ROLE OF HIV-1 AND BACTEREMIA CO-INFECTION IN PROMOTING INFLAMMATORY MEDIATOR PRODUCTION IN KENYAN CHILDREN WITH SEVERE MALARIAL ANEMIA

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Severe anemia is the primary outcome of childhood malaria in holoendemic malaria transmission regions such as western Kenya. HIV-1 and bacteremia are equally important diseases in Kenyan children. Anemia is also the hallmark trait of pediatric HIV-1 infection, and despite our previous reports of exacerbated anemia in malaria/HIV-1 co-infected childrena, we also observed significantly lower parasitemias without worsening anemia in malaria/bacteremia co-infected children. Abundant production of pro-inflammatory cytokines is known to adversely affect erythropoiesis and is common to malaria, HIV-1, and bacteremia. As such, we performed a comprehensive bead-based 25-plex Multiplex assay to identify cytokines patterns and profiles associated with negative and positive hematolgic outcomes in co-infected children. Children in Aims 1 and 2 infected with *P. falciparum* malaria (*Pf*[+], aged 3-36 mos., n=542) were stratified into three groups: HIV-1 negative (HIV-1[-]/*Pf*[+]); HIV-1 exposed (HIV-1[exp]/*Pf*[+]); and HIV-1 infected (HIV-1[+]/*Pf*[+]). In Aim 3, malaria and bacteremia co-infected children (n=192) were divided into three categories: malaria alone, *Pf*[+]; Gram negative bacteremia/malaria co-infected, G[-]/*Pf*[+]; and Gram positive bacteremia/malaria co-infected, G[+]/*Pf*[+]. Univariate, correlational, and hierarchical regression analyses were used to determine differences among the groups and to define predictors of worsening anemia. Aim 1 analyses revealed HIV-1[+]/*Pf*[+] children had significantly more malarial pigment-containing neutrophils (PCN), monocytosis, increased severe anemia (Hb<6.0g/dL), and ~10-fold greater mortality. Hierarchical multiple regression revealed that worsening anemia was associated with elevated pigment-containing monocytes, younger age, and increasing HIV-1 status (HIV-1[-]→HIV-1[exp]→HIV-1[+]). Aim 2,

addressing the inflammatory milieu, demonstrated that exacerbated anemia was associated with inflammatory mediator (IM) dysregulation, but not parasitemic or erythropoietic indices. A principal component analysis revealed that IL-12 was the most influential variable on Hb levels in HIV-1[+]/*Pf*[+] children, while the IL-1β:IL-10 ratio was most influenced by PCN. In Aim 3, both bacteremia co-infected groups had lower parasitemia compared to the *Pf*[+] group. A multiple mediation model examining IMs responsible for decreased parasitemia in the bacteremia co-infected groups identified IL-4, IL-10, IL-12, and IFN-γ as the key molecules in decreasing parasitemia. Thus, malaria/HIV-1 co-infection is defined by significantly enhanced anemia that is associated with unique IM profiles known to exacerbate anemia, while enhanced immune activation in malaria/bacteremia co-infected children appears to promote reduced parasitemia without adversely affecting anemia outcomes. By defining the inflammatory milieu associated with severe anemia, therapies can be developed to mitigate detrimental immune responses, thereby lessening the pediatric public health burden in western Kenya.

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PREFACE

The results of investigations conducted for this dissertation are presented as manuscripts that have either been published, or are ready to be submitted for publication. Permission has been obtained from all co-authors and from publishers of all journals in which this work has been published. The following is the full list of manuscripts presented in this dissertation:

1. Gregory C. Davenport, Collins Ouma, James B. Hittner, Tom Were, Yamo Ouma, John M. Ong'echa, and Douglas J. Perkins. Hematological predictors of increased severe anemia in Kenyan children co-infected with *Plasmodium falciparum* and HIV-1. *American Journal of Hematology.* 2010 Apr; 85(4):225-226.
2. Gregory C. Davenport, James B. Hittner, Collins Ouma, Tom Were, Yamo Ouma, John M. Vulule, John M. Ong'echa, and Douglas J. Perkins. Inflammatory mediator patterns associated with exacerbated severe anemia in Kenyan children with *Plasmodium falciparum* and HIV-1 co-infection. *Manuscript-in-preparation.*
3. Gregory C. Davenport, Tom Were, James B. Hittner, Collins Ouma, Yamo Ouma, John M. Vulule, John M. Ong'echa, and Douglas J. Perkins. *Plasmodium falciparum* and bacteremia co-infection in Kenyan children is characterized by reduced parasitemia in the absence of worsening anemia: an inflammatory mediator profile comparison. *Manuscript-in-preparation.*

LIST OF ABBREVIATIONS

Absolute reticulocyte number (ARN); analysis of variance (ANOVA); anemia of chronic disease (ACD); anti-retroviral (ARV); acquired immunodeficiency syndrome (AIDS); bone marrow derived macrophages (BMDM); burst forming unit-erythroid (BFU-E); Centers for Disease Control (CDC); cerebral malaria (CM); cluster determinant (CD); colony forming unit-erythroid; CpG oligodeoxynucleotides (CpG-ODN); C-reactive protein (CRP); cyclooxygenase (COX); deoxyribonucleic acid (DNA); Duffy antigen receptor for chemokines (DARC); entomologic inoculation rate (EIR); erythrocyte binding antigen (EBA); erythropoietin (EPO); ferriprotoporphyrin IX (FP-IX); glucose-6-phosphate dehydrogenase (G6PD); glycosylphosphatidylinositiol (GPI); Gram negative (G[-]); Gram positive (G[+]); granulocyte-colony stimulating factor (G-CSF); granulocyte macrophage-colony stimulating factor (GM-CSF); inducible nitric oxide synthase (iNOS); *Haemophilus influenzae* B (HiB); hemoglobin (Hb); hours (hrs); high-density parasitemia (HDP); highly active anti-retroviral therapy (HAART); human immunodeficiency virus-1 (HIV-1); HIV-1 negative (HIV-1[-]); HIV-1 exposed (HIV-1[exp]); HIV-1 positive (HIV-1[+]); human leukocyte antigen (HLA); immunoglobulin (Ig); inflammatory mediators (IM); interferon (IFN); interleukin (IL); intracellular adhesion molecule (ICAM); least significant difference (LSD); lipopolysaccharide (LPS); Kenya medical research institute (KEMRI); long terminal repeat (LTR); macrophage

inflammatory protein (MIP); macrophage migration inhibition factor (MIF); malaria parasites (MPS); maturation factor (MF); major histocompatibility complex (MHC); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); mean corpuscular volume (MCV); merozoite surface proteins (MSP); monocyte chemotactic protein (MCP); monokine induced by gamma interferon (MIG); mucosa-associated lymphoid tissue (MALT); myeloid dendritic cell (mDC); natural killer (NK); nitric oxide (NO); non-Typhi Salmonella (NTS); odds ratio (OR); parasite rate (PR); parasitized RBC (pRBC); peptidoglycan (PGN); peripheral blood mononuclear cell (PBMC); pigment-containing monocytes (PCM); pigment-containing neutrophils (PCN); plasmacytoid dendritic cell (pDC); *P. falciparum* erythrocyte membrane protein 1 (PfEMP1); *P. falciparum* hemozoin (*Pf*Hz); *P. falciparum* parasitemic (*Pf*[+]); polymerase chain reaction (PCR); principal component analysis (PCA); prostaglandin (PG); receptor (R); receptor antagonist (Ra); red blood cell (RBC); red blood cell distribution width (RDW); reticulocyte index (RI); reticulocyte production index (RPI); severe anemia (SA); severe malarial anemia (SMA); single nucleotide polymorphism (SNP); stem cell growth factor (SCGF); stem cell factor (SCF); synthetic hemozoin (sHz); Toll-like receptor (TLR); transforming growth factor (TGF); tumor necrosis factor (TNF); United Nations (UN); variable surface antigen (VSA); white blood cell (WBC); World Health Organization (WHO).

1.0 CHAPTER ONE: INTRODUCTION

Malaria, meaning 'bad air' in Italian, is an ancient protozoal pathogen that has ravaged the human population and shaped its genome for thousands of years. *Plasmodium* entered the realm of known infectious agents in 1880 during the inspection of an Algerian malaria patient's blood smear, when Alphonse Laveran noted a gametocyte amongst the red blood cells (RBCs) (Laveran 1880). Ronald Ross provided the next milestone in 1897 when, during the dissection of mosquitoes fed on parasitemic avian hosts, he observed Plasmodial oocysts in the guts of the insects, thereby linking malaria transmission to mosquitoes (Ross 1899). These findings were solidified by William George McCallum in 1897 when he verified that exflagellation was indeed the mechanism of Plasmodial sexual reproduction (McCallum 1897; McCallum 1898), and confirmation by Batistta Grassi *et al.* in 1900 (Grassi 1899) that anopheline mosquitoes transmitted human malaria. Currently there is an estimated one to two million deaths attributed to malaria annually worldwide, or 1.6% of the total global deaths, and *Plasmodium* infections constitute 2.3% of the global disease burden (Lewison and Srivastava 2008). Human malarial disease is caused by infection with four of the 100 species of *Plasmodium*, specifically *Plasmodium falciparum*, *P. vivax*, *P. ovale*, or *P. malariae*, as well as the recently recognized *P. knowlesi* (Cox-Singh, Davis *et al.* 2008;

Cox-Singh and Singh 2008). Falciparum malaria is the most severe of these and responsible for more than 95% of the global malaria morbidity and mortality (Playfair 1994). This single-celled eukaryotic vector-borne disease is transmitted by various species of the female anopheline mosquitoes and is endemic to 109 countries, thereby placing three billion people at risk each year (WHO 2008).

1.1 MALARIA LIFE CYCLE IN HUMANS

Malaria is transmitted by the female *Anopheles* mosquito, of which there are approximately 60 species competent for transmission out of the 400 *Anopheles* species worldwide. Sporozoites are contained in the mosquito's saliva and less than 100 are transferred to the human host during a blood meal (Vanderberg 1977; Rosenberg, Wirtz *et al.* 1990). These sporozoites then circulate through blood and lymph, with the majority arriving at the liver to infect hepatocytes. Even with approximately 80% of sporozoites reaching the liver, erythrocytic productive infection is low (Frevert, Engelmann *et al.* 2005), and in human volunteers, at least five infective bites were needed to guarantee parasitemia (Rickman, Jones *et al.* 1990; Verhage, Telgt *et al.* 2005). Recognition and binding of sporozoites to hepatocytes involves thrombospondin domains on the sporozoite binding to hepatocyte heparan sulfate proteoglycans to effect invasion (Frevert, Sinnis *et al.* 1993). After entry, the sporozoites begin asexual amplification, a stage known as exo-erythrocytic schizogeny, for 8 to 25 days to mature into schizonts, which then rupture releasing 1,000 to 30,000 merozoites per sporozoite

into the blood stream (Tuteja 2007). The exception being *P. vivax* and *P. ovale*, which are capable of lying dormant in liver cells, in the form of hypnozoites, and reactivating to continue infection weeks to months after the initial inoculation (Krotoski, Collins *et al.* 1982). The liver merozoites are actually released as clusters contained in a host cell membrane, termed merosomes, rather than individually as from the erythrocytic stage (Baer, Klotz *et al.* 2007). Merozoite invasion of the erythrocyte (Cowman and Crabb 2006) is a four-step process that involves recognition and temporary binding to the RBC; reorientation and permanent attachment of the apical end of the merozoite to the RBC; engulfment of the merozoite by invagination of the RBC membrane; and finally, closing of the parasitophorous vacuole and the RBC membrane (Miller, Baruch *et al.* 2002). Initial recognition of the RBC by the merozoite involves merozoite surface proteins (MSP)-1 through -4, while permanent docking employs erythrocyte binding antigen (EBA)-175 to form a junction with glycophorin A (Miller, Baruch *et al.* 2002). The merozoite grows by nuclear division, while catabolizing host cell hemoglobin (Hb) and glucose, and transitioning from ring stage, to trophozoite, and finally maturing into schizonts approximately 48 hrs from erythrocyte invasion. During intracellular growth, the infected erythrocytes display the parasite-encoded *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP1), which is responsible for parasite recognition by host receptors as well as pathogenesis, such as capillary occlusion, due to recognition and binding within capillaries (Su, Heatwole *et al.* 1995; Newbold, Craig *et al.* 1999; Chen, Schlichtherle *et al.* 2000; Duffy, Craig *et al.* 2001; Horrocks, Pinches *et al.* 2004; Kraemer and Smith 2006). The schizont-laden erythrocyte eventually ruptures,

releasing 6 to 40 merozoites into circulation to infect additional RBCs. Occasionally, sexual forms of the parasite develop into the gametocytes, typically requiring 7 to 15 days from liver merozoite release. Although gametocytogenesis is essential to the cycle between human and vector hosts, it still remains poorly understood (Drakeley, Sutherland *et al.* 2006). The male microgametocytes, and the female macrogametocytes, are ingested by the female *Anopheles* mosquito during subsequent blood meals, which initiates the sporogenic cyle in the mosquito and requires between ten and twelve days for *P. falciparum*. In the stomach of the mosquito the microgametocyte penetrates the macrogametocyte and fuses to form a zygote. As the zygotes mature, they become elongated and motile, which allows them to penetrate the midgut wall of the mosquito. Attached to the outer midgut wall, these ookinetes mature into oocysts in 24 hrs, which then rupture and release sporozoites. The sporozoites migrate through the hemocoel to the salivary glands of the mosquito (Vanderberg 1975), where they will be injected into the mammalian host during the next blood meal.

1.2 MALARIA BURDEN

1.2.1 Worldwide malaria burden

Approximately 247 million malaria infections are estimated annually, resulting in greater than one million deaths, primarily in African children under the age of five (WHO 2008). The 3.3 billion people at risk for malarial disease span the globe, but are focused in South and Central America, Africa, and South-East Asia (WHO 2008). Outside of sub-Saharan Africa, the greatest number of cases and deaths occur in India and Sudan. After accounting for infecting species, with *P. falciparum* responsible for the greatest mortality, it is transmission intensity of the region that dictates disease severity, clinical presentation, and age of presentation, and can be broadly grouped as either seasonal (unstable) or stable transmission. In areas of seasonal transmission, children typically are exposed at a later age, and the disease manifests as cerebral malaria (CM). Conversely, in regions of stable transmission, children are chronically exposed to the malaria parasite at a very young age, and this chronicity of infection leads to severe malarial anemia (SMA). Transmission intensity can be measured by a number of methods, but entomologic inoculation rate (EIR) and endemicity, which is derived from parasite rate (PR) (Gilles 1993), are the two most common. EIR is a metric that considers the ratio of human bites by competent vectors per person per day and bites by infectious mosquitoes per person (Birley and Charlewood 1987). For example, the average EIR estimation for the African continent is 121, and is expressed per annum, meaning 121 infective bites per person per year (Rogers, Randolph *et al.* 2002),

whereas the EIR of the rural research area presented herein, Siaya District, has been estimated at 60 to 300 (Beier, Oster *et al.* 1994). PR is defined by the proportion of individuals sampled, typically between the ages of two and ten, which have positive blood smears for malaria. The PR then translates into endemicity as follows: hypoendemic, <0.10 PR; mesoendemic, 0.11-0.50; hyperendemic, 0.51-0.75; and holoendemic, >0.75, in which only children one year of age are sampled (Metselaar 1959). Unfortunately, while substantial strides have been made in reducing or eliminating malaria in regions designated as epidemic (100% reduction), hypoendemic (66%), and mesoendemic (45%) from 1900 to 2002, the regions with the highest transmission rates, hyperendemic and holoendemic, had 16% and 0% reductions, respectively, during the same time period (Hay, Guerra *et al.* 2004). While malaria accounts for approximately 2% of the deaths and infectious disease burden worldwide, only about 0.4% of the global biomedical research is focused on this disease (Lewison and Srivastava 2008).

1.2.2 Malaria burden in sub-Saharan Africa

In 2006, 91% of the estimated malaria cases were caused by *P. falciparum* and 86% were located in Africa (WHO 2008). Additionally, 90% of deaths from malaria occurred in Africa, compared to just 4% in south-east Asia. Within Africa, nineteen countries bear the burden of 90% of the estimated cases, while eighteen countries are responsible for 90% of the deaths, with Kenya being fifth and seventh on these lists, respectively (WHO 2008). The at-risk population in sub-Saharan Africa has increased

ten-fold since 1900 due to population increase, and more than 80% of these individuals reside in hyper- or holoendemic areas (Hay, Guerra *et al.* 2004). Interestingly, because of the dynamics of malaria immunity acquisition, the risk of disease is actually lower in areas of high transmission, and higher risks are found in low transmission areas (Mbogo, Snow *et al.* 1993; McElroy, Beier *et al.* 1994; Mbogo, Snow *et al.* 1995; McElroy, Beier *et al.* 1997; Snow, Omumbo *et al.* 1997) because clinical immunity is never acquired in regions with low or sporadic transmission.

1.3 CLINICAL MANIFESTATIONS OF MALARIA

Malaria is unique in that the transmission intensity of the region dictates the clinical presentation observed in the resident population. Generally speaking, areas of low transmission have higher rates of hospital admission, primarily of older patients presenting with severe, acute disease manifesting as CM (Snow, Omumbo *et al.* 1997; Okiro, Al-Taiar *et al.* 2009). Areas of high transmission have lower rates of admission, with these patients being younger and experiencing a more chronic illness in the form of severe anemia, which can quickly escalate to life-threatening disease (Snow, Omumbo *et al.* 1997; Okiro, Al-Taiar *et al.* 2009). In the study conducted by Snow *et al.*, the mean age of admission in the low transmission area of Bakau, Gambia was 49 months, with 11% of children under one year of age admitted, versus Siaya, Kenya, where 52% of admissions were below the age of one and the mean age was 17 months (Snow, Omumbo *et al.* 1997; Okiro, Al-Taiar *et al.* 2009). Interestingly, although low

endemicity regions have higher rates of hospital admissions, the percentage of children diagnosed with severe disease is lower, while the converse is true in highly endemic areas. Okiro *et al.* reported this phenomenon with a meta-analysis of 17 regions of varying endemicity, where they noted a 4% diagnosis of CM in 10,642 children, compared to 17% of children reported with SMA (Okiro, Al-Taiar *et al.* 2009). In addition to endemicity, age plays a significant role as well, as children are more susceptible to severe anemia and hypoglycemia, while non-resident malaria-naïve adults are more likely to present with jaundice and progress to renal failure and respiratory distress due to pulmonary edema (White 1998).

Approximately 11 days after an infective bite, parasites reach a detectable level of 20 to 50 parasites/μL in the blood, which may or may not elicit mild symptomatology. The patient begins to feel overt symptoms on the 13th day post-inoculation such as fever, headache, malaise, myalgia, and weakness, which typically correlates with 20 to 20,000 parasites/μL. At this point, patients follow one of three paths that depends on regional endemicity, previous exposure, genetics, and age; eventual resolution or chronic low level parasitemia, severe disease manifesting as CM, or severe disease manifesting as SMA. In resident adults of endemic areas, it is possible for them to remain asymptomatic with parasite loads of 10,000/μL or more (Schellenberg, Smith *et al.* 1994; Smith, Genton *et al.* 1994).

1.3.1 Cerebral malaria and related sequelae

The CM manifestation of *P. falciparum* occurs in both children and adults, unlike SMA, which is predominantly a childhood manifestation. The diagnosis is one of exclusion, where all other potential diagnoses for altered consciousness in a patient with evidence of parasitemia have been ruled out (WHO 1990). Children with CM typically present with seizures and coma (Molyneux, Taylor *et al.* 1989), while adults are less likely to present with seizures but often have accompanying multi-organ failure that includes acute renal failure and respiratory distress from pulmonary edema (Warrell 1987). Mortality is dependent on the age of the patient, previous exposure, regional endemicity, and sophistication of medical care, and occurs in 10 to 50% of the CM cases (Rey, Nouhouayi *et al.* 1966; Bernard and Combes 1973; Stace, Bilton *et al.* 1982; Warrell, Looareesuwan *et al.* 1982). However, those who emerge from coma or altered consciousness, 90% of children and 98% of adults, typically recover with no long-term sequelae (WHO 1990). These statistics cast doubt on the widely touted mechanism for CM first described in the 1800s (Marchiafava 1894), that oxygen deprivation in the brain is due to vascular blockage with parasitized RBCs (pRBCs) (Berendt, Ferguson *et al.* 1994; Berendt, Tumer *et al.* 1994; Silamut, Phu *et al.* 1999; Beeson and Brown 2002). If vascular blockage, anoxia, and subsequent cell death were the cause of coma, one would expect outcomes similar to those seen in stroke, with little to no cognitive improvement following recovery (Clark, Alleva *et al.* 2008). Since recovery in these patients is nearly complete, and parasite sequestration is not always present (Turner 1997), it is more likely that soluble mediators, such as nitric oxide (NO), tumor necrosis

factor (TNF)-α, and macrophage migration inhibitory factor (MIF), may be affecting neurotransmission and consciousness (Clark, Awburn *et al.* 2003). For instance, antibodies to TNF-α administered to mice have been shown to be protective from cerebral manifestations (Grau, Fajardo *et al.* 1987). Similarly, another study in *P. berghei*-infected mice, which usually succumb to infection via neurological sequelae or severe anemia, found that administration of human recombinant interferon (IFN)-α reduced TNF-α, brain sequestration of pRBCs and white blood cells (WBCs), the expression of intercellular adhesion molecule (ICAM)-1, and circulating parasite numbers, which in turn reduced mortality from 87% in control mice to 6% in IFN-α-treated mice (Vigario, Belnoue *et al.* 2007). Studies such as those above, among others, suggest that cytokine modulation may be the key to pathogenesis, protection, and recovery from CM, and perhaps even the alleged mechanism of CM (Ringwald, Peyron *et al.* 1993; Prudhomme, Sherman *et al.* 1996), i.e., pRBC sequestration in the brain (Clark, Alleva *et al.* 2008).

1.3.1.1 Renal insufficiency Renal insufficiency and failure are primarily adult manifestations of severe disease, which are rarely seen in African children. A study published from Vietnam (Trang, Phu *et al.* 1992) found that half of the adult patients with severe malaria in their study had renal insufficiency, and mortality was reduced from 75% to 25% with peritoneal dialysis. Severe anemia, hemoglobinuria, and hypovolemia all contribute to the renal insufficiency caused by tubular necrosis (White 1998). Hypoxia and poor perfusion of the kidney are suspected to cause the necrosis,

which is precipitated by occlusion of the capillaries by pRBCs (Mishra and Das 2008) in addition to other immunologic mechanisms (Eiam-Ong 2002).

1.3.1.2 Metabolic acidosis Metabolic acidosis is a frequent result of severe malarial infection and cause of death, which is primarily caused by lactate accumulation (White 1998). Respiratory failure and salicylate intoxication can be other important contributors in malaria endemic regions (English, Marsh *et al.* 1996). The degree of lactic acidosis is proportional to disease severity, and is thought to stem from the sequestration and blockage of microvasculature. Pro-inflammatory cytokines, such as MIF (Calandra and Bucala 1995), increase metabolism, and combined with accelerated glycolysis, impairment of kidney and liver function, and restricted oxygen delivery, the anaerobic glycolysis produces more lactate than can be cleared by the impaired organs (Krishnan and Karnad 2003). In addition to increasing metabolism, the pro-inflammatory cytokine TNF-α, a molecule that plays a prominent role in the immune response to malaria, has been associated with hypoglycemia in African children with severe malaria (Grau, Taylor *et al.* 1989). Parasites contribute to the lactate accumulation as well, since pRBCs metabolize approximately 70 times more glucose than uninfected erythrocytes, the majority of which is catabolized to lactate (Maitland and Newton 2005; Planche, Dzeing *et al.* 2005). A similar process and consequence occurs in bacterial sepsis, with the exception being the septic metabolic acidosis is predominantly driven by hypermetabolism rather than anoxia (Jacobi 2002).

1.3.2 Severe malarial anemia

SMA is a manifestation of falciparum malaria predominantly seen in children and pregnant women residing in holoendemic transmission areas (Bloland, Ruebush *et al.* 1999; Ekvall 2003). These holoendemic regions, such as western Kenya, carry the risk of receiving an infective bite nearly every day of the year (Beier, Oster *et al.* 1994), and with antimicrobial resistance high and no effective vaccine available, long-term survival depends on acquisition of protective immunity through repeated infections during childhood. However, the repeated infections necessary to acquire this immunity takes a significant toll on children born and raised in these areas and manifests as severe anemia due to the chronicity. SMA has also been found to contribute heavily to hospital admissions in low and moderate regions of malaria transmission (Brewster and Greenwood 1993), making SMA a potential outcome in any falciparum-endemic region, and even in epidemic outbreaks (Idro, Bitarakwate *et al.* 2005). In Tanzania, 60% of the severe anemia cases in children were attributed to malaria [(Menendez, Kahigwa *et al.* 1997; Abdulla, Schellenberg *et al.* 2001), while malaria was responsible for only 18-39% of anemic pregnant women in that region (Shulman, Dorman *et al.* 1999; Brabin, Premji *et al.* 2001; Guyatt and Snow 2001; Marchant, Schellenberg *et al.* 2002), so clearly younger age predisposes to SMA. Studies in holoendemic regions of Kenya have found that the previous exposure and parasitemia levels were far more predictive of the current Hb level than the present infection's degree of parasitemia (Ekvall, Premji *et al.* 2001; McElroy, ter Kuile *et al.* 2001).

