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Biomolecular signatures of severe malarial anemia

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BIOMOLECULAR SIGNATURES OF SEVERE MALARIAL ANEMIA

by

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B.S., Cellular and Molecular Biology, Highlands University, 2006

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DEDICATIONS

For my parents: Ali Karim and Angelica Karim-Gonzales who I lost during my time as a graduate student. They gave me the tools to succeed and the love that I needed to become who I am today.

For the children of Siaya County.

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Abstract

Plasmodium falciparum-associated severe malarial anemia [SMA, hemoglobin (Hb)<5.0g/dL] is a leading cause of morbidity and mortality in African children. Examination of the host immune response and underlying genotypic traits that condition SMA can offer an improved understanding of malaria pathogenesis. Previous investigations have suggested that SMA in high transmission regions is due to, impaired erythropoiesis hemolysis, and erythrocyte destruction. In the holoendemic transmission region of western Kenya our studies have shown that dyserythropoiesis drives and conditions SMA through poorly understood molecular pathways. Pediatric studies have shown that early erythroid cell populations as well as reticulocyte production is greatly reduced in children with SMA. The driving force of malaria pathogenesis is multifaceted and the mechanisms that promote erythropoietic dysfunction are still being understood. We hypothesize that SMA is conditioned by the immune response through underlying genetic factors that influence erythropoiesis resulting in a exacerbated disease state. To that end the studies described in this manuscript focus on the inflammatory chemokines, monokine induced by gamma (MIG) and interferon gamma-induced protein (IP10) as well as the hemoglobin-coding gene, hemoglobin alpha 2 (HBA2) in the context of SMA

and dyserythropoiesis. The first study described herein, identified MIG and IP10 as important mediators associated with SMA. In this study we examined circulating MIG and I10 profiles in non-SMA and SMA children and then genotyped and constructed haplotypes between 5 individual polymorphic variants within the promoter regions for the *MIG* and *IP10* for the entire study cohort (*n*=1,600). The second study described in this manuscript, utilized a global approach where genome wide association studies as well as whole-genome transcriptomics identified novel molecular pathways between non-SMA and SMA children. These analyses revealed that the expression of the hemoglobin-coding gene, *HBA2* is significantly modulated between populations. We then examined these findings closer and genotyped a larger population for 2 promoter variants with the same approach described in the first study. Regression analysis of genotype and haplotype data for both studies revealed significant findings where several variant haplotypes correlated with SMA outcomes and erythropoietic response. Lastly, we used an *in vitro* model of erythropoiesis to determine if recombinant human MIG and IP10 as well as pediatric study serum (non-SMA and SMA) influence erythroid function. Our results from these studies revealed that pediatric serum from children with SMA influenced cell viability as well as erythroid lineage when we examined cell surface erythroid markers. A similar result was also seen in cell populations that were treated with the inflammatory mediators, MIG, and IP10 which reflected our proposal that serum from children with SMA and inflammatory mediators (MIG and IP10) infringe upon erythroid cell maturation and viability *in vivo*. Together the results from these studies independently verify the influence of biomolecular markers and mediators on SMA outcomes.

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1.0 CHAPTER ONE: INTRODUCTION

1.1 MALARIA PARASITE AND LIFE CYCLE

Malaria is a multifaceted disease that is caused by protozoan *Plasmodium* parasites. *Plasmodium* malaria is a vector borne disease transmitted by female *Anopheles* mosquitos. Currently, there are five human species in the *Plasmodium* genus: *P. falciparum*, *P. ovale*, *P. malariae*, *P. vivax* and the newly emerging zoonotic pathogen *P. knowlesi*. The falciparum species account for over 90% of all malaria infections and has the widest breath of infectious range, where infections have been found on five continents in the tropics, sub-tropics and temperate zones worldwide (Garcia 2010, Perkins 2011, Wassmer 2015). *P. falciparum* also accounts for the highest severity of disease pathogenesis (mortality and morbidity) where the bulk of transmission occurs in sub-Saharan Africa. In this region, it is estimated that 250 million infections occur annually with young children and pregnant women being at the highest risk for severe disease outcomes. Siaya County in western Kenya, where our study site is located, is holoendemic for malaria (80% infected from ages 1-4), which further drives high transmission rates year round (Bloland 1999, WHO 2014)

The life cycle of malaria starts when a parasite carrying female *Anopheline* mosquito feeds and transfers sporozoites into the human host. The sporozoites then migrate to hepatocytes within the liver where they proliferate into haploid merozoites. After several weeks, merozoites then emerge from the liver and move into the blood stream where they infect red blood cells (RBCs), undergo asexual replication, and mature into schizonts. At this stage of the parasitic life cycle, RBCs lyse and thousands of newly formed merozoites then invade neighboring non-parasitized RBCs. It is also at this stage

that the majority of pathology of malaria manifests in the human host. After asexual replication in RBCs, a portion of merozoites enter sexual replication forming gametocytes within the blood stream and bone marrow. When the *Anopheles* mosquitoes take a blood meal from an infected individual, they also ingest gametocytes. Gametocytes mature into male and female gametes and reproduce sexually in the mosquito's midgut to produce oocysts. The oocysts then divide and burst, yielding thousands of sporozoites that eventually migrate to the salivary glands of the mosquito, thus starting the transmission cycle again (Figure 1.1).

1.2 SEVERE MALARIAL ANEMIA AND PATHOGENESIS

P. falciparum infection manifests in a wide spectrum of disease pathogeneses that ranges from asymptomatic to minor complications such as fever, chills, headaches, and muscle aches to the more severe: severe anemia, hyperparasitemia, metabolic acidosis, and cerebral malaria (CM). Severe malaria anemia [SMA, Hemoglobin (Hb) < 5.0g/dL any parasitemic density] is a hallmark of malarial pathogenesis in sub-Saharan Africa with the highest burden of disease left to children under the age of five. Several studies have indicated that SMA in high transmission regions is due to dyserythropoiesis, impaired erythropoiesis (reduced red blood cell production), hemolysis, and erythrocyte destruction (Chang 2004, Perkins 2011). In the holoendemic transmission region of western Kenya, Siaya County, where our group has worked for the past 15 years, we have shown that ineffective erythropoiesis is a major underlying molecular mechanism that leads to SMA. Erythrocyte production is also reduced in children that have contemporaneous hemolytic malaria infections and is mechanistically driven by the system's inability to restore lost RBCs and therefore induces a more severe anemic state.

Furthermore, in pathology studies where bone marrow has been examined from pediatric patients with SMA, erythrocyte burst forming (BFU) and colony forming (CFU) units (Abdalla 2004) are reduced. We have also shown similar results in *in vitro* models of erythropoiesis using malaria parasite products and products of the inflammatory response (Awandare 2010). We have also demonstrated that reticulocyte production is correlated with the severity of anemia in Kenyan children with malaria (Were 2006). The driving force of malaria pathogenesis is multifaceted and the mechanisms that promote erythropoietic dysfunction are still being understood. We and others hypothesize that anemia during malaria infections is driven primarily by the host immune response and underlying genetic factors that can exacerbate pathogenesis (Figure 1.1).

1.3 MALARIAL PATHOGENESIS AND THE INNATE IMMUNE RESPONSE

Malarial pathogenesis primarily manifests during the erythrocytic replication stage of the parasite cycle where merozoites are rapidly and abundantly released into the blood stream. It is at this time that infected RBCs, parasites, and parasitic byproducts are phagocytosed by monocytes, causing hyper-activation of the innate immune response. Specifically, phagocytosis of the parasitic byproduct *P. falciparum* hemozoin (*Pf*Hz) has been characterized as an integral component stimulating the innate immune response at this time. The link between *Pf*Hz phagocytosis and SMA pathogenesis remains elusive, but several ongoing studies are attempting to bridge the gap-in-knowledge for these mechanisms. However, a variety of regulatory immune genes have been linked to SMA pathogenesis through erythropoietic suppression (Perkins 2011 and others). Key pro-inflammatory cytokines such as IL-12, TNF-α and IFN-γ are released early in parasitic

infection by activated phagocytes and other regulatory cells. Production of such cytokines induces inflammation and in response, increased pathogenesis. Also, overproduction of such mediators has been associated with the severity of *P. falciparum* infections in children (Perkins 2000), along with sustained phagocytic cell activation (Biemba 1998).

1.4 MONOKINE INDUCED BY GAMMA (MIG) AND INTERFERON GAMMA-INDUCED PROTEIN 10 (IP-10) GENETICS AND ROLE IN PATHOGENESIS

Interferon-gamma-induced monokine (MIG/CXCL9) and IFN-gamma-inducible protein (IP-10/CXCL10) belong to the IFN-γ chemo-attractant cytokines (chemokines) that are induced as part of the inflammatory immune response. More specifically, MIG and IP-10 serve as important mediators in the pro-inflammatory immune response by induction of their ligand CXCR3 receptor on type-1 helper (Th1) CD4+ T-cells, effector CD8+ T-cells, and Natural Killer (NK) T-cell innate-type lymphocytes (Blanchet 2012). Furthermore these chemokines are induced by the pro-inflammatory cytokines IFN-γ and TNF-α (Gasperini 1999, Wong 1994) and are produced by a host of cells including neutrophils, monocytes, and endothelial cells (Goebeler 2001, Gasperini 1999, Wong 1994, Farber 1990).

MIG and *IP-10* genes can be found amongst several other CXC family chemokines on chromosome 4 within about 22kb of each other's coding regions (**Figure 1.1**). Polymorphic variation of *MIG* and *IP-10* genes have been described by previous studies of infectious diseases to be associated with a hepatitis B (Xu 2013), HIV/HCV co-infection (Tenor 2009, 2015), tuberculosis (Tang 2009, Sheikh 2015), Chagas disease

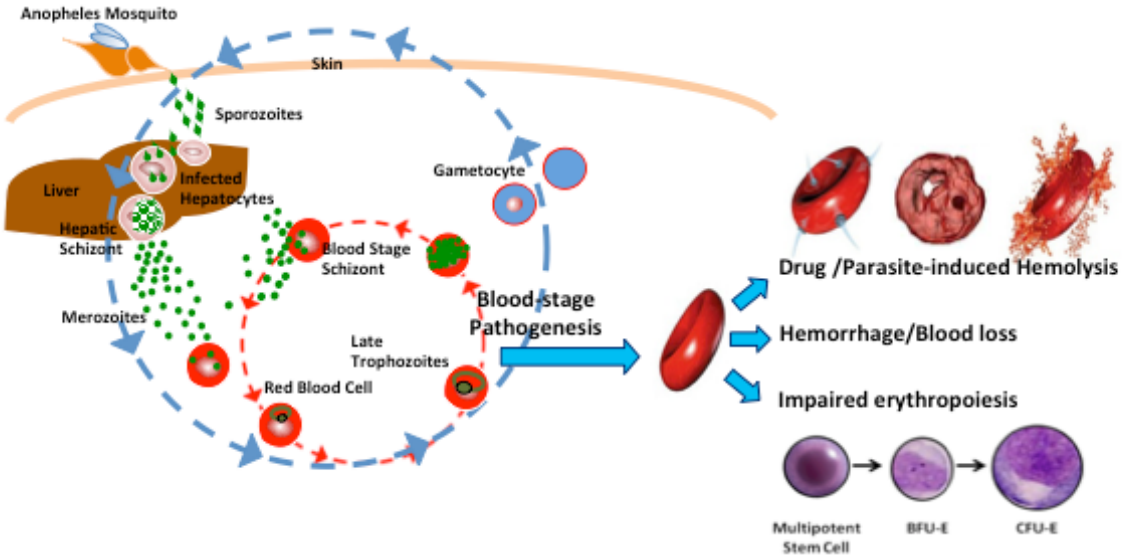
(Nogueira 2012), *Aspergillus fumigatus* (Mezger 2008) and *P. falciparum* malaria (Wilson 2013). Several autoimmune and inflammatory diseases such as Graves disease (Bruck 2010), multiple sclerosis (Galimberti 2007), Crohn's disease (Lacher 2007), and type 1 diabetes (Bruck 2009, Klich 2012), as well as cancers (Dimberg 2014, Nakata 2013) have also been linked to *MIG* and *IP-10* polymorphisms.

1.5 HEMOGLOBIN SUBUNIT ALPHA 2 (HBA2), α-THALASSEIMIA GENETICS AND ROLE IN PATHOGENEISIS

The hemoglobin subunit alpha 2 (*HBA2*) gene codes for one of the two beta chains that comprise hemoglobin. It is localized within a gene cluster on chromosome 16 of hemoglobin that contains associated genes such as *HBA1*, *HBB*, and *HBF* (**Figure 1.2**). The coding regions of *HBA1* and *HBA2* are identical, but vary throughout the 3' UTR as well as the 5' UTR. These two alpha chain-coding genes, along with two beta (HBB) coding genes form the HbA protein and constitute 97% of all hemoglobin. The remaining portion is primarily composed of fetal hemoglobin (HbF). The pathogenesis characterized by polymorphic variation of *HBA2* are primarily thalassemia-associated hemoglobinopathies. Deletions and non-deletions of both *HBA1* and *HBA2* drive the anemic phenotype of α-thalassemia disease. To date, polymorphic variation in *HBA2* alone has not been directly linked to disease pathogenesis apart from the α-thalassemia hemoglobinopathies. However, α-thalassemia has been studied in the context of malarial infection by several groups in a number of regions where malaria is prevalent (Evenfold 2008, Fowkes 2008, Williams 2005, Wambua 2006). It hypothesized that carriers of the

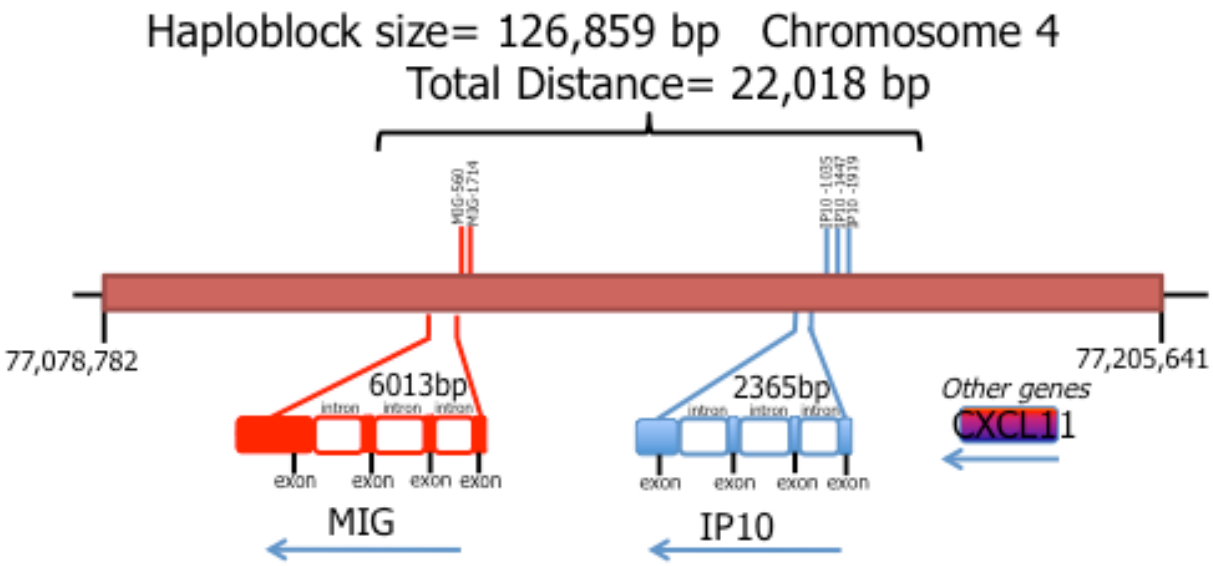
α-thalassemia trait are selectively protected in regions where malaria endemicity is high (Marteveld 2010).

Figure 1.1 Malarial Anemia: Pathogenesis



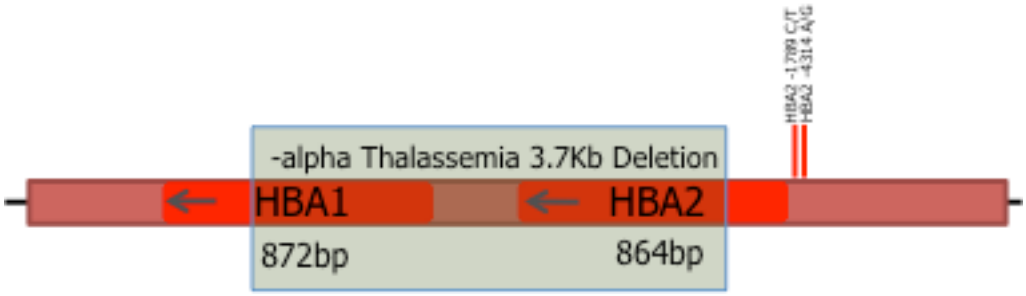
The malaria life cycle is biphasic involving the vector (female Anopheles mosquito) and host (human). The host portion of the life cycle begins when the mosquito takes a blood meal and inoculates the host with sporozoites. The sporozoites then migrate through the blood and into the liver where they undergo maturation into the schizont form. Schizont cells rupture and release merozoites into the periphery thus entering the erythrocytic portion of the malaria life cycle. It is at this point, that the bulk of human pathogenesis occurs resulting in anemia caused by parasitic hemolysis, hemorrhage, and dyserythropoiesis.

Figure 1.2 MIG and IP-10 Genes



The *MIG* (CXCL9) and *IP-10* (CXCL10) genes are both located on chromosome 4 along the same region of about 22kb. Each are composed of four exons and separated by three introns each. The promoter region (~1.5kb from the start codon) for *MIG* contains several well-characterized transcription factor-binding sites (TFBS) including STAT3 (Signal transducer and activator of transcription 3), IRF1 (Interferon regulatory factor 1), and p53 (Tumor protein 53). Similarly, the promoter region for *IP-10* contains STAT3 TFBS, as well as GATA1 (Erythroid transcription factor). Polymorphic variation at two sites (-560-560 G/A, -1714 A/T) in the *MIG* gene promoter and three sites in the *IP-10* gene promoter (-1035 G/A, -1447 A/G, -1919 T/G) are the focus of the studies described in Chapter 3.

Figure 1.3 HBA2 Gene and α-Thalassemia deletion



The *HBA2* gene is located amongst other similar hemoglobin coding genes (*HBA1* and *HBB*) on chromosome 16. These genes have minor differences in their introns and 5' untranslated regions, but have recognizable distinct features in the 3' untranslated regions. The promoter region for *HBA2* contains TCF-2 (hepatocyte nuclear factor 2), cJun, and NF-1 (Nuclear actor 1) sites for transcription factor binding. Polymorphic variations between two sites (-1789 C/T, -4314 A/G) in the *HBA2* gene promoter are the focus of the studies described in Chapter 4.

2.0 CHAPTER TWO: SPECIFIC AIMS

2.1 Specific Aim 1. To determine if the pro-inflammatory chemokines, i.e., monokine induced by gamma (MIG/CXCL9) and interferon-γ-induced protein (IP10/CXCL10), are biomolecular signatures for conditioning SMA.

Hypothesis: Delineation of specific genetic variants within the promoter regions of MIG and IP10 using high-throughput genotyping will reveal associations with phenotypic outcomes that influence SMA

These experiments were accomplished by genotyping the Kenyan pediatric cohort (n=1,600) for several polymorphic alleles located in the promoter regions of the pro-inflammatory chemokines, MIG and IP10. Genotypic data were used to construct intra- (extended) and inter-gene promoter extended haplotypes. Functional association of extended haplotypes was determined using clinical outcomes. Furthermore, circulating inflammatory mediator levels (i.e., MIG, IP10, IFN-γ) were evaluated in haplotype groups associated with SMA using the Cytokine 25-plex Ab Bead Kit, Hu (Invitrogen™).

2.2 Specific Aim 2. To identify novel biomolecular signatures, clustered genetic variants, and candidate genes associated with SMA using high-throughput genotyping and gene expression arrays.

