz enhanced HIV-1 replication in MDMs by day 3 and 5 post-infection compared to unstimulated control cells. In contrast, viral replication in cell cultures stimulated with Hz was comparatively lower than that of control cells. Treatment of the cells with a 1:1 dilution of the conditioned medium had an inhibitory effect on effect on HIV-1 replication (Table 3).

Table 3 HIV-1 Infectivity of Monocyte-Derived Macrophages (MDMs)

Treatment	Duration post-infection (days) and p24 levels (pg/mL)					
	Conditioned medium (-)			Conditioned medium (+)		
	0	**3**	**5**	**0**	**3**	**5**
Control	<limit*	142	21,985	<limit	92	1,084
Hemozoin (Hz)	<limit	131	3,884	<limit	98	241
Synthetic hemozoin (sHz)	<limit	589	202,903	<limit	247	1,197

To determine the effect of malarial pigment on HIV-1 replication, monocyte-derived macrophages, cultured in the absence (control) or presence of Hz (10μg/mL) or sHz (10 μg/mL) for seven days, were exposed to M-tropic HIV-1_{BAL} (MOI=0.1) for 2 hr. Infected cells were cultured for an additional five days either in fresh medium or medium containing a 1:1 dilution of culture supernatant harvested from control, Hz- or sHz-stimulated cells (i.e. conditioned medium). HIV-1 p24 antigen concentrations were determined in culture supernatants at days 0, 3 and 5 with a commercial ELISA. * Indicates levels below the assay detection limit (26 pg/mL)