The primary manifestations of SMA are fatigue, prostration, rapid breathing, and pulmonary edema. The direct cause of death, which is significantly more likely at Hb values less than 5.0g/dL, is believed to be a combination of tissue hypoxia and metabolic acidosis (English, Waruiru *et al.* 1996; English, Waruiru *et al.* 1996). Since 90% of the RBCs destroyed in a falciparum malaria infection are uninfected (Price, Simpson *et al.* 2001), due to nonspecific immune activation, treatment with anti-malarial drugs can only have a limited effect on the anemia present. Management of severe anemia currently is confined to blood transfusion, which still carries a considerable risk in areas where human immunodeficiency virus (HIV) and hepatitis prevalence is high [reviewed in (Jayaraman, Chalabi *et al.* 2010)].

SMA is caused by a myriad of mechanicsms, including RBC lysis of infected and uninfected RBCs (Dondorp, Angus *et al.* 1999; Dondorp, Chotivanich *et al.* 1999; Price, Simpson *et al.* 2001; Egan, Fabucci *et al.* 2002), splenic sequestration of RBCs (Buffet, Safeukui *et al.* 2009), dyserythropoiesis and marrow suppression (Abdalla, Weatherall *et al.* 1980; Phillips, Looareesuwan *et al.* 1986) by cytokines and hemozoin (*Pf*Hz), nutritional deficiencies (Berkley, Bejon *et al.* 2009), co-infections (Berkley, Lowe *et al.* 2005; Otieno, Ouma *et al.* 2006; Bassat, Guinovart *et al.* 2009; Davenport, Ouma *et al.* 2010), and the chronic transmission in a holoendemic region, all contribute to chronically low Hb values in children residing in these endemic regions. For this reason, degree of parasitemia is a poor indicator of disease severity in SMA, especially considering that it is a “snapshot” in time of a complex and continuously evolving disease state. However, while a low parasitemia may not necessarily indicate a mild

infection, high parasitemias in children or non-immune individuals can certainly lead to massive lysis and clearance of RBCs, resulting in profound anemia (Phillips, Looareesuwan *et al.* 1986; Molyneux, Taylor *et al.* 1989).

Since SMA only occurs in a subset of the population residing in a holoendemic region, genetic susceptibility plays a significant role in the development of, or protection from, severe disease [reviewed in (Verra, Mangano *et al.* 2009)]. For instance, the human leukocyte antigen (HLA) class I and class II alleles, HLA-B53 and HLA-DRB1*1302, respectively, were associated with protection from SMA in children with severe malaria in The Gambia (Hill, Allsopp *et al.* 1991). Two other studies from The Gambia found associations between single nucleotide polymorphisms (SNPs) in the TNF-α promoter region and CM (TNF-α -308) (McGuire, Hill *et al.* 1994) and SMA (TNF-α -238) (McGuire, Knight *et al.* 1999). Our group, working in a pediatric population located in western Kenyan, has identified multiple SNPs and haplotypes in cytokines associated with either SMA protection [interleukin (IL)-10 -1082G/ -819C/ -592C, odds ratio (OR) 0.68 (Ouma, Davenport *et al.* 2008)] or increased susceptibility [IL-1β -31C/ -511A, OR 1.98 (Ouma, Davenport *et al.* 2008); MIF -794CATT7,8/ -173C, OR 2.46 (Awandare, Martinson *et al.* 2009)]. These polymorphisms and haplotypes lead to dysregulation of cytokine pathways, which are believed to be an important underlying cause of SMA, i.e. dyserythropoiesis and erythropoietic suppression, since hemolysis cannot solely account for the severity of anemia.

1.4 ACQUIRED IMMUNITY TO MALARIA

Robert Koch, in 1900, compared peoples from West Java, an area of low endemicity, and Central Java, which was highly endemic, illustrating that chronic exposure to malaria was able to induce clinical immunity over time (Koch 1900; Koch 1900; Koch 1900; Koch 1900). Those residing in West Java showed similar disease patterns regardless of age, while those in Central Java also had similar clinical signs, but which were only focused in the young and rarely observed in those who survived childhood.

1.4.1 Semi-immune status

While sterilizing immunity is virtually unachievable in humans naturally, clinical immunity or the ability to tolerate parasitemia without signs or symptoms, as well as resistance to severe disease, is well documented [reviewed in (Doolan, Dobano *et al.* 2009)]. As early as 1920, the tenets of naturally acquired immunity were understood to be: present in adults that had endured a holoendemic environment since birth; specific to the Plasmodia species of that endemic region, and initially, to the strains in the immediate area; gained proportionally to the EIR of that region, i.e., acquired more quickly in a holoendemic region than in a mesoendemic region; and quickly lost upon leaving the endemic area (Sergente 1910; Schuffner 1919). Falciparum malaria is not only the most severe form of the human malarias, but is also the slowest to confer clinical immunity as well. The explanation for this phenomenon was put forth in a landmark paper in 1965 (Brown and Brown 1965) suggesting that the slow acquisition of immunity was due to the immense antigenic repertoire displayed by the parasite,

which necessitates many infections to develop adequate immunity. PfEMP1 is believed to be the dominant antigen in host immunity to *P. falciparum* [reviewed in (Scherf, Lopez-Rubio *et al.* 2008)], and is one of the proteins termed variant surface antigens (VSA). An immunoglobulin (Ig)G immune response to this group of VSA proteins has been shown to be associated with protection in various venues across sub-Saharan Africa (Giha, Staalsoe *et al.* 2000; Dodoo, Staalsoe *et al.* 2001; Staalsoe, Shulman *et al.* 2004; Joergensen, Turner *et al.* 2006). Unfortunately, studies using sera from children in The Gambia (Marsh and Howard 1986) and Papua New Guinea (Forsyth, Anders *et al.* 1989) previously infected with *P. falciparum*, as well as primate studies with rhesus macaques (Brown, Brown *et al.* 1968) and *Aotus* monkeys (James 1931), demonstrated that there was little to no cross-reactivity of antibodies between various strains, thereby confirming the need for multiple infections to develop a broad repertoire capable of recognizing the various strains present in the region. However, evidence exists that repeated infection with a homologous strain eventually confers some resistance against other heterologous strains (Sinton 1935; Cadigan and Chaicumpa 1969; Powell 1972).

Clinical immunity is acquired in two phases, with the first being ‘anti-disease immunity’ in young children, which eventually minimizes the likelihood of cerebral manifestations of disease and results in increasingly milder symptoms with subsequent infections. While anti-disease immunity is a relatively rapid development, the ability to control and tolerate parasitemia with few to no symptoms is established much more slowly. This ‘anti-parasite immunity’, achieved around puberty, not only controls parasitemia but protects from the more chronic manifestations of disease, such as SMA

(Marsh and Snow 1997). The acquisition of these two forms of immunity is estimated to require approximately five infections per year for 10 to 15 consecutive years (Doolan, Dobano *et al.* 2009). Once established, mathematical modeling has estimated the duration of these two 'forms' of immunity outside of the endemic region to be approximately five years for the anti-disease immunity and greater than 20 years for the anti-parasitic immunity (Filipe, Riley *et al.* 2007). While parasitemia does not always predict the degree of disease (Meerman, Ord *et al.* 2005), high-density parasitemia (HDP) does increase the risk of hypoglycemia, metabolic acidosis, respiratory distress, CM, and SMA (WHO 2000), as well as having some predictive ability for mortality (McElroy, Beier *et al.* 1994; Beadle, McElroy *et al.* 1995). Rather than a sterile immunity derived from the immune response, clinical immunity achieved in adulthood was originally termed "premunition" by Koch in 1900, and is believed to be parasite-driven, in that, the near-constant presence of parasitemia prevents re-infection (Sergent 1935). This was demonstrated in a holoendemic area of Northern Ghana where 97% of the adults were parasitemic during a 16-week follow-up during the wet season (Owusu-Agyei, Koram *et al.* 2001). In contrast, 32% of children less than 24 months had parasitemia >20,000/μL, while only 2% of the adults had this high level of parasitemia, and disease severity was nearly identical in both groups with high parasitemia.

In a holoendemic environment, the likelihood of severe disease begins at birth and does not subside until approximately five years of age. Initially the child is protected by the mother's antibodies (Fouda, Leke *et al.* 2006), but then susceptibility to severe disease and death increases dramatically at approximately six months after

these antibodies have cleared the child's system. In areas of seasonal transmission, the child is at the greatest risk for CM between two and four years of age. Depending on the endemicity of the region, resistance to severe disease can be observed from the ages of two to five years. Generally speaking, the susceptibility to SMA decreases with age, while risk of CM increases with age (Baird, Masbar *et al.* 1998; Marsh and Snow 1999). As emphasized above, parasitemia is not indicative of disease severity or prognosis, partially due to the fact that sequestration can potentially constitute the bulk of the parasite burden. By puberty, the risk of severe disease has fallen to almost nil, but debate exists concerning the significance of this milestone, that is, whether cumulative immunity merely coincides with puberty or pubescent changes solidify the immunity enjoyed by adults. A study of non-immune immigrants to Indonesian New Guinea showed that parasitemia prevalence was similar across age groups after eight months, but after 20 months prevalence peaked at 10 years of age, with a 40% prevalence, and decreased every five years to a low of 10% in the over-forty age group (Baird 1998).

1.4.2 Non-immune status

Non-immune status, as the name suggests, describes an individual with no exposure to malaria, or one who has been removed from a region of endemicity long enough that immunity wanes, leaving them susceptible to severe disease. While not corroborated in recent literature, reports from the 1800s of Europeans dying in Niger (McGregor 1993) and Senegal (Curtin 1994) from malaria-like illness suggests a fatality rate of 30%.

Although the African children are currently the hardest stricken by malaria, their estimated death rate is only 2% by comparison (Greenwood, Marsh *et al.* 1991). This disparity of severe disease between adults and children was verified in the Indonesian New Guinea study cited above when three months into residence, an epidemic struck the transmigrants (Baird, Jones *et al.* 1991). At the peak of the epidemic, 48 adults (60%) required emergency evacuation, compared to only seven children (40%), which translated into a relative risk of 2.7 for adults (Baird, Masbar *et al.* 1998). Similar findings were discovered in a rat model of lipopolysaccharide (LPS) exposure, in which the adult rats were found to be more sensitive than younger rats, which also applied to *P. berghei* infection in these animals (Clark 1982). So it would seem that the immunological advantage of adulthood is a double-edged sword, being protective if cultured in a holoendemic environment, while detrimental in a naïve host entering an endemic area.

1.5 HOST IMMUNE RESPONSE TO MALARIA

A successful type 1 response to malaria requires a well-timed and proportional release of IL-12, IFN-γ, and TNF-α to minimize parasitemia and preserve erythropoiesis (Crutcher, Stevenson *et al.* 1995; Stevenson, Tam *et al.* 1995). The pro-inflammatory phase should be followed by an equally timely abrogation of this response by type 2 cytokines such as IL-10, transforming growth factor (TGF)-β, and IL-4, to avoid inflammatory host damage (Clark, Budd *et al.* 2006).

1.5.1 Pro- and anti-inflammatory cytokines

IL-12 is a heterodimeric protein composed of 35 and 40 kDa subunits and the prototypical cytokine of the type 1 immune response (Gately, Renzetti *et al.* 1998; Trinchieri 1998). It is secreted from dendritic cells, monocytes, and B-cells in response to bacterial cell wall components, intracellular pathogens, and by CD40 ligation (Gately, Renzetti *et al.* 1998; Trinchieri 1998; Mosser and Karp 1999) and stimulates production of IFN-γ and TNF-α from T-cells and natural killer (NK) cells (Gately, Renzetti *et al.* 1998; Trinchieri 1998), thereby driving type 1 responses. Other cytokines and chemokines can either promote IL-12 [e.g., granulocyte macrophage-colony stimulating factor (GM-CSF) and IFN-γ] or hinder its production [e.g., IL-4, IL-10, IL-11, IL-13, monocyte chemotactic protein (MCP)-1/CCL2, and TGF-β] (Trinchieri 1998; Mosser and Karp 1999). While IL-12 has myriad crucial immune functions during infection, its role as a hematopoietic growth factor is particularly important in malaria infection (Bellone and Trinchieri 1994; Dybedal, Larsen *et al.* 1995). In concert with IL-3, IL-12 as well as IL-6, GM-CSF, and IL-11 can bolster colony formation in dormant hematopoietic progenitors during times of cytopenic crisis (Bellone and Trinchieri 1994; Dybedal, Larsen *et al.* 1995).

IFN-γ is produced by natural killer and $\alpha\beta$-T cells, as well as the regulatory $\gamma\delta$-T cells, early in the immune response to a malaria infection (Hensmann and Kwiatkowski 2001; Artavanis-Tsakonas and Riley 2002; D'Ombrain, Hansen *et al.* 2007). This prototypical type 1 cytokine has been shown to be a key molecule in protecting children

(D'Ombrain, Robinson *et al.* 2008) and pregnant women (Moore, Nahlen *et al.* 1999) from severe disease, as well as non-immune volunteers experimentally infected with malaria (Pombo, Lawrence *et al.* 2002). IFN-γ has recently been attributed to the Fulani tribe's significant resistance to *P. falciparum* malaria as compared to other tribes in the Mali, Burkina Faso, and Sudan regions. The authors found that, compared to the Dogon tribe, mononuclear cells exposed to asexual parasites *in vitro* produced more than ten times the amount of IFN-γ 2010(McCall, Hopman *et al.*).

TNF-α, the molecule typically associated with pathology in malaria disease, but also critical for parasite clearance, was first describe in 1975 (Carswell, Old *et al.* 1975) and hypothesized to be involved in host response to malaria in 1978 (Clark 1978). Indeed, correlations have been found between greatly elevated TNF-α levels and morbidity and mortality (Grau, Taylor *et al.* 1989; Kwiatkowski, Hill *et al.* 1990), as well as TNF-α being necessary for parasite killing and holding parasite replication in check (Grau, Taylor *et al.* 1989; Kern, Hemmer *et al.* 1989; Kwiatkowski, Cannon *et al.* 1989; Clark, Cowden *et al.* 1990; Kwiatkowski, Hill *et al.* 1990). In addition to its direct effects (Clark, Hunt *et al.* 1987; van Hensbroek, Palmer *et al.* 1996), TNF-α also mediates its effects by inducing production of MIF (Calandra and Bucala 1995; Lan, Yang *et al.* 1997) and inducible nitric oxide synthase (iNOS) (Rockett, Awburn *et al.* 1992), which through generation of NO also has direct parasite killing effects (Rockett, Awburn *et al.* 1991). TNF-α can also exacerbate inflammation by induction of cyclooxygenase (COX)-2 and hence prostaglandins, which is important in placental malaria (Perkins and Kniss 1997). In addition to promoting further pro-inflammatory mediator production, TNF-α

has also been shown to suppress IL-12 production as well, which is important for a strong erythropoietic response (Ma, Sun *et al.* 2000). Many of the signs and symptoms of malaria, as well as other systemic disease such as sepsis, can also be linked to TNF-α, such as fever, headache, nausea, vomiting, diarrhea, anorexia, myalgias, and thrombocytopenia (Schwartz, Scuderi *et al.* 1989). This was definitively proven in human clinical trials using recombinant TNF-α to treat cancer (Creagan, Kovach *et al.* 1988), as well as the inability to distinguish between those with malaria and/or sepsis by clinical signs alone in the field (Clark, Budd *et al.* 2006).

IL-10 is a powerful type 2 anti-inflammatory cytokine necessary to prevent damage from over-production of pro-inflammatory responses, such as TNF-α, during infection (Ho, Schollaardt *et al.* 1998). Unfortunately, an over-abundant type 2 response is just as detrimental to the resolution of, and recovery from, malaria (Ferrante, Staugas *et al.* 1990; Day, Hien *et al.* 1999), and in the case of IL-12 (Aste-Amezaga, Ma *et al.* 1998) and GM-CSF (Moore, de Waal Malefyt *et al.* 2001), can inhibit secretion necessary for erythropoiesis (Mohan and Stevenson 1998). We have previously shown that IL-10 suppresses IL-12 production using both *in vitro* experiments with *Pf*Hz and *in vivo* findings (Keller, Yamo *et al.* 2006). Studies conducted in Gabonese children with severe malaria by our group also showed low IL-12 levels in the presence of elevated circulating IL-10 and TNF-α concentrations (Luty, Perkins *et al.* 2000; Perkins, Weinberg *et al.* 2000).

MIF was the first soluble mediator described in malaria (Coleman, Bruce *et al.* 1976), but was only recently fully explored in the context of malaria (Awandare, Hittner

et al. 2006; Awandare, Ouma *et al.* 2006; Awandare, Ouma *et al.* 2007; De Mast, Sweep *et al.* 2008; Awandare, Martinson *et al.* 2009; Jain, McClintock *et al.* 2009). While MIF has been found in high levels in patients with Gram negative (G[-]) sepsis (Roger, Glauser *et al.* 2001), it is decreased in children with increasing malaria severity (Awandare, Hittner *et al.* 2006). MIF has also been shown to up-regulate toll-like receptor (TLR)-4 in response to endotoxin and G[-] bacteremia (Roger, David *et al.* 2001). In summary, severe malaria is a disease of inappropriate responses in magnitude, rather than inappropriate responses in type, much like bacterial sepsis.

1.5.2 Chemokines

Chemotactic cytokines, or chemokines, not only recruit leukocytes by chemotaxis, but also play a role in immune activation, hematopoiesis, angiogenesis, and have antimicrobial properties as well (Rollins 1997). These molecules have been found to be important in placental malaria (Abrams, Brown *et al.* 2003; Suguitan, Leke *et al.* 2003), CM (Sarfo, Singh *et al.* 2004; John, Opika-Opoka *et al.* 2006; Armah, Wilson *et al.* 2007; Jain, Armah *et al.* 2008), and SMA. Burgmann *et al.* (Burgmann, Hollenstein *et al.* 1995) first investigated chemokines in 1995 by measuring the C-C chemokine macrophage inflammatory protein (MIP)-1α/CCL3 and C-X-C chemokine IL-8/CXCL8 in the serum of acutely infected *P. falciparum* adult patients, finding a positive linear association between parasitemia and IL-8/CXCL8. Lyke *et al.* (Lyke, Burges *et al.* 2004) also found ten-fold higher concentrations of IL-8/CXCL8 in Malian children with severe malaria compared to either healthy controls or uncomplicated malaria. *Pf*Hz, one of the

primary parasitic products responsible for immune dysregulation, has been found to increase messenger RNA (mRNA) levels of MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2, and MCP-1/CCL2 in murine models (Jaramillo, Godbout *et al.* 2005). Similarly, our group reported elevated levels of MIP-1α/CCL3 and MIP-1β/CCL4 from children with mild and severe anemia, while regulated on activation normal T-cell expressed and secreted (RANTES)/CCL5 levels were inversely proportional to increasing disease severity (Ochiel, Awandare *et al.* 2005). These findings were confirmed by measuring transcripts in peripheral blood mononuclear cells (PBMCs), which mimicked the levels found in plasma samples from these same children (Ochiel, Awandare *et al.* 2005). In addition, RANTES/CCL5 levels were found to correlate with prior malaria disease severity, as children with a history of SMA had decreased circulating levels of RANTES/CCL5 compared to those with previous mild disease (Ochiel, Awandare *et al.* 2005). In a follow-up study, we found RANTES/CCL5 was associated with thrombocytopenia as well as suppression of erythropoiesis (Were, Hittner *et al.* 2006). The Duffy blood group antigen is also important in vivax malaria, since absence of this marker on RBCs confers resistance. In addition, the Duffy antigen receptor for chemokines (DARC) also serves as a receptor for IL-8/CXCL8, MCP-1/CCL2, and RANTES/CCL5, so it is relevant in other *Plasmodium* infections and general immune activation as well (Horuk, Chitnis *et al.* 1993). The population in western Kenya is mostly devoid of DARC [Davenport *et al.*, 2009, American Society of Tropical Medicine & Hygiene annual conference], which helps explain the absence of *P. vivax* in this region.

Since there is no signal transduction from chemokines binding to DARC, it is thought to function as a chemokine “sink” to regulate blood levels of these molecules (Comerford and Nibbs 2005).

1.5.3 Growth factors

Growth factors, such as GM-CSF (Liehl, Hildebrandt *et al.* 1994) and stem cell growth factor (SCGF) (Hiraoka, Yano Ki *et al.* 2001; Elliott, Pham *et al.* 2008), are key to erythropoiesis, and therefore, recovery from malarial anemia. Infections that influence the levels of these molecules can severely impact the generation of new RBCs that have been lysed, phagocytized, and cleared during malaria. TGF-β is a growth factor that has recently been found to be instrumental in malaria pathogenesis (Gourley, Kurtis *et al.* 2002; Chaiyaroj, Rutta *et al.* 2004; Prakash, Fesel *et al.* 2006), and also attributed to both positive (Zermati, Varet *et al.* 2000) and negative (Hino, Tojo *et al.* 1988; Sing, Keller *et al.* 1988; Zermati, Fichelson *et al.* 2000) effects on erythropoiesis. Similarly, granulocyte-colony stimulating factor (G-CSF) has been associated with complicated *P. falciparum* infections as well (Stoiser, Looareesuwan *et al.* 2000), which may stem from its negative impact on erythropoiesis (Van Zant and Goldwasser 1977; Kojima, Fukuda *et al.* 1991; Papaldo, Ferretti *et al.* 2006). GM-CSF, in addition to its beneficial effects on erythropoiesis, has been demonstrated to synergize with TNF-α to increase the killing capabilities of neutrophils on blood-stage malaria parasites (Kumaratilake, Ferrante *et al.* 1996). Lastly, we have previously shown that the circulating levels of the beneficial erythropoietic factor SCGF were proportional to Hb levels and the reticulocyte

production index (RPI). However, SCGF was inversely proportional to the number of pigment-containing monocytes (PCM), suggesting a mechanism for the inhibited erythropoiesis seen in children with SMA (Keller, Ouma *et al.* 2009).

1.5.4 Immune dysregulation by parasite products

1.5.4.1 Hemozoin Infection with malaria exerts its effects on the immune system through various mechanisms. As the organism grows within the erythrocyte, Hb is metabolized for amino acids (Krugliak, Zhang *et al.* 2002; Liu, Istvan *et al.* 2006), leaving the iron-rich heme portion designated ferriprotoporphyrin IX (FP-IX). The organism polymerizes these toxic free FP-IX molecules, using heme polymerase (Chou and Fitch 1992; Slater and Cerami 1992), into an insoluble product known as malarial pigment or *Pf*Hz (Slater, Swiggard *et al.* 1991; Egan 2002; Pandey, Babbarwal *et al.* 2003). Once the schizont has outgrown the host RBC, the erythrocyte ruptures (Omodeo-Sale, Motti *et al.* 2005), releasing *Pf*Hz along with merozoites, with newly-formed merozoites infecting other RBC, while *Pf*Hz is phagocytized by monocytes/macrophages and neutrophils.

The immune perturbances caused by monocytic and neutrophillic phagocytosis of *Pf*Hz have yet to be fully elucidated, although *in vitro* and *ex vivo* experiments have shown significant cytokine dysregulation in response to *Pf*Hz acquisition (Luty, Perkins *et al.* 2000; Casals-Pascual, Kai *et al.* 2006; Keller, Yamo *et al.* 2006; Awandare, Ouma *et al.* 2007; Ong'echa, Remo *et al.* 2008; Dostert, Guarda *et al.* 2009; Jaramillo, Bellemare *et al.* 2009; Were, Davenport *et al.* 2009; Cambos, Bazinet *et al.* 2010). For

instance, our lab (Keller, Yamo *et al.* 2006) and others (Aste-Amezaga, Ma *et al.* 1998; Casals-Pascual, Kai *et al.* 2006) have previously demonstrated that IL-12 is significantly reduced in children with SMA, as well as in *in vitro* experiments with *Pf*Hz, by an IL-10-dependent mechanism. Similarly, IL-12 can also be antagonized by high levels of TNF-α (Ma, Sun *et al.* 2000), which is a hallmark of malaria infections, as well as G[-] sepsis (Giroir 1993). In addition to the sub-therapeutic levels of IL-12 being produced, greatly elevated levels of TNF-α, IL-1, and IL-6 are associated with severe malaria (Grau, Taylor *et al.* 1989; Kwiatkowski, Hill *et al.* 1990; Kurtzhals, Adabayeri *et al.* 1998) and *Pf*Hz deposition (Sherry, Alava *et al.* 1995; Griffith, Sun *et al.* 2009) and are known to inhibit erythropoietin (EPO) secretion (Faquin, Schneider *et al.* 1992), erythropoiesis (Schooley, Kullgren *et al.* 1987; Means, Dessypris *et al.* 1992), and alter iron store regulation (Nemeth, Rivera *et al.* 2004), respectively.

Hemozoin, in addition to inducing erythropoiesis-inhibiting cytokines, can also directly cause apoptosis of erythroid precursor cells via oxidative stress (Lamikanra, Theron *et al.* 2009). Interestingly, while pigment-containing neutrophils (PCN) have been shown to be an indicator of disease severity, PCMs generally have the same amount of *Pf*Hz regardless of the degree of clinical manifestation (Amodu, Adeyemo *et al.* 1998). In addition, activated macrophages also phagocytize infected RBCs (Turrini, Ginsburg *et al.* 1992), which results in *Pf*Hz residing within macrophage endosomes where, among other effects, TLR-9 interacts with the *Pf*Hz (Latz, Visintin *et al.* 2004; Takeshita, Gursel *et al.* 2004; Coban, Igari *et al.* 2010). *Pf*Hz signaling via the TLR-9 receptor has been shown to elicit production of IL-6, IL-12p40, TNF-α, and MCP-

1/CCL2, which is nearly identical to the cytokines/chemokines produced in response to treatment with CpG oligodeoxynucleotide (ODN) (Coban, Ishii *et al.* 2005). *Pf*Hz has also been shown to mediate the release of TNF-α, MIP-1α/CCL3, and MIP-1β/CCL4 from both murine macrophages and human PBMCs (Sherry, Alava *et al.* 1995). Recent studies have suggested the effect of *Pf*Hz to be mediated by TLR-9 ligation of the parasitic nucleic acids embedded in the surface of *Pf*Hz (Parroche, Lauw *et al.* 2007), or bound in protein-DNA complexes (Wu, Gowda *et al.* 2010). The caveat to these findings is many of these studies were conducted in mouse myeloid (mDC) and plasmacytoid (pDC) dendritic cells, which display significantly different TLRs from human cells (Iwasaki and Medzhitov 2004).