Hypothesis: Novel biomolecular signatures will emerge from the high-throughput genome-wide association study (GWAS) and whole transcriptome analysis using the gene expression array

Genetic signatures for SMA were identified from genomic DNA samples from 48 phenotypically characterized children with malarial anemia using a high-throughput genotyping microarray [Illumina® Human Omni2.5 BeadChip with 2.44 x 10^6 markers, (GWAS)] to profile SNPs and copy number variation, and transcriptomics (Illumina® 'HumanHT-12 v4 Expression BeadChip', 47,231 probes covering 19,185 gene transcripts) to measure gene expression. In combination, the GWAS and transcriptomics provided insight into candidate genes associated with the immune response, signal transduction, and hematopoiesis in children with malaria. Children with malaria were stratified into two polarized extremes (non-SMA and SMA) controlling for covariates that influence anemic outcomes [i.e., age, gender, HIV-1, bacteremia, glucose-6-phospate dehydrogenase deficiency (G6PD), α-thalassemia, sickle-cell trait (HbS)].

2.3 Specific Aim 3. **To determine if the biomolecular markers characterized in Aim 1 influence erythropoiesis in an *in vitro* model.**

Hypothesis: Chemokine (MIG and IP10) treatment of CD34+ hematopoietic stem cells will alter an erythroid cell lineage, disrupt cell expansion, and reduce cell viability in our model for erythropoiesis

The effects of rh (recombinant human) MIG, rhIP-10, rhIFN-γ (Aim1), and pooled serum from non-SMA and SMA children on erythropoiesis was evaluated using an *in vitro* CD34+ hematopoietic stem cell model. Naïve CD34+ cells from US donors were inducted into the erythroid lineage and treated with biomolecular markers and pooled serum from disease categories, and their influence on erythropoietic proliferation,

survival, and differentiation was determined using biochemical, molecular, and flow cytometric methods.

3.0 CHAPTER THREE: RESULTS OF SPECIFIC AIM 1

Extended haplotypes in the *MIG* (CXCL9) and *IP-10* (CXCL10) promoters influence susceptibility to *Plasmodium falciparum-associated* severe malarial anemia

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ABSTRACT

Plasmodium falciparum-induced severe malarial anemia [SMA, hemoglobin (Hb)<5.0g/dl] is a leading cause of morbidity and mortality in African children. However, the underlying genotypic traits that condition SMA have not been fully elucidated. Chemokines such as monokine-induced by gamma (MIG/CXCL9) and interferon gamma-induced protein-10 (IP-10/CXCL10) are typically increased during a type 1 inflammatory response. We recently observed that circulating levels of MIG and IP-10 were significantly reduced in children with SMA compared to parasitized children with non-SMA (P<0.005 and P<0.001, respectively). To investigate the role of polymorphic variation in *IP-10* and *MIG* on susceptibility to SMA, extended haplotypes were constructed using five promoter variants [*MIG* -1714A/T (rs7670156), *MIG* -560G/A (rs6532083), *IP-10* -1035G/A (rs4257674), *IP-10* -1447A/G (rs4508917), and *IP-10* -1919T/G (rs4371639)] on chromosome 4. These particular variants were selected based on a high minor allelic distribution (>10%) in the population. Binary logistic regression was performed [controlling for age, gender, nutritional status, co-infections (HIV-1, bacteremia), G6PD deficiency, α-thalassemia and HbS]. Presence of the *IP-10*-1035G/-1447A (GA) haplotype was associated with increased susceptibility to SMA (OR, 1.62; 95% CI, 1.12-2.35; *P<0.01)* and inefficient erythropoiesis (RPI<2: OR 1.54; 95% CI 1.01-2.38; *P<0.05*), while the *MIG*-560G/-1714A (GA) haplotype was associated with susceptibility to SMA (OR, 1.73; 95% CI, 1.04-2.88; *P<0.05)* and an efficient erythropoietic response (RPI<2 OR, 0.41; 95% CI, 0.22-0.77; *P<0.01)*. Three extended intragenic haplotypes between the two promoters for each gene were associated with increased susceptibility to SMA (*MIG*-560G/*IP-10*-1447A [GG/GA] OR 1.34; 95%

CI 1.00-1.84; *P<0.05*, OR 1.47; 95% CI 1.00-2.19; *P<0.05*, and *MIG*-560G/*IP-10*-1035G-1447A [GGG] OR 1.41; 95% CI 1.02-1.96; *P<0.05*) and inefficient erythropoiesis (*MIG*-560G/*IP-10*-1447A [GG/GA] RPI<2 OR 1.63; 95% CI 1.08-2.45; *P<0.05*, OR 1.61; 95% CI 1.00-2.61; *P<0.05*, and *MIG*-560G/*IP-10*-1035G-1447A [GGG] OR 1.91; 95% CI 1.23-2.96; *P<0.01*). In addition, the extended haplotype [*MIG*-560G-1714A/*IP-10*-1035G (GAG)] showed increased susceptibility to SMA (OR 1.58; 95% CI 1.00-2.55; *P<0.05*) and protection against efficient erythropoiesis (RPI<2: OR 0.57; 95% CI 0.32-1.00; *P<0.05*). Carriage of two intragenic extended haplotypes (*MIG*-560G/*IP-10*-1447A[GG] and *MIG*-560G/*IP-10*-1035G-1447A [GGG]) showed significant variability in the production of several inflammatory mediators, including IP-10 in children with SMA. Taken together, these data illustrate that variation in *MIG* and *IP-10* are important in conditioning susceptibility to SMA and erythropoietic responses.

BACKGROUND

Plasmodium falciparum is responsible for >90% of all malarial infections. Of the estimated 250 million *P. falciparum* infections worldwide, 77% of these infections occur in Africa (WHO, 2014). Children (<5 years of age) in sub-Saharan Africa carry the highest burden of mortality and morbidity. Even though malarial infection contributes to several severe clinical manifestations, [e.g., hypoglycemia and cerebral malaria (CM)], the most common severe disease outcome in children living in holoendemic *P. falciparum* transmission areas is severe malarial anemia (SMA: Hb<5.0g/dL as defined by the WHO, 2000). In holoendemic regions, such as our study site in western Kenya, malaria-related infant mortality rates are up to 20% (Ongecha, 2006, Perkins 2011).

During malarial intraerythrocytic replication, monocyte/macrophage phagocytosis of the malarial parasitic byproduct hemozoin (*Pf*Hz) occurs, driving immune pathway dysregulation. Several immune modulatory genes have been identified as key factors that play roles in erythropoietic suppression and enhanced SMA pathogenesis (Perkins 2011). The initial host immune response is mediated by production of pro-inflammatory cytokines such as IL-12, TNF-α and IFN-γ to control parasitic infection. However, this response also induces enhanced pathogenesis as a result of monocyte/macrophage activation. Overproduction of these mediators has also been linked to *P. falciparum* infected children (Perkins 2000), as well as sustained phagocytic cell activation (Biemba 1998).

Monokine induced by gamma (MIG/CXCL9) and interferon gamma-induced protein (IP-10/CXCL10) are chemo-attractant cytokines (chemokines) that are induced as

part of the inflammatory immune response. More specifically, MIG and IP-10 serve as important mediators in the pro-inflammatory immune response by induction of their ligand CXCR3 receptor on type-1 helper (Th1) CD4+ T-cells, effector CD8+ T-cells, and Natural Killer (NK) T-cell innate-type lymphocytes (Blanchet 2012). Furthermore, these chemokines are induced by the pro-inflammatory cytokines IFN-γ and TNF-α (Gasperini 1999, Wong 1994) and are produced by a host of cells including neutrophils, monocytes, and endothelial cells (Goebeler 2001, Gasperini 1999, Wong 1994, Farber 1990). CXCR3 ligands are influential during inflammatory responses after cerebral injury in ischemic stroke (Mirabelli-Badenier 2011). In autoimmune diseases such as psoriasis, MIG is highly expressed (Goebeler 1998). Whereas in thyroid disorders, IP-10 and MIG activated through T-cells (Garcia-Lopez 2001) and similarly highly expressed in multiple sclerosis (Simpson 2000). Furthermore, chronic inflammation that is driven by the CXC family of cytokines (MIG and IP-10)has been implicated in ovarian cancer (Rainczuk 2012).

Previous studies from our group have shown that circulating MIG and IP-10 are significantly reduced in children with SMA with MIG being predictive of worsening anemia (Ongecha 2011). Additionally, MIG is down-regulated in experimental malaria infection in human volunteers (Dunachie 2010). Conversely, both MIG and IP-10 are up-regulated in murine models of cerebral malaria (Campanella 2008, Villegas-Mendez 2012) where chemokine expression is isolated primaraliy in the neural regions rather than in the periphery. Consistent with previous investigations in other diseases, we show that allelic variation in the *MIG* and *IP-10* genes play an essential role in conditioning susceptibility to SMA.

MATERIALS AND METHODS

Study participants. Children < 3 years of age with malaria (n=1,600) were recruited at Siaya County Hospital in western Kenya, where *P. falciparum* transmission is holoendemic (Ongecha, 2006). Study participants were primarily from the Luo ethnic group, which is the predominant population in this region (>96%). Children with *P. falciparum* malaria (any density parasitemia) were categorized into 2 groups: SMA (Hb level <5.0 g/dL) and non-SMA (Hb level ≥ 5.0 g/dL) according to WHO clinical guidelines. Children were excluded from the study if they had CM or non–*P. falciparum* malaria. In addition, all children were tested for HIV-1 and bacterial co-infections since these co-infections influence malarial anemia severity (Were 2011, Davenport 2012, Otieno 2006). Genetic determinants which influence anemic outcomes, glucose-6-phosphate dehydrogenase (G6PD) deficiency, α-thalassemia and sickle cell trait, were also characterized in the cohort. The study was approved by the ethical and scientific review committees at the Kenya Medical Research Institute (Kenya) and institutional review board at the University of New Mexico (United States).

Laboratory measures. Venous blood (<3.0 mL) was collected into EDTA vacutainer® tubes (Becton-Dickinson, NJ, USA) and used to determine complete hematological measurements (AcT diff2® Coulter, Beckman-Coulter Inc., Fullerton, USA) and laboratory investigations. Intraerythrocytic cycle asexual stage malaria parasites were counted against 300 leukocytes using Giemsa-stained blood films. Parasitic counts were expressed per mL of blood using the patient's absolute leukocyte count. HIV-1 status was determined according to our published methods (Otieno 2006). HIV-1 positive individuals were given trimethoprim-sulfamethoxazole and the appropriate anti-retroviral

therapies. Bacteremia was determined by microbial cultivation according to our standard methods (Were 2011). Alpha-thalassemia, sickle cell status and G6PD deficiency were determined as previously described (Anyona 2011). Intracellular pigment in monocytes and neutrophils was determined as previously described (Luty 2000, Lyke 2003). The erythropoietic response was determined by calculating the reticulocyte production index (RPI) using our previous methods (Were, 2006) and the absolute reticulocyte number (ARN) was calculated using reticulocyte and erythrocyte counts.

MIG and IP-10 genotyping. Genomic DNA was isolated from buccal swabs (Buccal Amp™, Epicentre Biotechnologies, Madison WI) and then amplified using GenomiPhi™ V2 DNA Amplification kit (GE Healthcare Life Sciences, Amersham, UK). *MIG* G-560A (rs6532083), A-1713-T (rs7670156), and *IP-10* G-1035-A (rs4257674), A-1447-G (rs4508917), T-1919-G (rs4371639) SNPs were genotyped using the Taqman® 5'-allelic discrimination Assay-by-design (ABI) on the StepOnePlus™ PCR platform.

Determination of circulating Cytokines, Chemokines, and Effector Molecules. Plasma samples obtained from venous blood were stored at -70°C until use. Soluble mediator concentrations were determined using the Human Cytokine Twenty-Five-Plex Antibody Bead Kit (Invitrogen International), in accordance with the manufacturer's instructions. Plates were read on a Luminex 100 system (Luminex) and analyzed using Bio-Plex Manager software (version IS 2.3; Bio-Rad Laboratories). The detection limits for MIG, IP-10, and IFN-γ were 4.0 , 5.0 and 5.0 pg/mL, respectively.

Statistical analyses. Statistical analyses were performed using SPSS software (version 19.0), and χ^2 and Mann-Whitney *U* tests were used to examine differences between

proportions and for pairwise comparisons of medians, respectively. *MIG* and *IP-10* extended haplotypes were constructed using PHASE® software (Stephens Laboratory). Bivariate logistic regression, controlling for age, sex, HIV-1 status (including both HIV-1 exposure and definitively HIV-1 positive results), bacteremia, and prevalence of sickle cell trait (HbAS) and α-thalassemia were used to examine associations of genotypes and extended haplotypes with SMA and RPI (<2). Statistical significance was defined as $P<0.05$. Haploview statistical software (Version 4.2) was used to determine SNP and haplotype distribution and linkage disequilibrium.

Longitudinal follow-up. Upon enrollment (Day 0), parents were asked to bring their children back to the study clinic every 3 months for the extent of the study (36 months).

RESULTS

Clinical and demographic characteristics of the study participants (Table 3.1). Study participants were (<36 months of age) were categorized into aparasitemic (*n*=300), non-SMA (*n*=1044; Hb ≥ 5.0 g/dL), and SMA (*n*=256; Hb<5.0 g/dL). Summaries of clinical and demographic measures are shown in **Table 3.1**. Gender ratios across the groups were normally distributed (*P*=0.370). The SMA group contained the youngest children (*P*<0.001).

As expected based on *a priori* grouping, hemoglobin (Hb), red blood cell (RBC) count, and hematocrit were significantly lower in the SMA group (*P*<0.001). The SMA group had the highest total white blood cell count (WBC) (*P*=0.011), as well as a higher lymphocyte count (*P*<0.001). Monocyte counts were highest in the non-SMA group (*P*<0.001). Mean parasite densities (MPS/μL) and high-density parasitemia (HDP; MPS ≥ 10,000/μL) were significantly lower in the SMA group (*P*<0.001 and *P*<0.05, respectively). The overall reticulocyte production index (RPI) and RPI<2 were lowest in the SMA group (*P*<0.001 and *P*<0.01, respectively). HIV-1 and bacterial co-infections were comparable between the groups. Glucose 6-phosphate dehydrogenase (G6PD) deficiency was equally distributed across the groups. Heterozygous sickle cell trait carriers and α-thalassemia trait carriers were represented the highest in the aparasitemic group (*P*<0.001).

Circulating MIG and IP-10 in children with non-SMA and SMA (Figure 3.1). Circulating chemokines (MIG and IP-10) were examined in the non-SMA (*n*=215) and SMA (*n*=62) groups prior to investigation of *MIG* and *IP-10* promoter variants. Co-

infected (HIV-1 and bacteremia) individuals were removed from the chemokine analysis because of potential impact on circulating chemokines. MIG and IP-10 levels were significantly lower in children with SMA compared to the non-SMA group ($P<0.005$ and $P<0.001$ respectively) **(Figure 3.1)**. To determine if these differences were associated with genetic variation, *MIG* and *IP-10* promoter variants were examined.

MIG and IP-10 genotype distributions (Table 3.2). Genotype frequencies for the two *MIG* and three *IP-10* promoter variants are stratified according to non-SMA and SMA (**Table 3.2**). Distributions of *MIG* variants were: -560G/A GG (*n*=943, 0.612), GA (*n*=554, 0.360), GG (*n*=43, 0.028) and -1714A/T GG (*n*=432, 0.284), AT (*n*=947, 0.622), TT (*n*=144, 0.095). Although both *MIG* promoter variants were not in Hardy-Weinberg equilibrium (HWE; χ^2=18.52, $P<0.0001$ and χ^2=180.34, $P<0.0001$, respectively), the genotypic distribution between the clinical groups (non-SMA and SMA) was not statistically different for either genotype (P=0.189 and P=0.166).

Distributions of the *IP-10* loci were: -1035G/A GG (*n*=889, 0.596), AG (*n*=487, 0.327), GG (*n*=115, 0.077); -1447A/G GG (*n*=969, 0.620), AG (*n*=435, (0.278), GG (*n*=159, 0.102); -1919G/T GG (*n*=880, 0.600), TG (*n*=462, 0.315), TT (*n*=125, 0.085). The IP-10 variants showed a departure from HWE (χ^2=15.39, $P<0.0001$; χ^2=30.18, $P<0.0001$; and χ^2=23.96, $P<0.0001$, respectively). No differences in genotypic differences were observed between the non-SMA and SMA groups (P=0.918, P=0.625, and P=0.869, respectively) (**Table 3.2**).

Association of MIG and IP-10 genotypes with susceptibility to SMA (Table 3.3). Logistic regression modeling, controlling for age, gender, nutritional status, co-infections (HIV-1, bacteremia), G6PD deficiency, and HbS, was used to examine the association between the genotypes and susceptibility to SMA. These analyses revealed that carriage of the homozygous mutant [MIG-1714 TT genotype (n=50)] was associated with protection against SMA [OR 0.43; 95% CI 0.19-0.98; *P*=0.04], whereas none of the other genotypes were significantly associated with susceptibility to SMA.

Association of MIG and IP-10 genotypes with susceptibility to inefficient erythropoiesis (RPI<2) (Table 3.3). To determine the association between genotypes and the erythropoietic response, a standard measure of reticulocyte production that corrects for anemia and premature release of reticulocytes from the bone marrow, i.e., reticulocyte production index (RPI<2) was determined. Binary logistic regression was performed as described above with the outcome variable defined by RPI≥2 (efficient erythropoiesis) and RPI<2 (inefficient erythropoiesis). These analyses revealed that only one genotype, i.e., carriage of the heterozygous MIG-560 GA genotype was associated with protection against inappropriate erythropoiesis [OR 0.60; 95% CI 0.40-0.89; *P*=0.01]**.**

MIG and IP-10 haplotypic distributions and linkage disequilibrium. Haplotypic constructs were made to further understand the influence of both intragenic and extended haplotypes of *MIG* and *IP-10* promoter variants on susceptibility to SMA. From these analyses, a total of 84 haplotypes (>10% distribution) emerged for intragenic (n=15) and extended (n=69) haplotypes. To determine if the alleles at different loci were in linkage

disequilibrium, LD was examined using the Haploview statistical package. Shown in Figure 3.4 are the D' values for the five SNPs that were used to create the different haplotypic constructs.

Association between MIG and IP-10 intragenic and extended haplotypes and SMA (Table 3.5). Binary logistic regression was performed [controlling for age, gender, nutritional status, co-infections (HIV-1, bacteremia), G6PD deficiency, and HbS] to determine the association of the haplotypes with SMA. There were four intragenic haplotypes associated with increased susceptibility to SMA [OR 1.35-1.73; 95% CI 1.00-2.88; (P=0.01-0.05)]. None of the intragenic haplotypes were associated with protection against SMA.

For the extended haplotypes, there were 17 constructs associated with increased susceptibility to SMA [OR 1.34-2.00; 95% CI (Range) 1.00-3.40; P=0.001-0.05]. In contrast, four extended haplotypes showed protection against SMA [OR 0.48-0.69; 95% CI 0.25-1.00; P<0.010-0.05].

Association between MIG and IP-10 extended haplotypes and RPI<2 (Table 3.5). Using the same binary regression model described above, we performed analyses using the parameters of RPI≥2 (efficient erythropoiesis) and RPI<2 (inefficient erythropoiesis). These analyses indicated that carriers of the intragenic *IP-10*-1035G/-1447A (GA and GG) haplotypes showed increased inefficient erythropoiesis [OR 1.35-1.62; 95% CI 1.00-2.35; P< 0.05], whereas carriers of the *MIG*-560/-1714A (GA) haplotype had protection against inefficient erythropoiesis [OR 0.41; 95% CI 0.22-0.77; P<0.01].

Analyses of the extended haplotypes revealed that there were three haplotypes associated with enhanced inefficient erythropoiesis [OR 1.61-1.91; 95% CI 1.00-2.96; *P*=0.001-0.05], while two haplotypes were associated with protection against inefficient erythropoiesis [OR 0.30-0.38; 95% CI 0.17-0.61; *P*<0.001].

MIG* and *IP-10* haplotypes associated with both SMA and erythropoietic responses *(Figure 3.3). A summary of the haplotypic constructs associated with significant susceptibility to both SMA and RPI<2 are shown in Figure 3.3 Although seven of the haplotypes showed the same directionality for susceptibility to SMA and an RPI<2, two of the haplotypes demonstrated increased susceptibility to SMA in the context of protective response on the RPI.