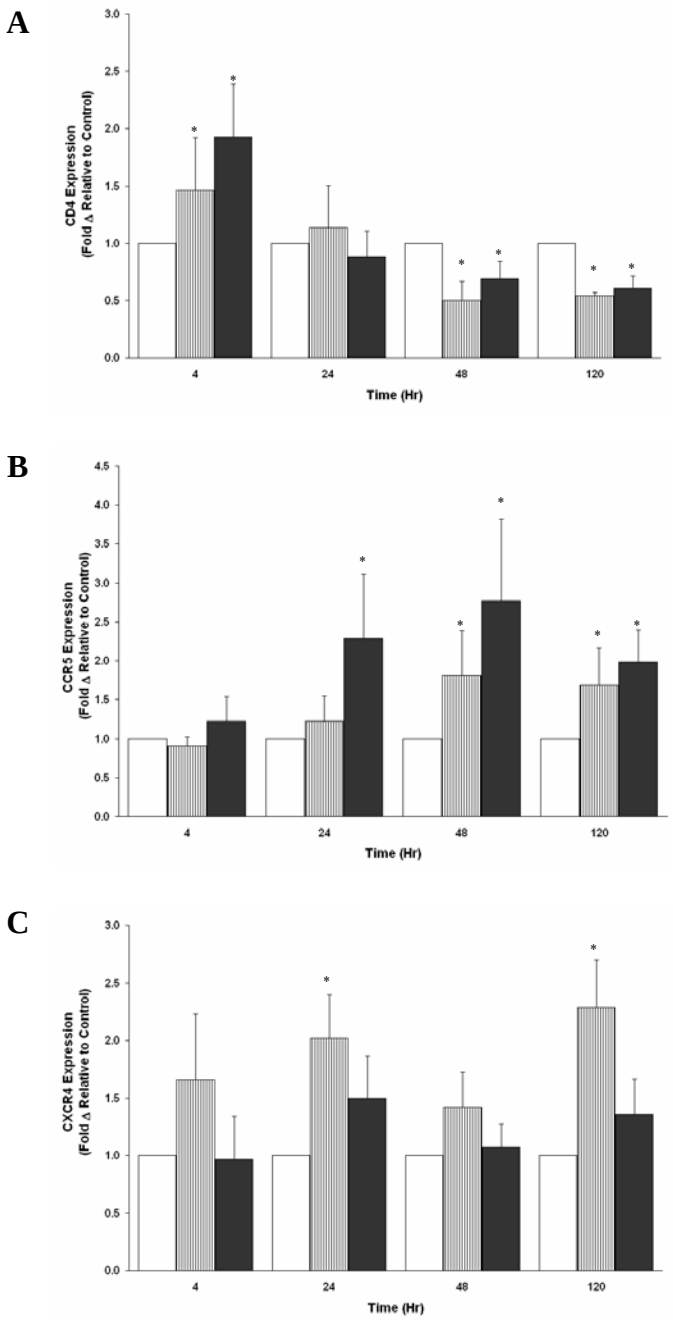

Figure 8. Expression of CD4, CCR5 and CXCR4 Transcripts in PBMCs.
Temporal profile of expression of mRNA transcripts for CD4 (A), CCR5 (B) and CXCR4 (C) determined in PBMCs from healthy adult volunteers (n=5) cultured either Hz (10 μg/mL) or sHz (10 μg/mL) for 4, 24, 48, and 120 hr. Unstimulated cells served as negative controls. Gene expression profiles were determined by real time RT-PCR using specific primer/probe sets on cDNA generated from the PBMCs RNA. Data were presented as fold-change in gene expression in stimulated cells relative to unstimulated controls. Clear bars (unstimulated controls), hatched bars (Hz) and filled bars (sHz). ** indicates statistical significance; $p < 0.01$, and * indicates statistical significance; $p < 0.05$ compared to non-stimulated controls.

CHAPTER 5

5.0 Discussion

5.1. Profile of β-Chemokines in Children with Malaria

We show here that acute falciparum malaria in Gabonese children is characterized by differential regulation of β-chemokines in which circulating levels of MIP-1α and MIP-1β are elevated and RANTES is reduced. Analysis of gene expression profiles revealed that altered patterns of circulating β-chemokines during different states of disease occur, at least in part, through changes in PBMC β-chemokine transcripts levels. Additional results demonstrated that healthy, malaria-exposed children with a history of severe malaria have significantly lower circulating levels of RANTES than children with previous mild malaria, suggesting that children who develop severe disease may have an inherent inability to produce elevated levels of RANTES. Experiments conducted in PBMCs from healthy, malaria-naïve adult donors further revealed that co-culture of Hz with monocytes/macrophages in PBMC elicits dysregulation in β-chemokine mRNA and protein.

The role of chemokines in the regulation of host-parasite interactions appears to be highly variable in protozoan infections (28). Chemokines have been shown to have direct antiprotozoal activity against *Toxoplasma gondi*, *Leishmania donovani*, and *Trypanosoma cruzi* (28). Although the ability of chemokines to regulate intraerythrocytic growth of the malaria parasite has not been reported, a functional role for altered expression patterns of chemokines during malaria has been associated with mononuclear cell infiltration during placental malaria (29, 30). In addition, increased expression of chemokines has been shown in both murine and human cerebral malaria (86, 87). Previous results also showed that serum concentrations of MIP-1α are

elevated in adults with *P. falciparum* malaria in Southeast Asia; levels of MIP-1α were highest on day 14 after admission and following treatment (32). Our studies show that MIP-1α is also elevated in children with acute falciparum malaria prior to treatment with antipyretics and/or antimalarials, demonstrating that elevated circulating concentrations of MIP-1α are the direct result of a malaria infection and are not due solely to treatment interventions. Analysis of MIP-1α transcripts in *ex vivo* PBMC illustrated that MIP-1α gene expression was greater than 50% higher in children with malaria than in their healthy, age-matched controls. These results suggest that changes in MIP-1α during acute disease occur, at least in part, through enhanced MIP-1α mRNA transcription in PBMC. Investigation of MIP-1β revealed similar results in which acute malaria was characterized by elevated circulating levels of MIP-1β that appear to result from malaria-induced up-regulation of MIP-1β gene expression in PBMCs.

Currently, it is not clear whether increased circulating levels of MIP-1α and MIP-1β in acute malaria are an appropriate physiologic response to the infection or if they contribute to enhanced pathophysiology. The previous observation that MIP-1α and IL-8 are induced in the acute phase of malaria and remain elevated even after parasite clearance (32), suggests that the immunologic effects of chemokines may occur over prolonged periods. Since a number of studies have shown that MIP-1α suppresses hematopoiesis (44, 88-92), sustained elevated levels of MIP-1α may contribute to malarial anemia. Evidence showing that enhanced MIP-1β production from bone marrow aspirates is associated with decreased hemoglobin concentrations in HIV positive adults subjects, further supports a potential role of chemokines in the promotion of anemia (93). However, results presented here do not support the premise that increased MIP-1α and MIP-1β during malaria promote anemia. It is important to note that disease severity in the present study was defined according to both parasitemia and anemia status. Thus, with

disease severity based on these two parameters, it may not be possible to fully elucidate the contribution of MIP-1α and MIP-1β to the etiology of malarial anemia. We are currently investigating the role of MIP-1α and MIP-1β in the promotion of malarial anemia in a pediatric population in western Kenya in which the primary clinical manifestation of severe malaria is defined by severe anemia.