A significant body of data also exists for similar immune disturbances being caused by the synthetically produced polymer, synthetic hemozoin (sHz), which also contains the heme components found in *Pf*Hz, but no parasitic or host products (Taramelli, Monti *et al.* 2001; Keller, Hittner *et al.* 2004; Coban, Ishii *et al.* 2005; Nti, Slingluff *et al.* 2005; Ochiel, Awandare *et al.* 2005; Huy, Trang *et al.* 2006; Keller, Yamo *et al.* 2006; Ong'echa, Remo *et al.* 2007). Newly published data have definitively shown, on a molecular and atomic level, that it is indeed the *Pf*Hz binding to, and causing conformational changes in, the TLR-9 receptor, identical to those induced by CpG-ODN, the classical TLR-9 ligand (Coban, Igari *et al.* 2010).

1.5.4.2 Glycosylphosphatidylinositol The other major category of malaria antigens that have been associated with altered immune responses are the glycosylphosphatidylinositols (GPIs) (Nebl, De Veer *et al.* 2005), which form the

connection between the parasite's cellular membrane and its external antigens. Krishnegowda *et al.* (Krishnegowda, Hajjar *et al.* 2005) demonstrated that GPIs act as a ligand for the TLR-4 receptor, as well as the TLR-2 receptor in concert with either TLR-1 or TLR-6. Using IFN-γ-primed murine bone marrow-derived monocytes/macrophages (BMDM) the authors demonstrated that increasing concentrations of GPI elicited TNF-α in a dose-dependent manner and treatment of these mouse BMDMs with either anti-TLR-2 or -4 reduced TNF-α production, as did TLR-2 and MyD88 gene knockouts. The bacterial products peptidoglycan (PGN) and LPS also bind these receptors, respectively, and induce powerful immune responses (Beutler 2002; Dziarski and Gupta 2005). Not surprisingly, ligation of TLR-2, -4, and -9 have also been implicated in HIV reactivation in latently infected mast cells (Sundstrom, Little *et al.* 2004), while *Mycoplasma* lipid extracts signaling through the same TLR as *P. falciparum* GPIs have also been found to activate the long terminal repeat (LTR) of HIV (Shimizu, Kida *et al.* 2004). Jarrossay *et al.* found that mDCs down-regulated the C-C chemokine receptor CCR5 in response to the TLR-2 and -4 ligands PGN and LPS, while pDC only responded to CpG-ODN by upregulating CCR7 and downregulating CXCR3 (Jarrossay, Napolitani *et al.* 2001), which may also have HIV-susceptibility implications.

1.6 ANEMIA AND ERYTHROPOIESIS

1.6.1 The erythropoietic response

The generation of new RBCs begins with the sensing of low oxygen tension by cells in the kidneys using heme-like molecule receptors (Goldberg, Dunning *et al.* 1988). The endothelial and interstitial cells of the kidneys then release EPO in response to the hypoxic conditions. Extrarenal sources account for up to 15% of the secreted EPO, and is released from hepatocytes and Kupffer cells in the liver, as well as small amounts released by monocytes (Rich, Heit *et al.* 1982), human placenta (Conrad, Benyo *et al.* 1996), and hair follicles (Bodo, Kromminga *et al.* 2007). Synthesis of EPO has been found to be inhibited by IL-1 and TNF-α (Fandrey and Jelkmann 1991), with conflicting reports on the ability of IL-6 to block production (Faquin, Schneider *et al.* 1992). EPO is a highly glycosylated heat- and pH-stable protein that exerts its effects by binding to specific receptors found on many cell types, including those unable to respond to EPO, presumably because of its extra-erythropoietic ability to reduce pro-inflammatory cytokines and oxidative damage (Kanzaki, Soda *et al.* 2005; Li, Takemura *et al.* 2006). EPO is primarily responsible for regulating differentiation and maturation of progenitor cells, which carry 300 – 3000 EPO receptor gene copies per cell, and synergizes with IL-4. The sensitivity of progenitor cells to EPO increases with maturation, and EPO may also play a role in their proliferation. As progenitor cells mature into burst-forming units, erythroid (BFU-E), which are the earliest dedicated erythroid cells, they become increasingly more dependent on EPO as well as IL-3, IL-4, IL-6, and IL-11, while

expressing CD33, CD34, and HLA-DR surface markers. IL-6 has the dubious distinction of both directly aiding in BFU-E maturation and progenitor cell proliferation (Pojda and Tsuboi 1990), while simultaneously causing hypoferremia by inducing hepcidin (Nemeth, Rivera *et al.* 2004). Hepcidin, which is secreted in response to free iron and inflammation, and suppressed in hypoxic conditions, exerts its effect by preventing the release of iron from macrophages and hepatocytes through degradation of ferroportin, which is the sole iron export channel (Nicolas, Bennoun *et al.* 2002; Nicolas, Chauvet *et al.* 2002; Nicolas, Viatte *et al.* 2002; Nemeth, Rivera *et al.* 2004). Similar to the contradictory roles of IL-6, IL-1 has the ability to promote the survival of BFU-E, while also inhibiting their growth (Smith, Knight *et al.* 1992). The effect of IL-1 on BFU-E is believed to be mediated through IFN-γ (Means, Dessypris *et al.* 1992). Similarly, while the survival and differentiation of BFU-E into hemoglobinized cells is dependent on EPO, in conjunction with IL-9, their growth is independent of this crucial protein. Continued development of the BFU-E also relies heavily on stem cell factor (SCF), which can substitute for EPO in the presence of IL-3. The chemokine MIP-1α/CCL3 (Su, Mukaida *et al.* 1997) also has the ability to inhibit colony formation of BFU-E, while TGF-β (Zermati, Fichelson *et al.* 2000), TNF-α (Roodman, Bird *et al.* 1987), TNF-β (lymphotoxin, LT) (Ratajczak and Ratajczak 1994), and MIF (Martiney, Sherry *et al.* 2000; McDevitt, Xie *et al.* 2006) can inhibit BFU-E growth. BFU-E give rise to the colony-forming unit erythroid (CFU-E), which have the densest expression of EPO receptors, while also expressing CD36. Exposure of CFU-E to EPO results in several rounds of cell division leading to terminal differentiation and the inability to undergo

further cell division. TNF-α may also inhibit colony formation of CFU-E (Allen, Breen *et al.* 1999), in addition to its effects on BFU-E and EPO (Pontikoglou, Liapakis *et al.* 2006). IFN-γ has been found to inhibit CFU-E indirectly (Means, Dessypris *et al.* 1992) through an IL-15-dependant mechanism (Mullarky, Szaba *et al.* 2007). Maturation of the CFU-E results in formation of proerythroblasts, which mature through ambiguous phases to reach polychromatophilic normoblasts, the first cell stage to contain Hb. These cell types are still capable of cell division until the nucleus is extruded, at which point they become reticulocytes and exit the bone marrow. Given the extensive beneficial and detrimental effects of cytokines, chemokines, and growth factors on erythropoiesis, with both effects occasionally being exhibited by the same molecule, exploring the inflammatory milieu elicited during co-infection will lead to insight into the origin of dyserythropoiesis associated with these co-infections.

1.6.2 Anemia of inflammation

The cytokines dysregulated and over-expressed in malaria are also those that pose the greatest impediment to erythropoiesis, which are not unique to malaria, but are a reoccurring theme in autoimmune-related anemia, as well as those diseases with infectious origins (Ferrucci, Guralnik *et al.* 2005). For example, in patients with chronic idiopathic neutropenia there is significantly reduced BFU-E, which rebounds upon treatment with anti-TNF-α and/or anti-IFN-γ (Pontikoglou, Liapakis *et al.* 2006). However, a study in which mice were treated with CpG-ODN, a TLR-9 agonist, and the same TLR shown to be targeted by *Pf*Hz, only neutralization of IFN-γ ameliorated the

erythropoietic suppression, while neutralization of IL-12, TNF-α, IFN-α, IL-1α, and IL-1β had no effect (Thawani, Tam *et al.* 2006). Similarly, addition of serum from patients with rheumatoid arthritis and anemia of chronic disease (ACD), as well as exogenous TNF-α and IFN-γ, inhibits EPO production from cobalt-induced human hepatic cancer cells and dose-dependently suppresses BFU-E and CFU-E in normal human bone marrow (Zhu, Ye *et al.* 2000). In addition, studies indicate that 5 – 10% of those suffering from chronic kidney disease are either refractory or poor responders to exogenous EPO (Eschbach, Downing *et al.* 1989). Cooper *et al.* found that poor responders to EPO therapy had at least twice the concentration of IFN-γ, TNF-α, IL-13, and IL-10 of either good responders or healthy controls (Cooper, Breen *et al.* 2003). C-reactive protein (CRP) has also been shown to be a predictor of response to EPO in anemia (Ortega, Rodriguez *et al.* 2002; Martone, Zanchi *et al.* 2003). A study in renal disease patients demonstrated that 80% higher doses of EPO were needed in those with CRP levels >20 mg/L, versus patients with EPO deficiency simply due to renal disease (Barany 2001). Similarly, MacDougall *et al.* (Macdougall and Cooper 2002; Macdougall and Cooper 2002) found that patients with CRP levels >50 mg/L failed to achieve the Hb concentrations of those with CRP levels <50 mg/L, despite receiving greater doses of EPO. Patients with anemia of inflammation also have a greater proportion of zinc, rather than iron, substituted into FP during heme synthesis, and this cannot be corrected by iron supplementation, as the additional iron simply becomes trapped in the reticuloendothelial system (Hume, Currie *et al.* 1965; Cartwright 1966; Beamish, Davies *et al.* 1971; Hastka, Lasserre *et al.* 1993). TNF-α is also known to

inhibit EPO production in addition to inducing NO, which has direct detrimental effects on stem cells (Shami and Weinberg 1996). Many of the cytokines common to inflammation-derived anemia are also central to the immune response towards various infectious diseases as well, such as IFN-γ, TNF-α, and IL-10, which are recurring themes in malaria cytokine dysregulation (Kwiatkowski, Hill *et al.* 1990; Sherry, Alava *et al.* 1995; Chaisavaneeyakorn, Moore *et al.* 2002; Deshpande and Shastry 2004; Lyke, Burges *et al.* 2004; Prato, Giribaldi *et al.* 2005; Keller, Yamo *et al.* 2006; Ong'echa, Remo *et al.* 2007).

1.6.3 Infectious disease-derived anemia

Malarial anemia stems from three distinct mechanisms: RBC destruction caused by the rupture of infected RBC, erythrophagocytosis of infected and uninfected RBC by activated macrophages (Clark and Chaudhri 1988; Waitumbi, Opollo *et al.* 2000; Evans, Hansen *et al.* 2006), and inhibition of erythropoiesis by a pro-inflammatory environment (Wickramasinghe and Abdalla 2000). In addition, the deposition of *Pf*Hz, the insoluble product of parasitic Hb digestion, has not only been implicated in producing the pro-inflammatory environment and cytokine dysregulation when deposited in phagocytic cells, but has been shown to directly inhibit erythropoiesis in children with *Pf*Hz bone marrow infiltration (Casals-Pascual, Kai *et al.* 2006; Lamikanra, Theron *et al.* 2009). Evidence also suggests that, while there is increased EPO secretion in asymptomatic children harboring malaria (Burchard, Radloff *et al.* 1995; Kurtzhals, Rodrigues *et al.* 1997), in symptomatic children, EPO secretion may be inappropriate for the degree of

anemia (Vedovato, De Paoli Vitali *et al.* 1999), as evidenced by RPI values below two. However, EPO secretion is only the beginning of the erythropoietic cascade to correct anemia. Human volunteers injected with LPS were found to release large amounts of IL-6 within three hours, and hepcidin at six hours, which elicited the expected hypoferremia (Kemna, Pickkers *et al.* 2005), and thereby demonstrates the contribution of G[-] bacteremia to anemia. Three other inhibitors of erythropoiesis were also increased, with IFN-γ and TNF-α peaking at 1.5 hours, while IL-10 peaked at 2 hours, followed by peak IL-6 levels at 4 hours. As noted above, TNF-α has the ability to inhibit the release of EPO, while IFN-γ thwarts erythropoiesis through an IL-15-dependant mechanism. This collection of *in vitro* experiments suggests some of the mechanisms that may promote anemia via erythropoiesis inhibition. However, a definitive inflammatory profile from children suffering from the co-infections investigated as part of this doctoral work has not been identified in a rural sub-Saharan pediatric population.

1.7 CO-INFECTIONS

The three primary infectious diseases plaguing developing nations are malaria, HIV, and bacteremia. Currently, sub-Saharan Africa is home to 90% of the world's *P. falciparum* infections (WHO 2008) and 64% of the HIV-1 infections (UNAIDS 2008). Children are the most effected group in the population, with malaria and HIV-1 being responsible for 18% and 6%, respectively, of deaths in sub-Saharan African children under the age of five (Rowe, Rowe *et al.* 2006). However, Berkely *et al.* concluded after surveying 19,339 Kenyan children that the major cause of death in that pediatric population was not malaria, acquired immunodeficiency syndrome (AIDS), or tuberculosis, but common bacterial infections (Berkley, Lowe *et al.* 2005). Suffice to say, all three have contributed significantly to the pediatric morbidity and mortality in this region of world, with the predominant clinical sequelae being severe anemia (Ellaurie, Burns *et al.* 1990; Otieno, Ouma *et al.* 2006).

1.7.1 Human immunodeficiency virus (HIV)

1.7.1.1 Global epidemiology of HIV Globally, there were approximately 33.2 million people living with HIV in 2007, of which 2.5 million were children (UNAIDS 2008). In that year alone, approximately 420,000 of 2.5 million people were newly infected with HIV (UNAIDS 2008). The epidemic is showing signs of slowing, mostly due to transmission reductions in India and sub-Saharan Africa, which resulted in a fall in prevalence from the 2001 adult prevalence of 5.8% to the 2007 level of 5.0%. A cure for HIV remains elusive, and while treatments are evolving along with viral

resistance, anti-retroviral (ARV) drugs are still unavailable in much of the developing world. This health care disparity resulted in 2.1 million AIDS deaths in 2007, or 5,700 per day, the majority of which, 76%, occurred in sub-Saharan Africa (UNAIDS 2008).

1.7.1.2 Pediatric HIV in east Africa The women of sub-Saharan Africa bear the brunt of the adult HIV prevalence, as illustrated by 61% of the adult infections being in women. Clearly the number of infected women will directly impact the number of HIV-infected children, however, HIV prevalence in pregnant Kenyan women residing in urban and rural areas has declined by 25% since 2001 (UNAIDS 2008). While transfusion still carries considerable risk in malaria-endemic areas, blood supply safety is improving, and vertical transmission still remains the main mode of HIV transmission to children (Wiktor, Ekpini *et al.* 1997). There is a 25-40% risk of virus transmission to a child from conception to cessation of breast feeding, which can be broken down into a 10% risk of *in utero* transmission, 10-20% peri-partum, and another 10-20% during breast feeding (Dabis and Ekpini 2002). In regions devoid of ARVs, children infected with HIV typically die before two years of age (Newell, Coovadia *et al.* 2004).

1.7.1.3 Overview of HIV pathogenesis The recent HIV literature indicates there are two distinct phases of the disease (Derdeyn and Silvestri 2005; Moanna, Dunham *et al.* 2005). Immediately after transmission, there is an acute phase lasting two to three weeks, during which ~60% of the memory T-cells in the mucosa-associated lymphoid tissue (MALT) are destroyed. The acute phase viremia that ensues shortly after infection is quickly brought under control, in part, by virus-specific cytotoxic T-

lymphocyte responses (Musey, Hughes *et al.* 1997), and is followed by a chronic phase lasting years and leading to AIDS. This latter phase is a state of chronic immune activation where a pro-inflammatory milieu taxes the immune system and eventually results in an immunocompromised condition due to infection of the activated immune cells and cellular fatigue because of their prolonged hyper-activated state (Picker 2006). The widespread infection of activated immune cells culminates in the loss of crucial CD4+ T-cells necessary to mount an immune response when encountering opportunistic pathogens (Veazey and Lackner 2003). Interestingly, although the typical opportunistic infections are encountered as an HIV-1[+] child approaches AIDS, the primary manifestation of pediatric infection is anemia (van Eijk, Ayisi *et al.* 2002). As in malaria, where parasite-mediated lysis of RBCs cannot account for the massive red cell loss that is observed, much of the anemia seen in pediatric HIV and malaria may be due to impairment of erythropoiesis in a pro-inflammatory milieu. Exposure of the bone marrow and erythropoietic cascade to a pro-inflammatory milieu likely contributes to the anemia noted in HIV-1, malaria, and bacteremia (Casals-Pascual, Kai *et al.* 2006).

1.7.1.4 Consequences of malaria and HIV co-infections *Plasmodium falciparum* and HIV-1 are co-endemic in many tropical and sub-tropical countries, with the potential risk for marked clinical, hematological, and parasitological complications. HIV is immunosuppressive and affects the backbone of the acquired immune response, CD4+ T-helper cells, impeding the development of a protective response that is vital to developing partial immunity to clinical malaria. Despite the geographical overlap between malaria and HIV, earlier studies revealed little or no definitive interactions

between the two infections (Colebunders, Bahwe *et al.* 1990; Muller, Musoke *et al.* 1990; Greenberg, Nsa *et al.* 1991; Kalyesubula, Musoke-Mudido *et al.* 1997). Previously, we have shown that there is a definitive interaction between HIV-1 and malaria in Kenyan children resulting in the exacerbation of anemia (Otieno, Ouma *et al.* 2006). There was a greater degree of SMA, defined as Hb <6.0g/dL, in children that were HIV-1 positive (HIV-1[+]) versus either the HIV-1 exposed (HIV-1[exp]) or HIV-1 negative (HIV-1[-]) children, and was independent of parasite load. Similarly, Grimwade *et al.* (Grimwade, French *et al.* 2004) evaluated the effect of HIV status on outcome of malaria infection, observing that the likelihood of a complicated malaria episode or death substantially increased in both scenarios, as indicated by adjusted odds ratios of 2.3 and 7.5, respectively. Epidemiological analyses of our SMA study cohort in western Kenya have also shown that these children typically die within one year of viral RNA copies being detected in their blood (Davenport *et al.*, 2008, unpublished observation), often due to the compounded anemia inflicted by HIV, as well as malaria. Exacerbating the anemia seen in each disease, epidemiological evidence also shows that both Ugandan HIV-1-infected and uninfected infants have iron-deficiency rates of 44.3% and 45.4%, respectively (Totin, Ndugwa *et al.* 2002). Iron-deficiency is, in part, caused and aggravated by IL-6 upregulation of hepcidin, which not only sequesters iron stores in the reticuloendothelial system (Jacober, Mamoni *et al.* 2007), but also prevents intestinal absorption of iron (Yamaji, Sharp *et al.* 2004). Although our group (Otieno, Ouma *et al.* 2006) and others (van Eijk, Ayisi *et al.* 2002)

have previously shown that anemia is worsened in pediatric malaria and HIV-1 co-infection, little is known about the cytokine milieu in these co-infected children.

In addition to the anemia caused by both HIV-1 and malaria, viral load, and hence, HIV-1 progression and transmission are also serious concerns. A prospective study published in 2005 by Kublin *et al.* (Kublin, Patnaik *et al.* 2005) showed that HIV-1[+] study participants doubled their viral load when they became infected with malaria, however, their viral copies declined to baseline levels approximately eight weeks after treatment, while those remaining aparasitemic during the study had no appreciable changes in their HIV-1 RNA levels from those measured at baseline. Additional recent reports indicate that the interaction may not be completely deleterious when malaria precedes HIV-1 infection. Moriuchi *et al.* (Moriuchi, Moriuchi *et al.* 2002) have shown that PBMC treated with supernatants from lysed schizonts (*Pf*-Ags) prior to HIV-1 infection caused a decrease in viral production by inducing cellular resistance to viral entry, even though the *Pf*-Ags up-regulated CCR5. These studies further showed that treatment of cells with IFN-γ in the absence of *Pf*-Ags produced the same resistance to HIV-1 infection, while adding IFN-γ blocking antibodies abrogated the protective effect. Similarly, Gurney *et al.* (Gurney, Colantonio *et al.* 2004) demonstrated that CpG-treated thymic cells inhibited HIV-1_{NL4-3} replication by increased IFN-α production. We have found similar results in our laboratory when PBMCs were treated with *Pf*Hz or sHz prior to exposing cells to HIV-$1_{89.6}$ dual-tropic virus. Intracellular p24 assays indicated that there was significantly less viral entry when cells were pre-treated

with *Pf*Hz or sHz indicating a cell membrane or receptor repertoire change (Davenport *et al.*, 2008 American Society of Tropical Medicine and Hygiene annual meeting).

Chemokine and chemokine receptor changes also play a significant role in the spread and amplification of HIV-1. Ochiel *et al.* (Ochiel, Awandare *et al.* 2005) have shown in both *ex vivo* PBMCs from malaria-infected children, as well as with *in vitro* experiments, that malaria parasites and their products are associated with decreased levels of RANTES/CCL5, which may have implications for accessibility of HIV-1 to CCR5. Indeed, previous studies have shown a correlation between the likelihood of being infected and the level of CC chemokines found in the periphery (Zagury, Lachgar *et al.* 1998; Wasik, Bratosiewicz *et al.* 1999). In addition, healthy children with previous episodes of SMA were also found to have decreased RANTES/CCL5 levels, while those children with a history of only mild malaria had normal levels (Ochiel, Awandare *et al.* 2005). Similar *in vitro* experiments using CD14+ cells and PBMCs treated with either *Pf*Hz or sHz and assayed for surface expression of CCR5, CXCR4, or CD4 over the course of five days revealed that the co-receptors were both decreased at 48 hrs and increased above control at 120 hrs, while CD4 was increased three-fold over control at 48 hrs and decreased by 50% at 120 hrs (Ochiel *et al.*, 2004, unpublished data).

1.7.2 Bacteremia

1.7.2.1 Bacteremia in sub-Saharan Africa Although malaria and HIV are still major infectious disease obstacles in sub-Saharan Africa, these two pathogens have received the majority of attention in the infectious disease realm. Bacteremia has

received far less attention, perhaps because it is not strictly associated with sub-Saharan African or is overshadowed by the devastation caused by HIV-1 and malaria. However, the need to address bacterial infection and elucidate its interactions with malaria are equally important, as emphasized by the findings of Berkely *et al.* (Berkley, Lowe *et al.* 2005). Their group found that 26% of hospital deaths were attributed to bacteremia and the mortality rate of children with an admitting diagnosis of bacteremia was 28%. In many of those cases the children had co-morbid conditions that contributed to their death such as HIV (18%) and malnutrition (37%). Okwara *et al.* (Okwara, Obimbo *et al.* 2004) found that the presence of malaria parasitemia is not specifically associated with increased bacteremia risk (OR 0.9%, 95% CI 0.4-0.7). In our cohort of western Kenyan children, we found no exacerbation of anemia, and surprisingly, a reduction in parasitemia in both Gram positive (G[+]) and Gram negative (G[-]) co-infected children (presented here). This striking finding deserves investigation to determine the inflammatory milieu that is controlling the parasitemia, while not exacerbating anemia.

1.7.2.2 Consequences of malaria and bacteremia co-infection Much focus has been given to malaria and HIV in sub-Saharan Africa, but the contribution of bacterial blood-borne infections to the death of 4.6 million African children per year has only been superficially explored. Berkley *et al.* (Berkley, Lowe *et al.* 2005) surveyed 19,339 Kenyan children in a malaria hyper-endemic region (10 – 100 infective bites annually) and found 12.8% of children less than 60 days of age had bacteremia, as well as an additional 5.9% of children over 60 days. The two organisms most frequently isolated

were *Streptococcus pneumoniae* and non-Typhi *Salmonella* (NTS) species, with the incidences greatest in the <1 year old group and <2 year old group, respectively. In the context of severe malaria, Brent *et al.* (Brent, Oundo *et al.* 2006) found that both anemia and malaria were independently associated with *Salmonella* bacteremia, and 75% of those had current or recent malaria infections and were less than two years of age. A similar study conducted in Malawi reported that both bacteremia and NTS prevalence were highest in children with SMA (11.7% and 7.6%, respectively), relative to CM, or CM with severe anemia (Bronzan, Taylor *et al.* 2007). In addition, the increased incidence of sickle cell trait, which is tightly associated with malaria endemic areas due to its protective effects against malaria, also predisposes native Africans to bloodstream infections with encapsulated organisms (Overturf, Powars *et al.* 1977; Pearson 1977; Lobel and Bove 1982; Zarkowsky, Gallagher *et al.* 1986; Okuonghae, Nwankwo *et al.* 1993), as well as *Salmonella* bacteremia (Workman, Philpott-Howard *et al.* 1994; Wright, Thomas *et al.* 1997). These data collected in a similar region to our study area indicate a significant association between malaria and NTS, and suggests an interaction and exacerbation of malarial anemia by the presence of G[-] bacteremia. Given the high incidence of NTS in our study population (44%; Were *et al.*, 2009, unpublished results), and sub-Saharan Africa as a whole, the need to investigate the interaction with malaria is critical to ameliorating morbidity in pediatric populations in sub-Saharan Africa.