Functional association between haplotypic variants and circulating inflammatory mediators (MIG, IP-10, and IFN-γ) (Table 3.6). The functional association between haplotypes associated with susceptibility to both SMA and erythropoietic responses (n=9) was investigated by comparing MIG, IP-10, and IFN-γ between carriers and non-carriers of the haplotypes.

The GG haplotype at the *MIG*-560G/*IP-10*-1447A locus was associated with increased IFN-γ levels [non-GG Median: 11.17, IQR: 26.24; GG Median: 13.15, IQR: 22.94, (*P*=0.858)] and a significantly reduction in IP-10 levels [non-GG Median: 240.59, IQR:606.38; GG Median: 235.74 IQR: 336.76, (*P*=0.05)] in circulation. Furthermore, the pro-inflammatory eosinophil cytokine IL-5 ([non-GG Median: 2.11, IQR: 3.70; GG

Median: 3.00 IQR: 3.51, (*P*=0.046); ([non-GGG Median: 2.01, IQR: 3.66; GGG Median: 3.40 IQR: 3.54, (*P*=0.012)] was elevated where as TNF-α [non-GG Median: 24.70, IQR: 51.04; GG Median: 19.90 IQR: 29.40, (*P*=0.025); non-GGG Median: 24.30, IQR: 48.90; GGG Median: 18.52 IQR: 29.40, (*P*=0.017)] was significantly decreased in circulation for SMA/RPI associated haplotypes. Interestingly, the anti-inflammatory cytokine IL-10 was significantly lower [non-GG Median: 258.77, IQR: 680.14; GG Median: 178.42 IQR: 311.95, (*P*=0.031); non-GGG Median: 94.51, IQR: 196.80; GGG Median: 71.20 IQR: 225.58, (*P*<0.001)] for GG and GGG haplotypes.

DISCUSSION

Chemokines serve as chemotactic signaling cytokines, which stimulate cell migration in response to several types of induction. In the context of infectious disease, chemokines are produced during the pro-inflammatory immune response and serve as activators of several types of immune cells (Zlotnik 2012). Our previous studies reported high induction of MIP-1α and MIP-1β and suppression of RANTES in children with SMA (Ochiel 2005, Were 2006). In this study, we report an association of the chemokines, MIG and IP-10, with SMA and furthermore illustrate how chromosome 4 *MIG* and *IP-10* promoter variation influence erythropoiesis and SMA outcomes. In agreement with our previous study and that of another, children with severe malaria had low levels of MIG and IP-10 in circulation (Dunachie 2010 and Ongecha 2011). However, contrary to these findings, studies conducted using murine models for cerebral malaria (CM) show an up-regulation of both MIG and IP-10 (Villegas-Mendez 1990, Campanella 2008). Furthermore, investigations in India and Ghana have also shown strong association with fatal *P. falciparum* human CM and increased IP-10 (Armah 2007, Jain 2008). This

demonstrates that disease outcomes (SMA vs CM) may be influenced differently by the initial innate immune response, and where CM phenotypes are the results of central nervous system influence, SMA is a result of erythropoietic, hemolysis, and red blood cell dysregulation. Also, these results provide further evidence that phenotypic outcomes are conditioned based on underlying genetic traits of a population.

There have been several studies that have investigated polymorphic variation for the CXCR3 ligands (MIG, IP-10) and pathogenesis. A study by Deng et al. showed an positive association between a *IP-10* promoter polymorphism and progression of hepatitis B virus infection (Deng 2008). Polymorphisms in *MIG* and *IP-10* are also correlated positively with the intensity of myocarditis in Chagas disease (Nogueira 2012). A longitudinal study investigating a single *IP-10* polymorphism predicted increased survival in bone-marrow transplant recipients (Nakata 2013). In the context of autoimmunity, carriage of particular *IP-10* haplotypes is associated with the progression of multiple sclerosis (Galimberti 2007), and several *MIG* and *IP-10* haplotypes were shown to be associated with type-1 diabetes in a German population (Bruck 2009). *MIG* polymorphisms have also been associated with Crohn's disease (Lacher 2007).

To date, there have been no investigations focusing on *MIG* and *IP-10* promoter polymorphic variation and susceptibility to SMA. Using logistic regression modeling, we demonstrate that two haplotype constructs (*MIG*-560A/-1714T and *IP-10*-1447A/-1919T) for promoter variants of *MIG* and *IP-10* are significantly associated with susceptibility to SMA. Conversely, *MIG*-560G/-1714T carriers showed protection against SMA. Interestingly, examination of seven intragenic extended haplotypes showed increased susceptibility to SMA, whereas ten extended haplotypes indicated

protection against SMA. To further understand the impact of the erythropoietic response on the pathogenesis of SMA in the context of the study, the association between haplotypic groups that showed association with susceptibility to SMA were also utilized as a measure for efficient erythropoiesis, RPI, as the outcome variable. These analyses revealed that presence of the *MIG*-560G/-1714A (AT) and *IP-10*-1447A/-1919T (AT) haplotypes were associated with increased susceptibility to SMA and an elevated risk of inefficient erythropoiesis. Similarly, six extended intragenic haplotypes between the two promoters were associated with enhanced susceptibility to and inefficient erythropoiesis. In contrast, an extended haplotype (*MIG*-560G *IP-10*-1447A: GA) showed protection against both SMA and inefficient erythropoiesis.

Our analysis of the functional association of haplotypic groups with SMA and erythropoietic response identified several circulating biomolecular mediators in our population. Through these measures, the pro-inflammatory mediator IFN-γ was reduced in GG haplotype carriers. In comparison, IP-10 was significantly unregulated in the same population. However, there is a temporal disconnection between the influence of IFN-γ and its downstream-mediated chemokine, IP-10.

Concomitantly, these results demonstrate an association of circulating MIG and IP-10 with SMA. These data further illustrate how several haplotypic groups within the locus of MIG and IP-10 promoters influence erythropoiesis and SMA, and more specifically, how extended haplotypes between promoter regions show association with differential regulation of soluble mediators, including IP-10.

COMPETING INTERESTS

None of the authors of the manuscript have a personal or financial competing interest that influences the interpretation of data or presentation of any information contained herein.

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TABLE AND FIGURE LEGENDS

Table 3.1. Clinical and Demographic Characteristics.

Parasitemic children (*n*=860) were categorized into SMA (Hb<5.0 g/dL' *n*=152) and non-SMA (Hb≥ 5.0 g/dL; *n*=708) according to WHO guidelines. Data are presented as the median (Interquartile range, IQR) unless stated otherwise. Reticulocyte production index (RPI) were calculated using previously described methods (reticulocyte index (RI)= (reticulocyte count × hematocrit)/30.7 (average hematocrit of children <5.0 years of age in Siaya District); maturation factor (MF)= 1+0.05(30.7-hematocrit); RPI=RI/MF; Were 2009) [a]Chi-Square test was used to determine differences in proportions . [b] Kruskal-Wallis test was used to determine differences in medians. $P<0.05$ was considered significant

Figure 3.1. Circulating chemokine levels in parasitemic children.

Concentrations of chemokines (pg/mL) were measured in plasma from children <3.0 years with *P. falciparum* malaria using a Human Cytokine 25-plex Antibody Bead Kit and Luminex® 100™ system (See materials and methods for description). Concentrations of circulatingIP-10, and MIG (pg/mL) were determined for 277 children [SMA (*n*=62) and non-SMA (*n*=215)]. Box-plots depict the data where the box represents the interquartile range, the line through represents the median, and whiskers represent the 10th and 90th percentile. When compared to the non-SMA group, circulating levels of MIG and IP-10 were significantly reduced in children with SMA relative to those without SMA ($P<0.005$ and $P<0.001$ respectively) Differences between groups were compared by Mann-Whitney *U* test.

Table 3.2. Association of *MIG* and *IP-10* Genotypes and SMA/RPI.

Binary logistic regression analyses for haplotypes were conducted in SPSS for parasitemic children controlling for covariates (age, gender, HIV-1, parasite count, G6PD, bacteremia, α-Thalassemia, and sickle-cell trait). Data presented as percentages and p-values from x^2 analysis.

Table 3.3. Association of *MIG* and *IP-10* Haplotypes and SMA/RPI.

Binary logistic regression analyses for haplotypes was conducted in SPSS for parasitemic children controlling for covariates (age, gender, HIV-1, parasite count, G6PD, bacteremia, α-Thalassemia, and sickle-cell trait). Indicates significance ($P<0.05$). Data are presented as Odds Ratios (95% Confidence Interval).

Figure 3.3 and Table 3.4. Association between *MIG/IP-10* extended haplotypes and SMA (Top) and RPI (Bottom).

The cross-sectional analysis between extended haplotypes and SMA (Hb<5.0g/dL) and RPI (<2) were determined in parasitemic children (n=860). Binary logistic regression analysis, controlling for age, gender, the coinfections of HIV-1 and bacteremia, and genetic variants of G6PD deficiency, Sickle cell, and α-Thalassemia traits were used to determine odds ratios (ORs,) and 95% confidence intervals (CI). Individuals not carrying haplotype were used as reference group. Significance for analysis was set at P<0.05. n values beneath the figure represent the number of individuals with corresponding haplotype you may need to submit a supplemental table with all the haplotypes that had over 5% representation in the population.

Figure 3.4 Linkage disequilibrium between *MIG and IP-10* promoter gene polymorphisms.

Genotypic data for each promoter SNP variant was examined for LD using Haploview statistical package (see materials and methods). D' values are given in the intersecting boxes where the darker red boxes signify a stronger linkage disequilibrium between alleles. SNP position relative to each gene is indicated (1-5).

Figure 3.5. Circulating biomolecular molecules (25-plex) and Haplotypic variants.

Concentrations of chemokines (pg/mL) were measured in plasma from children <3.0 years with *P. falciparum* malaria using a Human Cytokine 25-plex Antibody Bead Kit and Luminex® 100™ system (See materials and methods for description). Children (*n*=184) were categorized into those the GG haplotype (*n*=88) and non-GG carriers (*n*=96) and GGG haplotype (*n*=78) and non-GGG carriers (*n*=82). Box-plots depict the data where the box represents the interquartile range, the line through represents the median, and whiskers represent the 10th and 90th percentile. Differences between groups were compared by Mann-Whitney *U* test[a].

TABLES AND FIGURES

Table 3.1. Clinical and Demographic Characteristics.

Characteristic	Aparasitemic	non-SMA	SMA	*P-value*
Number, *n*	300	1044	256	N/A
Gender, %F:M	49:51	49:51	53:47	0.370[a]
Age, months	11.3 (13.3)	12.8 (10.5)	10.1 (10.5)	**<0.001**[b]
Hematological Indices				
Hemoglobin (g/dL)	10.3 (2.5)	7.6 (2.9)	4.2 (1.0)	**<0.001**[b]
RBC count, x 10^{12}/L	4.6 (1.0)	3.5 (1.9)	3.4 (1.8)	**<0.001**[b]
Hematocrit, (%)	32.6 (7.3)	23.8 (12.2)	22.8 (10.8)	**<0.001**[b]
WBC count (10^3/µL)	11.0 (7.3)	11.7 (6.2)	14.9 (9.8)	**<0.05**[b]
Lymphocytes (10^3/µL)	43.2 (22.1)	48.5 (18.9)	55.8 (20.9)	**<0.001**[b]
Monocytes (10^3/µL)	7.5 (5.4)	8.5 (5.8)	7.5 (3.9)	**<0.001**[b]
Parasitological Indices				
Parasitemia, MPS/µL	-	28,675 (79,130)	22,294 (66,103)	**<0.01**[b]
High-density Parasitemia, *n* (%)	-	747 (70.5)	168 (63.9)	**<0.05**[a]
Reticulocyte Indices				
Reticulocyte Production Index (RPI)	0.60 (0.69)	0.70 (0.99)	0.57 (0.83)	**<0.001**[b]
RPI<2.0, *n* (%)	19 (7.7)	140 (13.8)	22 (9.3)	**<0.01**[a]
Co-infections				
HIV-1 Status, *n* (%)	12 (4.0)	31 (3.0)	13 (5.2)	0.193[a]
Bacteremia, *n* (%)	39 (12.7)	98 (9.3)	21 (8.0)	0.128[a]
Genetic Variants				
G6PD deficiency, *n* (%)	10 (4.0)	38 (4.1)	6 (3.0)	0.666[a]
Sickle cell trait, *n* (%)	54 (17.7)	169 (16.2)	18 (7.1)	**<0.001**[a]
α-thalassemia, *n* (%)	68 (26.4)	168 (18.7)	45 (19.2)	**<0.05**[a]

Figure 3.1. Circulating chemokine levels in parasitemic children.

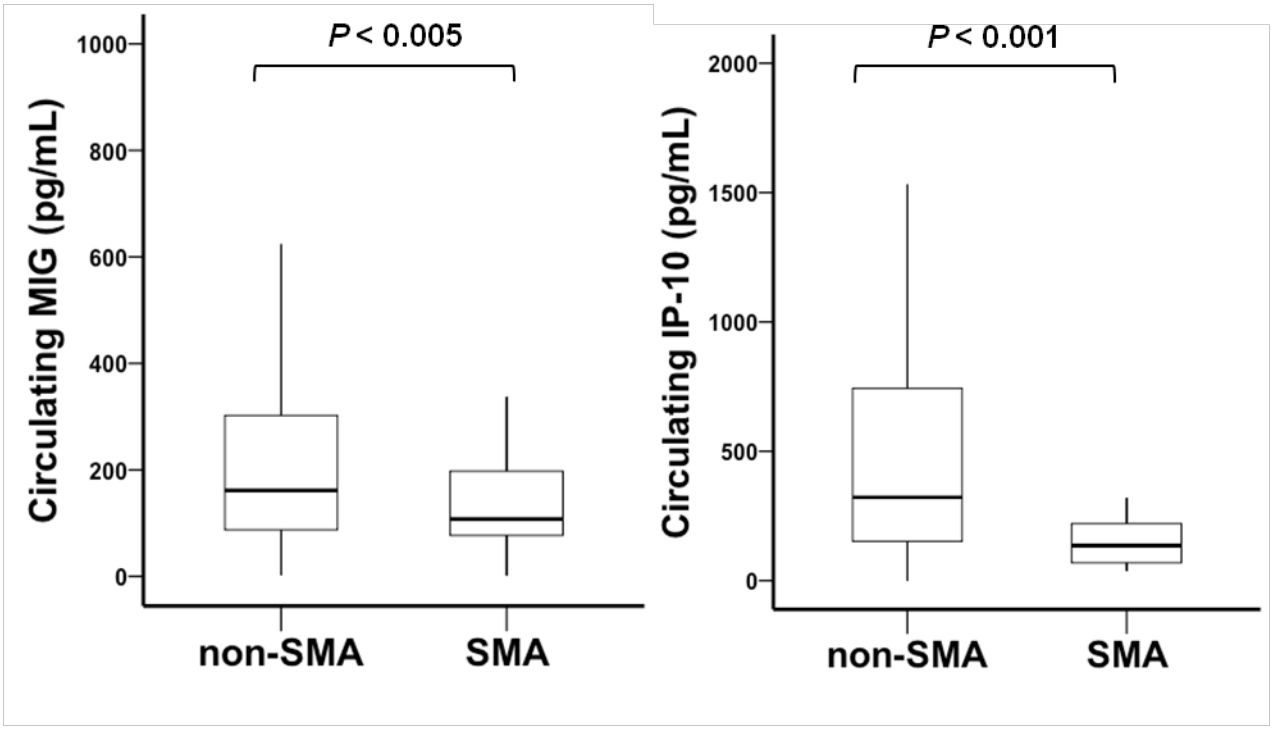

Table 3.2. *MIG* and *IP-10* genotype distributions

	Aparasitemic	non-SMA	SMA	Total	*P* value
***MIG* (G-560A)**					
GG, *n* (%)	161 (57.3)	613 (61.9)	169 (63.1)	943 (61.2)	0.189
GA, *n* (%)	116 (41.3)	348 (35.1)	90 (33.6)	554 (36.0)	
AA, *n* (%)	4 (1.4)	30 (3.0)	9 (3.4)	43 (2.8)	
***MIG* (A-1714T)**					
AA, *n* (%)	64 (22.7)	290 (29.6)	78 (30.0)	432 (28.4)	0.166
AT, *n* (%)	185 (65.6)	604 (61.6)	158 (60.8)	947 (62.2)	
TT, *n* (%)	33 (11.7)	87 (8.9)	24 (9.2)	144 (9.5)	
***IP10* (G-1035A)**					
GG, *n* (%)	166 (60.4)	569 (59.5)	154 (59.5)	889 (59.6)	0.918
AG, *n* (%)	85 (30.9)	315 (32.9)	87 (33.6)	487 (32.7)	
AA, *n* (%)	24 (8.7)	73 (7.6)	18 (6.9)	115 (7.7)	
***IP10* (A-1447G)**					
AA, *n* (%)	188 (65.7)	618 (61.7)	163 (59.3)	969 (62.0)	0.625
AG, *n* (%)	72 (25.2)	281 (28.0)	82 (29.8)	435 (27.8)	
GG, *n* (%)	26 (9.1)	103 (10.3)	30 (10.9)	159 (10.2)	
***IP10* (G-1919T)**					
GG, *n* (%)	160 (60.6)	564 (59.9)	156 (59.8)	880 (60.0)	0.869
TG, *n* (%)	82 (31.1)	297 (31.5)	83 (31.8)	462 (31.5)	
TT, *n* (%)	22 (8.3)	81 (8.6)	22 (8.4)	125 (8.5)	

Table 3.3. Association of *MIG* and *IP-10* Genotypes with SMA and RPI<2.

Genotype	SMA Odds Ratio	95% CI	*P* value	RPI Odds Ratio	95% CI	*P* value
	***MIG* -560 G>A**			***MIG* -560 G>A**		
GG (n=782)	1.00	-	-	1.00	-	-
GA (n=438)	0.86	0.61-1.22	0.39	0.60	0.40-0.89	**0.01**
AA (n=39)	0.71	0.26-1.93	0.51	1.72	0.39-7.52	0.47
	***MIG* -1714 A>T**			***MIG* -1714 A>T**		
AA (n=172)	1.00	-	-	1.00	-	-
AT (n=573)	0.99	0.69-1.43	0.97	0.87	0.56-1.36	0.54
TT (n=50)	0.43	0.19-0.98	**0.04**	1.09	0.47-2.52	0.84
	***IP-10* -1035 G>A**			***IP-10* -1035 G>A**		
GG (n=488)	1.00	-	-	1.00	-	-
GA (n=240)	0.96	0.67-1.37	0.81	1.42	0.89-2.24	0.14
AA (n=61)	0.65	0.32-1.32	0.23	0.69	0.35-1.37	0.29
	***IP-10* -1447 A>G**			***IP-10* -1447 A>G**		
AA (n=586)	1.00	-	-	1.00	-	-
AG (n=190)	1.20	0.84-1.72	0.32	1.40	0.88-2.25	0.16
GG (n=47)	0.97	0.54-1.72	0.90	1.07	0.54-2.14	0.85
	***IP-10* -1919 G>T**			***IP10* -1919 G>T**		
GG (n=463)	1.00	-	-	1.00	-	-
GT (n=227)	0.79	0.42-1.49	0.48	0.88	0.44-1.79	0.73
TT (n=68)	0.98	0.68-1.40	0.90	1.52	0.94-2.47	0.09

Figure 3.2. Linkage disequilibrium between *MIG* and *IP-10* promoter gene polymorphisms.

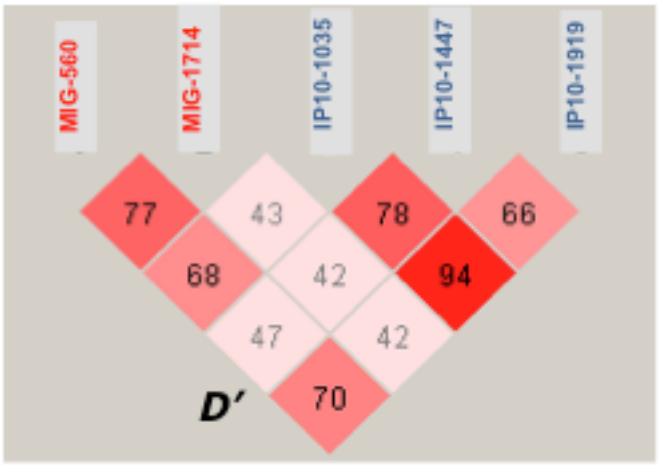

Table 3.4. Association of *MIG* and *IP-10* Haplotypes with SMA and RPI<2.