Of particular interest in the current study was the finding that RANTES is suppressed in children with severe malaria at both the mRNA and protein level. There is growing evidence that RANTES provides protection against protozoan diseases (94-96). Although the molecular basis of this protection remains unclear, it may be related to bridging innate and adaptive immune responses, since RANTES is a specific chemoattractant for memory T cells, and augments polarization of a T helper 1 (Th1) response (97). Thus, in the context of malaria, reduced RANTES production may lead to an ineffective immunologic response. This hypothesis is supported by the fact that immunosuppression is a prominent feature of malaria, as evidenced by increased susceptibility to a number of bacterial and viral infections, including Salmonellosis (98), and reactivation of chronic and latent *Herpes zoster* (99), *Herpes simplex* (100) and Epstein–Barr virus (101, 102). Data presented here showing that circulating levels of RANTES are significantly reduced in healthy children with a history of severe malaria relative to those that previously experienced mild malaria suggests that elevated levels of RANTES may provide protection against severe disease. Interestingly, transcriptional analysis of RANTES revealed that children with PSM had significantly higher RANTES mRNA expression than those with PMM. Additional experiments are required to determine if there is an inherent deficiency in RANTES transcript stability and/or post-translational modifications that lead to high levels of RANTES message, but low levels of circulating protein in children with PSM.

Although a number of parasite-derived factors, such as glycosylphospatidylinositols, soluble antigens, and Hz, could account for alteration of β-chemokine expression, our work using PBMCs from healthy, malaria-naïve adults illustrate that malarial pigment appears to be, at least in part, responsible for the altered production of β-chemokines by PBMC. Comparable results with a crude Hz preparation and a sHz moiety prepared in a cell-free solution demonstrate that the core heme polymer, FP-IX, and not additional parasite-derived molecules, is likely responsible for altered β-chemokine mRNA and protein expression. Another prominent feature of the current work is that, unlike many of the previous studies, a physiologically relevant concentration of *P. falciparum*-derived Hz and sHz (34) was used for the in vitro studies. The profile of MIP-1α and MIP-1β mRNA and protein in the in vitro experiments corresponds more closely to the results observed in children with acute malaria than the results for RANTES. For example, protein levels of RANTES decreased under baseline conditions by 48 hr, while RANTES increased in cells treated with Hz and sHz at the same time points. Analyses of RANTES transcripts, however, revealed that the mRNA declined by 48 hr in culture, suggesting that there would be a subsequent decrease in RANTES protein over prolonged periods. In the current studies, in vitro experiments were not conducted beyond 48 hr since there was decreased viability in cells treated with Hz and sHz after several days, making the experiments difficult to interpret (data not presented).

Taken together, our results provide the first reported evidence that children with acute falciparum malaria have dysregulation of β-chemokines characterized by elevated MIP-1α and MIP-1β and decreased RANTES at the mRNA and protein level. This distinct profile of β-chemokine expression appears to be an effect of Hz PPIX on PBMC. Additional studies aimed at defining the role of β-chemokines in the promotion of malarial anemia and the impact of

decreased RANTES on malaria-induced immunosuppression may provide important information about the pathophysiology of malaria.

5.2. Modulation of CD4 and Chemokine Receptors by Hemozoin

Our results show that the *P. falciparum*-derived product, Hz and sHz modulate expression of CD4, CCR5 and CXCR4 both at the protein and transcript level in human PBMCs. Hz and sHz also exerted a differential effect on the pattern of surface expression of these receptors on monocyte and lymphocyte subsets, implying that there are cell-specific effects of malarial pigment. In addition, malarial pigment-induced patterns of expression of these surface molecules appear, at least in part, to influence susceptibility of human mononuclear cells to infection with M-tropic strains of HIV-1.