1.8 CONCLUSION

Given the paucity of literature available on the inflammatory milieu of malaria co-infections with HIV-1 and bacteremia, the current study contributes to the body of knowledge by characterizing the inflammatory milieu of co-infected Kenyan children living in a holoendemic region of malaria transmission. Furthermore, through extensive parasitological, hematological, clinical, and genetic analyses of these children, as well as advanced statistical techniques, the following investigations have suggested the predictors of anemia common to these co-infections.

2.0 CHAPTER TWO: SPECIFIC AIMS

Severe anemia is the primary outcome of childhood malaria in holoendemic malaria transmission regions such as Kenya, where inhabitants may endure up to 300 infective bites annually. Malaria is characterized by abundant production of pro-inflammatory cytokines, as well as a perturbed immune response caused by the deposition of the *Pf*Hz in monocytes and neutrophils. The anemia stems from three distinct mechanisms resulting from a pro-inflammatory milieu and exposure to *Pf*Hz: RBC phagocytosis and destruction of infected and uninfected RBCs, dyserythropoiesis due to impaired development of erythroid precursors, and inhibition of erythropoiesis by a pro-inflammatory environment. Two diseases with equal importance in the Kenyan pediatric population are HIV-1/AIDS and bacteremia. Similar to malaria, anemia is the hallmark trait of pediatric HIV-1 infection. The chronic immune activation phase of HIV-1 infection is also characterized by elevated levels of pro-inflammatory cytokines, as is bacteremia. We have found that in HIV-1 and malaria co-infection, as well as in HIV-1[-] children exposed to HIV-1 *in utero*, the anemia was substantially more severe than in malaria infection alone. The opposite phenomenon was observed in bacteremia and malaria co-infection, with co-infected children having the same degree of anemia, but significantly lower parasitemias. The association of a pro-inflammatory milieu with

anemia and the resistance to erythropoietin therapy has also been noted in chronic diseases with non-infectious origins, such as kidney disease and Lupus. Rather than simply correlate individual inflammatory mediators with anemia, we sought to identify cytokines patterns and profiles associated with negative and positive hematolgic outcomes in co-infected children through the use of complex statistical modeling to identify the inflammatory milieus that are most conducive to controlling and clearing infection without adverse clinical consequences.

2.1 SPECIFIC AIM 1

To determine the parasitologic and hematologic predictors of anemia exacerbation in malaria and HIV-1 exposed and infected children residing in a holoendemic environment of western Kenya.

Hypothesis 1: *Anemia will increase proportionally with the density of hemozoin-containing monocytes and neutrophils, due to their induction of pro-inflammatory responses and thereby, the inihibition of erythropoiesis.*

Parasitologic, hematologic, microbiologic, and clinical data collected from malaria-infected (*Pf*[+]) children between the ages of 3 - 36 months of age enrolled in the Severe Malarial Anemia study in Siaya District of western Kenya were analyzed to determine the predictors of worsening anemia. HIV-1 exposure and HIV-1 infection

were determined using two rapid serological immunoassays and a pro-viral DNA PCR assay, respectively. The children were stratified into three categories: HIV-1 negative (HIV-1[-]), no evidence of circulating HIV-1 antibodies on two serologic assays; HIV-1 exposed (HIV-1[exp]), a positive result on at least one of two serologic assays, and a negative HIV-1 DNA PCR result; and HIV-1 positive (HIV-1[+]), a positive result on at least one of two serologic assays for HIV-1 antibodies, and two positive consecutive HIV-1 DNA PCR results three months apart. All children used in this analysis were free of bacteremia and hookworm infection. Using previously established known predictors of anemia (i.e., age and HIV status), a Pearson correlation of potential factors with Hb values, and a hierarchical multiple regression model we identified and quantitated the factors contributing to worsening anemia in HIV-1 co-infected children.

2.2 SPECIFIC AIM 2

To determine the inflammatory mediator profile associated with exacerbated severe anemia in children co-infected with *Plasmodium falciparum* malaria and HIV-1.

Hypothesis 1: *Infection with Plasmodium falciparum causes enhanced anemia in HIV-1-infected children through exacerbation of the chronic immune activation phase.*

Plasma samples were collected from Kenyan children at their first acute malaria hospital contact and stratified into the following categories: HIV-1[-], HIV-1[exp], and HIV-1[+]. Circulating inflammatory mediator levels (cytokines, chemokines and growth factors) were measured using a BioSource™ human 25-plex Luminex® assay. The data from these experiments, analyzed with a principal component factor analysis, identified the inflammatory mediator profile associated with enhanced severe anemia in HIV-1 and malaria co-infected children.

2.3 SPECIFIC AIM 3

To determine the inflammatory mediator profile associated with lower parasitemia levels in children co-infected with *Plasmodium falciparum* and bacteremia without worsening anemia.

Hypothesis 1: *Blood borne bacterial infections elicit a unique profile of inflammatory mediators resulting in lower parasitemia levels without promoting enhanced anemia in co-infected children.*

Circulating blood was collected from Kenyan children at their first hospital contact with acute malaria with and without bacteremia. Only HIV-1 negative children were included in this aim. Presence of bacteremia were determined by microbial isolation using Wampole™ pediatric Isostat® Isolator™ tubes and standard microbiologic media. Circulating inflammatory mediators (cytokines, chemokines and growth factors) were measured using a BioSource™ human 25-plex Luminex® assay. Children were then divided into three groups; malaria mono-infected (*Pf*[+]), Gram positive bacteremia and malaria co-infected (G[+]/*Pf*[+]), and Gram negative bacteremia and malaria co-infected (G[-]/Pf[+]). Using a multiple mediation model, these experiments identifed the unique inflammatory profile associated with decreased parasitemia, without worsening anemia, in children co-infected with malaria and bacteremia.

3.0 CHAPTER THREE: RESULTS, SPECIFIC AIM 1

To determine the hematologic and parasitologic predictors of exacerbated severe anemia in HIV-1[+] and HIV-1[exp] co-infected children.

3.1 HYPOTHESIS 1, PRESENTATION OF MANUSCRIPT ENTITLED:

HEMATOLOGICAL PREDICTORS OF INCREASED SEVERE ANEMIA IN KENYAN CHILDREN CO-INFECTED WITH PLASMODIUM FALCIPARUM AND HIV-1

Hypothesis 1: Anemia will increase proportionally with the density of hemozoin-containing monocytes and neutrophils, due to their induction of pro-inflammatory responses and thereby, the inihibition of erythropoiesis.

To address hypothesis 1, children enrolled in the SMA study and without bacteremia were divided into three groups according to their HIV-1 status: HIV-1[-], HIV-1[exp], and HIV-1[+]. The clinical, hematologic, and parasitologic parameters were collected and then ranked using correlational analysis. Parameters demonstrating high correlation with Hb were included in a hierarchical multiple regression analysis to determine their influence on Hb values in HIV-1[exp] and HIV-1[+] children with more

severe anemia. The findings from these analyses are presented in the following manuscript published in the American Journal of Hematology [*Am J Hematol*. 2010 Apr; 85(4):227-33; reproduced with permission from Wiley-Blackwell Publishing].

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3.2 ABSTRACT

Malaria and HIV-1 are co-endemic in many developing countries, with anemia being the most common pediatric hematological manifestation of each disease. Anemia is also one of the primary causes of mortality in children mono-infected with either malaria or HIV-1. Although our previous results showed HIV-1 positive (HIV-1[+]) children with acute *Plasmodium falciparum* malaria (*Pf*[+]) have more profound anemia, potential causes of severe anemia in co-infected children remain unknown. As such, children with *P. falciparum* malaria (aged 3-36 mos., n=542) from a holoendemic malaria transmission area of western Kenya were stratified into three groups: HIV-1 negative (HIV-1[-]/*Pf*[+]); HIV-1 exposed (HIV-1[exp]/*Pf*[+]); and HIV-1 infected (HIV-1[+]/*Pf*[+]). Comprehensive clinical, parasitological, and hematological measures were determined upon enrollment. Univariate, correlational, and hierarchical regression analyses were used to determine differences among the groups and to define predictors of worsening anemia. HIV-1[+]/*Pf*[+] children had significantly more malarial pigment-containing neutrophils (PCN), monocytosis, increased severe anemia (Hb <6.0g/dL), and nearly ten-fold greater mortality within three months of enrollment. Common causes of anemia in malaria-infected children, such as increased parasitemia or reduced erythropoiesis, did not account for worsening anemia in the HIV-1[+]/*Pf*[+] group, nor did carriage of sickle cell trait or G6PD deficiency. Hierarchical multiple regression analysis revealed that more profound anemia was associated with elevated PCM, younger age, and increasing HIV-1 status (HIV-1[-] → HIV-1[exp] → HIV-1[+]). Thus,

malaria/HIV-1 co-infection is characterized by more profound anemia and increased mortality, with acquisition of monocytic pigment having the most detrimental impact on Hb levels.

3.3 BACKGROUND

Plasmodium falciparum and human immunodeficiency virus-1 (HIV-1) are co-endemic in many tropical and sub-tropical countries, with the potential risk for enhanced clinical, hematological, and parasitological complications. Approximately 250 million malaria infections are reported annually, resulting in greater than one million deaths, primarily in African children less than five years of age (WHO 2008). Sub-Saharan Africa also contains approximately 67% of the global HIV population, with an estimated 22 million HIV infections (UNAIDS 2008). In 2007 alone, this region accounted for 75% of the global AIDS deaths and over 1.9 million individuals were newly infected (UNAIDS 2008). Despite the geographical overlap between malaria and HIV, a number of earlier studies revealed little or no definitive interactions between the two infections (Nguyen-Dinh, Greenberg *et al.* 1987; Colebunders, Bahwe *et al.* 1990; Muller, Musoke *et al.* 1990; Greenberg, Nsa *et al.* 1991; Kalyesubula, Musoke-Mudido *et al.* 1997). However, more recent literature shows that malaria and HIV-1 co-infection results in adverse pathological outcomes in both diseases, such as, increased HIV-1 viral loads following acute malaria, increased malaria episodes in HIV-1 positive (HIV-1[+]) individuals, reduced hemoglobin (Hb) concentrations during malaria and HIV-1 co-infection, and

reduced efficacy of antimalarial drugs (Hoffman, Jere *et al.* 1999; Whitworth, Morgan *et al.* 2000; van Eijk, Ayisi *et al.* 2002; Grimwade and Swingler 2003; Grimwade and Swingler 2003; Grimwade, French *et al.* 2004; Mwapasa, Rogerson *et al.* 2004; Cohen, Karstaedt *et al.* 2005; Kublin, Patnaik *et al.* 2005).

Mono-infection with either malaria or HIV-1 is associated with hematological complications, including anemia, monocytosis, and hemolysis (Abdalla 1990; Sen, Tewari *et al.* 1994; Shearer, Rosenblatt *et al.* 2003; Belperio and Rhew 2004). Additional hematological complications in malaria include splenomegaly, leukopenia, leukocytosis, eosinophilia, and thrombocytopenia (Abdalla 1990), while HIV-1 infection is characterized by suppression of all three major blood cell lineages (Shearer, Rosenblatt *et al.* 2003). Although most interactions between malaria and HIV-1 have been described in adults [reviewed in (Brentlinger, Behrens *et al.* 2007)], our recent investigation demonstrated that HIV-1[+] children had a significantly higher risk of developing severe anemia (SA) during acute falciparum malaria than HIV-1 negative (HIV-1[-]) children (Otieno, Ouma *et al.* 2006). In addition, enhanced pathogenesis has been extensively described in pregnant women with malaria and HIV co-infection (ter Kuile, Parise *et al.* 2004; Briand, Badaut *et al.* 2009).

Our previous findings (Keller, Yamo *et al.* 2006; Awandare, Ouma *et al.* 2007), and those of others (Nguyen, Day *et al.* 1995; Amodu, Adeyemo *et al.* 1998; Lyke, Diallo *et al.* 2003), revealed that pigment-containing monocytes (PCM) and pigment-containing neutrophils (PCN) are important markers of malaria disease severity. Malarial pigment (hemozoin, *Pf*Hz) is a crystalline structure formed from monomeric heme and released

as a by-product of parasitic proteolysis of host Hb (Egan 2002). Phagocytic cells acquire *Pf*Hz through ingestion of parasitized red blood cells (pRBC) (Arese and Schwarzer 1997) and by scavenging free *Pf*Hz released into circulation following rupture of pRBC (Schwarzer, Alessio *et al.* 1998). To date, the impact of naturally-acquired intraleukocytic *Pf*Hz on Hb concentrations in co-infected individuals has not been reported.

To investigate etiologies of worsening anemia in HIV-1[+]/*Pf*[+] children, we performed comprehensive hematological analyses and examined potential causes of anemia in three groups of children (n=542; 3-36 mos. of age): HIV-1[-]/*Pf*[+]; HIV-1 exposed [exp]/*Pf*[+], and HIV-1[+]/*Pf*[+]. The study was conducted in a holoendemic *P. falciparum* transmission area of western Kenya in which SA is the primary manifestation of severe malaria in children less than 48 mos. of age, with pediatric cerebral malaria occurring only in rare cases (Snow, Omumbo *et al.* 1997; McElroy, Lal *et al.* 1999).

3.4 METHODS

3.4.1 Study site and participants

Children aged 3-36 months (n=542) with *P. falciparum* parasitemia (*Pf*[+], any density) were recruited at Siaya District Hospital, western Kenya, during their first hospital contact for malaria from March 2004 to January 2006. Siaya District is a holoendemic *P. falciparum* transmission area where residents receive up to 300 infective bites per annum (Beier, Oster *et al.* 1994). None of the children in the study had bacteremia, cerebral malaria, or non-falciparum malarial infections. A detailed description of the study area and pediatric population can be found in our previous publication (Ong'echa, Keller *et al.* 2006).

Children with *P. falciparum* malaria were divided into three groups: HIV-1 negative (HIV-1[-], negative HIV serological results by both Determine® and Uni-Gold™ assays); HIV-1 exposed (HIV-1[exp], at least one (of two) positive serological tests with Determine® and Uni-Gold™ and negative HIV-1 DNA PCR); and HIV-1 positive (HIV-1[+], at least one (of two) positive serological result with the Determine® and Uni-Gold™ tests, and positive HIV-1 DNA PCR results on two consecutive HIV-1 DNA PCR assays three months apart according to our previously published methods (Otieno, Ouma *et al.* 2006)). For the PCR analyses, HIV-1 gp41 primers were selected for highly conserved HIV-1 group M, N, and O sequences for use in western Kenya (Cappadoro, Giribaldi *et al.* 1998; Ruwende and Hill 1998). Although the maternal HIV-1 status was unknown, based on the approved informed consent process for

enrollment into the study, HIV-1[exp] children presumably acquired HIV-1 antibodies from their mother during gestation and/or through breastfeeding. It is important to note that none of the children in the cohort had received prior blood transfusions. Pre- and post-test HIV counseling were provided for the parents/guardians of all participants. Children positive for one or both HIV-1 serological tests were prophylactically treated with trimethoprim–sulfamethoxazole from the time of enrollment onward. None of the children had been initiated on antiretroviral (ARV) therapy at the time of enrollment since ARVs were not available during the study period. Children in all three groups were followed for three months post-enrollment to examine mortality. The parents/guardians of all children were asked to report to the hospital three months post-enrollment. For those children that did not report, members of the study team traveled to the residence and inquired about the child's health status. In addition, parents/guardians were asked to return to the hospital during each febrile episode that their child experienced prior to the three month follow-up visit.

Severe anemia was categorized according to a geographically appropriate definition for this holoendemic region (i.e., Hb <6.0g/dL) based on previous longitudinal Hb measures in children (<48 months of age; n>14,000) (McElroy, Lal *et al.* 1999). The WHO standard of SA (i.e., Hb <5.0g/dL) (2000) was also utilized to frame the current findings into a broader global context. Children were treated according to Ministry of Health, Kenya, guidelines that included intravenous quinine for the treatment of severe malaria and oral Coartem^{TM} for non-severe malaria. Written informed consent

was obtained from the participants' parents/guardians. Approval for the study was granted by the ethical and scientific review committees at the University of Pittsburgh, University of New Mexico, and the Kenya Medical Research Institute.

3.4.2 Laboratory methods

Asexual malaria trophozoites were determined with thick and thin Giemsa-stained peripheral blood smears prepared from venous blood samples for malaria parasite identification and quantification according to our previous methods (Otieno, Ouma *et al.* 2006). Complete blood counts were performed with a Beckman Coulter© A^C-T diff2™ (Beckman Coulter, Inc.) on blood obtained prior to administration of antimalarials and/or antipyretics. Glucose-6-phosphate dehydrogenase (G6PD) deficiency was assessed using the G6PDH Screening Kit (Trinity Biotech, PLC) according to the manufacturer's instructions. The presence of Hb variants in sample hemolysates was detected using a Hb electrophoresis kit, which allowed for detection of HbS, HbC, HbF, and HbA, in either the heterozygous or homozygous states (Helena laboratories). The reticulocyte count, absolute reticulocyte number (ARN), reticulocyte production index (RPI), and prevalence and quantity of pigment-containing neutrophils (PCN) and pigment-containing monocytes (PCM) were determined per our previous methods (Were, Hittner *et al.* 2006).

Presence of bacteremia was performed by blood culture according to our previous report (Awandare, Ouma *et al.* 2007). Since bacteremia is a common cause of anemia in African children (Brent, Oundo *et al.* 2006; Calis, Phiri *et al.* 2008), which may have potentially confounding effects on results presented here, all children found to have bacteremia were excluded from the current study.

3.4.3 Statistical analyses

Data were analyzed using SPSS (version 15.0). Inter-group clinical, demographic, and hematological measures were compared by either analysis of variance (ANOVA) or Kruskal-Wallis tests, followed by pair-wise post-hoc comparison with Student's t-test or Mann-Whitney U test, respectively. Pearson's Chi Square (χ^2) or Fisher's exact test was used for comparing proportions. The conventional level of statistical significance was set at $P \leq 0.05$. Pearson correlations were performed to select (i.e., $P \leq 0.05$) and prioritize potential predictors of Hb to be entered into a hierarchical multiple regression model.

3.5 RESULTS

3.5.1 Demographic, clinical, and hematologic characteristics

Children were stratified into three categories: HIV-1[-]/*Pf*[+], n=406; HIV-1[exp]/*Pf*[+], n=112; and HIV-1[+]/*Pf*[+], n=24. The demographic, clinical, and hematological characteristics of the study participants are listed in Table 1. Age (mos.) differed across the groups ($P=0.055$) with the HIV-1[+]/*Pf*[+] group being the oldest. Additional significant inter-group differences included absolute monocyte count ($\times 10^3/\mu L$; $P=0.007$), Hb concentration (g/dL; $P=0.009$), hematocrit (Hct, %; $P=0.012$), red blood cell (RBC) count ($\times 10^6/\mu L$, $P=0.022$), mean corpuscular Hb concentration (MCHC, g/dL, $P=0.069$), and RBC distribution width (RDW; $P=0.018$). Post-hoc testing of these significant values revealed that, relative to the HIV-1[-]/*Pf*[+] and HIV-1[exp]/*Pf*[+]

groups, the HIV-1[+]/*Pf*[+] group had higher monocyte counts (P=0.002 and P=0.006, respectively), decreased Hb levels (P=0.004 and P=0.012, respectively), reduced RBC counts (P=0.011 and P=0.045, respectively), lower MCHCs (P=0.066 and P=0.027, respectively), and greater RDWs (P=0.007 and P=0.041, respectively). Evaluation of malaria parasitological indices revealed that median peripheral (/µL) and geometric mean (/µL) parasitemias were not significantly different across the groups (P=0.205 and P=0.123, respectively).

3.5.2 Genetic variants

Certain genetic traits confer protection against severe malaria, namely sickle cell trait and G6PD deficiency (Min-Oo and Gros 2005; Cappellini and Fiorelli 2008). As shown in Table 1, HIV-1[+]/*Pf*[+] children had a lower incidence of sickle cell trait than either the HIV-1[-]/*Pf*[+] or HIV-1[exp]/*Pf*[+] children (P=0.198). In contrast, prevalence of G6PD deficiency increased across the groups, with the HIV-1[+]/*Pf*[+] group having the highest proportion of G6PD deficiency (P=0.084, Table 1).

3.5.3 Severe anemia distribution

Severe anemia is the primary manifestation of severe malaria and accounts for a substantial proportion of mortality in western Kenya, with the peak incidence occurring between ages 7-24 mos. (Bloland, Ruebush *et al.* 1999; McElroy, Lal *et al.* 1999). As such, children were classified according to a geographically appropriate definition of SA (i.e., Hb <6.0g/dL) (McElroy, Lal *et al.* 1999) and the WHO definition (i.e., Hb <5.0g/dL) (2000). The proportion of SA at Hb <6.0g/dL progressively increased with HIV-1 status (P=0.020), while the proportion of SA at Hb <5.0g/dL was comparable in the HIV-1[-]/*Pf*[+] and HIV-1[exp]/*Pf*[+] groups, and highest in co-infected children (P=0.026, Fig. 1). Differences between the HIV-1[+]/*Pf*[+] and the HIV-1[-]/*Pf*[+] groups were significant for both Hb <6.0g/dL (P=0.008) and Hb <5.0g/dL (P=0.025), and between the HIV-1[+]/*Pf*[+] and HIV-1[exp]/*Pf*[+] groups (P=0.016) with the WHO standard (Fig. 1).

3.5.4 Mortality associated with co-infection

In holoendemic *P. falciparum* transmission areas such as Siaya District, approximately 30% of the mortality in children less than three years of age is due to *P. falciparum*-promoted SA (Waitumbi, Opollo *et al.* 2000). Examination of the three-month, post-enrollment mortality revealed that HIV-1[+]/*Pf*[+] children had significantly more mortality than both the HIV-1[-]/*Pf*[+] and HIV-1[exp]/*Pf*[+] groups ($P<0.001$, for both categories, Fig. 1). The number of children that died during the three-month follow-up period was 13 (3.2%) in HIV-1[-]/*Pf*[+] group, 6 (5.4%) in the HIV-1[exp]/*Pf*[+] group, and 8 (33.3%) in the HIV-1[+]/*Pf*[+] group.

3.5.5 Erythropoietic indices

To determine if altered erythropoietic responses were responsible for more profound anemia in co-infected children, measures of erythropoiesis were examined in the three groups. As shown in Table 2, the reticulocyte count and ARN did not significantly differ across the groups. We have previously shown that children with severe malarial anemia (SMA) have suppression of erythropoiesis (Awandare, Goka *et al.* 2006), as evidenced by an RPI<2 (Lee 1999). Although the majority of children in all three groups had suppression of erythropoiesis, the inter-group proportions were not significantly different ($P=0.766$, Table 2).

3.5.6 Intraleukocytic hemozoin

Previous investigations illustrated that intracellular *Pf*Hz levels in circulating neutrophils and monocytes are associated with malaria disease severity (Nguyen, Day *et al.* 1995; Amodu, Adeyemo *et al.* 1998; Luty, Perkins *et al.* 2000). As shown in Fig. 2, the percentage of children with PCN was highest in the HIV-1[+]/*Pf*[+] group (P=0.029, inter-group difference). Post-hoc testing revealed that PCN was greater in the HIV-1[+]/*Pf*[+] group than the HIV-1[exp]/*Pf*[+] group (P=0.016), while the differences between the HIV-1[+]/*Pf*[+] and HIV-1[-]/*Pf*[+] groups, and HIV-1[exp]/*Pf*[+] and HIV-1[-]/*Pf*[+] groups were not significant (P=0.079 and P=0.121, respectively). In addition, the median concentration of PCN (/μL) was highest in children co-infected with malaria and HIV-1, but the across-group differences were not significant (P=0.249, Fig. 2). Examination of intra-monocytic *Pf*Hz revealed that the percentage of children with PCM and the median concentration of PCM (/μL) were similar across the groups (P=0.852 and P=0.456, respectively, Fig. 2).

3.5.7 Linear correlation analyses

As an initial step to explore variables that were potentially important in predicting Hb levels, Pearson correlations were performed in the full sample (n=542) between the following measures: HIV-1 status (i.e., HIV-1[-]/*Pf*[+], HIV-1[exp]/*Pf*[+], and HIV-1[+]/*Pf*[+]); age; PCM; PCN; and Hb. There were significant correlations between progressing HIV-1 status (HIV-1[-] → HIV-1[exp] → HIV-1[+]) and age (r= 0.091, P=0.034) as well as Hb (r= -0.105, P=0.017). Significant relationships with age were

also identified for PCM (r= -0.099, P=0.021) and Hb (r= 0.193, P<0.001). The strongest relationship observed was between PCM and Hb (r= -0.413, P<0.001).

3.5.8 Predictors of Hb concentrations

Following the bivariate correlation analyses, a hierarchical multiple regression analysis was performed to identify predictors of Hb concentrations (Table 3). The influence of HIV-1 was determined by entering HV-1 status (HIV-1[-] → HIV-1[exp] → HIV-1[+]) into the model. Variables were entered as predictors in two sequential blocks with Hb as the dependent variable. Block 1 consisted of age and HIV-1 status (HIV-1[-] → HIV-1[exp] → HIV-1[+]) as covariates, while block 2 was comprised of PCN and PCM. Prior to performing the regression, both PCM and PCN were inverse-transformed to approximate univariate normality. The hierarchical multiple regression model demonstrated that both age (P<0.001) and HIV-1 status (P=0.011) were significant predictors of Hb with block 1 being highly significant (P<0.001) and accounting for 4.4% of the variability in Hb. For block 2, PCM was also a significant predictor of Hb (P<0.001), whereas PCN did not significantly influence Hb levels (P=0.138). This second block was highly significant (P<0.001) and accounted for 14.8% of the variability in Hb. Examination of the squared semipartial correlations indicated that age, HIV-1 status, and PCM accounted for 2.8%, 1.2%, and 14.4% of the unique variance in Hb, respectively.