Haplotypes	SMA Odds Ratio	95% CI	*P* -value	RPI Odds Ratio	95% CI	*P*- value
IP10-1035G/-1447A						
GA (n=629)	0.876	0.590-1.300	0.510	0.497	0.338-0.731	**0.000**
AA (n=292)	1.137	0.835-1.549	0.416	1.177	0.892-1.552	0.249
GG (n=221)	0.838	0.603-1.165	0.293	0.973	0.725-1.305	0.854
AG (n=5)	0.977	0.198-4.818	0.977	-	-	0.999
IP10-1035G/-1919G						
GG (n=661)	0.838	0.499-1.408	0.506	0.536	0.345-0.834	**0.006**
AT (n=268)	1.231	0.908-1.668	0.181	1.262	0.986-1.614	0.065
GT (n=29)	0.938	0.428-2.059	0.874	1.640	0.873-3.084	0.124
AG (n=14)	1.331	0.434-4.080	0.616	6.357	1.970-20.509	**0.002**
IP10-1447A/-1919G						
AG (n=607)	0.873	0.599-1.273	0.481	0.582	0.419-0.808	**0.001**
AT (n=287)	1.341	0.990-1.818	**0.058**	1.439	1.117-1.854	**0.005**
GG (n=210)	0.881	0.638-1.215	0.440	1.082	0.822-1.424	0.576
GT (n=10)	0.529	0.166-1.680	0.280	0.902	0.286-2.847	0.861
MIG-560G/-1714A						
GA (n=712)	1.191	0.693-2.047	0.526	0.600	0.359-1.002	**0.051**
GT (n=327)	0.618	0.458-0.833	**0.002**	0.992	0.772-1.274	0.949
AT (n=299)	1.411	1.039-1.917	**0.028**	1.506	1.172-1.936	**0.001**
AA (n=26)	1.619	0.656-3.993	0.296	2.271	1.028-5.013	**0.042**
MIG-560G/IP10 -1035G						
GG (n=702)	1.010	0.597-1.710	0.970	0.527	0.321-0.865	**0.011**
AA (n=243)	1.390	1.008-1.917	**0.044**	1.379	1.060-1.793	**0.017**
GA (n=98)	0.660	0.437-0.997	**0.048**	1.250	0.851-1.836	0.255
AG (n=72)	1.168	0.705-1.935	0.546	1.391	0.909-2.130	0.129
MIG-560G/IP10 -1447A						
GA (n=674)	0.657	0.422-1.023	**0.063**	0.641	0.449-0.915	**0.014**
AA (n=307)	1.336	0.988-1.805	**0.060**	1.520	1.185-1.950	**0.001**
GG (n=228)	1.177	0.860-1.611	0.309	0.958	0.733-1.253	0.754
AG (n=10)	-	-	0.999	1.485	0.558-3.954	0.429
MIG-560G/IP10 -1919G						
GG(n=666)	0.970	0.572-1.643	0.909	0.540	0.333-0.875	**0.012**
AT (n=235)	1.512	1.089-2.101	**0.014**	1.398	1.071-1.826	**0.014**
GT (n=97)	0.772	0.510-1.168	0.221	1.250	0.854-1.830	0.251
AG (n=62)	1.228	0.711-2.123	0.461	1.330	0.840-2.105	0.224
MIG-1714A/IP10 -1447A						
AA(n=698)	0.934	0.571-1.530	0.787	0.766	0.506-1.159	0.207
TA (n=425)	1.104	0.822-1.483	0.511	1.297	1.019-1.650	**0.035**
TG (n=203)	0.761	0.552-1.051	0.098	1.141	0.867-1.502	0.347
AG (n=47)	1.874	0.924-3.800	0.082	1.125	0.685-1.850	0.642
MIG-1714A/IP10 -1035G						
AG (n=661)	0.955	0.605-1.509	0.845	0.702	0.471-1.045	0.081
TG (n=331)	0.711	0.528-0.958	**0.025**	1.087	0.849-1.391	0.507
TA (n= 268)	1.310	0.998-1.852	**0.051**	1.330	1.029-1.718	**0.029**
AA (n=66)	1.292	0.749-2.230	0.357	1.242	0.787-1.961	0.352
MIG-1714A/IP10 -1919G						
AG (n=632)	0.973	0.515-1.838	0.933	0.616	0.350-1.084	0.093
TT (n=525)	1.260	0.778-2.041	0.348	0.692	0.459-1.044	0.079
TG (n=313)	1.284	0.840-1.964	0.249	0.915	0.640-1.308	0.625
AT (n=71)	1.542	0.694-3.424	0.287	1.430	0.758-2.698	0.270

Haplotypes	SMA Odds Ratio	95% CI	*P* -value	RPI Odds Ratio	95% CI	*P*- value
MIG-560G/-1714A/IP10-1035G						
GAG (n=614)	1.067	0.701-1.622	0.763	2.458	1.731-3.491	**0.000**
GTG (n=61)	0.594	0.366-0.966	**0.036**	1.114	0.710-1.747	0.638
MIG-560G/-1714A/IP10-1447A						
GAA (n=628)	0.848	0.569-1.264	0.418	0.645	0.461-0.903	**0.011**
ATA (n=285)	1.432	1.035-1.981	**0.030**	1.546	1.198-1.995	**0.001**
GTA (n=181)	0.719	0.511-1.013	**0.059**	1.144	0.855-1.531	0.365
GTG (n=150)	0.699	0.489-0.999	**0.049**	0.865	0.631-1.186	0.368
GAG (n=82)	1.685	1.011-2.808	**0.045**	1.241	0.847-1.819	0.268
MIG-560G/-1714A/IP10-1919G						
GAG (n=591)	1.263	0.855-1.866	0.240	0.671	0.469-0.959	**0.029**
GTG (n=290)	0.575	0.422-0.783	**0.000**	0.988	0.762-1.282	0.929
MIG-560G/-IP10-1035G-1447G						
GGA (n=609)	0.998	0.688-1.449	0.993	0598	0.430-0.832	**0.002**
AAA (n=240)	1.273	0.925-1.753	0.139	1.457	1.118-1.898	**0.005**
GGG (n=199)	0.779	0.563-1.079	0.133	0.931	0.703-1.233	0.616
GAA (n=91)	0.656	0.431-0.998	**0.049**	1.254	0.846-1.858	**0.259**
MIG-560G/-IP10-1035G-1919G						
GGG (n=659)	0.863	0.632-1.179	0.355	0.641	0.480-0.854	**0.002**
AAA (n=154)	1.304	0.879-1.933	0.187	1.199	0.850-1.691	0.302
MIG-560G/-IP10-1447A-1919G						
GAG (n=587)	0.827	0.562-1.216	0.333	0.590	0.424-0.821	**0.002**
AAT (n=122)	1.384	0.919-2.082	0.120	1.397	0.999-1.954	**0.051**
GGG (n=108)	0.957	0.636-1.442	0.835	1.018	0.715-1.449	0.922
MIG-1714A/-IP10-1035G-1447G						
AGA (n=586)	0.997	0.695-1.432	0.989	0.615	0.450-0.840	**0.002**
TAA (n=263)	1.250	0.910-1.716	0.168	1.369	1.058-1.772	**0.017**
TGG (n=155)	0.780	0.546-1.116	0.174	0.959	0.709-1.299	0.789
AGG(n=78)	1.126	0.701-1.809	0.623	1.256	0.848-1.859	0.256
AAA (n=65)	1.224	0.708-2.117	0.468	1.329	0.836-2.114	0.229
MIG-1714A/-IP10-1035G-1919G						
AGG (n=612)	0.935	0.588-1.489	0.778	0.614	0.410-0.919	**0.018**
TGG (n=296)	0.684	0.503-0.932	**0.016**	1.026	0.793-1.326	**0.847**
TAT (n=124)	1.099	0.727-1.661	0.653	1.174	0.829-1.664	0.366
MIG-1714A/-IP10-1447A-1919G						
AAG (n=569)	0.991	0.686-1.431	0.962	0.570	0.413-0.786	**0.001**
TAT (n=117)	1.198	0.794-1.807	0.389	1.366	0.972-1.922	0.073
TGG (n=97)	0.887	0.578-1.361	0.583	1.100	0.763-1.586	0.609
TAG (n=85)	0.758	0.487-1.179	0.220	0.997	0.676-1.471	0.988
IP10-1035G/-1447A/-1919G						
GAG (n=589)	0.922	0.533-1.597	0.773	0.597	0.370-0.962	0.034
AAT (n=263)	1.193	0.769-1.849	0.431	1.317	0.912-1.902	0.142
GGG (n=196)	0.819	0.515-1.304	0.401	1.016	0.681-1.515	0.939

Haplotypes	SMA Odds Ratio	95% CI	*P* -value	RPI Odds Ratio	95% CI	*P*- value
MIG-560G/-1714A/IP10-1035G/1447A						
GAGA (n=567)	1.000	0.695-1.438	0.999	0.620	0.453-0.849	**0.003**
ATAA (n=227)	1.401	1.002-1.960	**0.049**	1.427	1.089-1.868	**0.010**
GTGG (n=147)	0.707	0.491-1.016	0.061	0.834	0.609-1.141	0.256
GTGA (n=142)	0.817	0.560-1.190	0.292	1.101	0.804-1.506	0.549
MIG-560G/-1714A/IP10-1035G/1447A						
GAGG (n=575)	1.297	0.871-1.932	0.201	0.747	0.531-1.051	0.094
MIG-560G/-1714A/IP10-1447A/1919G						
GAAG (n=545)	1.106	0.740-1.655	0.623	1.052	0.753-1.469	0.765
ATAT (n=218)	1.388	0.971-1.983	0.072	0.683	0.483-0.842	**0.001**
GTGG (n=144)	0.781	0.543-1.123	0.183	0.906	0.658-1.246	0.543
GTAG (n=124)	0.379	0.267-0.538	**0.000**	1.752	1.239-2.478	**0.002**
MIG-560G/IP10-1035G/1447A/1919G						
GGAG (n=571)	0.818	0.548-1.221	0.325	0.0706	0.510-0.978	**0.036**
GGGG (n=67)	1.211	0.712-2.059	0.479	0.932	0.605-1.438	0.751
MIG-1714A/IP10-1035G/1447A/1919G						
AGAG (n=555)	0.982	0.674-1.431	0.927	0.603	0.453-0.834	**0.002**
TAAT (n=241)	1.205	0.872-1.665	0.259	1.352	1.035-1.765	**0.027**
TGAG (n=159)	0.700	0.492-0.995	**0.047**	1.048	0.774-1.418	0.764
TGGG (n=146)	0.792	0.548-1.145	0.215	0.951	0.696-1.299	0.751
MIG-560G/-1714A/IP10-1035G/1447A/1919G						
GAGGA (n=322)	1.027	0.760-1.388	0.862	0.737	0.578-0.941	**0.014**
AATAT (n=107)	1.181	0.772-1.807	0.444	1.417	1.009-1.990	**0.044**
GGGT (n=74)	0.835	0.509-1.369	0.475	0.909	0.605-1.366	0.645
GAGGT (n=67)	0.828	0.519-1.320	0.427	0.796	0.537-1.179	0.255

Figure 3.3. Association between *MIG/IP-10* extended haplotypes and SMA (Top) and RPI (Bottom).

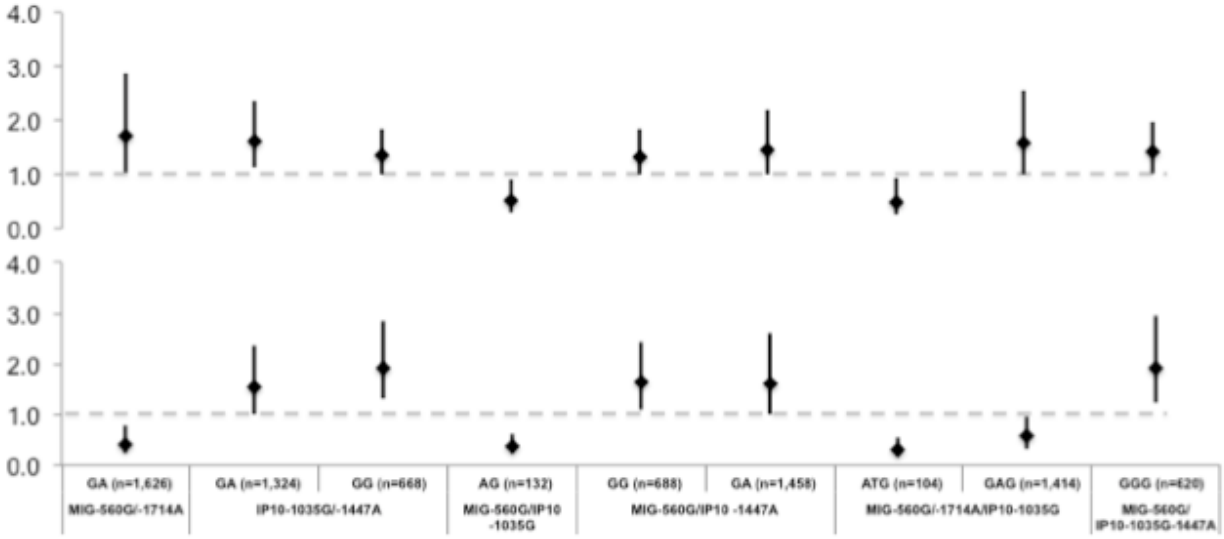

Table 3.5. Circulating inflammatory mediators (25-plex) and haplotypic variants.

Circulating Mediator (pg/mL)	IP-10			MIG			IFN-γ		
	Median	IQR	*P*-Value	Median	IQR	P-Value	Median	IQR	*P*-Value
Intragenic Haplotypes									
MIG-560G/-1714A									
GA (n=52)	224.5	626.9	0.629	161.2	310.7	0.832	11.7	28.1	0.52
non-GA (n=176)	253.8	294.2		187	166.7		18.2	28.5	
IP10-1035G/-1447A									
GA (n=54)	215.5	280.9	0.384	155.9	178.4	0.717	11.7	35.5	0.955
non-GA (n=144)	263.6	466.5		193.8	182.3		16.3	21.5	
GG (n=66)	282.4	292.5	0.028	203.1	182.8	0.050	22.9	29.4	0.852
non-GG (n=132)	203	274.9		152.2	154.7		18.2	20.3	
Extended Haplotypes									
MIG-560G/IP10 -1035G									
AG (n=30)	241.4	304.5	0.998	161.2	201.9	0.773	13.4	24.63	0.561
non-AG (n=168)	216.4	302.7		158.4	178.6		14.8	31.4	
MIG-560G/IP10 -1447A									
GA (n=43)	208.7	286.7	0.081	159.8	182.2	0.9	13.8	29.6	0.564
non-GA (n=158)	265	422.04		194.3	184.4		16.7	25.7	
GG (n=71)	274.9	322.9	**0.005**	153.6	159.4	**0.068**	19.6	27.7	**0.005**
non-GG (n=130)	199.2	259.6		210	187.5		8.55	21.4	
MIG-560G/-1714A/IP10-1035G									
GAG (n=146)	231.6	285.03	0.78	162.9	169.4	0.76	14.5	31.04	0.52
non-GAG (n=42)	183.9	537.4		155.9	153.75		15.3	22.2	
ATG (n=21)	240	302.3	0.88	127.01	161.5	0.69	12.3	22.5	0.6
non-ATG (n=141)	224.8	313.8		165.8	175.2		14.8	28.2	
MIG-560G/IP10-1035G-1447A									
GGG (n=47)	274.9	270.2	**0.024**	196.1	171.97	**0.050**	24.31	27.16	0.085
non-GGG (n=118)	205.4	298.13		152.3	153.2		20.12	21.6	

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4.0 CHAPTER FOUR: RESULTS OF SPECIFIC AIM 2

Hemoglobin subunit alpha 2 (HBA2) promoter variation influences susceptibility to *Plasmodium falciparum-associated* anemia

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ABSTRACT

Plasmodium falciparum-induced severe malarial anemia [SMA, hemoglobin (Hb)<5.0 g/dL] is a leading cause of morbidity and mortality in African children. However, the underlying genotypic traits that condition SMA have not been fully elucidated. Recent studies in our laboratory from high-throughput genotyping and transcriptomics revealed that >2 copies of hemoglobin-α 2 (*HBA2*) and 1.81-fold increase in gene expression, respectively, were significantly associated with enhanced susceptibility to SMA in a stratified cohort of Kenyan children (3-36mos.) with polarized clinical phenotypes: SMA (n=70) and non-SMA (n=74). These signals were validated using *in silico* analysis for *HBA2*, and identified two single nucleotide polymorphisms (SNPs), -1789C/T and -4314A/G, which were then genotyped in the larger cohort (n=1,314). The deletion for -$\alpha^{3.7}$thalassemia was also determined for the population. Promoter genotypes and -$\alpha^{3.7}$ thalassemia deletions were used in the construction of haplotypes, followed by binary logistic regression analysis, controlling for covariates (age, gender, G6PD, HIV-1, bacteremia, and HbAS status). This model revealed that carriage of the *HBA2* TT genotype protected against SMA (OR: 0.60, 95%CI: 0.37-0.966, P<0.05). In addition, several haplotypic constructs between the promoter variants and α-thalassemia showed significantly altered susceptibility to SMA and reduced erythropoietic responses (RPI<2) . In contrast, several haplotypes were significantly associated with protection against SMA. Taken together, these results demonstrate that variation in the *HBA2* promoter, and *HBA2*/-$\alpha^{3.7}$-thalassemia haplotypes are associated with susceptibility to both SMA and altered erythropoietic responses.

BACKGROUND

Between 124-283 (198) million cases of malaria with an estimated 367,000-755,000 (584,000) malaria-associated deaths occur each year worldwide [WHO 2013]. *Plasmodium falciparum* malarial infections in sub-Saharan Africa account for approximately 82% of all malarial infections globally. Furthermore, children under the age of five and pregnant women carry the highest burden of disease. This burden is particularly prominent in holoendemic areas of *P. falciparum* such as our study site in western Kenya where severe disease primarily manifests as severe malarial anemia [SMA (any parasitemia and Hb<5.0 g/dL)] (Perkins 2011, Novelli 2010, Ongecha 2006, Bloland 1999, Obonyo 2007, Zucker 1997).

Thalassemias are among the most prevalent hemoglobinopathies induced by a defect in the synthesis of either α or β globin chain (Higgs 2008 Cohen 2004). Reduction in the production of either (or both) globin chains results in anemia caused by abnormal hemoglobin synthesis. The thalassemia disease state is characterized by hemolysis that is caused by imbalanced and mismatched globin chains, resulting in weak linkage between clusters (Higgs 1989)Alpha (α) thalassemia is an inherited disorder caused by deletions and/or mutations between the two α-globin coding genes, *HBA1* and *HBA2*. Variation in these genes can result in abnormal hemoglobin syntheses, often leading to mild to severe anemia (Yap 2013 Harteveld 2010). Ordinarily, hemoglobin molecules contain four α-globin genes at two per haploid genome (αα/αα). When both α globin genes are suppressed, the resultant genotype is heterozygous (--/αα), whereas one α globin gene inactivation results in heterozygous (-α/αα) (Higgs DR 1989). Mild to severe α-globin chain defects are caused by the inactivation of any or all of the four α-globin genes,

further inducing the severe anemia phenotype depending on the relative number of genes inactivated. Previous studies suggest that homo- and heterozygous thalassemia carriers are protected from *P. falciparum* infection (Williams 2005, Wambua 2006, May 2007 , Allen 1997, Mockenhaupt 2004, Fowkes 2008).