Despite growing epidemiological evidence supporting an interaction between malaria and HIV infection, limited data exist that define the underlying biological interactions during co-infection. Chemokine receptors serve as co-receptors for HIV-1 entry into susceptible cells, and their regulation forms a critical determinant of HIV-1 pathogenesis (13). Our study provides evidence for changes in the regulation of CD4, CCR5 and CXCR4 expression by malaria parasite-derived products, and suggests a potential mechanism by which malaria can impact on HIV infection. The pattern of expression of CCR5 and CXCR4 in this study is consistent with previously reported findings with soluble *P. falciparum*-derived antigens (79). Similarly, in the context of placental malaria, accumulation of Hz in placental macrophages enhances CCR5 mRNA expression (103). In our study, however, increased CCR5 mRNA expression did not correlate with surface expression of CCR5 on monocytes. A possible explanation for this discordance is receptor-ligand mediated internalization. CCR5 undergoes rapid internalization

and recycling following interaction with β-chemokines; MIP-1α, MIP-1β and RANTES (104). Our group, as well as others have shown that stimulation of human mononuclear cells with Hz induces secretion of β-chemokines, the primary ligands for CCR5 (38, 105). The precise mechanism by which Hz and sHz modulates cellular expression of CCR5 is remains unclear but may be through a TNF-α-mediated mechanism (106). TNF-1α acts via p80 type II TNF receptor (TNFR2) to induce NF-κB with resultant enhanced secretion of MIP-1α, MIP-1β and RANTES which directly down-regulates CCR5. The similar pattern in expression observed with Hz and sHz stimulation indicate that the polymerized iron core structure, ferriprotoporphorin (FP)-IX may mediate these effects.

The current study also determined the profile of CXCR4 expression. This study demonstrated that CXCR4 expression is differentially regulated on $CD14^+$ and $CD3^+$ cell subsets, being down-regulated on monocytes and up-regulated on T cell subsets following Hz stimulation. CXCR4 is expressed predominantly on naïve subsets of $CD4^+$ T cells (107, 108) and serves as a co-receptor for T-tropic HIV-1 strains (109). One potential mechanism for decreased surface expression of CXCR4 occurs through interactions with stromal cell derived factor 1 (SDF-1), the only known ligand, and a factor that that upon binding to the receptor, leads to consequent receptor internalization (110). Our previous results showed that stimulation of human PBMCs with Hz or sHz failed to induce SDF-1 transcript and protein (data not presented), suggesting that this mechanism is not responsible for down-regulation of CXCR4 expression on monocytes. It is possible that CXCR4 down-regulation on monocytes observed in our study is due to the recently described IFN-γ and/or TNF-α induced down-regulation of the receptor on human blood cell subsets (111, 112). However, enhanced expression of this receptor on T cell subsets, reported in the present study, following stimulation with Hz and sHz against a

background of undetectable SDF-1 could lead to *in vivo* selection of T tropic HIV-1 variants in malaria/HIV co-infection, with the consequential rapid clinical progression of HIV to AIDS (77, 113).

Another critical question that this study addressed is whether modulation of surface receptor expression alters susceptibility of human mononuclear cells to HIV-1 infection. Using MDMs stimulated with either Hz or sHz, and subsequently infected with M-tropic strain of HIV, our preliminary data indicates differential effects of Hz and sHz on HIV-1 replication. Whereas sHz enhanced HIV-1 replication, Hz stimulation had an opposite inhibitory effect. A previous study performed with soluble *P. falciparum*-derived soluble antigens showed a similar inhibitory effect attributed to IFN-γ–induced suppression of HIV-1 replication (79). More recently, enhanced infectivity of human mononuclear cells with HIV-1 has been reported following activation with soluble malarial antigens (69). It is possible that these conflicting *in vitro* findings reflect inherent differences in type of malarial products used for cellular stimulation, which ultimately determines the nature of the extra-cellular milieu defined by specific patterns of cytokines and chemokines which can exert both inhibitory and stimulatory effects on HIV-1 replication in mononuclear cells.

In conclusion, our study demonstrates that the malaria-parasite derived product, Hz, modulates the expression of CD4, CCR5 and CXCR4, the principal receptors required for the initial infection of target cells by HIV-1 and subsequent *in vivo* dissemination of the virus. Further experiments using this model may provide important details about the molecular mechanisms responsible for observed effects of malaria on HIV pathogenesis.

5.3. Future Perspectives

The pathogenesis of *P. falciparum* infection is complex and little is known about underlying molecular determinants. This is further obscured by existing interactions between malaria and other co-infections such as HIV. It is, however, widely recognized that a delicate balance between pro-inflammatory and anti-inflammatory cytokines influence malaria severity (21, 23-25). Chemokine-chemokine receptor axis, the principal regulator of leukocyte chemotaxis, could also play a role in *P. falciparum* infection. Chemokines induced at local sites of infection creates a gradient that results in attraction of immune cells (26).