3.6 DISCUSSION

The current investigation presents a comprehensive examination of the hematological factors that contribute to worsening anemia in Kenyan children residing in a holoendemic *P. falciparum* environment with a high prevalence of HIV-1 infection. Co-infection with malaria and HIV-1 was associated with significantly higher rates of SA, regardless of whether the geographically-relevant definition (Hb <6.0g/dL) of anemia, or the WHO definition (Hb <5.0g/dL), was applied. Results presented here show that two prominent causes of anemia (i.e., malaria parasitemia and reduced erythropoiesis) were *not* responsible for worsening anemia in co-infected children. However, results presented here show that acquisition of *Pf*Hz by monocytes appears central to the pathogenesis underlying more profound anemia in HIV-1[+]/*Pf*[+] children with falciparum malaria. This study is also the first investigation showing that malaria and HIV-1 co-infection is associated with significantly higher rates of pediatric mortality.

Results presented here demonstrate a progressive decline in Hb levels and increased SA in the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups, with HIV-1[+]/*Pf*[+] children having the greatest degree of SA. Malaria contributes to anemia through a number of different mechanisms, including RBC lysis, organ sequestration and destruction of erythrocytes, phagocytosis of uninfected and infected RBCs, and dyserythropoiesis [reviewed in (Ghosh 2007)]. Although all three groups in this study had decreased MCV, normal MCH values, and MCHC in the low normal range, none of these values were significantly different across the groups, with the exception of decreased MCHC values in the HIV-1[+]/*Pf*[+] group. This hematological profile is

suggestive of a microcytic hypochromic anemia, a finding typically attributed to iron deficiency, which may be a consequence of sequestered iron due to elevated levels of IL-6, and consequently hepcidin (Nemeth, Valore *et al.* 2003; Nemeth, Rivera *et al.* 2004; Ganz 2006). Studies are currently ongoing in our laboratory to confirm this hypothesis. The RDW was substantially greater in the HIV-1[+]/*Pf*[+] group, suggesting that, despite the relative consistency in the RPIs across the groups, children with the greatest anemia also had the greatest compensation for their anemia, as indicated by variability in RBC size. Calis *et al.* (Calis, Phiri *et al.* 2008) reported similar RDWs in their study participants, but concluded that folate and iron deficiency were not contributing factors in Malawian children with SA. However, it remains to be determined if folate and iron deficiency are important contributors to worsening anemia in malaria and HIV-1 co-infected children.

It is estimated that 15 to 20% of untreated HIV-1[+] infants will progress to AIDS, and ultimately death, by four years of age in developed nations (Mayaux, Burgard *et al.* 1996), although the rate is likely much higher in African children. A study with participants from the U.S. and Puerto Rico reported 32% had progressed to CDC Clinical Category C or had died by 18 months (Rich, Fowler *et al.* 2000). Consistent with these data, the HIV-1[+]/*Pf*[+] group in our study had nearly 10 times more mortality (33.3%) over the three month follow-up period. However, while the proportion of SA cases and mortality was significantly increased in the HIV-1[+]/*Pf*[+] children, there was no correlation between death and Hb. Furthermore, upon longitudinally examining deceased children from the three groups, there were no significant differences between

acute (febrile, non-parasitemic) or parasitemic visits, median Hb over those visits, or age at which children died. These data may be explained by the fact that substantially more children died at home versus at hospital, and therefore, cause of death, parasitemia status, and Hb levels were not available.

While not reaching statistical significance, there was a progressive increase in G6PD deficiency across the groups. The 7.5% prevalence of G6PD deficiency in our overall study population was similar to the 8.5% reported in a meta-analysis of two previous Kenyan studies (Nkhoma, Poole *et al.* 2009). Interestingly, the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] individuals in the present study had greater than twice the frequency of G6PD deficiency than the HIV-1[-]/*Pf*[+] children. This X-linked deficiency provides mild protection against malaria infection (Cappadoro, Giribaldi *et al.* 1998; Ruwende and Hill 1998), due to stunted parasite growth inside the G6PD-deficient RBC (Clark and Hunt 1983; Miller, Golenser *et al.* 1984). However, oxidative stress caused by infections and various drugs can result in acute hemolysis and subsequent chronic anemia (Cappellini and Fiorelli 2008). Therefore, while this deficiency may provide some protection against acquisition of malarial infection and hyperparasitemia, anemia may be exacerbated once the host becomes infected and treatment ensues.

Although the proportion of children with sickle cell trait was not significant across the groups, there was a three-fold decrease in carriage of HbAS in HIV-1[+]/*Pf*[+] children. Failure to achieve statistical significance may be due to the sample size in the HIV-1[+]/*Pf*[+] group. Since HbAS confers 90% protection against severe malaria and

mortality (Aidoo, Terlouw *et al.* 2002), sickle cell trait may be bolstering the more favorable outcomes (i.e., less severe anemia and fewer fatalities) seen in the HIV-1[-]/*Pf*[+] and HIV-1[exp]/*Pf*[+] groups. However, when G6PD deficiency and sickle cell status were entered into the multiple regression analysis, neither of these factors significantly predicted Hb levels.

Our previous study showed that SMA was associated with suppression of erythropoiesis (Were, Hittner *et al.* 2006). However, the erythropoietic response was not significantly different between the groups examined here, suggesting that neither HIV-1 exposure nor HIV-1 positivity antagonized erythropoiesis beyond that observed in malaria mono-infection. In addition, parasitemia levels were 46% and 37% lower in the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups, respectively, than in the HIV-1[-]/*Pf*[+] children, demonstrating that the degree of parasitemia does not correlate with the severity of anemia, as we (Awandare, Ouma *et al.* 2007) and others (Waitumbi, Opollo *et al.* 2000) have previously reported. However, the fact that the erythropoietic indices are nearly identical, while the degree of anemia is substantially more severe across the groups, indicates that a proportional response to the worsening anemia had not been achieved in the HIV-1[+]/*Pf*[+] group.

By examining *Pf*Hz burden in neutrophils and monocytes, it is possible to determine if individuals suffer from recent (acute) versus prolonged (chronic) malaria infection, since monocytes remain in circulation longer than neutrophils (Tomas Ganz 1995). Our previous study in Kenya demonstrated that SMA is characterized by increased chronicity of infection, resulting in higher levels of PCM and lower levels of

PCN (Awandare, Ouma *et al.* 2007). However, our present data show that co-infection is associated with a higher percentage of PCN, suggesting that HIV-1[+]/*Pf*[+] children suffer from more acute malaria. Although the reason for this finding remains unclear, HIV-1[+]/*Pf*[+] children presumably had worse overall health status, which may result in more rapid treatment-seeking behavior by their caregiver. Additional longitudinal follow-up studies with active surveillance are required to confirm this hypothesis.

To investigate predictors of Hb levels, we first performed Pearson correlations followed by a hierarchical multiple regression analysis to determine those variables with the greatest influence on Hb. Results of the regression model indicated that increasing age was significantly associated with higher Hb levels. In contrast, increasing HIV-1 status (i.e., HIV-1[-] → HIV-1[exp] → HIV-1[+]) in children with falciparum malaria was a significant predictor of worsening anemia. This finding supports the higher proportion of SA found in HIV-1[+]/*Pf*[+] children. Increasing PCM was associated with worsening anemia, and of all the factors examined, was the strongest predictor of Hb. The negative correlation between PCM and Hb supports our previous findings (Keller, Yamo *et al.* 2006; Awandare, Ouma *et al.* 2007) and those of others (Lyke, Diallo *et al.* 2003) illustrating that PCM is a significant predictor of anemia. Moreover, results presented here extend previous findings by demonstrating that monocytic acquisition of *Pf*Hz is also a significant predictor of Hb in children with malaria and HIV-1 co-infection.

Taken together, results presented here demonstrate that children with malaria and HIV-1 co-infection have more profound anemia and increased mortality relative to HIV-1[-]/*Pf*[+] children. These findings also demonstrate that unlike parasitemia levels

and suppression of erythropoiesis, *Pf*Hz may play a central role in the pathogenesis of anemia in children co-infected with malaria and HIV-1. Since phagoctyosis of *Pf*Hz by monocytes and neutrophils promotes dysregulation in inflammatory mediators known to cause inflammatory-derived anemia (Sherry, Alava *et al.* 1995; Perkins, Moore *et al.* 2003; Keller, Kremsner *et al.* 2004; Casals-Pascual, Kai *et al.* 2006), it will be important to determine if altered expression of cytokines, chemokines, and hematopoietic growth factors are responsible for more profound anemia in co-infected children.

3.7 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.

3.8 AUTHORS' CONTRIBUTIONS

GCD collected and analyzed the data and prepared the manuscript. CO conducted the HIV testing. JBH designed the data analysis strategy and provided statistical support. TW conducted the microbiological testing. YO analyzed the blood smears for parasitologic indices. JMO aided in the study design and interpretation of the results. DJP designed the study, supervised the activities in New Mexico, Pennsylvania, and Kenya, and co-wrote the manuscript with GCD.

3.9 ACKNOWLEDGMENTS

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Table 1. *Clinical, demographic, and hematologic characteristics of the study participants.*

Characteristic	HIV-1[-]/*Pf*[+]	HIV-1[exp]/*Pf*[+]	HIV-1[+]/*Pf*[+]	*P*
Number of subjects	406	112	24	N/A
Age (mos.)	10.5 (10.5)	9.0 (6.2)	12.1 (9.6)	**0.055**
Gender, male/female [a]	219/187	57/55	14/10	0.755
Axillary temperature (°C)	37.5 (1.7)	37.5 (1.6)	37.6 (1.0)	0.913
Glucose (mMol/L)	5.1 (1.3)	4.9 (1.5)	4.8 (1.2)	0.544
Hematologic Indices				
WBC ($\times 10^9$/µL)	11.2 (6.6)	11.7 (6.7)	13.3 (8.4)	0.144
Lymphocytes ($\times 10^3$/µL)	50.0 (19.2)	51.9 (17.4)	49.4 (16.7)	0.163
Monocytes ($\times 10^3$/µL)	8.9 (5.8)	8.8 (5.2)	13.0 (6.3)	**0.007**
Granulocytes ($\times 10^3$/µL)	41.0 (22.8)	38.0 (18.6)	35.6 (21.1)	0.124
Platelets ($\times 10^3$/µL)	163 (124)	140 (108)	158 (82)	0.129
Hemoglobin (g/dL)	6.9 (3.5)	6.2 (2.7)	5.2 (2.9)	**0.009**
Hematocrit (%)	22.0 (10.4)	20.5 (7.8)	17.7 (6.5)	**0.012**
RBC ($\times 10^6$/µL)	3.2 (1.8)	3.1 (1.5)	2.5 (1.6)	**0.022**
MCV (fL)	70.3 (12.3)	68.8 (11.8)	73.3 (11.4)	0.361
MCH (fL/cell)	22.6 (4.4)	22.2 (3.5)	22.1 (4.1)	0.899
MCHC (g/dL)	32.1 (2.6)	32.3 (2.3)	30.5 (4.1)	**0.069**
RDW	21.3 (4.7)	22.2 (5.2)	23.5 (7.6)	**0.018**
Parasitologic Indices				
Parasitemia (/µL)	22,281 (51,064)	15,299 (37,040)	16,220 (43,127)	0.205
Geomean parasitemia (/µL) [b]	15,739	12,736	11,850	0.123
Genetic Variants				
Sickle cell trait, n (%) [c]	57 (14.1)	18 (16.1)	1 (4.3)	0.198
G6PD deficiency, n (%) [c,d]	25 (6.7)	13 (12.5)	3 (15.0)	0.084

Data are presented as median (interquartile range) and compared using the Kruskal-Wallis test unless stated otherwise. [a] Differences in the proportion of gender were compared using Pearson's χ^2 test. [b] Geometric mean parasitemia was compared using ANOVA. [c] Differences in the proportion of individuals with sickle cell trait (HbAS) and G6PD deficiency were compared using Pearson's χ^2 test. [d] G6PD deficiency was defined as hemizygous in males and homozygous in females, since it is an X-linked gene. Children were grouped as follows: HIV-1[-]/*Pf*[+], negative reaction on the Determine® and Uni-Gold™ HIV-1 serology tests; HIV-1[exp]/*Pf*[+], positive reaction on one or both serology tests and a negative reaction for HIV-1 by PCR; HIV-1[+]/*Pf*[+], positive reaction on one or both serology tests and a positive reaction on two consecutive HIV-1 PCR assays three months apart. The number and species of asexual *Plasmodium* organisms per 300 white blood cells (WBC) were determined using Giemsa-stained thin and thick smear venous blood. Complete blood counts were determined in venous blood using a Coulter® AcT diff2™ (Beckman Coulter Corp.). Mean Corpuscular Volume (MCV); Mean Corpuscular Hemoglobin (MCH); Mean Corpuscular Hemoglobin Concentration (MCHC); and Red Blood Cell Distribution Width (RDW).

Table 2. *Erythropoietic indices.*

Characteristic	HIV-1[-]/*Pf*[+]	HIV-1[exp]/*Pf*[+]	HIV-1[+]/*Pf*[+]	*P*
Number of subjects	406	112	24	N/A
Reticulocyte Count (%) [b]	2.7 (4.1)	3.0 (3.9)	3.3 (6.7)	0.319
ARN ($\times 10^{12}$/L) [a]	0.065 (0.08)	0.064 (0.08)	0.066 (0.10)	0.966
RPI (/µL) [a]	1.45 (1.90)	1.45 (1.94)	1.76 (2.66)	0.974
RPI<2, n (%) [b]	234 (61.6)	66 (62.9)	12 (54.5)	0.766

[a] Data are presented as median (interquartile range) and differences between the three groups were compared using Kruskal-Wallis test. [b] Differences in the reticulocyte count (%) were compared using Pearson's χ^2 test. Reticulocyte production index (RPI) and absolute reticulocyte number (ARN) were calculated as follows: Reticulocyte Index (RI) = reticulocyte count × hematocrit / 30.7 (average hematocrit of children <5yrs of age in Siaya district); Maturation Factor (MF) = 1 + 0.05 (30.7 – hematocrit); RPI = RI / MF; ARN = (RI × RBC count) / 100.

Table 3. *Predictors of hemoglobin concentrations*.

Variable	β-weight	Semipartial r^2	*P*	Block Δ statistics
Age	0.133	0.017	**0.004**	
HIV-1 Status	-0.113	0.013	**0.014**	
Block 1 Summary:				R^2=0.033
				***P*<0.001**
PCM	-0.415	0.155	**<0.001**	
PCN	0.071	0.005	0.102	
Block 2 Summary:				R^2=0.159
				***P*<0.001**

Hierarchical multiple regression analysis was performed to determine predictors of Hb levels. Age (mos.) and HIV-1 status (i.e., HIV-1[-] → HIV-1[exp] → and HIV-1[+]) were entered in block 1; and pigment-containing monocytes (PCM) and pigment-containing neutrophils (PCM) were entered into block 2. The full model was significant at $F(5, 512)= 18.748$, $P<0.001$, $R=0.441$, $R^2=0.194$.

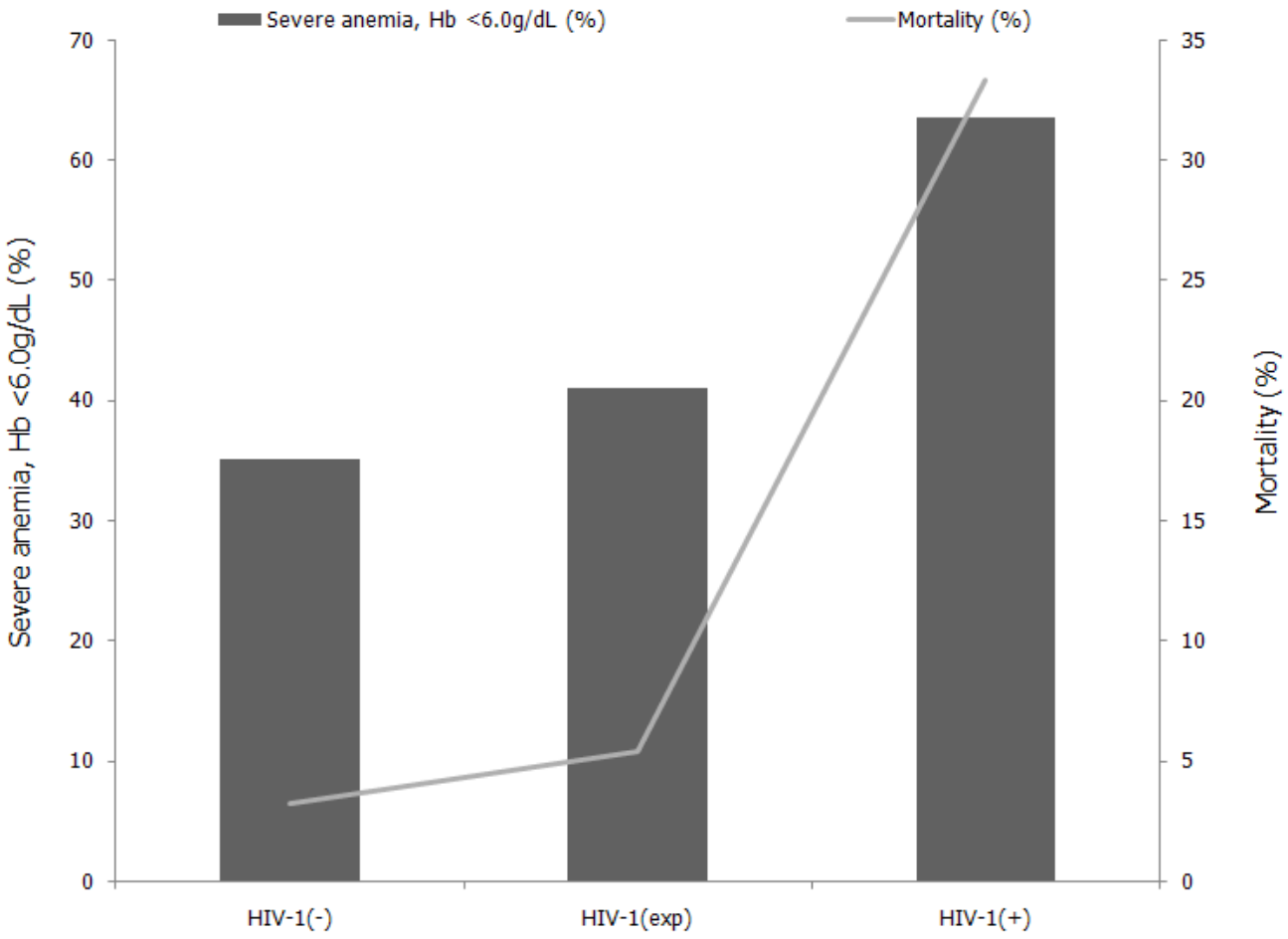

Figure 1. ***Increased anemia and mortality in HIV-1(+) children.*** Bars represent percentage of children with SA (left y-axis), while lines depicts mortality percentage (right y-axis) in each HIV status. Differences in the proportion of individuals with SA (P=0.020) and those deceased (P<0.001) were compared using Pearson's χ^2 test. Pair-wise comparison revealed significant differences between proportions of children with SA and mortality in the HIV-1[-]/*Pf*[+] vs. HIV-1[exp]/*Pf*[+] groups (P=0.045 and P<0.001, respectively) and HIV-1[-]/*Pf*[+] vs. HIV-1[+]/*Pf*[+] groups (P=0.008 and P<0.001, respectively). There were 13 (3.2%) HIV-1[-]/*Pf*[+], 6 (5.4%) HIV-1[exp]/*Pf*[+], and 8 (33.3%) HIV-1[+]/*Pf*[+] children that died within the three-month follow-up period after enrollment. SA (Hb <6.0g/dL) cases were as follows: 137 (35.2%) HIV-1[-]/*Pf*[+]; 44 (41.1%) HIV-1[exp]/*Pf*[+]; and 14 (63.6%) HIV-1[+]/*Pf*[+].

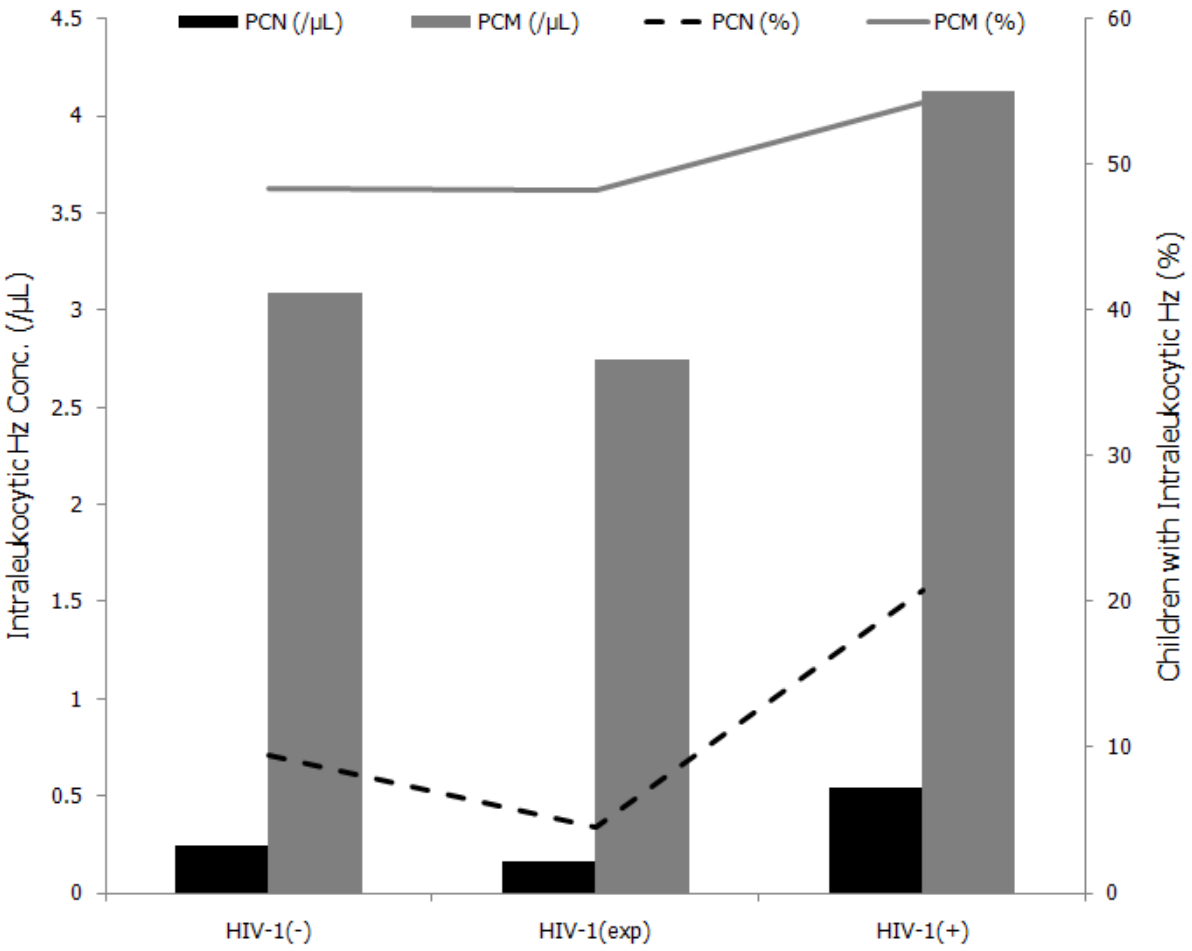

Figure 2. ***Increased intraleukocytic* Pf*Hz in HIV-1[+]\|*Pf*[+] children.*** Bars represent mean PCN/µL (black) and PCM/µL (grey) concentrations and are associated with the left y-axis, while percentage of children with intraleukocytic *Pf*Hz are depicted with a broken line (PCN) and grey line (PCM) and associated with the right y-axis. Differences in the proportion of individuals with PCN (9.4% HIV-1[-]/*Pf*[+], 4.5% HIV-1[exp]/*Pf*[+], 20.8% HIV-1[+]/*Pf*[+]; *P*=0.029) and PCM (48.3% HIV-1[-]/*Pf*[+], 48.2% HIV-1[exp]/*Pf*[+], 54.2% HIV-1[+]/*Pf*[+]; *P*=0.852) were compared using Pearson's χ^2 test, while PCN (HIV-1[-]/*Pf*[+], 0.24/µL; HIV-1[exp]/*Pf*[+], 0.16/µL; HIV-1[+]/*Pf*[+], 0.54/µL; *P*=0.249) and PCM concentrations (HIV-1[-]/*Pf*[+], 3.09/µL; HIV-1[exp]/*Pf*[+], 2.75/µL; HIV-1[+]/*Pf*[+], 4.13/µL; *P*=0.456) were compared across the groups using the Kruskal-Wallis test. Pair-wise comparisons were performed using either Pearson's χ^2 test, for categorical variables, or Mann-Whitney U, for continuous variables. A total of 30 monocytes and 100 neutrophils were examined per slide and expressed as a percentage of the counted monocytes and neutrophils, and then PCM and PCN concentrations were derived by multiplying the percentages by the total absolute monocyte and neutrophil counts, respectively.