The most common α-thalassemias are $-\alpha^{3.7}$ and $-\alpha^{4.2}$ characterized by 3.7kb and 4.2kb deletions, respectively. Worldwide, there is a distribution of the heterozygous ($-\alpha^{3.7}/\alpha\alpha$) and homozygous ($-\alpha^{3.7}/-\alpha^{3.7}$) α-thalassemia. However prevalence for the $-\alpha^{3.7}$ deletion is found in African and Mediterranean populations where the $-\alpha^{4.2}$ is found primarily in Asian and Mediterranean populations (Friedman S 1974, Kattamis AC 1996, Pirastu M, 1982). As such, this study will focus on the -3.7kb ($\alpha/-\alpha^{3.7}$) deletion. During meiosis, the $-\alpha^{3.7}$ deletion occurs as a result of a reciprocal crossover and misalignment of homologous block (Z-blocks) overlapping parts of the *HBA1* and *HBA2* genes on chromosome 16. This results in the thalassemia phenotype, with heterozygous ($-\alpha^{3.7}/\alpha\alpha$) and homozygous ($-\alpha^{3.7}/-\alpha^{3.7}$) individuals. The -3.7kb deletion removes the promoter region of hemoglobin subunit alpha 1 (*HBA1*), and a portion of the coding region itself leaving the promoter for the hemoglobin subunit alpha 2 (*HBA2*) largely intact. *HBA2* codes for one of the two α-chains of hemoglobin clustered with related genes (e.g., *HBA1* and *HBB*).

In a concurrent study (unpublished), high-throughput genotyping [Illumina® Human Omni2.5-8v1 BeadChip' ($>2.45 \times 10^6$ markers)] and global gene expression arrays [HumanHT-12 v4 BeadChip (47,231 probes)] were utilized to identify genetic variants associated with malaria clinical outcomes in a stratified subset of Kenyan children (3-36mos.) with SMA (n=70) and non-SMA (n=74). The genotyping studies revealed that

>2 copies of HBA2, and several polymorphic variants in the promoter region of the gene were significantly associated with enhanced susceptibility to SMA. In addition, global gene expression profiling showed a 1.81-fold increase for *HBA2* in the SMA group. To extend and validate the whole genome findings, we performed *in silico* analysis for *HBA2* and identified two (potentially) functional SNPs (-1789C/T, rs2974771 and -4314A/G, rs1203833) that were then genotyped in a larger cohort of children with malaria (n=1,314). The current study investigates the influence of *HBA2* promoter polymorphisms and α-thalassemia variation on susceptibility to SMA and inefficient erythropoiesis characterized by a reticulocyte production index (RPI)<2 in children with falciparum malaria.

METHODS

Study participants. Children aged 3-36 mos. (n=1,314) presenting with *P. falciparum* malaria at Siaya County Hospital (SCH) were recruited for the study. Children with non-*falciparum* malaria species, cerebral malaria, or previous hospitalization (for any reason) were excluded from the study. Upon enrollment, heel/finger-prick of <100μL blood was taken to evaluate Hb and parasitemia status. Parasitemic children were stratified into SMA (Hb<5.0 g/dL, any density parasitemia) and non-SMA (Hb≥5.0 g/dL, any density parasitemia).

Sample collection. Venous blood (1-3 mL) was obtained from each study participant. Hematological measures, including a complete blood count (CBC) and reticulocyte count, along with HIV-1 status and bacteremia status were determined. A portion of the venipuncture blood was centrifuged and isolated plasma was stored at -80^{o}C until use. For DNA isolation, buccal cells were collected using MasterAmp^{TM} buccal swab kits (Epicenter Biotechnologies, USA).

Laboratory evaluation. For determination of parasitemia, thin and thick blood smears were prepared, stained with Giemsa reagent and examined under oil immersion. Trophozoites were counted against 300 leukocytes and parasite densities estimated using the WBC counts from the CBC.

High-throughput genotyping: whole genome genotyping. Genomic DNA was isolated from buccal swabs using the MasterAmp^{TM} Buccal swab DNA extraction kit (Epicentre Biotechnologies, Madison, WI), cleaned and concentrated using the genomic DNA kit (Zymo Research, CA, USA. Parasitemic children (non-SMA and SMA) that were

selected for this study were age and gender matched in equal proportions (n=48). Children that were aparasitemic, HIV-1 and/or bacteremia-infected, sickle-cell disease (SSD) positive, and glucose-6-phosphatase dehydrogenase (G6PD) deficient were excluded from these analysis. Children were categorized into low Hb levels (SMA cases; Hb<5.0g/dL; n=22, avg. Hb=4.1) and high Hb levels (non-SMA, controls, Hb= 8.0-10.9g/dL; n=26, avg. Hb=10.8)]. Genomic DNA sample quality was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA). Quantitative analysis of genomic DNA was measured using the Qubit®2.0 fluorimeter and also with the dsDNA BR assay kit (Invitrogen®). Concentrated DNA (50 ng) was used for the Illumina® Infinium® HD Super Assay in conjunction with Illumina's® Human Omni2.5-8v1 BeadChip (Illumina,CA, USA) which is coated with >2.45M markers to profile single nucleotide polymorphisms (SNPs). The array was scanned on the Illumina® 'iScanSQ' platform and the genotype calls were made using Illumina's® GenomeStudio® genotyping module. For quality control, those samples with call rates of less than 95% and MAFs less than 5% were excluded from the analysis. GWAS data analyses were performed using a 'full model of regression' controlling for covariates with 'SNP and Variation Suite (SVS) 7.7' (Golden Helix, Inc, MT, USA).

Gene expression array (transcriptome analysis). Seventy two additional subjects from the overall pediatric cohort (n=1,654) were chosen using the same selection criteria of Hb levels: SMA (cases, n=21, avg. Hb=5.13) and non-SMA (controls, n=51, avg. Hb=9.43). White blood cell (WBC) pellets were used to extract total RNA with the GITC organic method (Chomczynski 1987; Keller 2004). Total RNA was quantitatively measured using the Qubit®2.0 fluorimeter and also with the RNA BR assay kit (Invitrogen®).

Global gene expression profiling was conducted on the Illumina® HumanHT-12 v4 beadchip array (Illumina,CA, USA) covering >47,000 where transcripts were read on the Illumina® "iScanSQ" platform. The Illumina GenomeStudio® was used to eliminate internal assay background noise this yeildinge 60 samples with 19,185 transcripts expressed overall. Data were further filtered using step-wise procedures to remove transcripts that were identified as having an "absent" expression/flag quality control step. Genes that were differentially expressed between two clinical category groups (>1.5 fold change) and statistically significant between populations were selected for validation. Correlation tests for gene expression data were conducted using the GraphPad Prism 5® (GraphPad Software Inc. La Jolla, CA) software. Microarray gene expression data were processed and examined using GeneSpring GX v13 software (Agilent, CA USA).

HBA2 -1789C/T and -4314A/G, and α-thalassemia genotypes and haplotypes. DNA was extracted using BuccalAmp™ DNA extraction kits. Genotyping of *HBA2* -1789C/T (rs2974771) and *HBA2* -4314A/G (rs1203833) was carried out using Taqman® 5' allelic discrimination Assay-By-Design℠ methods according to manufacturer's protocol (Applied Biosystems, Inc.). The α- thalassemia $-\alpha^{3.7}$ deletion was determined using nested PCR. Primers used for nested PCR were; Forward :5'-AAG TCC ACC CCT TCC TTC CTC ACC-3', Reverse 1: 5'-ATG AGA GAA ATG TTC TGG CAC CTG CAC TTG 3', and Reverse 2: 5'- TCC ATC CCC TCC TCC CGC CCC TGC CTT TTC 3'. The PCR reaction was performed in a 20 μL reaction mixture containing 100 ng genomified DNA, 50mmol $MgSO_4$, 10 mmol dNTPs, 12.5 ng and 5 units/μL of PlatinumTaq DNA polymerase (Promega, Madison, WI) in buffer per manufactures instructions. Nested PCR conditions were an initial 2 minute denaturation at 94°C,

followed by 44 cycles of denaturation at 94 °C for 30 seconds, annealing at 65 °C for 1 minute and extension at 72 °C for 2 minutes followed by a final extension at 72 °C for 5 min. PCR products were separated on 3% high resolution agarose gel (Peqlab, Germany) containing ethidium bromide and visualized under UV illumination. Haplotypes were constructed using PHASE software (version 2.1).

Statistical Analyses. Comparisons of non-parametric data were determined by Chi-Square test and Mann-Whitney U test, respectively. The association between the haplotypes and SMA were examined by binary logistic regression, controlling for confounders of age, gender, HIV status, bacteremia, sickle cell trait (HbAS), and G6PD deficiency status. Statistical significance was set at $P \leq 0.05$.

RESULTS

The influence α-thalassemia ($-\alpha^{3.7}$) deletion and variation in the promoter region of the HBA2 gene was investigated by stratifying parasitemic children into two clinical categories: non-SMA (Hb≥5.0 g/dL, n = 1,053) and SMA (Hb< 5.0 g/dL, n = 261). Demographic, laboratory, and clinical measures for the study participants are summarized in **Table 4.1**. Children with SMA were younger (*P*<0.001) and had lower admission temperature (°C, *P*< 0.05). Consistent with *a priori* grouping, hemoglobin (Hb; g/dL), red blood cell count, and hematocrit were lower in children with SMA (*P*<0.001). Children with SMA had elevated white blood cell counts, along with marked leukocytosis and monocytosis (*P*<0.001). Peripheral malaria parasite density (MPS/μL) and high-density parasitemia (HDP; >10,000/μL) were highest in the non-SMA group (P=0.003 and P=0.030, respectively). The absolute reticulocyte number (ARN) and reticulocyte production index (RPI) were higher in the SMA group (*P*=0.007 and *P*<0.001, respectively). The percentage of children with inefficient erythropoiesis (RPI<2) was highest in children with SMA (*P*=0.024). The impact of genetic variants on the development of SMA was also examined. Sickle cell trait and α-thalassemia deletion carriers were higher in the non-SMA group (16.4% vs. 8.9%; *P*=0.025 and 22.5% vs. 19.2%; *P*<0.001, respectively). HIV-1 co-infection was higher in children with SMA (5.2% vs. 3.9%; *P*<0.001), whereas bacteremia was similar between the two groups (8.1% vs. 9.3%; *P*=0.225).

Distribution of *HBA2* genotypes and α-thalassemia -3.7kb deletion in children with malarial anemia (Table 4.2). Prevalence of the *HBA2*-1789C/T (rs2974771) genotype in

parasitemic children was 43.9% CC, 38.7% CT and 17.4% TT with overall allele frequencies of C = 0.62 and T = 0.38, respectively. The combined distribution of the *HBA2*-1789C/T genotypes displayed significant departure from Hardy-Weinberg Equilibrium (HWE, c2=2.88, *P*=0.010). However, the overall genotypic distribution for the *HBA2*-1789C/T allele between non-SMA and SMA groups was not statistically significant (*P*=0.122). The *HBA2*-4314A/G (rs1203833) genotype in parasitemic children was 55.1% AA, 34.2% AG and 10.8% GG with overall allele frequencies of A=0.69 and G=0.31, respectively. The distribution of the *HBA2*-4314A/G genotypes did not depart from Hardy-Weinberg Equilibrium (HWE, c2=2.46, P = 0.117). Likewise, genotypic distribution for the *HBA2*-4314A/G allele between non-SMA and SMA groups was not statistically significant (*P*=0.774). The α-thalassemia deletion was distributed as 42.3% for αα/αα, 38.9% for -α/αα and 18.8% -α/-α respectively. The distribution of the *HBA2*-4314A/G genotypes did not depart from Hardy-Weinberg Equilibrium (HWE, c2 = 3.11, P = 0.083) and were normally distributed between clinical categories (P = 0.234).

Distribution of *HBA2* promoter and α-Thalassemia -3.7kb deletion haplotypes in children with severe malarial anemia (Table 4.3). Haplotype constructs for the two *HBA2* polymorphisms displayed the overall frequencies of: 0.58 for -1789C/-4134A (CA), 0.18 for TG, 0.15 for TA, and 0.09 for CG. Haplotype distribution for children with SMA (n= 261) was 0.51 for CA, 0.15 TG, 0.24 for TA, and 0.10 CG. This distribution frequency was not significantly different for haplotype carriers (non-CA versus CA, P = 0.66; non-TG versus TG, P = 0.28; and non-CG versus CG, P = 0.65)

when compared between non-SMA and SMA groups. However, TA haplotype carriers were significantly less represented in children with SMA (P = 0.01).

Haplotypes between both *HBA2* polymorphisms and the α-Thalassemia -3.7kb deletion were also constructed. Haplotypes were distributed for the -1789C/T and -3.7kb αα/αα combination at: 0.36 -1789 for C/α, 0.30 for C/-α, 0.25 for T/α, and 0.08 for T/-α amongst parasitemic children. When compared between non-SMA and SMA children, frequencies for these haplotypes were not significantly different for 0.23 -1789C/α and 0.23 for C/-α, and 0.21 for T/α. Conversely, T/-α haplotypes were significantly lower in SMA children (0.18; P = 0.05). In parasitemic children, -4134A/G and -3.7kb αα/αα haplotypic combination showed frequency of 0.49 for A/α, 0.24 for A/-α, 0.13 for G/α, and 0.14 G/-α. When categorized into non-SMA and SMA children, these 0.22 for A/α, 0.22 for A/-α, 0.23 for G/α, and 0.24 G/-α, haplotypes were not significantly distributed between groups. Both polymorphisms were combined with the α-Thalassemia -3.7kb deletion to create for CA/α (0.33) CA/-α (0.26), CG/α (0.03), CG/-α (0.04), TA/α (0.12), TA/-α (0.03), TG/α (0.14), and TG/-α (0.05) distributions. These haplotypes were then categorized into non-SMA and SMA and showed the following CA/α (0.49), CA/-α (0.27), CG/α (0.04), CG/-α (0.05), TA/α (0.11), TA/-α (0.01), TG/α (0.12), and TG/-α (0.06) distributions.

Association of *HBA2* genotypes and α-thalassemia deletion variants with parasitemia, SMA, and RPI (Table 4.4). Logistic regression analysis was performed to investigate the association of *HBA2* -1789C/T (rs2974771), *HBA2* -4314A/G (rs1203833), and α-thalassemia -3.7kb deletion with parasitemia, SMA, and RPI . Covariates that could influence the different clinical outcomes such as age, sex, parasite

count, co-infections (bacteremia and HIV-1), and genetic variables (sickle-cell trait and glucose-6 deficiency) were controlled for in the regression model. There was no significant association between *HBA2* -1789 genotypes and either parasitemia or RPI<2. However, carriers of the wild-type (TT) were significantly less likely to develop SMA (41%, OR=0.59 [95%CI, 0.37-0.97], *P*=0.04).

Carriers of the *HBA2* -4314 AG genotype had a 38% increase in the risk of developing parasitemia (OR=1.38 [95%CI, 0.99-1.92], *P*=0.05). However, none of the *HBA2* -4314 genotypes were associated with susceptibility to SMA or inefficient erythropoiesis.

Interestingly, none of the α-thalassemia -3.7kb deletion variants were associated with susceptibility to SMA or inefficient erythropoiesis. There was, however, a non-significant 30% protective advantage against parasitemia for homozygous carriers of the α-thalassemia -3.7kb deletion (-α/-α) (OR=0.70 [95%CI, 0.48-1.03], *P*=0.07).

Association between *HBA2* and α-thalassemia -3.7kb deletion haplotypes and SMA (Table 4.5). After determining the association between genotypes and the various clinical outcomes, the influence of haplotypic variance on SMA and RPI<2 was investigated. Logistic regression modeling of *HBA2* and α-thalassemia -3.7kb deletion haplotypes, controlling for covariates, was performed as described above. Carriers of the *HBA2*-1789C/*HBA2*-4134A (CG) haplotype had a 67% increase in susceptibility to SMA (OR=1.67 [95%CI, 1.21-2.30], *P*<0.01), and a 115% enhanced susceptibility to inefficient erythropoiesis (OR=2.15 [95%CI, 1.26-3.67], *P*<0.01). In contrast, carriers of the *HBA2*-1789C/*HBA2*-4134A (TA) haplotype showed a 38% protection against the

development of SMA (OR=0.62 [95%CI, 0.46-0.82], *P*<0.001), and no significant association with altered erythropoietic responses (OR=1.28 [95%CI, 0.91-1.79], *P*=0.16).

The influence of haplotypic constructs between the two individual HBA2 SNPs and the α-thalassemia -3.7kb deletions was then examined. When the *HBA2*-1789C allele was examined within the context of the α-thalassemia -3.7kb deletions, C/-α carriers showed a 3-fold increased susceptibility to SMA (OR=3.23 [95%CI, 1.64-6.37], *P*<0.01). In contrast, carriers of the *HBA2*-1789T allele and a -3.7kb deletion (T/-α) had a 71% reduction in susceptibility to SMA (OR=0.29 [95%CI, 0.18-0.48], *P*<0.01). None of the haplotypic combinations between the *HBA2*-1789 alleles and -3.7kb deletions had significantly altered susceptibility to altered erythropoiesis. Moreover, none of the haplotypic constructs for *HBA2*-4134 alleles and α-thalassemia -3.7kb deletions showed any significant changes in susceptibility to either SMA or erytropoietic outcomes.

After completion of these analyses, full haplotypic constructs between both HBA2 combinations and α-thalassemia -3.7kb deletions were investigated. These analyses revealed that carriers of the CG alleles with and without -3.7kb deletions were 83% and 81% more likely to develop SMA (OR=1.83 [95%CI, 1.01-3.31], *P*=0.04; and OR=1.81 [95%CI, 1.79-2.77], *P*<0.01, respectively). In contrast, TA carriers with and without -3.7kb deletions showed 27% and 68% reduced risk of developing SMA (OR=0.73 [95%CI, 0.53-0.99], *P*=0.04; and OR=0.32 [95%CI, 0.16-0.63], *P*<0.01, respectively). There was also a 41% reduction in susceptibility to SMA in carriers of the TG alleles in the context of a -3.7kb deletion (TG/-α) that was of borderline significance (OR=0.59 [95%CI, 0.35-1.00], *P*=0.05). Of all of the full constructs examined, only CG/-α carriers

had altered susceptibility to erythropoietic responses (OR=1.84 [95%CI, 1.02-3.65, *P*=0.04).

DISCUSSION

The pathways that connect genetic variants to phenotypic disease outcomes are often complicated and poorly understood. In this study, we further the results from a previous study followed by the candidate gene approach, which has been successful in characterizing determinants of SMA (Ouma 2008, Ouma 2010, Awandare 2009, Ongecha 2011, Anoya 2011, Kempaiah 2012, Okenyo 2013). This method for discovery, aids in the development of information for mechanisms and pathways that influence progression of children to SMA after *P. falciparum* infection. We hypothesized that underlying genetic factors that influence hemoglobin regulation and production contribute to the phenotypic outcomes of SMA. This includes the amelioration of a population specific α-thalassemia 3.7kb deletion, in combination with HBA2 genotypes and haplotypic constructs. Children who were enrolled in this study were evaluated for falciparum, bacterial, and HIV-1 infections, as well as a full clinical evaluation that included tests for common genetic diseases (G6PD, HbS) within the sub-Saharan population. These measures were corrected and controlled for in our regression model due to possible influences on disease outcomes, specifically anemia. For instance, anemia is a hallmark of both HIV-1 infection (Oteino 2006, Bailey 2015) and Sickle-cell disease (Chakravorty 2015). Hypoglycemia, respiratory stress, and hyperparasitemia also contribute to falciparum related disease, but severe anemia in children is often the result in holoendemic infection regions such as western Kenya (Perkins 2011 review) where cerebral malaria is a rare occurrence (Ongecha 2006 Novelli 2010).