A fundamental question that still needs to be defined is the functional role of circulating levels of chemokines in malaria. Our study has shown that CC-chemokines namely, MIP-1α, MIP-1β and RANTES are differentially modulated in children with acute *P. falciparum* malaria. However, no association was established with either parasitemia or hemoglobin concentrations. Induced MIP-1α and MIP-1β could potentially promote severe malaria anemia by via inhibitory effect on development and differentiation of hematopoeitic stem cells (HSCs) (44). Additional studies are required to define the functional relevance of MIP-1α and MIP-1β with respect to anemia particularly in settings where severe malaria anemia is the predominant clinical outcome. Further, persistent high levels of MIP-1α and MIP-1β even after parasite clearance (32) could lead to heightened state of *in vivo* cellular activation, and facilitate HIV-1 replication in malaria-HIV co-infected individuals (114). In addition, circulating levels of chemokines could also influence HIV pathogenesis via competitive receptor binding or through regulation of HIV replication (115).

In attempt to define interaction between HIV and malaria examined modulation chemokine receptors HIV and chemokines, this study has demonstrated modulation of expression of CD4, CCR5 and CXCR4 by Hz and sHz. An essential question that still needs to be addressed is the mechanism by which this modulatory effect is exerted. It could be either a direct effect of the pigment or an indirect effect mediated by soluble factors released upon cellular stimulation with the pigment. Cytokine have been shown to differentially regulate expression of chemokine receptors on human peripheral blood cells (PBLs). For example, TNF-α, IL-4 and IL-3 reduce surface expression of CD4, CCR5 and CXCR4 on human macrophages correlates which correlates with reduced HIV-1 infectivity (106, 116, 117). IL-10 enhances expression of CCR5 on human macrophages (118) while IL-12, on the other hand, inhibits its expression on T cells (118). IFN-γ and IL-6 have been shown to enhance CXCR4 (119).

Another unexpected finding in this study is the differential effect of Hz and sHz on HIV replication in MDMs. There is considerable structural similarity between Hz and sHz in terms of organization of the core iron constituent. However, Hz has in addition, has lipids, proteins, and nucleic acids which are associated with the polymerized iron core structure, ferriprotoporphorin (FP)-IX. It is probably that these subtle structural differences may account for the observed different effects of Hz and sHz on HIV-1 replication. Given that Hz and sHz induced similar profile of chemokines, CD4 and chemokine receptor expression, post-entry regulatatory mechanisms may account for the differences. A previous study showed a dichotomous effect of soluble malaria antigen in which stimulation of PBMCs resulted in modulation of expression of chemokine receptors with no corresponding increase in HIV-1 infectivity (79). This effect was shown to be IFN-γ-mediated. It is Possible that different cytokines induced by Hz and sHz could account for the differential effects of these molecules on HIV-1 replication. It is therefore,

apparent that relative balance of cytokines in circulation induced by an infectious agent such as *P. falciparum* potentially has an effect on HIV pathogenesis. With regards to malaria in children, cytokine profile characterized by overproduction of TNF-α and lower IL-10/IL-12 ratio is associated with severe disease (18). It remains to be determined if such a profile has any impact on HIV pathogenesis in malaria-HIV co-infected children