4.0 CHAPTER FOUR: RESULTS, SPECIFIC AIM 2

To determine the inflammatory mediator profiles in HIV-1[exp]*Pf*[+] and HIV-1[+]*Pf*[+] co-infected children with exacerbated severe anemia.

4.1 HYPOTHESIS 1, PRESENTATION OF MANUSCRIPT ENTITLED:

INFLAMMATORY MEDIATOR PATTERNS ASSOCIATED WITH EXACERBATED SEVERE ANEMIA IN KENYAN CHILDREN WITH PLASMODIUM FALCIPARUM AND HIV-1 CO-INFECTION.

Hypothesis 1: Infection with Plasmodium falciparum causes enhanced anemia in HIV-1-infected children through exacerbation of the chronic immune activation phase.

To address this hypothesis, a subset of children from the previous aim were randomly selected in each group while preserving the overall median Hb value so the results were generalizable to the entire study population. The plasma from these children were analyzed using a multiplex bead-array assay to determine the concentrations of 25 cytokines, chemokines, and growth factors. In addition to assessing the difference in the 25 inflammatory mediators across the three groups, a

principal component factor analysis procedure was used to mathematically group related cytokines into components based on their variance relatedness. In this way, the components, and ultimately, the cytokines, having the most influence on RPI and Hb values, and were most influenced by PCN and PCM, for each HIV-1 status were determined. The results of these findings are presented in the following manuscript prepared for submission.

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4.2 ABSTRACT

Malaria and HIV-1 are co-endemic in many developing countries, with anemia being the most common pediatric hematological manifestation of each disease. Since we previously demonstrated that malaria/HIV-1 co-infection was associated with exacerbation of severe anemia (SA), immunological mechanisms that promote anemia were investigated. Kenyan children (aged 3-36 mos., n=542) infected with *P. falciparum* and residing in a holoendemic transmission area were stratified into three groups: HIV-1 negative (HIV-1[-]/*Pf*[+]); HIV-1 exposed (HIV-1[exp]/*Pf*[+]); and HIV-1 infected (HIV-1[+]/*Pf*[+]). Clinical and hematological measures were determined by standard methods and immunological profiles were determined with a human 25-plex bead array assay. Worsening anemia was associated with dysregulation in inflammatory mediators (IM), but not indices of either parasitemia or erythropoiesis. Malaria/HIV-1[+] co-infected children had elevated levels of IL-12, IFN-γ, MIG/CXCL9, eotaxin/CCL11, and GM-CSF. To identify unique IM profiles associated with anemia, a principle components factor analysis was performed with 25 effector molecules and 5 inflammatory ratios. This analysis revealed six principal components that explained 69% of the variance in the IM inter-correlation matrix. Correlation analyses between the principal components and HIV-1[+]/*Pf*[+] co-infected children revealed an inverse relationship between component 2 (IL-1β, IL-2, GM-CSF, IL-1β:IL-10, IL-1β:IL-1Ra, IL-2:IL-2R) and pigment-containing neutrophils (PCN), and a direct relationship between component 4 (IL-4, IL-5, IL-12, Eotaxin/CCL11) and hemoglobin (Hb). Post-hoc analysis in the HIV-1[+]/*Pf*[+] group revealed that IL-12 was the most influential variable on Hb

levels, while the IL-1β:IL-10 ratio was most influenced by PCN. Thus, malaria/HIV-1 co-infection is defined by significantly enhanced anemia that is associated with unique inflammatory profiles known to exacerbate anemia.

4.3 INTRODUCTION

Currently, sub-Saharan Africa is home to 90% of the world's *Plasmodium falciparum* infections (WHO 2008) and 64% of the HIV-1 infections (UNAIDS 2008). Children are the most affected group in the population, with malaria and HIV-1 being responsible for 18% and 6%, respectively, of deaths in sub-Saharan African children under the age of five (Rowe, Rowe *et al.* 2006). The primary pediatric manifestation of each of these diseases is anemia.

One important mechanism common to both diseases, which may substantially contribute to the development of severe anemia (SA), is perturbation in inflammatory mediator (IM) production (Luty, Perkins *et al.* 2000; Perkins, Weinberg *et al.* 2000; Lyke, Burges *et al.* 2004; Awandare, Kremsner *et al.* 2007; Awandare, Ouma *et al.* 2007). Results from our laboratory and others have demonstrated that dysregulation in the balance of type 1 [e.g., interleukin (IL)-1β, IL-6, IL-12, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α] and type 2 (e.g., IL-4 and IL-10) cytokines is associated with enhanced immuno-pathogenesis of severe malarial anemia (SMA) (Mshana, Boulandi *et al.* 1991; Kurtzhals, Akanmori *et al.* 1999; Luty, Perkins *et al.* 2000; Perkins, Weinberg *et al.* 2000; McDevitt, Xie *et al.* 2004; Prakash, Fesel *et al.* 2006). We have also shown

that children with malaria have altered levels of β-chemokines, including macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, and regulated upon activation, normal T-cell expressed and secreted (RANTES/CCL5) (Ochiel, Awandare *et al.* 2005; Awandare, Goka *et al.* 2006). Changes in the relative levels of IMs may promote the pathogenesis of SMA by altering the erythropoietic cascade. For example, TNF-α rapidly and irreversibly inhibits human erythroid progenitors (Means, Dessypris *et al.* 1992), while IFN-γ directly suppresses human erythroid colony-forming units (CFU-E) (Means, Dessypris *et al.* 1992). IFN-γ also upregulates IL-15, which in turn, inhibits erythropoiesis at the CFU-E stage (Mullarky, Szaba *et al.* 2007). In addition, our previous results showed that pediatric malarial anemia is characterized by suppression of IL-12 (Luty, Perkins *et al.* 2000; Perkins, Weinberg *et al.* 2000).

Presence of malarial pigment (hemozoin, *Pf*Hz) within macrophages, monocytes, and neutrophils may also contribute to impairment of the immune response commonly observed in *P. falciparum* infections (Sherry, Alava *et al.* 1995; Perkins, Moore *et al.* 2003; Deshpande and Shastry 2004; Moore, Chaisavaneeyakorn *et al.* 2004; Coban, Ishii *et al.* 2005; Nti, Slingluff *et al.* 2005; Huy, Trang *et al.* 2006; Keller, Yamo *et al.* 2006; Awandare, Ouma *et al.* 2007). Hemozoin is a crystalline structure formed from monomeric heme and released as a by-product of parasitic proteolysis of host erythrocyte hemoglobin (Hb) [reviewed in (Hanscheid, Egan *et al.* 2007)]. Our previous findings (Keller, Yamo *et al.* 2006; Awandare, Ouma *et al.* 2007), and those of others (Nguyen, Day *et al.* 1995; Amodu, Adeyemo *et al.* 1998; Lyke, Burges *et al.* 2004; Hanscheid, Langin *et al.* 2008), revealed that pigment-containing monocytes (PCM) and

pigment-containing neutrophils (PCN) are important markers of malaria disease severity. We previously found in the cohort of children investigated here that PCM was the primary predictor of Hb concentrations in malaria/HIV-1 co-infected children, after HIV status and age had been accounted for as co-variates (Davenport, Ouma *et al.* 2010). However, the mechanism by which intraleukocytic *Pf*Hz acts on the erythropoietic cascade, and ultimately affects Hb levels, has yet to be elucidated.

Shortly after HIV-1 infection, resolution of the transient viremia is followed by a state of chronic immune activation characterized by increased pro-inflammatory cytokine release (Barcellini, Rizzardi *et al.* 1996), which potentially depletes CD4+ T-cells required to mount an effective immune response (Hazenberg, Otto *et al.* 2003; Deeks, Kitchen *et al.* 2004; Appay and Sauce 2008). The chronic immune activation phase is associated with elevated levels of IL-1β (Molina, Scadden *et al.* 1989; Roux-Lombard, Modoux *et al.* 1989), IL-6 (Nakajima, Martinez-Maza *et al.* 1989; Scala, Ruocco *et al.* 1994), IL-10 (Clerici, Seminari *et al.* 2000), IFN-γ (Clerici, Seminari *et al.* 2000), TNF-α (Molina, Scadden *et al.* 1989; Roux-Lombard, Modoux *et al.* 1989), MIP-1α/CCL3 (Canque, Rosenzwajg *et al.* 1996; Muthumani, Kudchodkar *et al.* 2000), MIP-1β/CCL4 (Muthumani, Kudchodkar *et al.* 2000), and RANTES/CCL5 (Canque, Rosenzwajg *et al.* 1996; Schmidtmayerova, Sherry *et al.* 1996; Cremer, Vieillard *et al.* 2000; Muthumani, Kudchodkar *et al.* 2000), and decreased IL-12 levels (Chehimi, Starr *et al.* 1994; Daftarian, Diaz-Mitoma *et al.* 1995; Chougnet, Wynn *et al.* 1996; Vigano, Balotta *et al.* 1996). Thus, changes in IM production are common to both malaria and HIV-1 mono-infections.

Given the immunological complexity of malaria and HIV-1, and their anemia commonality, our goal was to define the soluble mediator milieu in co-infected children associated with the failure to mount an effective erythropoietic response to worsening anemia. As such, parasitological, hematological, and immunological profiles were investigated in malaria-infected children with and without HIV-1 from a holoendemic *P. falciparum* transmission area.

4.4 METHODS

4.4.1 Study participants

Children aged 3 - 36 months (n=542) with *P. falciparum* parasitemia (any density) were recruited at Siaya District Hospital, western Kenya, during their first hospital contact for malaria, and matched for age and gender. Siaya District is a holoendemic *P. falciparum* transmission area where residents receive up to 300 infective bites per annum (Beier, Oster *et al.* 1994). None of the children included in the data analysis plan had cerebral malaria, non-falciparum malarial infections, bacteremia, or hookworm infections. A detailed description of the study area and pediatric anemia in the population can be found in our previous publication (Ong'echa, Keller *et al.* 2006).

All children in the study had *P. falciparum* infections. Children with malaria were divided into three groups: HIV-1 negative (HIV-1[-]/*Pf*[+], negative HIV-1 serological results, n=148]; HIV-1 exposed (HIV-1[exp]/*Pf*[+], at least one (of two) positive serological test and negative HIV-1 DNA PCR on two separate blood draws ~3 mos.

apart, n=30); and HIV-1 positive (HIV-1[+]/*Pf*[+], at least one (of two) positive serological tests and positive HIV-1 DNA PCR results on two separate blood draws ~3 mos. apart, n=16). Although the HIV-1 status of mothers was unknown, HIV-1[exp]/*Pf*[+] children presumably acquired HIV-1 antibodies from their mothers during gestation and/or through breastfeeding. It is important to note that none of the children in the cohort had received prior blood transfusions. HIV-1[+]/*Pf*[+] children were not further stratified according to disease stages due to the reduced sample size (i.e., n=16). Pre- and post-test HIV counseling were provided for the parents/guardians of all participants. Children positive for one or both HIV-1 serological tests were prophylactically treated with trimethoprim–sulfamethoxazole. At the time of enrollment, none of the children had been initiated on antiretroviral therapy. Children were also stratified into SA (Hb <6.0g/dL) and non-SA (Hb 6.0–10.9g/dL and free from the symptoms of severe malaria, such as hypoglycemia). Definitions of SA were based on previous longitudinal Hb measures (>14,000) in children (<48 mos. of age) in western Kenya (McElroy, Lal *et al.* 1999). Children were treated according to Ministry of Health, Kenya, guidelines and written informed consent was obtained from the participants' parents/guardians. Approval for the study was granted by the University of New Mexico, University of Pittsburgh, and the Kenya Medical Research Institute (KEMRI).

4.4.2 Laboratory measurements

Asexual malaria trophozoites (Were, Hittner *et al.* 2006) and the number of PCM and PCN were determined according to previous methods (Nguyen, Day *et al.* 1995; Lyke,

Diallo *et al.* 2003). Briefly, thick and thin peripheral blood smears were prepared from venous blood samples and stained with Giemsa reagent for malaria parasite identification and quantification by microscopy. Asexual malaria trophozoites were counted against 300 leukocytes based on absolute counts of white blood cells (WBC)/μL of blood, and parasite density was estimated as follows: parasites/μL = WBC count/μL x trophozoites/300. Complete blood counts were performed with a Beckman Coulter© Ac-T diff2™ (Beckman Coulter, Inc.) on blood obtained prior to administration of antimalarials and/or antipyretics. The reticulocyte count was determined using a thin smear slide stained with New Methylene Blue. The following indices were then calculated to ascertain the reticulocyte production index (RPI): Reticulocyte Index (RI) = reticulocyte count x hematocrit / 30.7 (average hematocrit of children <5yrs in Siaya district); Maturation Factor (MF) = 1 + 0.05 (30.7 – hematocrit); RPI = RI / MF; ARN (absolute reticulocyte number) = (RI x RBC count) / 100. HIV-1 status was determined using two rapid serological antibody tests (i.e., Uni-Gold™, Trinity Biotech and; Determine®, Abbott Laboratories) and HIV-1 DNA PCR analysis was performed according to our previously published methods (Davenport, Ouma *et al.* 2010).

4.4.3 Multiplex assay

Venous blood samples were centrifuged immediately and plasma was separated, aliquoted, and stored at -70°C until use. Samples were then thawed and clarified by centrifugation (14,000 rpm for 10 min) before assaying. IM levels were determined by the Cytokine 25-plex Antibody Bead Kit, Human (BioSource™ International) according

to the manufacturer's instructions. Plates were read on a Luminex® 100™ system (Luminex® Corporation) and analyzed using the Bio-plex manager software (Bio-Rad Laboratories). Analyte detection limits were: 3pg/mL (IL-5, IL-6, IL-8/CXCL8); 4pg/mL [monokine induced by IFN-γ (MIG/CXCL9)]; 5pg/mL (IL-1α, IL-3, IL-4, IL-10, eotaxin-1/CCL11, IFN-γ, IP-10/CXCL10); 6pg/mL (IL-2); 10pg/mL (IL-7, IL-13, IL-15, IL-17, MIP-1α/CCL3, MIP-1β/CCL4, monocyte chemotactic protein [MCP-1/CCL2], TNF-α); and 15pg/mL (IL-1β, IL-12p40/p70, granulocyte-macrophage colony-stimulating factor [GM-CSF], IFN-α, RANTES/CCL5).

4.4.4 Statistical analyses

Data were analyzed using SPSS (version 15.0). Clinical, demographic, hematological, and immunological measures were compared inter-group by either analysis of variance (ANOVA) or Kruskal-Wallis tests, followed by pairwise post-hoc comparison with student's t-test or Mann-Whitney U test, respectively. Pearson's Chi-Square ($\chi 2$) or Fisher's exact test were used for comparing proportions. Statistical significance was determined at $P \leq 0.05$. However, in light of the small number of SA children available in our secondary analyses and, hence, the correspondingly lowered statistical power, we adopted a more liberal *P*-value criterion ($P \leq 0.075$) when interpreting the Kruskal-Wallis results. Our rationale for utilizing a more liberal criterion when interpreting the omnibus Kruskal-Wallis test is to minimize the probability of committing Type II errors. In order to reduce and synthesize the large number of IMs from the multiplex assay, the IMs were subjected to a principal components factor analysis (PCA), and the resulting

components were correlated with Hb, RPI, and *Pf*Hz burden for the entire sample of children and for the three HIV-1 status groups separately. Analyses of variance were also performed to examine whether, and to what extent, mean levels of the principal components differed across the three HIV-1 status groups.

4.5 RESULTS

4.5.1 Demographic, clinical, and hematologic characteristics

Children with falciparum malaria were stratified into three categories: malaria alone (HIV-1[-]/*Pf*[+], n=148); HIV-1[exp]/*Pf*[+], n=30; and co-infected (HIV-1[+]/*Pf*[+], n=16). Study participant demographics and clinical characteristics are listed in Table 4. Children in the three groups had similar ages (P=0.592) and gender proportions (P=0.921). Hematologically, Hb concentrations differed across the groups (P=0.017) and progressively declined with increasing HIV-1 status (HIV-1[-]→HIV-1[exp]→HIV-1[+]) and therefore, more cases of SA (Hb<6.0, P=0.023) were seen in the HIV-1[+]/*Pf*[+] group, however, RPI was not different (P=0.554). White blood cell (WBC) and granulocyte counts were also similar across the groups, although, there was a distinct monocytosis in the HIV-1[+]/*Pf*[+] group (P=0.033). Finally, parasitemia (P=0.112), geometric mean parasitemia (P=0.086), and PCM (P=0.728) were comparable among the groups. However, a marked increase in the concentration of PCN was noted in the HIV-1[+]/*Pf*[+] group (P=0.018).

4.5.2 Pro- and anti-inflammatory cytokine production

Since dysregulation in IM production plays an important role in promoting anemia (Clark and Chaudhri 1988; Ratajczak and Ratajczak 1994; Lyke, Burges *et al.* 2004; Awandare, Goka *et al.* 2006; Thawani, Tam *et al.* 2006; Ong'echa, Remo *et al.* 2007), circulating levels of cytokines, chemokines, and growth factors were determined with a hu 25-plex bead array assay. Our previous investigations (Perkins, Weinberg *et al.* 2000; Keller, Davenport *et al.* 2006; Keller, Yamo *et al.* 2006; Ong'echa, Remo *et al.* 2007), and those of others (Luty, Perkins *et al.* 2000; Lyke, Burges *et al.* 2004), demonstrated that childhood malaria is characterized by altered production of pro- and anti-inflammatory cytokines, which is closely associated with disease severity. However, the profile of cytokine production in HIV-1[exp] and HIV-1[+] children with falciparum malaria is largely unreported. Analyses of circulating cytokines revealed that IL-12 was significantly different across the groups (P=0.003, Table 5). Relative to the HIV-1[-]/*Pf*[+] group, IL-12 was increased in the HIV-1[exp]/*Pf*[+] group (P=0.055), and highest in the HIV-1[+]/*Pf*[+] group (P=0.002, Table 5). In addition, the difference in IFN-γ across the groups was of borderline significance (P=0.050), with the highest levels observed in HIV-1[exp]/*Pf*[+] children, followed by the HIV-1[+]/*Pf*[+] group (P=0.031 and P=0.220 vs. HIV-1[-]/*Pf*[+], respectively, Table 5). None of the other pro- or anti-inflammatory cytokines showed significant inter-group differences (Table 5).

4.5.3 Chemokine and growth factor production

Previous investigations in our laboratory demonstrated that β-chemokines are dysregulated in children with falciparum malaria (Ochiel, Awandare *et al.* 2005; Were, Hittner *et al.* 2006). Although altered regulation of chemokines is well-documented in HIV-1[+] children (Resino, Bellon *et al.* 2001; Tiemessen and Kuhn 2006), the pattern of chemokine production in co-infected children has not been characterized. Results presented here demonstrate that eotaxin/CCL11 levels differed across the three groups (P=0.003, Table 5). Relative to the HIV-1[-]/*Pf*[+] group, eotaxin/CCL11 was 1.3-fold higher in the HIV-1[exp]/*Pf*[+] group (P=0.156) and 1.6-fold higher in HIV-1[+]/*Pf*[+] children (P=0.001, Table 5). Circulating concentrations of the hematopoietic and immune-regulatory cytokine, GM-CSF, also differed across the groups (P=0.011, Table 5), with 4.5-fold and 6.8-fold higher concentrations in the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups compared to the HIV-1[-]/*Pf*[+] group (P=0.020 and P=0.030, respectively, Table 5). MIG/CXCL9 also showed a significant inter-group difference (P=0.027) and was increased in the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups relative to HIV-1[-]/*Pf*[+] children (P=0.038 and P=0.058, Table 5). No significant inter-group differences were observed for the other chemokines.

4.5.4 Inflammatory ratios

We (Perkins, Weinberg *et al.* 2000; Awandare, Goka *et al.* 2006; Keller, Yamo *et al.* 2006; Ouma, Davenport *et al.* 2008) and others (May, Lell *et al.* 2000; Dodoo, Omer *et al.* 2002) have previously shown that ratios of pro- and anti-inflammatory mediators

(e.g., TNF-α:IL-10, IL-1β:IL-1Ra, and IL-2:IL-2R) are more biologically informative than their absolute values. Of the five biologically relevant inflammatory ratios examined, only the IL-2:IL-2R ratio approached significance (P=0.063, Table 5). However, there was a trend towards a more pro-inflammatory environment in the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups.

4.5.5 Principal component analysis of inflammatory mediators

To identify unique profiles of IMs associated with malaria/HIV-1 co-infection, twenty-five IMs and five IM ratios were subjected to a principal component factor analysis (PCA) with a varimax rotation and Kaiser normalization. The purpose of PCA is to reduce a complex multivariable set of data into a smaller, and more manageable, number of composite variables. This is accomplished by forming weighted linear composites of variables such that each composite (or factor/component) contains highly interrelated variables thought to measure a common construct or phenomenon. When the components are subjected to an orthogonal (i.e., uncorrelated) mathematical rotation, as is performed with a varimax rotation, inter-component variance is minimized. This process enhances the distinctness and interpretability of each component.

The initial analysis of our PCA results, based on the scree plot and eigenvalue-greater-than-one rule, suggested a seven component solution. However, given that only two IMs loaded unambiguously on component 7, and considering the minimal variance accounted for by the 7th component, the PCA was re-computed with a six component solution. As shown in Table 6, PCA revealed the following six components:

component 1 (IL-6, IL-8, IL-15, TNF-α, MIP-1β/CCL4, and MCP-1/CCL2); component 2 (IL-1β, IL-2, GM-CSF, IL-1β:IL-10, IL-1β:IL-1Ra, and IL-2:IL-2R); component 3 (IL-1Ra, IL-7, IL-17, IFN-α, IFN-γ, and MIG/CXCL9); component 4 (IL-4, IL-5, IL-12, and Eotaxin/CCL11); component 5 (IP-10/CXCL10, IL-10, and TNF-α:IL-10); and component 6 (IL-2R, IL-13, MIP-1α/CCL3, and MIP-1α:MIP-1β). Component scores were calculated for each child by first multiplying the IMs and IM ratios by their respective factor loadings and then, for each component, summing the relevant IMs and IM ratios. Descriptive analyses of the resulting component score distributions indicated that all six components were normally distributed, as evidenced by small and non-significant skewness and kurtosis estimates. The six principal components provided an excellent fit of the data and accounted for 69.03% of the variance in the IM inter-correlation matrix. The eigenvalues and variance estimates for each component are shown in Table 6.

4.5.6 Unique associations between the principal components and HIV-1 status

A series of ANOVAs were then performed to examine whether the mean levels of the six components differed across the three HIV-1 status groups. Results indicated that components 2 [$F(2, 207)=5.95$, $P=0.003$], 3 [$F(2, 194)=4.72$, $P=0.010$], and 4 [$F(2, 207)=4.16$, $P=0.017$] significantly differed across the HIV-1 status groups. Post hoc pairwise comparisons, using Fisher's Least Significant Difference (LSD) test, indicated that the HIV-1[exp]/*Pf*[+] group (M=3.22) scored higher on component 2 than the HIV-1[-]/*Pf*[+] group (M=1.09, $P=0.001$). For component 3, the HIV-1[exp]/*Pf*[+] group

(M=6.14) scored higher than the HIV-1[-]/*Pf*[+] group (M=5.27, *P*=0.006), while on component 4, the HIV-1[+]/*Pf*[+] group (M=4.01) scored higher than the HIV-1[-]/*Pf*[+] group (M=3.46, *P*=0.006). Thus, there are unique profiles of IM production that differ according to HIV-1 status, suggesting that the inflammatory pathways differ in children co-infected with malaria and HIV-1.

4.5.7 Relationship between inflammatory mediator profiles, intraleukocytic *Pf*Hz, and anemia

Since there were unique patterns of IM production that differed according to HIV-1 status, Pearson correlation analyses were then conducted to examine the associations between the six IM-derived components and key indices of malaria-related morbidity and mortality. In particular, we examined *Pf*Hz burden (i.e., PCM and PCN) and anemia status (i.e., RPI and Hb). Analyses that included all of the children, revealed that PCN was negatively correlated with both components 2 (r=-0.187, *P*=0.007) and 4 (r=-0.157, *P*=0.023), RPI was negatively correlated with component 5 (r=-0.200, *P*=0.004), and Hb was positively correlated with component 3 (r=0.167, *P*=0.019).

For the HIV-1[-]/*Pf*[+] group, PCN was negatively correlated with component 4 (r=-0.200, *P*=0.013), RPI was negatively correlated with component 5 (r=-0.209, *P*=0.010), and Hb was positively correlated with component 3 (r=0.170, *P*=0.040, Table 7). There were no significant correlations for the HIV-1[exp]/*Pf*[+] group (Table 7). For the HIV-1[+]/*Pf*[+] group, PCN was negatively correlated with component 2 (r=-0.671, *P*=0.004), while Hb was positively correlated with component 4 (r=0.490, *P*=0.054, Table 7).

The two correlations for the HIV-1[+]/*Pf*[+] group are particularly impressive given their large magnitudes and the small number of HIV-1[+]/*Pf*[+] children in the analysis (n=16). To further probe these correlations, a leave-one-out-method was adopted whereby, for each component, each IM was systematically removed from the component and the correlation between the component and Hb (or PCN) was re-calculated. The purpose of these post hoc analyses is to ascertain which IMs exert the greatest influence on the PCN- and Hb-component correlations. For example, if Hb is correlated with a component consisting of four IMs (labeled 1, 2, 3, and 4), in accordance with the leave-one-out-method, four new components would be created (i.e., 123, 124, 134, and 234), and each new component would then be correlated with Hb. The correlation showing the largest decrement in relation to the original component correlation would indicate which constituent IM is most important in driving the Hb correlation. When applying this methodology to the significant HIV-1[+]/*Pf*[+] correlations, we found that of the four IMs that comprise component 4, IL-12 exerted the greatest influence on the correlation between Hb and this component. Upon exclusion of IL-12 from component 4, the magnitude of the correlation decreased from 0.490 to 0.340, which represents a 30.6% reduction. For the relationship between the HIV-1[+]/*Pf*[+] group's IM profiles and PCN, we found that of the six IMs that comprise component 2, the IL-1β:IL-10 ratio exerted the greatest influence on the correlation between PCN and this component. When the IL-1β:IL-10 was excluded from component 2, the absolute magnitude of the correlation changed from -0.671 to -0.587, which represents a 12.5% reduction.