This study examined the role of *HBA2* SNPs (-1789C/T, rs2974771 and -4314A/G, rs1203833) in combination with the $-\alpha^{3.7}$thalassemia deletion, and their

individual and combined influence on severe malarial anemia. *HBA2* functionally codes for one of two alpha globin chain in hemoglobin and is expressed 2-3 fold higher than the *HBA1* counterpart (Liebhaber 1986). As such, *HBA2* gene expression is paramount to proper hemoglobin synthesis. Furthermore, polymorphic variation within the coding, as well as promoter regions of *HBA2* has been characterized within α-thalassemia disease (Megawati 2014, Qadah 2014, Hamid 2014) where anemia is a key clinical finding. Megawati et. al., investigated a novel mutation within the coding region of *HBA2* that was found prenatally in a Sudanese family that had a history of α-thalassemia (Megawati 2014). Another study characterized three *HBA2* promoter variants (*HBA2* -59C>T,-81C>A, and -91G>A) in transcription and translation in α-thalassemia patients. Investigators found that *in vivo*, *HBA2* -59C>T and -91G>A, mutations were significantly associated with HBA2 transcript levels (Qadah 2014). A novel nonsense mutation (*HBA2* c.382A>T) was found in a smaller study (n=4) of α-thalassemia patients in Iran. This study found that carriers the nonsense mutation had microcytic anemia and moderate hypochromia. Furthermore, these individuals were unrelated, but were of the same ethnic group (Hamid 2014). Previous findings by Ya et al, have shown that carriage of rs2974771 (*HBA2* -4314A/G) prenatally can be potentially used to diagnose α-thalassemia (Ya 2011).

The manifestations of falciparum malaria are multifaceted with disease severity influenced by many factors (e.g., co-infections, age, endemicity, ethnicity, nutrition, and genetic variation (Perkins 2011 review).

Several studies have attempted to bridge the understanding of malarial infection and α-thalassemia disease (Opoku-Okrah 2013, Mockenhaupt 2004, Williams 2005,

Allen 2007, Evenvold 2008). This study was designed to help further unravel the complexity of hemoglobinopathies by using our discovery of novel variation in HBA2 in the context of α-thalassemia for examination of the influence of the individual and combined variants on susceptibility to SMA. Protection against severe malaria disease is a characteristic that α-thalassemia has been associated with in several populations worldwide (Wambua 2006, May 2007, Williams 2005, Allen 1996, Mockenhaupt 2004, Fowkes 2008). The α-thalassemia genotype for our cohort was distributed as 43% normal (αα/αα), 37% heterozygous (-α/αα), and 20% homozygous deletions (-α/-α) (*P*=0.016). A study in 382 children living off the coast of Kenya showed similar distributions [32% (αα/αα), 49% (-α/αα), and 19% (-α/-α)] for the α-thalassemia trait (Wambua 2006). Williams et. al., described a large study for the same Kenyan coastal cohort where homozygous and heterozygous carriers (-α/-α and -α/αα) were associated with reduced risk for severe disease (Williams 2005). In contrast to the study presented, Wambua and Williams et. al., showed a significant association with α-thalassemia and severe malaria disease outcomes (Wambua 2006). We found 30% protection against overall parasitemia (**Table 4**) in children with the -$\alpha^{3.7}$kb deletion (-α/-α) that was borderline significance (*P*=0.07), but no association with the -$\alpha^{3.7}$kb and susceptibility to SMA was discovered.

In a large prospective study of Thai children (<120 mos.) infected with falciparum malaria, there was a strong link between severe anemia and cerebral malaria with (-α/αα and -α/-α) carriers (May 2007). The investigators suggested that the anemic association with α-thalassemia carriers seen in their study was due to the hypoxic effects of abnormally low hemoglobin that is often countered by a significant increase in erythrocyte overcompensation. Another study that examined the anemic findings in a

Papau New Guinea pediatric population with malaria found significantly reduced Mean Cell Volume (MCV), Mean Cell Hemoglobin (MCH), and Hemoglobin (Hb) (Fowkes 2008). Our study showed similar findings overall when examining these measures in α-thalassemia carriers alone. We extended previous findings by others in our study by examining an inefficient erythropoietic response (RPI<2) as a categorical outcome variable in the logistic regression models. When examining polymorphic variation within the promoter of *HBA2*, children with homozygous wild-type haplotypic constructs (HBA2-1789/-4134 CG) are significantly more likely to get SMA, whereas their homozygous polymorphic counterparts (HBA2-1789/-4134 TA) are significantly less likely to develop the disease. Furthermore, we discovered that children who are carriers of the homozygous -1789 C/T (C) allele and $-\alpha^{3.7}$kb deletion (C/-α) are 3-times more likely to develop SMA. In addition, when a child is also a carrier of the -4134 G/A (G) allele and $-\alpha^{3.7}$kb deletion (CG/-α), they are more susceptible to SMA and have a 2-fold greater risk of an inefficient erythropoietic response. Conversely, those who are carriers of the polymorphic TA/-α haplotype are protected against SMA, but have (2-fold) reduced erythroid response.

In conclusion, anemia induced by falciparum malaria can result from both dysregulation in erythroid function and hemolysis of RBCs through parasite invasion (Perkins Review 2011, Menendez 2000). Furthermore, anemia in the context of α-thalassemia diseases has been shown in several studies. Data presented here demonstrate that α-thalassemia, in concert with polymorphic variation within the *HBA2* promoter, significantly influence severe malaria disease outcomes, specifically anemia and erythropoietic responses. Results from this study further illustrate the impact of

hemoglobinopathies and polymorphic variation within genes that regulate hemoglobin on susceptibility to malaria and the differing nature of these variants on disease outcomes in regions with holoendemic *P. falciparum* transmission.

COMPETING INTERESTS

None of the authors of the manuscript have a personal or financial competing interest that influences the interpretation of data or presentation of any information contained herein.

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TABLE AND FIGURE LEGENDS

Table 4.1. Demographic, laboratory and clinical measures of parasitemic children enrolled in the study.

Data are represented as median (IQR). Children with acute malaria at the Siaya District Hospital were stratified into two groups: non-severe malarial anemia (Non-SMA; Hb≥5.0 g/dL) and severe malarial anemia (SMA; Hb<5.0 g/dL). [a] Differences in the proportion of gender was determined using Chi-square test. [b] Differences in the median ages, parasitemia, red blood cell (RBC) counts, hemoglobin levels and reticulocyte production index (RPI) between the groups were determined using Mann-Whitney U test.

Table 4.2. Distribution of *HBA2* genotypes and α-Thalassemia deletions in children with malaria.

Data are shown as proportion (n,%) of children into aparasitemic, parasitemic, non-SMA and SMA groups. Children were categorized based on *P. falciparum* malaria infection and according to SMA classification with SMA status (Hb < 5.0 g/dL) and non-SMA status (Hb ≥ 5.0g/dL). Chi-Square test was used to determine differences in proportions and significance was set at P<0.05.

Table 4.3. Distribution of *HBA2* promoter and α-Thalassemia deletions haplotypes in children with severe malarial anemia.

Data are shown as proportion (n,%) of children into aparasitemic, parasitemic, non-SMA and SMA groups. Children were categorized based on *P. falciparum* malaria infection and according to SMA classification with SMA status (Hb < 5.0 g/dL) and non-SMA

status (Hb ≥ 5.0g/dL). Children were further stratified according to their HBA2 and α-Thalassemia haplotypes where chi-Square test was used to determine differences in proportions and significance was set at P<0.05.

Table 4.4. Figure 4.1 Cross-sectional association of *HBA2* -1789C/T, *HBA2* -4314A/G and α-Thalassemia deletion haplotypes with SMA and RPI.

Binary logistic regression analyses were conducted in parasitemic children (*n*=1,314) to determine relationship between *HBA2* -1789C/T, *HBA2* -4314A/G and α-Thalassemia haplotypes and SMA **(A)** and and RPI **(B)** . For each test, the age, gender, HIV status, bacteremia, sickle cell trait (HbAS), and G6PD deficiency status were controlled for in the analyses. Presented as odds ratio (OR; 95% confidence interval), with *P*<0.05 considered statistically significant.

TABLES AND FIGURES

Table 4.1. Demographic, laboratory and clinical measures of parasitemic children enrolled in the study.

Characteristic	Non-SMA (Hb≥5.0 g/dL)	SMA (Hb<5.0 g/dL)	*P*-value
Number, *n*	1053	261	N/A
Gender, %F:M	51:49	47:53	0.137[a]
Age, months	12.8 (10.5)	10.0 (10.5)	**<0.001**[b]
Admission Temperature, °C	38.0 (2.0)	37.5 (1.0)	**0.026**
Hematological Indices			
Hemoglobin (g/dL)	7.6 (2.9)	4.2 (1.0)	**<0.001**[b]
Red Blood Cell count, (10^{12}/L)	3.7 (1.5)	1.8 (0.7)	**<0.001**[b]
Hematocrit, (%)	25.1 (9.1)	13.9 (3.6)	**<0.001**[b]
White Blood Cell count (10^3/µL)	11.7 (6.4)	15.0 (9.8)	**<0.001**[b]
Lymphocytes (10^3/µL)	46.4 (21.0)	50.9 (16.7)	**0.001**[b]
Monocytes (10^3/µL)	7.9 (5.4)	9.0 (6.5)	**<0.001**[b]
Parasitological Indices			
Parasitemia, MPS/µL	28,902 (79,044)	22,267 (63,516)	**0.003**[b]
High Density Parasitemia (>10,000/µL) , n (%)	731 (79)	168 (64)	**0.030**[a]
Reticulocyte Indices			
Absolute Reticulocyte Number (ARN)	1.4 (2.0)	2.8 (4.0)	**0.007**
Reticulocyte Production Index (RPI)	1.2 (1.6)	1.3 (2.1)	**<0.001**[b]
RPI<2.0,n (%)	867 (86)	223 (91)	**0.024**[a]
Genetic Variants			
Sickle Cell Trait, n (%)	173 (16.4)	23 (8.9)	**0.025**[a]
Glucose-6-phosphate deficiency, n (%)	38 (4.2)	6 (2.9)	0.143[a]
Alpha-Thalassemia Trait, n (%)	168 (22.5)	45 (19.2)	**<0.001**[a]
Co-infections			
HIV-1, n (%)	31 (3.9)	13 (5.2)	**<0.001**[a]
Bacteremia , n (%)	98 (9.3)	21 (8.1)	0.225[a]

Table 4.2. Distribution of HBA2 genotypes and α-Thalassemia deletions in children with malaria.

Genotype	Aparasitemic	non-SMA	SMA	Total	*P*-value
		***HBA2* -4314 A>G**			0.176
AA, *n* (%)	176 (60.9)	544 (55.2)	146 (54.7)	866 (56.2)	
AG, *n* (%)	77 (26.6)	334 (33.9)	94 (35.2)	505 (32.7)	
GG, *n* (%)	36 (12.5)	108 (10.9)	27 (10.1)	171 (11.1)	
		***HBA2* -1789 C>T**			0.408
CC, *n* (%)	140 (45.1)	444 (42.9)	134 (47.7)	718 (44.1)	
CT, *n* (%)	109 (35.0)	409 (39.5)	101 (35.9)	619 (38.0)	
TT, *n* (%)	62 (19.9)	183 (17.7)	46 (16.4)	291 (17.9)	
		α-Thalassemia			
α/α, *n* (%)	113 (43.8)	382 (43.3)	98 (39.0)	593 (42.6)	**0.016**
α/-α, *n* (%)	77 (29.8)	337 (38.2)	104 (41.4)	518 (37.2)	
-α/-α, *n* (%)	68 (26.4)	164 (18.6)	49 (19.5)	281 (20.2)	

Table 4.3. Distribution of *HBA2* promoter and α-Thalassemia deletions haplotypes in children with severe malarial anemia.

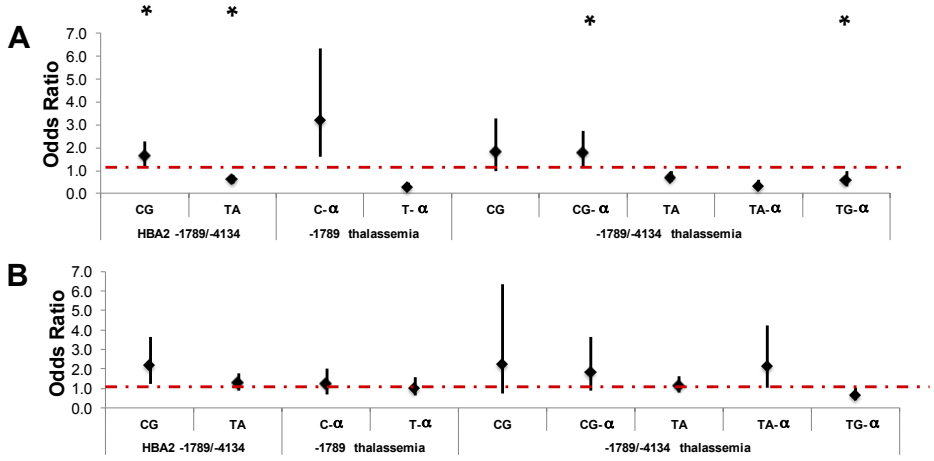
Haplotypes	Carriers Frequency		non-Carriers Frequency		
***HBA2* -1789C/-4134A**	non-SMA	SMA	non-SMA	SMA	*P*-value
CA	0.78	0.22	0.79	0.21	0.66
CG	0.76	0.24	0.79	0.21	0.28
TA	0.82	0.18	0.77	0.23	**0.01**
TG	0.79	0.21	0.78	0.22	0.65
***HBA2* -1789C/α-Thalassemia**					
C/α	0.77	0.23	0.78	0.22	0.33
C/-α	0.77	0.23	0.78	0.22	0.33
T/α	0.79	0.21	0.76	0.24	0.15
T/-α	0.82	0.18	0.77	0.23	0.05
***HBA2* -4134A/α-Thalassemia**					
A/α	0.77	0.23	0.78	0.22	0.51
A/-α	0.77	0.23	0.78	0.22	0.46
G/α	0.80	0.20	0.77	0.23	0.12
G/-α	0.77	0.23	0.78	0.22	0.68
***HBA2* -1789C/-4134A/α-Thalassemia**					
CA/α	0.77	0.23	0.78	0.22	0.49
CA/-α	0.76	0.24	0.78	0.22	0.27
CG/α	0.74	0.26	0.77	0.23	0.54
CG/-α	0.74	0.26	0.78	0.22	0.27
TA/α	0.79	0.21	0.77	0.23	0.36
TA/-α	0.89	0.11	0.77	0.23	**0.00**
TG/α	0.80	0.20	0.77	0.23	0.11
TG/-α	0.75	0.25	0.78	0.22	0.43

Table 4.4 Cross-sectional association of *HBA2* -1789C/T, *HBA2* -4314A/G and α-Thalassemia deletion haplotypes with SMA and RPI.

Genotype	Parasitemia Odds Ratio	95% CI	*P* value	SMA Odds Ratio	95% CI	*P* value	RPI Odds Ratio	95% CI	*P* value
	HBA2 -1789 C>T			**HBA2 -1789 C>T**			**HBA2 -1789 C>T**		
CC	1.00	-	-	1.00	-	-	1.00	-	-
CT	1.08	0.79-1.47	0.63	0.83	0.59-1.16	0.27	1.09	0.73-1.64	0.68
TT	1.01	0.68-1.51	0.95	0.59	0.37-0.97	**0.04**	0.68	0.42-1.10	0.114
	HBA2 -4314 A>G			**HBA2 -4314 A>G**			**HBA2 -4314 A>G**		
AA	1.00	-	-	1.00	-	-	1.00	-	-
AG	1.38	0.99-1.92	**0.053**	1.13	0.81-1.59	0.48	1.12	0.74-1.69	0.59
GG	1.01	0.63-1.61	0.98	0.98	0.57-1.68	0.93	0.84	0.46-1.51	0.56
	α-Thalassemia			**α-Thalassemia**			**α-Thalassemia**		
α/α	1.00	-	-	1.00	-	-	1.00	-	-
α/-α	1.28	0.89-1.83	0.177	0.84	0.58-1.21	0.34	0.89	0.58-1.39	0.63
-α/-α	0.70	0.48-1.03	**0.069**	0.95	0.60-1.50	0.83	0.83	0.49-1.40	0.49

Table 4.4/Figure 4.1 Cross-sectional association of *HBA2* -1789C/T, *HBA2* -4314A/G and α-Thalassemia deletion haplotypes with SMA and RPI.

Haplotypes	SMA Odds Ratio	95% CI	*P* value	RPI Odds Ratio	95% CI	*P* value
HBA2 -1789C/-4134A						
CA	1.17	0.89-1.54	0.24	0.96	0.70-1.31	0.78
CG	1.67	1.21-2.30	0.0017	2.15	1.26-3.67	<0.01
TA	0.62	0.46-0.82	0.001	1.28	0.91-1.79	0.16
TG	0.87	0.67-1.11	0.26	0.86	0.64-1.14	0.29
HBA2 -1789C/α-Thalassemia						
C/α	1.30	0.96-1.77	0.09	1.05	0.74-1.49	0.79
C/-α	3.23	1.64-6.37	<0.01	1.22	0.73-2.02	0.45
T/α	0.80	0.62-1.04	0.08	1.00	0.73-1.36	0.99
T/-α	0.29	0.18-0.48	<0.01	1.01	0.64-1.58	0.98
HBA2 -4134A/α-Thalassemia						
A/α	1.09	0.70-1.71	0.69	1.05	0.63-1.76	0.84
A/-α	0.98	0.69-1.39	0.93	1.05	0.70-1.57	0.81
G/α	0.93	0.68-1.26	0.61	1.35	0.92-1.98	0.13
G/-α	1.05	0.76-1.44	0.77	1.08	0.75-1.56	0.68
HBA2 -1789C/-4134A/α-Thalassemia						
CA/α	1.11	0.83-1.49	0.47	0.91	0.64-1.28	0.58
CA/-α	1.36	0.93-2.00	0.12	0.83	0.54-1.29	0.41
CG/α	1.83	1.01-3.31	0.04	2.22	0.78-6.36	0.14
CG/-α	1.81	1.79-2.77	<0.01	1.84	1.02-3.65	0.04
TA/α	0.73	0.53-0.99	0.04	1.15	0.79-1.66	0.46
TA/-α	0.32	0.16-0.63	<0.01	2.13	0.97-4.26	0.08
TG/α	0.87	0.66-1.15	0.33	0.95	0.68-1.32	0.76
TG/-α	0.59	0.35-1.00	0.05	0.65	0.39-1.06	0.08



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5.0 CHAPTER FIVE: RESULTS OF SPECIFIC AIM 3

Effect of *Plasmodium falciparum* patient serum, MIG, IP-10, and IFN-γ on erythroid lineage development

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ABSTRACT

The pathogenesis of severe malarial anemia (SMA) occurs as the result of increased red blood cell (RBC) destruction and a decrease in RBC production and ultimately chronic anemia. Previous studies from our laboratory have shown that dysregulation in circulating pro- and anti-inflammatory mediators influence the development of SMA. In this current study we postulate that malarial anemia pathogenesis is driven by key inflammatory mediators such as (recombinant human) rhMIG, rhIP-10, and rhIFN-γ as well as unknown factors in circulating in the serum from SMA category children. To test this hypothesis we used a CD34+ hematopoietic stem cell *in vitro* model for investigating erythroid development in which cells were either contidioned with serum from pediatric patients (non-SMA and SMA) or rhMIG, rhIP-10, and rhIFN-γ. .Examination of circulating inflammatory meditators (25-plex) in serum before the treatment of the *in vitro* model with serum showed a significant reduction in MIG and IP-10 in children with SMA. Results from the erythroid model indicated that treatment of erythroid cells with rhMIG, rhIP-10, and rhIFN-γ as well as conditioning cells with pooled serum from pediatric SMA patients (n=10) reduced the relative number of stage specific markers (CD71+/CD45+ and CD71+/CD235a+) in the erythrocytic model. These results reveal that complex nature of inefficient erythropoietic response in children might in part be caused by inflammatory mediators found in circulation and serum.