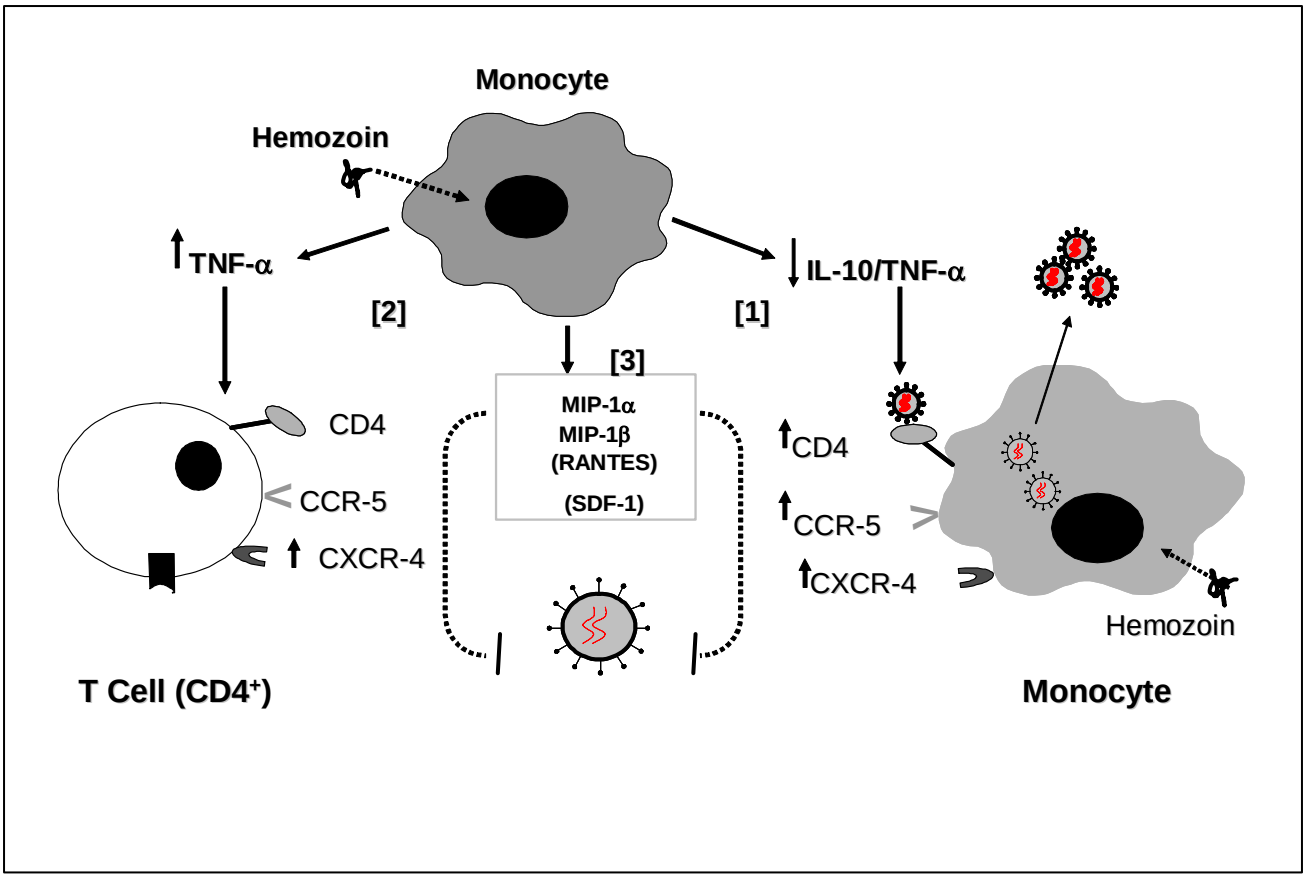

Figure 9. Proposed Model for Interaction of Malaria and HIV-1.
Potential mechanisms by which hemozoin (Hz) stimulation of human monocytes regulates HIV-1 replication. (1) Ingestion of Hz by monocytes induces secretion of IL-10 and TNF-α. A low ratio of IL-10 to TNF-α may be associated with increased HIV-1 replication in monocytes. (2) Hemozoin-induced secretion of TNF-α may increase surface expression of CD4, CCR5 and CXCR4 on monocytes, and CXCR4 expression on $CD4^+$ T cells, significantly increasing the proportion of cellular targets for HIV-1. In contrast, hemozoin-induced secretion of β-chemokines, MIP-1α and MIP-1β, the natural ligands for CCR5, may inhibit initial entry and intracellular replication of R5 HIV-1 strains. Up-regulation of CXCR4 on T cells in the absence of absence of induction of SDF-1 secretion may promote selection of X4 HIV-1 strains, and consequently rapid clinical progression. The relative balance between chemokine levels and expression of chemokine receptors may thus determine the overall effect of hemozoin on HIV-1 infection.

Based on the findings of this study and previously reported work, the proposed model for malaria-HIV interaction is illustrated in figure 9. Ingestion of Hz by monocytes induces secretion of IL-10 and TNF-α. A low ratio of IL-10 to TNF-α reported in children with severe malaria (18) may be associated with increased HIV-1 replication in monocytes. Hz-induced secretion of TNF-α may increase surface expression of CD4, CCR5 and CXCR4 on monocytes, and CXCR4 expression on $CD4^+$ T cells, significantly increasing the proportion of cellular targets for HIV-1. In contrast, Hz-induced secretion of β-chemokines, MIP-1α and MIP-1β, natural ligands for CCR5, may inhibit initial entry and intracellular replication of R5 HIV-1 strains. Up-regulation of CXCR4 on T cells in the absence of induction of SDF-1 secretion may promote selection of X4 HIV-1 strains, and consequently rapid clinical progression. The overall effect of malaria on HIV may therefore depend, to a large extent, on nature of soluble factors induced by *P. falciparum* infection, which in turn may exert enhancing and/or inhibitory effect on HIV-1 replication.

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