4.6 DISCUSSION

Our recent investigations in Kenyan children demonstrated that co-infection with malaria and HIV-1 was associated with increased susceptibility to SA (Otieno, Ouma *et al.* 2006; Davenport, Ouma *et al.* 2010). However, the underlying mechanisms of worsening anemia in co-infected children are largely undetermined. Since perturbations in the inflammatory milieu promote enhanced anemia through a number of different mechanisms (Macdougall and Cooper 2002; Macdougall and Cooper 2002; Tilg, Ulmer *et al.* 2002; Mullarky, Szaba *et al.* 2007), comprehensive inflammatory mediator analyses were performed.

We and others have shown that acquisition of *Pf*Hz by phagocytic cells is an important source of IM dysregulation (Amodu, Adeyemo *et al.* 1998; Awandare, Ouma *et al.* 2007) and a significant predictor of SMA (Day, Pham *et al.* 1996; Casals-Pascual, Kai *et al.* 2006). By examining *Pf*Hz burden in neutrophils and monocytes, it is possible to determine if individuals suffer from recent (acute) versus prolonged (chronic) malaria infection, since monocytes remain in circulation approximately 10 times longer than neutrophils (Tomas Ganz 1995). Our previous study in western Kenya demonstrated that SMA is characterized by increased chronicity of infection, resulting in higher levels of PCM and lower levels of PCN (Awandare, Ouma *et al.* 2007). Consistent with this investigation, a follow-up study in malaria and HIV-1 co-infected children in the same region revealed that PCM was the strongest predictor of reduced Hb levels, whereas parasitemia was largely unrelated to anemia status (Davenport, Ouma *et al.* 2010). Thus, it appears that phagocytosis of *Pf*Hz by circulating monocytes may be a primary

mechanism through which cytokine dysregulation promotes enhanced anemia in children with malaria mono-infection and in those co-infected with HIV-1.

Recent literature outlining the causes of 'anemia of inflammation' suggests that immune activation has a significant and lasting effect on Hb concentrations (reviewed in (Bertero and Caligaris-Cappio 1997; Ganz 2006)). Although studies by our group (Perkins and Kniss 1999; Luty, Perkins *et al.* 2000; Perkins, Kremsner *et al.* 2001; Keller, Hittner *et al.* 2004; Ochiel, Awandare *et al.* 2005; Awandare, Goka *et al.* 2006; Awandare, Hittner *et al.* 2006; Awandare, Ouma *et al.* 2006; Awandare, Kremsner *et al.* 2007) and others (Ammann 1993; Chaisavaneeyakorn, Moore *et al.* 2002; McDevitt, Xie *et al.* 2004; Moore, Chaisavaneeyakorn *et al.* 2004; Tiemessen and Kuhn 2006) demonstrated that both malaria and HIV-1 cause significant changes in inflammatory mediator production, a comprehensive evaluation of the inflammatory milieu in co-infected children was previously not reported. Newer technologies, such as the micro bead assays, that concomitantly measure an inclusive panel of inflammatory mediators, in small volumes of blood available from anemic children, allowed us to determine the inflammatory profile in malaria/HIV-1 co-infected children.

Of the 16 pro- and anti-inflammatory cytokines investigated, only IL-12 and IFN-γ emerged as markedly different across the HIV-1 status groups. While we and others have previously shown that children with malarial anemia have progressively declining IL-12 levels with increasing anemia severity (Luty, Perkins *et al.* 2000; Perkins, Weinberg *et al.* 2000; Keller, Yamo *et al.* 2006), our findings here showed increasing concentrations of IL-12 across the HIV status groups despite the worsening anemia. A

study in HIV/tuberculosis co-infected patients from Zimbabwe reported similar findings, with IL-12 levels increasing with the acquisition of serological HIV positivity (Gordeuk, Moyo *et al.* 2009). Since children in the current study were in the early phases of HIV disease in which non-specific immune activation is ongoing, this may explain elevated IL-12 levels that differ from findings in the malaria literature and those of chronically-infected HIV patients. For example, Byrnes *et al.* (Byrnes, Harris *et al.* 2008) found significantly elevated IL-12p70 levels in acutely- (<2 months post-infection) and early (2-12 months)-infected HIV patients compared to healthy controls. Circulating IL-12 levels in both of these groups remained significantly elevated six months after diagnosis, regardless of whether the patient received highly active anti-retroviral therapy (HAART) treatment. Framed in the context of acute/early HIV-1 infection, elevated IL-12 levels are not surprising in the current study in children that were less than one year of age. These results highlight the fact that elevated IL-12 levels alone cannot salvage a child from worsening anemia.

Following the same pattern as IL-12, IFN-γ was lowest in the HIV-1[-]/*Pf*[+] group and elevated in both the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups. IFN-γ is important for a robust response to infection and has been shown to decrease with HIV disease progression (Clerici, Seminari *et al.* 2000). Previous studies also illustrated that IFN-γ suppresses erythropoiesis through up-regulation of IL-15 (Mullarky, Szaba *et al.* 2007). Studies in murine models demonstrated that blockade of IFN-γ, but not IL-12, TNF-α, IFN-α, IL-1α, or IL-1β, ameliorated suppression of erythropoiesis induced by CpG-oligodeoxynucleotide (CpG-ODN) administration (Thawani, Tam *et al.* 2006).

However, results presented here showing that IFN-γ was highest in the HIV-1[exp]/*Pf*[+] group and elevated in the HIV-1[+]/*Pf*[+] children, both of which have progressively worsening anemia, but comparable reticulocyte responses relative to the HIV-1[-]/*Pf*[+] children, suggests that IFN-γ is not responsible for the worsening anemia in co-infected children. One possible explanation for the current findings may be due to the fact that IFN-γ alters the erythropoietic cascade via an indirect fashion through perturbations in a number of other cytokines, all of which were not significantly different between the groups that showed progressively worsening degrees of anemia with increasing HIV-1 status (i.e., HIV-1[-]→HIV-1[exp]→HIV-1[+]).

Examination of chemokines and growth factors demonstrated that eotaxin/CCL11, MIG/CXCL9, and GM-CSF progressively increased across the groups. While little has been reported on the role of eotaxin/CCL11 in either malaria or HIV-1, decreased circulating eosinophils have been reported in children with *P. falciparum*, suggesting that tissue penetration is presumably directed by a chemotactic agent, such as eotaxin/CCL11 (Kurtzhals, Adabayeri *et al.* 1998). In addition, eosinophilia during the convalescent phase of disease is associated with a favorable recovery from malarial anemia (Camacho, Wilairatana *et al.* 1999). The impact of increased circulating eotaxin/CCL11 levels on recovery from malarial anemia in co-infected children, however, could not be determined using the current cross-sectional data. The discovery of increased MIG/CXCL9 in co-infected children is consistent with the fact that this group had monocytosis. In addition, progressively higher levels of GM-CSF across the groups, with decreasing Hb concentrations, likely reflects an attempt to compensate for the

worsening anemia, since GM-CSF stimulates erythroid progenitor expansion and maturation (Wu, Liu *et al.* 1995). Although the clinical impact of increased eotaxin/CCL11, MIG/CXCL9, and GM-CSF in co-infected children remains to be determined, these novel findings provide a foundation for future investigations.

The principal component factor analysis mathematically determined six principal components from the 25 cytokines, chemokines, growth factors, and five inflammatory ratios that we examined. Comparing the mean levels of these six components within the three HIV-1 groups revealed that the HIV-1[exp]/*Pf*[+] group had higher levels of components 2 and 3 than the HIV-1[-]/*Pf*[+] children, while the HIV-1[+]/*Pf*[+] group had higher levels of component 4 than the HIV-1[-]/*Pf*[+] children. Since components 2 and 3 are predominantly pro-inflammatory, elevation of these components in the HIV-[exp]/*Pf*[+] group indicates a type 1 inflammatory process even in the absence of viremia. Moreover, although the HIV-1[+]/*Pf*[+] group had the highest values for seven of 12 IMs in these two components, the HIV-1[exp]/*Pf*[+] group had a significant representation with five of the greatest mean values in IL-1β:IL-10, IL-2:IL-2R, IL-7, IL-17, and IFN-γ, indicating that circulating virus is not necessary for a pro-inflammatory millieu, and the subsequent anemia associated with such an environment. Finally, component 4 is comprised of the prototypical type 1 and type 2 cytokines, IL-12 and IL-4, respectively, as well as the two eosinophil modulators, IL-5 and eotaxin/CCL11. While the interpretability of this predominantly type 2 component in the HIV-1[+]/*Pf*[+] group is likely complex, it may suggest a waning of type 1 non-specific immune

activation (often seen early in HIV-1 infection), and the initiation of a type 2 skewing of the immune response (Clerici and Shearer 1993; Klein, Dobmeyer *et al.* 1997).

Pearson correlations were then used to identify associations between these components and PCM and PCN, as well as RPI and Hb. A model that included all of the children identified significant negative correlations between PCN and components 2 (IL-1β, IL-2, GM-CSF, IL-1β:IL-10, IL-1β:IL-1Ra, IL-2:IL-2R) and 4 (IL-4, IL-5, IL-12, Eotaxin/CCL11), indicating that as PCN concentration increases, the cytokine milleu is skewed towards a type 2 immune response. A negative correlation was also identified between RPI and component 5 (IP-10/CXCL10, IL-10, IL-10:TNF-α), suggesting a type 2 immune response is associated with dyserythropoiesis and a poor response to anemia. In addition, there was a positive correlation between Hb and component 3 (IL-1Ra, IL-7, IL-17, IFN-α, IFN-γ, MIG/CXCL9), indicating that healthier Hb levels are associated with a more pro-inflammatory environment. Overall, these results suggest that a potent type 1 pro-inflammatory immune response is necessary to maintain an appropriate erythropoietic response to anemia, and hence greater Hb levels, and that elevated PCN in co-infected children may be driving this phenomenon.

Analyses of the HIV-1[+]/*Pf*[+] group demonstrated that PCN was significantly correlated with component 2, as seen in results that included all of the children. In addition, Hb was significantly associated with component 4 (IL-4, IL-5, IL-12, Eotaxin/CCL11). The most interesting finding of this analysis is the transition of Hb correlating with component 3 (IL-1Ra, IL-7, IL-17, IFN-α, IFN-γ, MIG/CXCL9) in the HIV-1[-]/*Pf*[+] children ($P<0.050$), with no evidence of correlation ($P>0.500$) with

component 4 (IL-4, IL-5, IL-12, Eotaxin/CCL11), and vice versa in the HIV-1[+]/*Pf*[+] group. While components 3 and 4 each contain type 1 (IFN-γ, IFN-α, IL-12, IL-17) and type 2 (IL-4, IL-5, IL-7) cytokines, component 3 is comprised of the potent erythropoietic inhibitors IL-17, IFN-α, and IFN-γ, which will be increasing with Hb concentrations because of the positive association and thwarting erythropoiesis, while component 4 contains IL-12, also increasing due to a positive association, but supporting erythropoiesis (Dybedal, Larsen *et al.* 1995; Mohan and Stevenson 1998; Mohan and Stevenson 1998). Further analysis of the HIV-1[+]/*Pf*[+] children revealed that Hb was strongly positively associated with component 4, with IL-12 being the greatest contributor to that component. These findings parallel the patterns seen in the IL-12 concentrations in the three groups, with IL-12 increasing from the levels found in HIV-1[-]/*Pf*[+] children to the HIV-1(+)/*Pf*[+] group, but differ from studies previously reported by our group (Perkins, Weinberg *et al.* 2000; Keller, Yamo *et al.* 2006) and others (Luty, Perkins *et al.* 2000; Malaguarnera, Pignatelli *et al.* 2002; Malaguarnera, Pignatelli *et al.* 2002), showing that IL-12 is crucial and directionally proportional to hemoglobin levels in children recovering from malaria. Clearly, the presence, or even exposure, to HIV-1 has a significant impact on the IL-12 levels in these children, and while IL-12 is linked to positive hematologic outcomes in malaria [reviewed in (Stevenson, Su *et al.* 2001)], elevated IL-12 levels, along with IL-1, IL-6, TNF-α, and IFN-γ, have been linked to poor responses to erythropoietin in dialysis patients [reviewed in (Macdougall and Cooper 2002; Macdougall and Cooper 2002)]. PCN showed negative associations with components 2 in the HIV-1[+]/*Pf*[+] group and

component 4 in the HIV-1[-]/*Pf*[+] group. In addition, the negative association between PCN and component 2 was found to be most influenced by the IL-1β:IL-10 ratio. This finding suggests that PCN is associated with a type 2 skewing of the immune response, which agrees with previous findings of increased IL-10 upon exposure to *Pf*Hz (Peyron, Burdin *et al.* 1994; Keller, Yamo *et al.* 2006). Conversely, there were no components associated with PCM, however, both the HIV-1[-]/*Pf*[+] and HIV-1[exp]/*Pf*[+] groups approach significance with component 1, while the HIV-1[+]/*Pf*[+] group showed no association. This result was surprising given our previous findings by multiple regression analysis that PCM is the strongest predictor of Hb, but given the lack of PCM concentration differences across the groups, the failure to find significant correlations with the components is expected.

Taken together, these results demonstrate that children with malaria and HIV-1 have enhanced anemia severity and more acute malaria infections relative to HIV-1[-]/*Pf*[+] children. Although suppression of erythropoiesis did not account for enhanced anemia in co-infected children, unique patterns of inflammatory mediator dysregulation were observed. Elevated IL-2, IFN-α, and IFN-γ levels may be important causes of anemia exacerbation in co-infected and exposed children. In addition to potential erythropoietic consequences of IM dysregulation, cytokines such as IL-1 and TNF-α may also be encouraging splenic (Clark, Alleva *et al.* 2008; Buffet, Safeukui *et al.* 2009) and capillary sequestration of RBCs in SA through upregulation of receptors, such as thrombospondin, CD36, and intercellular adhesion molecule [reviewed in (Chulay and Ockenhouse 1990; Clark, Alleva *et al.* 2004)]. Furthermore, downstream molecules,

such as nitric oxide induced by IFN-α, reduces RBC deformability and oxygen delivery, and may contribute to RBC capture in the spleen (Kor, Van Buskirk *et al.* 2009). Increased eotaxin/CCL11, MIG/CXCL9, and GM-CSF levels may also play an important role in promoting enhanced HIV-1 pathogenesis (Reinhart 2003; McClure, van't Wout *et al.* 2007). In addition to the direct influence of the pathogens on immune dysregulation presented here, additional factors (not examined here), such as the potential for poor nutritional status in the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups, may indirectly affect soluble mediator production and enhance anemia. Future investigations focusing on the significantly dysregulated inflammatory mediators and the underlying cause(s) of immune dysregulation may offer novel insight into the molecular mechanisms responsible for enhanced SA in co-infected children.

4.7 ACKNOWLEDGMENTS

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GCD collected and analyzed the data and prepared the manuscript. JBH designed the data analysis strategy and provided statistical input. CO conducted the HIV testing. TW conducted the microbiological testing. YO analyzed the blood smears for parasitologic indices. JMV aided in the study design and supervision of the work in Kenya. JMO aided in the study design and interpretation of the results. DJP designed the study, supervised the activities in New Mexico, Pennsylvania, and Kenya, and co-wrote the manuscript with GCD.

4.8 TABLES

Table 4. *Clinical characteristics of study participants.*

Characteristic	HIV-1[-]/*Pf*[+]	HIV-1[exp]/*Pf*[+]	HIV-1[+]/*Pf*[+]	*P*
Number of subjects	148	30	16	N/A
Age (mos) [a]	10.2 (8.8)	9.2 (7.3)	12.1 (9.2)	0.592
Gender, male/female [b]	77 / 71	15 / 15	9 / 7	0.921
Severe anemia, n (%)[b]	136 (35.8)	44 (41.9)	14 (63.6)	**0.023**
Hemoglobin (Hb, g/dL) [a]	7.0 (4.0)	6.2 (2.4)	5.2 (3.6)	**0.017**
RPI [a]	1.4 (2.2)	2.0 (1.7)	1.4 (2.6)	0.554
WBC ($x10^9$/µL) [a]	11.7 (6.2)	11.1 (6.2)	12.0 (6.4)	0.784
Granulocytes ($x10^3$/µL) [a]	42.3 (23.0)	40.3 (15.0)	37.4 (19.0)	0.436
Monocytes ($x10^3$/µL) [a]	8.5 (5.0)	9.1 (5.0)	12.6 (7.0)	**0.033**
Parasitemia (/µL) [a]	25,619 (59,721)	25,659 (35,865)	14,405 (25,599)	0.112
Geomean parasitemia (/µL) [c]	18,615	17,658	7,861	0.086
PCM (/µL) [c]	3.32 (0.47)	2.40 (0.65)	3.81 (1.11)	0.728
PCN (/µL) [c]	0.38 (0.12)	0 (0)	0.81 (0.50)	**0.018**

Data are presented as median (interquartile range) unless stated otherwise. [a] Differences in the age, reticulocyte production index (RPI), Hb levels, white blood cell (WBC) count, monocytes, granulocytes, and parasitemia were compared using the Kruskal-Wallis test. [b] Differences in the proportions of gender and severe anemia were compared using Pearson's χ^2 test. [c] Data are presented as mean (SEM) and differences in the geometric mean parasitemia, pigment-containing monocytes (PCM), and pigment-containing neutrophils (PCN) were compared using ANOVA.

Table 5. *Multiplex analyses of inflammatory mediators.*

Analyte	HIV-1[-]/*Pf*[+]	HIV-1[exp]/*Pf*[+]	HIV-1[+]/*Pf*[+]	*P*
Cytokines (pg/mL)				
IL-1β	148.7 (282.8)	224.4 (579.0)	227.0 (393.6)	0.105
IL-1Ra	1,717 (2,306)	1,894 (5,012)	3,566 (4,763)	0.116
IL-2	29.7 (69.6)	39.6 (168.0)	47.3 (78.5)	0.149
IL-2R	1,713 (1,725)	1,425 (1,531)	1,590 (3,008)	0.643
IL-4	4.1 (15.3)	6.3 (19.7)	13.3 (15.4)	0.123
IL-5	1.7 (3.0)	1.3 (2.8)	3.0 (3.7)	0.503
IL-6	89.8 (160.4)	76.8 (189.0)	87.9 (198.6)	0.421
IL-7	1.4 (34.0)	25.7 (48.9)	11.7 (36.3)	0.166
IL-10	262.9 (658.2)	246.9 (473.9)	254.1 (292.1)	0.833
IL-12	362.0 (222.2)	475.7 (362.7)	604.9 (798.8)	**0.002**
IL-13	28.1 (40.0)	30.5 (41.6)	44.8 (79.3)	0.282
IL-15	29.4 (57.4)	33.2 (202.6)	46.0 (83.6)	0.314
IL-17	5.6 (15.1)	5.7 (22.8)	5.4 (14.5)	0.772
TNF-α	28.6 (36.7)	22.1 (75.7)	36.0 (44.5)	0.553
IFN-α	10.6 (63.0)	5.0 (48.0)	26.4 (70.0)	0.391
IFN-γ	4.6 (22.6)	17.9 (48.5)	11.1 (28.5)	**0.050**
Chemokines & Growth Factors (pg/mL)				
Eotaxin/CCL11	39.0 (24.1)	48.9 (40.8)	62.3 (26.2)	**0.003**
IL-8/CXCL8	14.7 (24.0)	12.9 (16.7)	19.0 (42.9)	0.146
IP-10/CXCL10	254.0 (683.8)	220.9 (343.5)	363.5 (455.2)	0.444
MCP-1/CCL2	196.0 (226.6)	211.7 (279.5)	207.5 (401.4)	0.529
MIG/CXCL9	131.8 (162.8)	198.8 (261.9)	209.2 (125.2)	**0.027**
MIP-1α/CCL3	115.4 (97.7)	124.3 (149.5)	109.2 (84.8)	0.380
MIP-1β/CCL4	391.3 (387.6)	357.6 (450.8)	449.7 (390.2)	0.874
RANTES/CCL5	15,195 (64,333)	9,679 (17,061)	30,851 (124,301)	0.171
GM-CSF	36.6 (150.1)	170.3 (531.0)	259.1 (352.5)	**0.011**
Inflammatory Ratios				
IL-1β:IL-1Ra	0.070 (0.190)	0.141 (0.260)	0.061 (0.060)	0.185
IL-2:IL-2R	0.017 (0.080)	0.046 (0.160)	0.014 (0.190)	0.063
IL-1β:IL-10	0.489 (1.770)	0.793 (1.930)	0.894 (1.33)	0.139
TNF-α:IL-10	0.098 (0.230)	0.113 (0.180)	0.149 (0.140)	0.461
MIP-1α:MIP-1β	0.269 (0.360)	0.439 (0.400)	0.269 (0.210)	0.355

Data are presented as median (interquartile range) and compared using Kruskal-Wallis test. Plasma serum samples collected at the initial parasitemic visit were snap frozen at -70°C until the day of assay. Samples were thawed and centrifuged at 14,000 rpm for 10 minutes and stored on ice until assayed. Twenty-five effector molecules were measured with the Cytokine 25-plex Ab Bead Kit, Hu (BioSource™ International) according to the manufacturer's instructions. Plates were read on a Luminex® 100™ system (Luminex® Corporation) and analyzed using the Bio-plex manager software (Bio-Rad Laboratories).

Table 6. *Rotated component matrix for the principal component factor analysis.*

Component	Inflammatory Mediators	Loading Factor	Cumulative Eignevalue	Percent Variance	Cumulative Percent Variance
	IL-6	0.835			
	IL-8	0.605			
	IL-15	0.507			
	TNF-α	0.790			
	MIP-1β/CCL4	0.843			
1	MCP-1/CCL2	0.696	7.702	25.673	25.673
	IL-1β	0.902			
	IL-2	0.871			
	GM-CSF	0.669			
	IL-1β:IL-10	0.783			
	IL-1β:IL-1Ra	0.769			
2	IL-2:IL-2R	0.852	4.999	16.664	42.337
	IL-1Ra	0.712			
	IL-7	0.617			
	IL-17	0.558			
	IFN-α	0.406			
	IFN-γ	0.654			
3	MIG/CXCL9	0.604	2.769	9.229	51.565
	IL-4	0.517			
	IL-5	0.642			
	IL-12	0.684			
4	Eotaxin/CCL11	0.769	2.012	6.708	58.273
	IP-10/CXCL10	0.488			
	IL-10	0.642			
5	TNF-α:IL-10	0.854	1.944	6.479	64.752
	IL-2R	0.583			
	IL-13	0.742			
	MIP-1α/CCL3	0.555			
6	MIP-1α:MIP-1β	0.483	1.283	4.278	69.030

Twenty-five inflammatory mediators and five inflammatory ratios were entered into the factor analysis model. Variables were placed into six components based on a total per component Eigenvalue >1 (range 1.283-7.702) value.

Table 7. *Association between the principal components and disease outcomes.*

Component / Variable		1	2	3	4	5	6
HIV-1[-]/*Pf*[+] CHILDREN							
PCM	Pearson Corr.	0.141	-0.037	0.027	-0.016	0.014	0.067
	P-value	0.081	0.650	0.749	0.843	0.867	0.408
	N	154	155	146	154	155	156
PCN	Pearson Corr.	-0.022	-0.107	-0.027	-0.200	0.018	0.013
	P-value	0.788	0.186	0.748	**0.013**	0.826	0.873
	N	154	155	146	154	155	156
RPI	Pearson Corr.	-0.033	0.022	0.105	0.093	-0.209	-0.059
	P-value	0.687	0.785	0.212	0.260	**0.010**	0.475
	N	149	150	142	149	150	151
Hb	Pearson Corr.	-0.029	0.007	0.170	0.047	0.002	-0.063
	P-value	0.724	0.932	**0.040**	0.562	0.981	0.438
	N	154	155	146	154	155	156
HIV-1[exp]/*Pf*[+] CHILDREN							
PCM	Pearson Corr.	-0.260	-0.069	-0.120	-0.059	-0.149	-0.042
	P-value	0.105	0.678	0.479	0.717	0.358	0.796
	N	40	39	37	40	40	40
PCN	Pearson Corr.	N/A	N/A	N/A	N/A	N/A	N/A
	P-value	N/A	N/A	N/A	N/A	N/A	N/A
	N	40	39	37	40	40	40
RPI	Pearson Corr.	-0.192	-0.033	-0.188	-0.072	-0.272	-0.227
	P-value	0.235	0.844	0.264	0.661	0.090	0.158
	N	40	39	37	40	40	40
Hb	Pearson Corr.	0.039	-0.060	0.207	0.074	0.254	-0.140
	P-value	0.812	0.717	0.219	0.648	0.114	0.388
	N	40	39	37	40	40	40
HIV-1[+]/*Pf*[+] CHILDREN							
PCM	Pearson Corr.	0.005	-0.347	0.248	-0.054	0.254	-0.092
	P-value	0.986	0.187	0.392	0.841	0.361	0.735
	N	15	16	14	16	15	16
PCN	Pearson Corr.	0.122	-0.671	0.191	-0.134	0.434	0.190
	P-value	0.664	**0.004**	0.513	0.621	0.106	0.482
	N	15	16	14	16	15	16
RPI	Pearson Corr.	-0.236	-0.466	-0.126	0.285	-0.242	-0.114
	P-value	0.396	0.069	0.667	0.285	0.385	0.675
	N	15	16	14	16	15	16
Hb	Pearson Corr.	-0.074	0.001	0.175	0.490	-0.134	0.153
	P-value	0.793	0.997	0.549	**0.054**	0.634	0.572
	N	15	16	14	16	15	16

Pearson correlations between the six components from the PCA analysis and four predictors/outcomes were performed using each HIV-1 status to explore the unique associations with the principal components in each subset. Post-hoc analysis of the HIV-1[+]/*Pf*[+] group employing a leave-one-out method revealed IL-1β:IL-10 ratio was the most influential variable (12.52%) in the PCN/component 2 correlation, and IL-12 was the most influential variable (30.61%) in the Hb/component 4 correlation. The components contain the following effector molecules and inflammatory ratios: **1)** IL-6, IL-8/CXCL8, IL-15,

TNF-α, MIP-1β/CCL4, MCP-1/CCL2; **2)** IL-1β, IL-2, GM-CSF, IL-1β:IL-10, IL-1β:IL-1Ra, IL-2:IL-2R; **3)** IL-1Ra, IL-7, IL-17, IFN-α, IFN-γ, MIG/CXCL9; **4)** IL-4, IL-5, IL-12, Eotaxin/CCL11; **5)** IP-10/CXCL10, IL-10, IL-10:TNF-α; **6)** IL-2R, IL-13, MIP-1α/CCL3, MIP-1α:MIP-1β.