BACKGROUND

Severe malaria anemia (SMA, Hb<5.0 g/dL) is a hallmark of *Plasmodium Falciparum* infection in sub-Saharan Africa (Perkins 2011). In this region, mortality and morbidly of malaria infection has the highest health burden in young children. The onset of malaria pathogenesis begins at the erythrocytic stage of the parasite infection where severe disease can manifest as cerebral malaria or anemia (Haldar 2009). Malarial anemia is multifaceted where immune as well as non-immune mechanisms work in concert to drive pathogenesis. Altered erythropoietic responses and hemolysis of parasitized and non-parasitized erythrocytes by innate immune cells, along with cytokine-driven inflammation are factors that contribute to malarial anemia (Were 2006, Keller 2009). Several investigations have linked inflammatory mediators to dyserythropoiesis (Chasis 2006, Awandare 2010) where the erythroid lineage development is disrupted or impaired. For example, studies have shown that increased production of IL-6 alters hepcidin expression, which results in fewer iron stores accessible for erythrocyte formation (Wang 2011). The proinflammatory cytokine, TNF-α, has been shown to regulate the GATA-1 erythrocytic transcription factor (Singh 2000), and mitigate parasite replication during active infection (Kwiatkowski 1989). Another proinflammatory cytokine, IFN-γ, provides protective effects against malaria in children and in experimental human *P. falciparum* infections (D'Ombrain 2008, Pombo 2002). However, in severe disease states, over-expression of such proinflammatory cytokines can lead to hyper-activation of the innate immune response, stimulating inflammation in the peripheral tissue, damaging bone marrow, and eventually causing loss of functional erythrocytic cells (Clark 2003). The chemoattractant cytokines (Chemokines), MIG and IP-10 play a vital role in the IFN-

γ proinflammatory pathway. They are both secreted by IFN-γ activated neutrophils, monocytes, fibroblasts and endothelial cells, and act as a positive feedback mechanism for inflammation (Jinquan 2000, Rossi 2000, Taub 1999).

Previous examinations have shown that rhMIG negatively influences erythroid progenitors from primary bone marrow cultures (Schwartz 1997). A study by Dickinson-Copeland *et al* (2015) indicated a strong relationship between suppression in erythroid cells in older Ghanaian children with malaria and increased production of circulating IP10 through heme-driven apoptosis. Several studies from our laboratories and others have shown a hemozoin-driven model of cytokine/chemokine dysregulation that results in erythropoietic suppression in children with the SMA phenotype (Awandare 2007 2010, Cascals-Pascual 2006). Additional studies have also shown that inflammatory mediator activation through phagocytosis of hemozoin results in an anemic state (Ongecha 2008, Were 2009).).

The development of an *in vitro* erythropoietic model by our group (Awandare 2010) avoids the ethical and practical difficulties of obtaining bone-marrow aspirates from children to isolate erythroid progenitor cells. The model relies on isolation and culture of CD34+ cells from peripheral blood from malaria-naïve donors, followed by the induction of erythroid progenitors with soluble mediators that promote erythroid development. Studies conducted here utilized the *in vitro* model of erythropoiesis to examine the influence of chemokines (MIG and IP-10), cytokines (IFN-γ), patient serum isolated from children with malaria (non-SMA and SMA).

MATERIALS AND METHODS

Clinical Measures

Study participants. Children aged 3-36 mos. (n=20) presenting with *P. falciparum* malaria at Siaya County Hospital were recruited for the study. Children with non-*falciparum* malaria species, cerebral malaria, co-infections (HIV-1 or bacteremia), sickle-cell anemia, and α-thalassemia deletions and glucose-6-phosphate dehydrogenase deficiency were not included in the study. The study site and population has been described in detail by (Ong'echa 2006).

Sample collection and laboratory evaluation. 1-3 mL of venous blood was obtained from each child enrolled in the study where hematological measures, including a complete blood count (CBC) were determined. A fraction of blood was also centrifuged to isolate serum, which was snap frozen and stored at -80^{o}C. Parasitemia was determined by thin and thick blood smears prepared from finger/heal prick blood (~100uL). Blood smears were then stained for microscopy with Giemsa reagent (Sigma), trophozoites were counted against 300 leukocytes, and parasite densities estimated using the WBC counts from the CBC. Sample collection and laboratory evaluation procedures are previously described here (Novelli 2010).

Determination of circulating cytokines and chemokines. Plasma samples obtained from venous blood were stored at -70°C until use. Soluble mediator concentrations were determined using the Human Cytokine Twenty-Five-Plex Antibody Bead Kit (Invitrogen International), in accordance with the manufacturer's instructions. Plates were read on a Luminex 100 system (Luminex) and analyzed using Bio-Plex Manager software (version

IS 2.3; Bio-Rad Laboratories).

CD34+ Cell Isolation. CD34+ cells were enriched from peripheral blood mononuclear cells (PBMCs) that were isolated from whole blood leukopaks (500mL) from malaria-naïve donors using protocols approved by the University of New Mexico Institutional Review Board. PBMCs were separated from whole blood using ficoll-plaque (GE Healthcare Life Sciences) gradient separation. Cells were washed and suspended in phosphate buffered saline solution (PBS, 0.5% bovine serum albumin, 0.6% anticoagulant citrate dextrose). CD34+ progenitor cells were enriched from PBMC populations using anti-CD34 magnetic microbeads and magnetic separator columns per the manufacturer's recommendations (Miltenyi Biotec, Auburn, CA). CD34+ purity was determined to be >90% after isolation.

Erythroid progenitor cell lineage growth media. Basic cell culture media for CD34+ cells used in this study was based on previous methods (Awandare 2010, Freyssinier 1999, Neildez-Nguyen 2002) and contained Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen), 15% BIT Serum Substitute (Stem Cell Technologies), 100U/mL penicillin/streptomycin (Sigma), and 2 mM L-glutamine. Secondary culture contained basic media 10 ng/mL interluken-3 (IL-3), 10 ng/mL IL-6, and 100 ng/mL stem cell factor (SCF) (R&D Systems). Erythrocytic lineage induction media was added at day 3 and contained 1.0 U/mL erythropoietin (R&D Systems). CD34+ cells were plated into 12-well plates at $1.5x10^5$ cells/well and incubated at 37° C with 5% CO_2, 5% O_2 (balanced with N_2) in a humidified chamber. Viable cell counts were determined by trypan blue staining.

Serum conditioned media and CD34+ cell Treatment. Serum was pooled from children with non-SMA (n=10) and SMA (n=10) and then added to secondary media (described above) at 5% of the total serum concentration. Pooled serum in media was then added to CD34+ cells at day 3 for the respective conditions.

Cell Viability. Cell viability was determined using the CellTiter Glo ® (Promega) assay per the manufactures recommended methods and protocol. 50,000 cells from CD34+ assayed conditions were added to prepared CellTiter-Glo® Reagent in 96-well plates (duplicate) and rocked gently at room temperature for 15 minutes to induce cell lysis. Luminescence counts per second (CPS) for samples was determined using the PerkinElmer^{TM} Victor 3 1420 Multilabel Counter plate reader and data were captured using Wallac 1420 Workstation (Perkin Elmer, MA USA).

Immunophenotyping. At days 7, 10, and 14, cell surface markers were stained using conjugated antibodies [CD34 (MACs), CD45, CD71, and CD235a [Glycophorin A (GPA); Invitrogen] and then measured by flow cytometry. Cells were also concurrently stained for viability using 7-amino actinomycin (7AAD; eBioscience) DNA antibody. Cells were suspended in 0.5% BSA/PBS and incubated with respective antibodies for 25 minutes at 4°C. Following incubation, cells were washed twice and fixed in 1%PFA/PBS for flow cytometric capture. Populations were measured using the BD Fortessa flow cytometer and analyses were conducted using Flowjo (v7) software.

Statistical analyses. Non-parametric data were compared by Chi-square test and Mann-Whitney U test between treatment and disease categories for the different conditions. Data presented in this study for cell culture experiments are representative of three

independent experiments conducted in duplicate. Treatment conditions (EPO, non-SMA, and SMA) were compared and tested for statistical significance using analysis of variance (ANOVA) and students T-test, and normalized relative to EPO treatments. Statistical analyses for serum samples from children were performed using SPSS software (version 19.0), whereas cell culture experiments were amalyzed using GraphPad Prism 5 software. Statistical significance was set at as $P<0.05$.

RESULTS

Clinical characteristics of the study participants (Table 5.1)

Children (n=20) with active *P. falciparum* infections were stratified into non-SMA (Hb<5.0 g/dL, n=10) and SMA (Hb>5.0 g/dL, n=10) based on Hb levels. The clinical characteristics of the study participants from whom serum was isolated are summarized in **Table 1**. There was a non-significant difference in gender distribution with slightly more females than males in the two groups (P=0.085). Children with SMA were significantly younger (P=0.016), and based on the grouping strategy had lower hemoglobin (Hb), red blood cell (RBC) count, and hematocrit. The white blood cell (WBC), lymphocyte and monocyte counts were elevated in children with SMA ($P<0.001$, $P<0.005$, and $P<0.001$). Peripheral parasitic burden was lower in the non-SMA group ($P< 0.01$). The reticulocyte production index (RPI) was lower in children with SMA ($P<0.001$), whereas an RPI<2 (a corrected indicator of inefficient erythrocyte production) was higher in this group ($P<0.001$).

Circulating inflammatory mediators (25-plex) (Figure 5.1)

Levels of circulating (plasma) soluble mediators for children (non-SMA and SMA) who donated serum were examined using a human cytokine 25-plex-bead kit. From the panel of 25 mediators, four proteins were significantly different across the groups. IL-12 [non-SMA Median: 443.23, IQR: 550.23; SMA Median: 239.26, IQR: 301.23, (P=0.009)], IL-10 [non-SMA Median: 790.48, IQR: 921.02; SMA Median: 185.75, IQR: 207.37, (P=0.004)], MIG [non-SMA Median: 221.41, IQR: 285.26; SMA Median: 109.62, IQR: 125.26, (P=0.007)], and IP-10 [non-SMA Median: 708.23, IQR: 810.97; SMA Median: 327.82, IQR: 365.24, (P<0.001)] were all down-regulated in children with SMA.

Gating Strategy (Figure 5.2)

Forward (FSC) and side (SSC) scatter plots were generated for CD34+ populations. Viable cell populations were selected by gating for cell debris (or doublets) by selecting events that were higher in FSC and lower in SSC versus non-viable cells found in the lower portions of both FSC, and some portions of higher SSC. Thus, viable cell populations were selected based on higher FSC and lower SSC. To verify that live events were (indeed) captured, a viability stain, 7AAD, was added to flow cytometry panels (see methods). This particular marker binds free DNA which generated by lysed or dead cells, thus, leaving live (viable) cell populations unstained. The final selection strategy for live populations involved gating off of $7AAD^+$ cells with negative selection. A typical representation of these experiments is shown in **Figure 5.3** in which 62.2% of the live population was selected.

Effects of SMA and non-SMA serum on erythroid cell populations (Figures 5.3 and 5.4)

Children with SMA have been shown to have altered erythropoiesis by several mechanisms Wickramasinghe 2000, Nussenblatt 2001, Fowkes 2008, Awandare 2011, Anyona 2012). To further our understanding of the erythropoietic process, the *in vitro* model was exposed to pooled serum from children with non-SMA (n=10) and SMA (n=10). Erythroid development was characterized by examining cell surface markers as defined in Figure 5.3. Specifically, CD45, CD71, and CD235a were examined. CD45 (leukocyte common antigen) is a glycoprotein that is present on immature erythroid cells and is lost with progression to later lineages. CD71 (transferrin receptor) regulates the uptake of transferrin-iron and gradually increases throughout erythroid lineage until it declines after D12. CD235a (glycophorin a receptor) is an erythrocytic membrane protein that increases around D10 where it continues to rise, indicating later-stage erythroid lineages. Cells were treated with IL-3, IL-6, and SCF at D0 to induce erythroid lineage development. EPO was then added on D3, and every three days thereafter, to enhance lineage development. In addition, the pooled serum (non-SMA and SMA) and control serum were added to the cultures at D3. The first measurement of erythroid lineage markers was performed by flow cytometry on D7. Experimental conditions were compared to the control serum with EPO. As shown in Figure 5.4, the CD71+/CD45+ cell population was significantly lower in cultures treated with serum from children with SMA on D7 ($P<0.05$), and significantly lower for both non-SMA and SMA serum on D1 and 14 compared to the EPO control ($P<0.05$). Cells expressing CD71+/CD235a+ were significantly lower in cultures treated with serum from children with SMA at all time

points, and higher, but still significantly reduced in response non-SMA serum relative to EPO ($P<0.05$).

Effects of inflammatory mediators on erythroid cell populations (Figure 5.5)

Previous investigations have shown that pro-inflammatory mediators influence both the erythropoietic response and hemolysis which culminate in the development of anemia. As such, we investigated the influence of three pro-inflammatory mediators, MIG, IP-10, and IFN-γ to determine their impact on erythroid development.

Consistent with the experiments outlined above with serum from children with malarial anemia, cultures were treated MIG, IP-10, and IFN-γ on D3, and erythroid cell development was measured with stage-specific markers until D14.

CD71+/CD45+ populations were normally distributed (39-43%) for all treatment conditions when normalized and compared to EPO. At D10, IP-10 treated cells were significantly lower (76%) when compared to IFN-γ (90%), MIG (91%) and EPO conditions ($P< 0.05$). A similar result was seen at D14, even though IP-10 treated cells recovered to about 80%. In addition, CD71+/CD235+ populations were examined after pro-inflammatory mediator treatments. IP-10 and MIG treated cells (36-40%) were significantly lower at D7 when compared the IFN-γ treatment, as well as to normalized EPO control cells. MIG treated cells were 90% CD71+/CD235+ at D10 when compared to IP-10 (78%) and IFN-γ treatments. By D14, IFN-γ, IP-10, and MIG conditions were 75%, 80%, and 84% for CD71+/CD235+ cells when compared to the EPO treated control condition ($P<0.05$).

Effects of SMA and non-SMA serum conditioned media and inflammatory mediators on erythroid cell viability (Figure 5.6)

Erythroid lineage cells were examined and monitored at D7, D10 and D14 for viability after disease category serum treatment as well as inflammatory mediator stimulation independently. Cells grown in the presence of SMA serum were significantly less viable (60%: D7, 48%: D10, 18%: D14, $P<0.05$) at all three time-points examined, and had a marked decrease in viability by D14 when compared to non-SMA conditions (80%: D7, 82%: D10, 61%: D14, $P<0.05$) and both were also significantly less viable throughout the course when compared to EPO control cells. At D7, mediatory treated cells (IFN-γ, MIG, IP-10) had a marked decrease (71%, 75%, 67% respectively) in viability but increased in viability by D10 (91%, 88%, 80%). This increase was limited though and cell viability was reduced (79%, 87%, 78%) by D14.

DISCUSSION

Erythropoietic dysfunction during malarial infection in young children leads to a severe anemic state. We and others have described several pathways in which dyserythropoiesis is a key aspect of SMA pathogenesis (Were 2006, Awandare 2011, Keller 2009 OTHERS). While there are still significant gaps in our understanding of SMA pathogenesis, it is clear that immunity plays a vital role in directly regulating erythropoietic functions (Were 2006, Awandare 2011, Keller 2009, Lyke 2003, Anyona 2011, Ong'echa 2011, Ouma 2010). To overcome the difficulties of understanding the basic aspects of erythropoiesis, we have developed a novel model of erythroid lineage development in which we stimulate CD34+ cells isolated from peripheral blood with specific grow factors (Awandare 2011, Freyssinier 1999, Neildez-Nguyen 2002). This model of erythropoiesis is advantageous in that we are able to use readily available immunophenotyping techniques, as well as viability assays to monitor lineage without the necessity of relying on bone marrow aspirates.

Previously, our investigations and others have shown the influence of inflammatory mediators such as TNF-α, IL-12, Nitric Oxide (NO), MIF, and IFN-γ on severe disease outcomes in children with malaria (Singh 2000, Keller 2004, Awandare 2006, Ongecha 2011, Clark 2003, Lyke 2004). Furthermore, increased chemokines in plasma such as IL-8, MIP1-α, and RANTES have been linked to malarial pathogenesis. (Were 2006, Kremsner 1995, Burgmann 1995, Lyke 2004). As such, the mediators that are present within serum and/or plasma appear to directly contribute to disease outcomes. Based on this rationale, we utilized pooled serum at physiological concentrations from two disease categories (children with SMA and non-SMA) in our model system to

determine the impact on soluble mediators on the erythropoietic response with immunophenotyping and cell viability assays.

The clinical aspects of the serum samples that were pooled from the study participants can be found in **Table 1**. Children from the non-SMA group had significantly different indices of anemia ($P<0.01$), characterized by better hematological indices and more efficient erythropoiesis (RPI>2). Consistent with the selection criteria, results from the *in vitro* model showed a significantly delayed erythropoiesis in cultures treated with serum from children with SMA that was revealed upon measurement of CD235a and CD71 at 7, 10 and 14 days after induction of erythroid development. Moreover, there was also a loss in the leukocyte antigen marker, CD45, further confirming a delay in erythrocyte maturation. It is important to note that both non-SMA and SMA serum displayed maturational delays compared to the mock (EPO only control) cell populations. However, the results were more pronounced in children with SMA.

Additional experiments under the same conditions were used to investigate the importance of IFN-γ, IP10, and MIG on erythroid development. When cells were treated with IFN-γ, IP10, and MIG, only cultures treated with IP10 recovered by D14. This result in the context of reduced IP10 in the pooled serum from children with SMA suggests that reduced IP10 may be central to the erythoid development deficiencies observed in this group of children.

We also examined the viability of cells at different time points in the erythrocyte model. These experiments demonstrated that cultures treated with SMA serum, IFN-γ, IP10, and MIG treated cells were significantly less viable through the entire time course. This signifies that serum from severely ill children, as well as specific inflammatory

mediators can cause cell maturation dysfunction and delays in maturation as indicated through immunophenotyping. Several previous reports suggest that chemokines, specifically MIG and IP10, alter erythrocyte maturation and influence the pathogenesis of severe malaria (Schawartz 1997, Dickenson-Copeland 2015, Ongecha 2011). The key proinflammatory type-1 cytokine, IFN-γ, acts directly on both MIG and IP10 signaling pathways and has been characterized as a major cytokine in immunopathogenesis (D'Ombrain 2008, Pombo 2002). Results from this study suggest that the influence of these pathways is also important in erythroid development. Here, we demonstrate that serum from children with SMA, as well as specific mediators known to influence disease pathogenesis (i.e., MIG, IP10, and IFN-γ) impact on erythroid cell maturation and cell viability. Taken together, results presented here support our hypotheses that inefficient erythropoietic responses in children with malaria are associated with inflammatory mediators found in circulation during an active infection.

COMPETING INTERESTS

None of the authors of the manuscript have a personal or financial competing interest that influences the interpretation of data or presentation of any information contained herein.

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TABLE AND LEGENDS

Table 5.1. Clinical characteristics of the Study Participant Serum.

Parasitemic children (*n*=20) were categorized into SMA (Hb<5.0 g/dL) and non-SMA (Hb≥ 5.0 g/dL) according to WHO guidelines. Data are presented as the median (Interquartile range, IQR) unless stated otherwise. Reticulocyte production index (RPI) were calculated using previously described methods (reticulocyte index (RI)= (reticulocyte count x hematocrit)/30.7 (average hematocrit of children <5.0 years of age in Siaya County); maturation factor (MF)= 1+0.05(30.7-hematocrit); RPI=RI/MF; Were 2009) [a]Chi-Square test was used to determine differences in proportions. [b] Mann-Whitney test was used to determine differences in medians. *P*<0.05 was considered significant.

Figure 5.1. Circulating chemokine levels in parasitemic children.

Concentrations of chemokines (pg/mL) were measured in plasma from children <3.0 years with *P. falciparum* malaria using a Human Cytokine 25-plex Antibody Bead Kit and Luminex® 100™ system (See materials and methods for description). Concentrations of circulating,IP-10, and MIG (pg/mL) in children (*n*=20) were categorized into SMA (*n*=10) and non-SMA (*n*=10) groups based on the WHO definition of SMA (i.e., Hb <5.0 g/dL, with any density of parasitemia). Box-plots depict the data where the box represents the interquartile range, the line through represents the median, and whiskers represent

the 10th and 90th percentile. When compared to the non-SMA group, circulating MIG and IP10 are significantly reduced in the SMA category ($P<0.005$ and $P<0.001$ respectively)

Differences between groups were compared by Mann-Whitney *U* test.

Figure 5.2. *In Vitro* Model of erythropoiesis.

Hemaptopoetic progenitor cells (CD34+) were isolated from peripheral donor blood. CD34+ cells were positively selected from other cell types using a magnetic anit-CD34+ conjugated antibody platform (see materials and methods). CD34+ cells were expanded into erythroid lineage for three days using rhIL-3, rhIL-6, and rhSCF. At day 3 media was replaced with complete stem-cell media containing EPO, rhIL-3, rhIL-6, and rhSCF and patient serum (non-SMA and SMA) or rhMIG, rhIP10, and rhIFN-γ for respective experiments. Cells were collected at days 7, 10, and 14 cells in which immunophenotyping and cell viability assays were conducted.

Figure 5.3. Gating Strategy.

CD34+ cells were stimulated with EPO, rhIL-6, rhIL-3, and rhSCF. Cells were then stained for CD45, CD71 , and CD235 markers a Day 7, 10, and 14. Populations were measured using the BD Fortessa flow cytometer and analysis was conducted using Flowjo (v7) software. Erythroid progenitor cell flow cytometry plots were generated from live cell populations using 7AAD for negative selection.

Figure 5.4. Effects of SMA and non-SMA serum conditioned media on erythroid cell populations

CD34+ cells were stimulated with EPO, IL-6, IL-3, and SCF and then conditioned with 5% pooled serum from non-SMA (Hb≥5.0 g/dL) and SMA (Hb<5.0 g/dL) children respectively. Cells were then stained for CD45, CD71 , and CD235 markers a Day 7, 10, and 14. Populations were measured using the BD Fortessa flow cytometer and analysis were conducted using Flowjo (v7) software. Data represented as normalized to EPO. Treatment conditions (EPO, non-SMA, SMA) were compared and tested for statistical significance using analysis of variance (ANOVA) and students T-test and normalized relative to EPO treatments. Significance was determined at P< 0.01*.

Figure 5.5. Effects of inflammatory mediators on erythroid cell populations.

CD34+ cells were stimulated with EPO, IL-6, IL-3, and SCF and then treated with rhIFN-γ, rhIP10, and rhMIG. Cells were then stained for CD45, CD71 , and CD235 markers a Day 7, 10, and 14. Populations were measured using the BD Fortessa flow cytometer and analysis were conducted using Flowjo (v7) software. Data represented as normalized to EPO. Treatment conditions (EPO, non-SMA, SMA) were compared and tested for statistical significance using analysis of variance (ANOVA) and students T-test and normalized relative to EPO treatments. Significance was determined at P< 0.01*.

Figure 5.6. Effects of SMA and non-SMA serum conditioned media and of inflammatory mediators on erythroid cell viability.

CD34+ cells were stimulated with EPO, IL-6, IL-3, and SCF and then treated with 100ng/mL of IFNγ, IP10, and MIG. Cells were harvested at days 7, 10, and 14 and cell viability was measured by luminescence using the CellTiter-Glo assay. Data represented as means ± SD and were normalized to EPO. Treatment conditions (EPO, non-SMA, SMA, rhIFN-γ, rhIP10, and rhMIG) were compared and tested for statistical significance using analysis of variance (ANOVA) and students T-test and normalized relative to EPO treatments. Significance was determined at $P< 0.01$*.

Tables and Figures

Table 5.1. Clinical characteristics of the Study Participant Serum.

Characteristic	Non-SMA Patients	SMA Patients	*P*
Number, *n*	10	10	N/A
Gender, %F:M	40:60	35:65	0.085[a]
Age, months	12.0 (7.5)	7.4 (5.4)	**0.016**[b]
Hematological Indices	Median (IQR)		
Hemoglobin (g/dL)	11.1 (0.4)	4.4(0.7)	**<0.001**[b]
RBC counts, x 10^{12}/L	4.6 (0.56)	1.7 (0.4)	**<0.001**[b]
Hematocrit, (%)	33.7 (5.1)	13.5 (2.3)	**<0.001**[b]
WBC count (10^3/µL)	12.1 (5.9)	13.6 (4.6)	**<0.001**[b]
Lymphocytes (10^3/µL)	38.7 (27.2)	54.5 (9.4)	**<0.005**[b]
Monocytes (10^3/µL)	7.0 (4.7)	11.4 (5.4)	**<0.001**[b]
Parasitological Indices			
Parasitemia, MPS/µL	44,455 (71,536)	19,994(30,484)	**<0.01**[b]
Reticulocyte Indices			
Reticulocyte Production Index (RPI)	0.97(0)	0.69(0.65)	**<0.001**[b]
RPI<2.0,n (%)	**8(80)**	**10 (100)**	**<0.001**[a]

Figure 5.1. Circulating Mediators Mulitplex Data in Kenyan Children for Pulled serum.

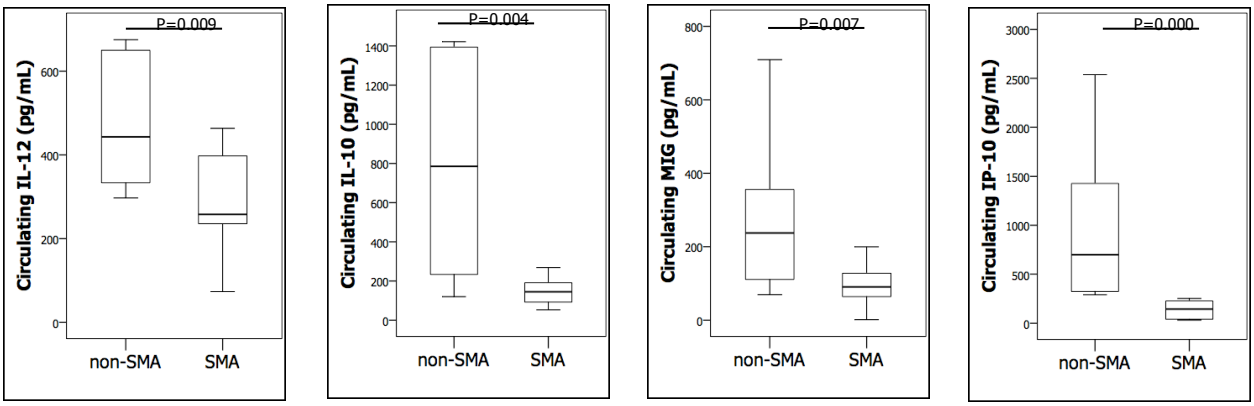


Figure 5.2. Gating Strategy.

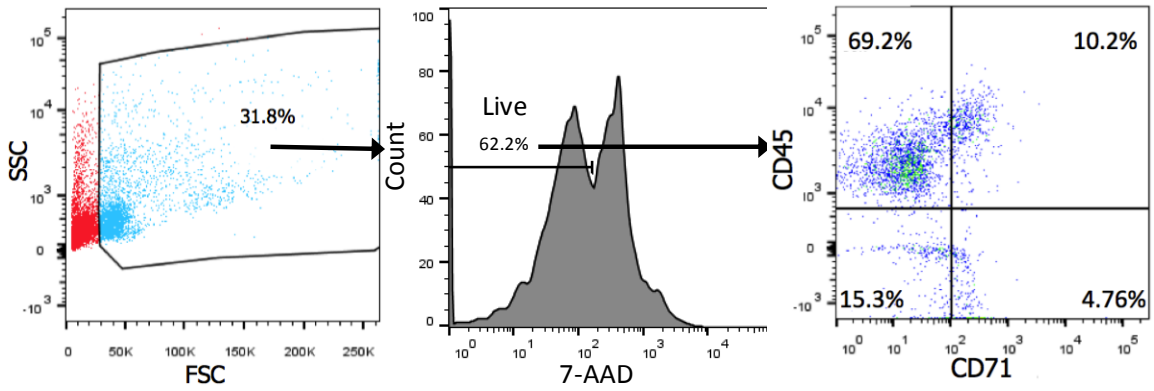


Figure 5.3. *In vitro* model: Phenotypic Markers at different development stages

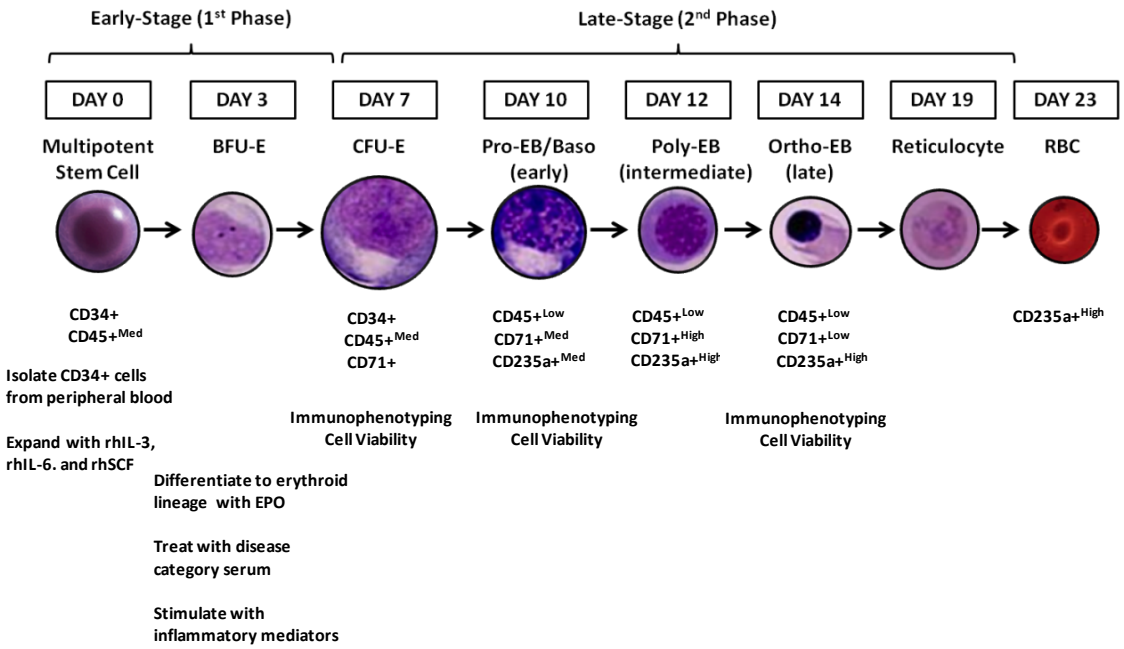


Figure 5.4. Effects of SMA and non-SMA serum conditioned media on erythroid cell populations

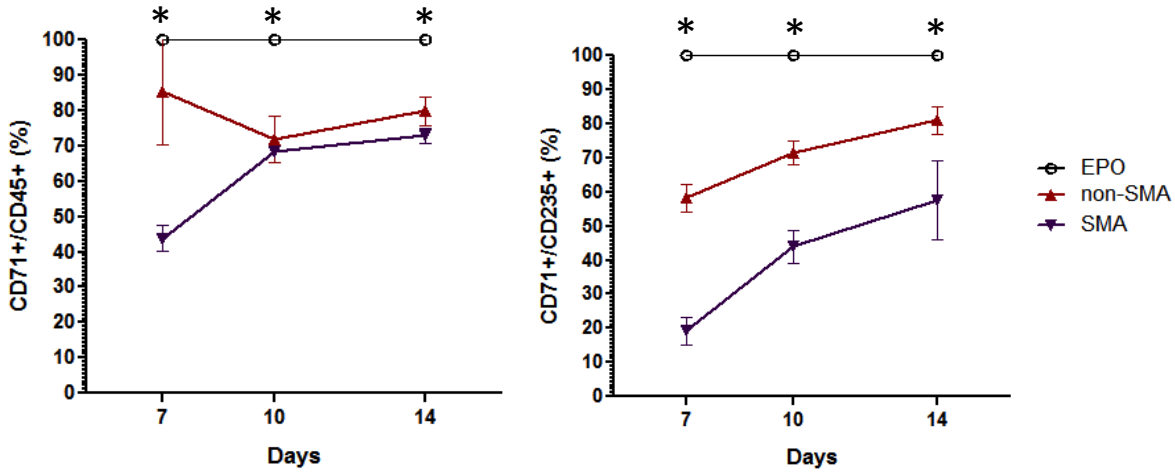


Figure 5.5. Effects of inflammatory mediators on erythroid cell populations.

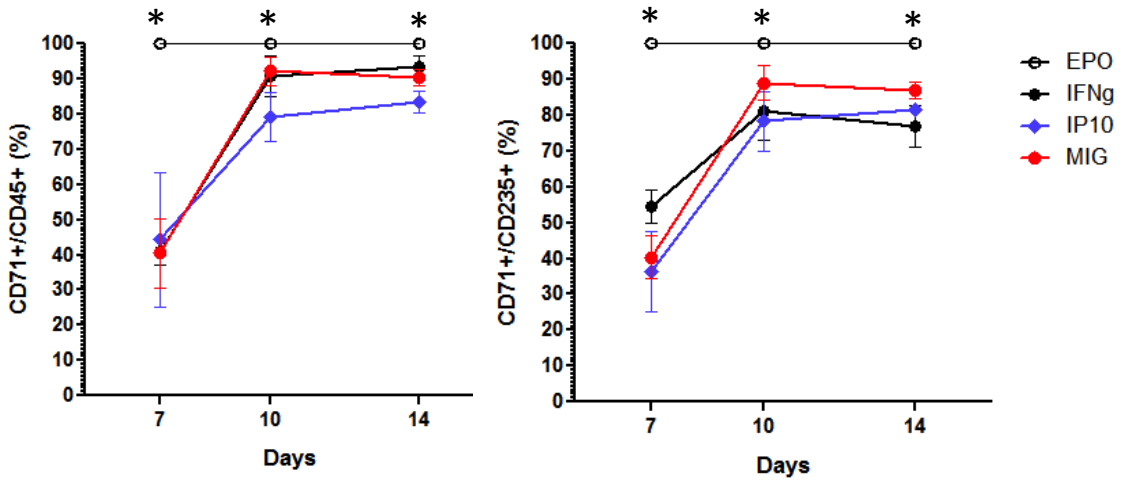
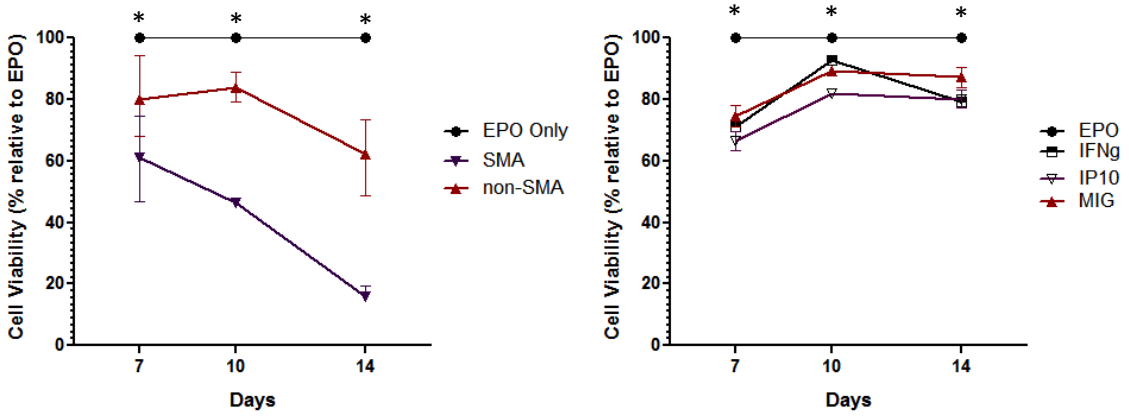


Figure 5.6. Effects of SMA and non-SMA serum conditioned media and of inflammatory mediators on erythroid cell viability.



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6.0 CHAPTER SIX: CONCLUSIONS

CONCLUSIONS

The first study in this dissertation examined the influence of promoter variants for the chemokines *MIG* and *IP10* and their association with SMA. We used regression analysis to show that variation within the promoter regions for MIG and IP10 (*MIG*-560A/-1714T and *IP-10*-1447A/-1919T) is significantly associated with susceptibility or protection against SMA. Several intragenic haplotypic constructs between promoters also showed either an in increased susceptibility to SMA whereas or protection against. We then examined erythropoietic response (RPI) in the context of SMA pathogenesis in which we cross-referenced regression analyses between groups. This analysis showed that 6 extended haplotypes had functional correlations between erythropoietic responses and SMA in intragenic haplotype carriers. To further these findings we examined MIG, IP-10 and IFN-γ which showed significant association of two extended haplotypes (GG and GGG) with SMA and erythropoietic response and these inflammatory mediators.

The second study in this dissertation was focused on the the hemoglobinopathy α-Thalassemia and hemoglobin coding gene promoter variants (HBA2) in the context of SMA. We were able to show protection against malaria infection (borderline significant; P=0.07) in children with the $-\alpha^{3.7}$kb deletion (-α/-α); however, no association with the $-\alpha^{3.7}$kb and SMA was seen independently. We also examined HBA2 promoter variants, $-\alpha^{3.7}$kb deletion, and the combination haplotypes between deletion and promoter SNPs in the context of SMA and erythropoietic response (RPI) using our regression model. In this study, we found that carriers of homozygous wild-type haplotypic constructs (HBA2-

1789/-4134 CG) were significantly more likely to develop SMA. In contrast, their homozygous polymorphic counterparts (HBA2-1789/-4134 TA) were significantly less likely to develop SMA. We also found that that children who carried the homozygous -1789 C/T (C) allele and had the $-\alpha^{3.7}$kb deletion (C/-α) were likely to have SMA. When considering erythropoietic response children who were carriers of the -4134 G/A (G) allele and had the $-\alpha^{3.7}$kb deletion (CG-α), were more susceptible to SMA and have a 2-fold decrease in erythropoietic response. These results demonstrate the influence of polymorphic variability within hemoglobin genes and hemoglobinopathies on SMA outcomes.

The third study in this dissertation examined the impact of MIG, IP10, and IFN-γ recombinant proteins and pediatric malaria serum on erythroid lineage using an *in vitro* model for erythropoiesis. We proposed that the mediators present in clinical serum mediate disease outcomes and, as such, we used physiological serum concentration in our model for erythropoiesis for each disease category: non-SMA and SMA serum. In a similar fashion we treated this model with the chemokines MIG and IP10 as well as the cytokine IFN-γ to examine their impact on erythroid lineage and viability. Our results from these studies revealed that pediatric serum from children with SMA influenced cell viability as well as erythroid lineage when we examined cell surface erythroid markers. A similar result was also seen in cell populations that were treated with the inflammatory mediators, MIG, IP10, and IFN-γ which reflected our proposal that serum from children with SMA and inflammatory mediators (MIG, IP10, and IFN-γ) infringe upon erythroid cell maturation and viability *in vivo*. Together the results from these studies

independently verify the influence of biomolecular markers and mediators on SMA outcomes.

Future Directions and Implications

The results presented in this dissertation offer a genetic connection between the biomolecular markers, MIG, IP-10, and HBA2 to clinical outcomes SMA pathogenesis. This dissertation utilizes the information from a well-defined pediatric enrollment study in combination in which clinical data, circulating mediator data, genetic variation data, and regression analysis link phenotypic outcomes presented here (SMA and RPI<2.0). Binary logistical regression analysis was performed in Aim 1 and 2 in which our dependent variable (Genotype or Haplotype) was utilized to estimate the probability of a conditioning the independent disease outcome variable (SMA and RPI<2). To overcome false discovery or reduced statistical impact, we used two disease categories, SMA and RPI<2, to indicate the most significant findings. This was important for determining the impact of genotypes or haplotypes of both immune (MIG and IP-10) and hemoglobin based (HBA2) genes and influential role in both SMA and erythropoietic response.

There are several pathways yet to be examined more closely within the context of mediator influence on severe disease. The use of cell models in which gene expression is measured based on disease category or variation within specific gene regions could prove useful to determine causality. For example, the use of gene reporter plasmid assays that are specific for gene regions that vary between genotypes could demonstrate loss of gene function *in vitro*. Similar experiments [electrophoretic mobility shift assay (EMSA)] focusing on transcription factor binding elements and genotype specific regions presented

in this work could reveal the influence of transcription factors on protein expression. Currently, we are investigating gene transcripts and pathways using RNA sequencing data collected from the CD34+ *in vitro* experiments presented here. The results collected from these and future studies will be beneficiary in advancing our understanding of the biomolecular pathways that constitute erythropoietic response in children with SMA.