5.0 CHAPTER FIVE: RESULTS, SPECIFIC AIM 3

To determine the cytokine milieu responsible for lower parasitemia without worsening anemia in malaria and bacteremia co-infected children.

5.1 HYPOTHESIS 1, PRESENTATION OF MANUSCRIPT ENTITLED:

PLASMODIUM FALCIPARUM AND BACTEREMIA CO-INFECTION IN KENYAN CHILDREN IS ASSOCIATED WITH DECREASED PARASITEMIA IN THE ABSENCE OF WORSENING ANEMIA

Hypothesis 1: Blood borne bacterial infections elicit a unique profile of inflammatory mediators resulting in lower parasitemia levels without promoting enhanced anemia in co-infected children.

To address this hypothesis, we grouped the HIV-1[-] children enrolled in the Severe Malarial Anemia study into three groups based on their bacteremia status on initial contact: malaria infection only, *Pf*[+]; malaria and bacteremia with a G[+] organism (G[+]/*Pf*[+]); and malaria and bacteremia with a G[-] organism (G[-]/*Pf*[+]). Plasma from these children was assayed using a multiplex bead-array assay for 16

cytokines to determine the inflammatory mediator profile for each group. Using a multiple mediation model, with infection status as the independent variable and parasitemia as the dependent variable, we identified the cytokines responsible for decreased parasitemia in the bacteremia co-infected children. The results of these findings are presented in the following manuscript prepared for submission.

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5.2 ABSTRACT

Bacteremia is associated with malnutrition in resource-poor settings and has been shown to exacerbate malarial anemia. We previously demonstrated that children co-infected with malaria and bacteremia had significantly decreased parasitemia, but no exacerbation of anemia. Our objective was to determine the inflammatory mediator profile associated with the reduced parasitemia in co-infected children. *P. falciparum*-infected children (n=192, aged <3yrs) were divided into three categories: malaria alone, *Pf*[+]; Gram negative bacteremia plus malaria, G[-]/*Pf*[+]; and Gram positive bacteremia plus malaria, G[+]/*Pf*[+]. Since a pro-inflammatory milieu is known to control parasitemia, but adversely affect erythropoiesis, circulating levels of 16 inflammatory mediators were determined with a human bead-based Multiplex assay. *Staphylococcus aureus* and non-Typhi *Salmonella* were the two most frequently isolated G[+] and G[-] organisms, respectively. Compared to the *Pf*[+] group, both co-infected groups had lower parasitemia and mean corpuscular hemoglobin concentration. In addition, both co-infected groups had significantly increased IL-4, IL-5, IL-7, IL-12, IL-15, IL-17, IFN-γ, IFN-α, and decreased TNF-α levels relative to the *Pf*[+] group. Additional significant findings included higher IL-1β, IL-1Ra, and lower IL-10 in the G[-]/*Pf*[+] versus the *Pf*[+] group. The G[-]/*Pf*[+] group also had significantly higher levels of WBC, granulocytes, and IFN-γ than the G[+]/*Pf*[+] group. A multiple mediation model examining the molecules responsible for decreased parasitemia in the bacteremia co-infected groups, but not the malaria-only group, identified IL-4, IL-10, IL-12, and IFN-γ as the key molecules in decreasing parasitemia while not exacerbating

anemia. Enhanced immune activation in co-infected children appears to promote reduced parasitemia without adversely affecting anemia outcomes. By comprehensively examining the inflammatory profile in malaria mono-infected and co-infected children we can begin to elucidate both common and unique inflammatory pathways responsible for differing clinical outcomes in children with malaria and bacteremia.

5.3 INTRODUCTION

Co-infection with bacteremia is a common cause of malaria severity in and mortality among children in sub-Saharan Africa (Berkley, Bejon *et al.* 2009). Studies in malaria endemic settings have estimated 3.1 - 5.4% of children with malaria are co-infected with bacteremia resulting in 18 - 22% mortality (Brent, Ahmed *et al.* 2006; Bassat, Guinovart *et al.* 2009; Sigauque, Roca *et al.* 2009). In western Kenya, an area of *Plasmodium falciparum* holendemic transmission, severe malaria is primarily characterized by severe malarial anemia (SMA, Hb <6.0g/dL) (McElroy, Lal *et al.* 1999). In this population, childhood anemia is exercabated by co-infection with bacteremia, human immunodeficiency virus (HIV)-1, hookworm, and high levels of malnutrition co-endemic in this region (Brent, Ahmed *et al.* 2006; Ong'echa, Keller *et al.* 2006; Otieno, Ouma *et al.* 2006; Berkley, Bejon *et al.* 2009; Sigauque, Roca *et al.* 2009; Davenport, Ouma *et al.* 2010).

Bacteremia is an important co-morbid factor in *P. falciparum* malaria and is associated with severe life-threatening manifestations such as SMA, cerebral malaria,

respiratory distress, and hepatosplenomegaly (Bassat, Guinovart *et al.* 2009). In addition, bacteremia is involved in increasing the mortality rates associated with these syndromes (Berkley, Bejon *et al.* 2009). Gram negative (G[-]) bacteremia caused by non-Typhi *Salmonella* (NTS) is the most common and important cause of severe syndromes and mortality in malaria and bacteremia co-infected children (Rabsch, Tschape *et al.* 2001; Morpeth, Ramadhani *et al.* 2009; Roux, Butler *et al.* 2010). A number of studies from across Africa demonstrated that SMA, cerebral malaria, respiratory distress, and malnutriution are associated with increased prevalence of NTS bacteremia in children with malaria (Friedland 1992; Walsh, Phiri *et al.* 2000; Bahwere, Levy *et al.* 2001; Bwibo and Neumann 2003; Berkley, Lowe *et al.* 2005; Sigauque, Roca *et al.* 2009). Our recent studies showed an 11.7% prevalence of bacteremia in *P. falciparum*-infected children, with NTS (42.4%) and *Staphylococcus aureus* (35.6%) being the predominant G[-] and Gram positive (G[+]) isolates, respectively (Were *et al.*, unpublished results). Despite the apparent importance of bacteremia, little is known about the underlying pathophysiological mechanisms governing the interactions between bacteremia and malaria. Accumulating evidence indicates that both diseases elicit potent type 1 inflammatory responses characterized by increased production of TNF-α, IL-1β, IL-6 cytokines that may lead to septic shock in the early stages of disease and suppression of erythropoiesis in the chronic stage (Suarez-Santamaria, Santolaria *et al.* ; Dahmer, Randolph *et al.* 2005; Bozza, Salluh *et al.* 2007). Previous studies demonstrated that a successful type 1 response to malaria infection is characterized by controlled-release of interleukin (IL)-12, interferon (IFN)-γ and tumor necrosis factor

(TNF)-α, which aids in the control of parasitemia and preservation of erythropoiesis (Crutcher, Stevenson *et al.* 1995; Stevenson, Tam *et al.* 1995; Stevenson, Su *et al.* 2001). Subsequently, these responses are followed by a controlled type 2 cytokine response that abrogates the pro-inflammatory response and prevents inflammatory-mediated damage to the human host (Clark, Budd *et al.* 2006). Our previous studies, and those of others, demonstrated suppression of IL-12 through intra-leukocytic accumulation of hemozoin (*Pf*Hz) promotes elevated production of IL-10 in children with SMA (Kalyesubula, Musoke-Mudido *et al.* 1997; Aste-Amezaga, Ma *et al.* 1998; Casals-Pascual, Kai *et al.* 2006; Keller, Yamo *et al.* 2006). In addition, elevated circulating levels of TNF-α, IL-1β, and IL-6 are associated with severe malaria (Grau, Taylor *et al.* 1989; Kwiatkowski, Hill *et al.* 1990; Kurtzhals, Adabayeri *et al.* 1998). The host immune response to bacteremia is characterized by an early secretion of IL-1, IL-6, IL-8, IL-10, and TNF-α (Pinsky, Vincent *et al.* 1993; Blackwell and Christman 1996; Bertero and Caligaris-Cappio 1997). Previous studies showed that elevated production of IL-6 occurs within three hours of lipopolysaccharide (LPS) injection in humans volunteers (Bertero and Caligaris-Cappio 1997). In addition, peak elevations of IFN-γ and TNF-α are present at 1.5 hours, IL-10 at 2 hours, and peak IL-6 at 4 hours (Bertero and Caligaris-Cappio 1997). Thus, both severe malaria and bacteremia are diseases that result from the inappropriate timing and magnitude of cytokine responses.

Many cytokines and effector molecules are known to be instrumental in inhibiting parasite growth. Unfortunately, these same molecules can have detrimental effects on erythropoiesis. The findings in this research study are unique in that reduced

parasitemia is observed with no significant effect on hemoglobin (Hb) levels, and in the absence of appreciable increases in body temperature. The primary goal of this study was to determine the inflammatory milieu responsible for a lower parasitic burden in co-infected children, without resulting in worsening anemia.

5.4 METHODS

5.4.1 Study participants

Children aged 3-36 months (n=192) with *P. falciparum* parasitemia (any density) were recruited at Siaya District Hospital, western Kenya, during their first hospital contact for malaria, and matched for age and gender. Siaya District is a holoendemic *P. falciparum* transmission area where residents receive up to 300 infective bites per annum (Beier, Oster *et al.* 1994). None of the children had cerebral malaria or non-falciparum malarial infections. A detailed description of the study area and population can be found in our recent publication (Ong'echa, Keller *et al.* 2006).

Children with malaria were divided into three groups: malaria only (Parasitemic, *Pf*[+], n=156); co-infected with Gram negative bacteremia (G[-]/*Pf*[+], n=22); and co-infected with Gram positive bacteremia (G[+]/*Pf*[+], n=14). In addition, a healthy control group (n=14) composed of afebrile, uninfected children with Hb >11.0g/dL were enrolled during routine immunization visits. All children were tested for antibody and viral evidence of HIV-1 exposure and infection using two rapid serological assays and pro-viral HIV DNA PCR, respectively. HIV-1 serological status was determined using

two rapid antibody tests (i.e., Uni-Gold™, Trinity Biotech; and Determine®, Abbott Laboratories), while HIV-1 positivity was determined by HIV-1 DNA PCR analysis according to our previously published methods (Davenport, Ouma *et al.* 2010). All children with positive HIV-1 serology or HIV-1 viremia were excluded from the study, since our previous investigation demonstrated significant inflammatory mediator perturbations in children exposed or infected with HIV-1 (Davenport *et al.*; in preparation). Pre- and post-test HIV counseling were provided for the parents/guardians of all participants.

Children were also stratified into SMA (Hb <6.0g/dL and <5.0g/dL) and non-SMA (Hb 6.0–10.9g/dL and free from the symptoms of severe malaria, such as hypoglycemia). Definitions of malarial anemia were based on previous longitudinal Hb measures (>14,000) in children (<48 months of age) living in western Kenya (McElroy, Lal *et al.* 1999) (Hb <6.0g/dL), and the World Health Organization definition of SMA (Hb <5.0g/dL) (WHO 2000). Children were treated according to Ministry of Health, Kenya, guidelines which included the use of Coartem® (artemether/lumefantrin) for uncomplicated malaria, quinine for severe malaria, and broad spectrum antibiotics such as trimethoprim/sulfamethoxazole for bacterial infections. Written informed consent was obtained from the participants' parents/guardians before enrollment. Approval for the study was granted by the University of Pittsburgh, University of New Mexico, and the Kenya Medical Research Institute (KEMRI).

5.4.2 Blood culture

Blood culture was performed upon enrollment and during acute visits whenever a child showed signs of sepsis. The phlebotomy site and Wampole Isostat® Pediatric 1.5 tube stopper were cleaned with 70% ethanol prior to venipuncture. The blood was processed according to the manufacturer's instructions and plated on standard microbiological media. Briefly, the entire Isostat supernatant was plated on Chocolate Agar and incubated for 18-24 hrs and then inspected for growth. Unique colonies were sub-cultured to standard microbiological media (Chocolate Agar, MacConkey's Agar, Mannitol Salt Agar, and Blood Agar) and incubated for 18-24 hrs in a 35°C CO_2 incubator. If no growth was detected after the initial incubation, an additional incubation period of four days was performed with daily inspection for growth. Unique colonies from sub-culture were identified using standard microbiological and biochemical techniques (i.e., Gram stain, colony characteristics, API biochemical panels, and agglutination tests). Specifically, G[-] isolates were identified using API® 20E for oxidase negative Enterobacteriaceae, or 20NE for oxidase positive organisms (bioMerieux, Inc.), while G[+] organisms were identified using various biochemical techniques (i.e. catalase, hemolysis patterns, serological grouping, and coagulase test). Gram positive bacilli and coagulase-negative *Staphylococcus* were considered laboratory/skin contaminants and were not included in analyses.

5.4.3 Laboratory measurements

Asexual malaria trophozoites (Were, Hittner *et al.* 2006) and the number of pigment-containing monocytes (PCM) and pigment-containing neutrophils (PCN) were determined according to previous methods (Nguyen, Day *et al.* 1995; Lyke, Diallo *et al.* 2003). Complete blood counts were performed with a Beckman Coulter© Ac-T diff2™ (Beckman Coulter, Inc.) on blood obtained prior to administration of antimalarials and/or antipyretics. The reticulocyte count, absolute reticulocyte number (ARN), reticulocyte production index (RPI) were determined per our previous methods (Were, Hittner *et al.* 2006). Glucose-6-phosphate dehydrogenase (G6PD) deficiency was assessed using the G6PDH Screening Kit (Trinity Biotech, PLC) according to the manufacturer's instructions. The presence of Hb variants in sample hemolysates was detected using a Hb electrophoresis kit, which allowed for detection of HbS, HbC, HbF, and HbA, in either the heterozygous or homozygous states (Helena laboratories).

5.4.4 Multiplex assay

Venous blood samples were centrifuged and plasma was separated immediately, aliquoted, and stored at -70°C until used for inflammatory mediator measurements. Samples were thawed and clarified by centrifugation (14,000 rpm for 10 min) before assaying. Inflammatory mediator levels were determined by the Cytokine 25-plex Antibody Bead Kit, Human (BioSource™ International) according to the manufacturer's instructions. Plates were read on a Luminex 100™ system (Luminex Corporation) and analyzed using the Bio-plex manager software (Bio-Rad Laboratories). Analyte

detection limits were: 3 pg/mL (IL-5, IL-6); 5 pg/mL (IL-1Ra, IL-3, IL-4, IL-10, IFN-γ); 6 pg/mL (IL-2); 10 pg/mL (IL-7, IL-13, IL-15, IL-17, TNF-α); and 15 pg/mL (IL-1β, IL-12p40/p70, GM-CSF, IFN-α).

5.4.5 Statistical analyses

Data were analyzed using SPSS (version 15.0). Clinical, demographic, hematological, and immunological measures were compared across groups by either analysis of variance (ANOVA) or Kruskal-Wallis tests, followed by pair-wise post-hoc comparison with student's t-test or Mann-Whitney U test, respectively. Pearson's chi square (χ^2) or Fisher exact test were used for proportion comparisons. In the event that the cell sum of categorical analyses was less than 20, as in the mortality data, pair-wise contrasts were employed to determine significance. Statistical significance was set at $P \leq 0.05$.

A multiple mediation model was performed to determine the inflammatory mediators (IMs) responsible for decreased parasitemia in co-infected children. The SPSS macro set (indirect.sps) and details for its use are located on the following website http://www.comm.ohio-state.edu/ahayes/SPSS%20programs/indirect.htm (Preacher and Hayes 2008).

5.5 RESULTS

5.5.1 Distribution of bacterial isolates

The two predominant blood culture isolates were *Staphylococcus aureus* (36.1%) and *Salmonella enterica* ssp. *enterica* ser. Typhimurium (44.4%), while NTS comprised 52.8% of the overall isolates (Table 8). Children selected for this study were free of other co-infections (i.e., HIV-1 and hookworm) and had the following bacterial isolate distribution: 61.2% G[-] (*Acinetobacter sp*., *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* ssp. *enterica* ser. Arizonae, *S. enterica* ssp. *enterica* ser. Enteritidis, *S. enterica* ssp. *enterica* ser. Typhimurium) and 38.9% G[+] (*Enterococcus sp*. and *S. aureus*) organisms.

5.5.2 Demographic, clinical, and hematologic characteristics

Children with falciparum malaria were stratified into three categories: malaria alone (Parasitemic, *Pf*[+], n=156); G[-]/*Pf*[+] co-infected (n=22); and G[+]/*Pf*[+] co-infected (n=14) (Table 9). A healthy control group was also included for comparison (n=14), but were not included in the omnibus statistical comparisons due to their ability to skew the results. Study participant demographics and clinical characteristics are listed in Table 2. The two co-infected groups had 38.5% and 28.6% more females than males for the G[-]/*Pf*[+] and G[+]/*Pf*[+] groups, respectively, while age, axillary temperature, and glucose were comparable across the groups. Hematologically, there were significant inter-group differences in white blood cell counts (WBC, P=0.047), absolute

granulocyte counts (P=0.010), and mean corpuscular hemoglobin concentrations (MCHC, P=0.003, Table 9). Post-hoc testing revealed that, relative to the parasitemic and G[-]/*Pf*[+] groups, the G[+]/*Pf*[+] group had lower WBC counts (P=0.022 and P=0.026, respectively) and granulocyte counts (P=0.003 and P=0.007, respectively), while the MCHC of both the G[-]/*Pf*[+] and G[+]/*Pf*[+] groups (P=0.008 and P=0.016, respectively) were lower than those of the parasitemic group. In addition, the red cell distribution width (RDW) approached statistical significance with higher values in the co-infected children, potentially indicating that they are either recovering from greater anemia, or they have a more robust erythropoietic response (P=0.053). Finally, while there were no fatalities in the G[+]/*Pf*[+] group, the greatest percentage of mortality was observed in the G[-]/*Pf*[+] children. Pair-wise contrasts revealed a significant difference in mortality between the *Pf*[+] and G[+]/*Pf*[+] groups (P=0.013, Table 9). Although previous reports have suggested an association between G6PD deficiency and altered cytokine expression (Wilmanski, Villanueva *et al.* 2007), as well as increased susceptibility to bacteremia in those carrying sickle cell alleles (Williams, Uyoga *et al.* 2009), no significant differences were found between the three parasitemic groups for either G6PD deficiency (χ^2=2.505, P=0.644) or sickle cell trait/disease (χ^2=0.158, P=0.924).

5.5.3 Parasitologic indices

Across-group comparison of the three parasitemic groups showed a significant difference in parasitemia densities (P=0.001, Figure 3A). Pair-wise comparison revealed

that children with G[-]/*Pf*[+] co-infections (6,461 malaria parasites [MPS]/µL), but not G[+]/*Pf*[+] co-infected children (8,704 MPS/µL, *P*=0.217), had lower parasite densities relative to *Pf*[+] group (25,619 MPS/µL, *P*<0.001, Figure 3A). In addition, the geometric mean parasitemia was greater in children with malaria alone relative to those with G[-]/*Pf*[+] co-infection (*P*=0.001, Figure 3B). Similarly, the *Pf*[+] group had a much greater percentage (72.4%) of children with high-density parasitemia (HDP, >10,000 parasites/µL, *P*=0.002 inter-group), compared to the G[-]/*Pf*[+] (40.9%, *P*=0.004) and G[+]/*Pf*[+] (42.9%, *P*=0.031) groups (Figure 3C).

5.5.4 Erythropoietic indices and intraleukocytic hemozoin

Our previous studies and those of others (Schellenberg, Menendez *et al.* 1999; Keller, Yamo *et al.* 2006; Ong'echa, Keller *et al.* 2006; Awandare, Ouma *et al.* 2007; Dondorp, Lee *et al.* 2008; Davenport, Ouma *et al.* 2010), showed that susceptibility to SMA is independent of the degree of parasitemia in holoendemic *P. falciparum* transmission regions. While there was reduced parasitemia in the bacteremic children, the percentage of children with SMA remained equivocal between the three groups, regardless of the appropriate regional standard (i.e., Hb <6.0g/dL, *P*=0.628, Table 3) (McElroy, Lal *et al.* 1999) or the WHO standard (Hb <5.0g/dL, *P*=0.562) (WHO 2000). Moreover, there were no significant differences in the reticulocyte production index (RPI, *P*=0.837, Table 10), or the percentage of children with an RPI <2 (*P*=0.453), which is a metric for an appropriate erythropoietic response for the degree of anemia. Although there were prominent differences in parasitemia levels and HDP between the

Pf[+] mono-infected and co-infected groups, no difference in the percent of children with pigment deposition, which has been correlated with disease severity (Nguyen, Day *et al.* 1995; Amodu, Adeyemo *et al.* 1998; Luty, Perkins *et al.* 2000; Davenport, Ouma *et al.* 2010), was seen across the groups for either PCM (P=0.957, Table 10) or PCN (P=0.455).

5.5.5 Pro-inflammatory cytokine production

Since IM dysregulation is an integral part of both malaria and bacteremia pathogenesis and associated sequelae (Clark and Chaudhri 1988; Ratajczak and Ratajczak 1994; Lyke, Burges *et al.* 2004; Ulloa and Tracey 2005; Awandare, Goka *et al.* 2006; Thawani, Tam *et al.* 2006; Bozza, Salluh *et al.* 2007; Ong'echa, Remo *et al.* 2007), circulating levels of cytokines were determined with a 25-plex bead array assay. Moreover, cytokine dysregulation is central to the pathological processes of SMA, cerebral malaria, and sepsis [reviewed in (Clark, Alleva *et al.* 2008)]. We (Perkins, Weinberg *et al.* 2000; Keller, Davenport *et al.* 2006; Keller, Yamo *et al.* 2006; Ong'echa, Remo *et al.* 2007) and others (Luty, Perkins *et al.* 2000; Lyke, Burges *et al.* 2004) have demonstrated that childhood malaria and the subsequent process of anemia is influenced by dysregulation of pro- and anti-inflammatory cytokines. However, the circulating inflammatory mediator profile in malaria and bacteremia co-infected children has yet to be characterized. Measurement of circulating inflammatory mediators reveled that IL-1β and its receptor antagonist (IL-1Ra) were significantly different across groups (P=0.027 and P=0.005, respectively). Pair-wise analysis showed elevated levels of IL-1β and IL-

1Ra in children with G[-]/*Pf*[+] co-infection (Figure 4A, *P*=0.009 and Figure 4B, *P*=0.001, respectively) compared to the *Pf*[+]-only group. However, the ratio for these inflammatory mediators (IL-1:IL-1Ra, *P*=0.854, Figure 4C), and that of IL-2 (*P*=0.068, Figure 4D) and the IL-2:IL-2R ratio (Figure 4E, *P*=0.226), showed no significant differences across the three groups. The highest levels of IL-6 were also seen in the G[-]/*Pf*[+] co-infected children, which parallels the pattern of axillary temperature (see Table 9). However, the difference in IL-6 levels across the groups was not significant (*P*=0.115, Figure 3F). IL-12 has been reported to be a key molecule in the appropriate response and recovery from malaria (Luty, Perkins *et al.* 2000; Perkins, Weinberg *et al.* 2000; Malaguarnera, Pignatelli *et al.* 2002). There was a significant difference in IL-12 across the groups (*P*<0.001, Figure 5A). The highest IL-12 concentrations were found in the G[-]/*Pf*[+] co-infected group (*P*<0.001 vs. *Pf*[+]), followed by the G[+]/*Pf*[+] co-infected group (*P*=0.034 vs. *Pf*[+]), and lowest in the *Pf*[+] group. The healthy control group also had greater IL-12 values than the *Pf*[+] children (*P*=0.036). An identical pattern and magnitude was observed in IL-15 levels (across-group *P*<0.001, Figure 5B), with the G[-]/*Pf*[+] and G[+]/*Pf*[+] groups both having higher levels than the *Pf*[+] children (*P*<0.001 and *P*<0.001, respectively), as well as the healthy controls (*P*<0.001 and *P*=0.002, respectively). There were similar levels of IL-17 (inter-group *P*=0.002, Figure 5C) in the healthy controls, G[-]/*Pf*[+] co-infected, and G[+]/*Pf*[+] co-infected groups, compared to the significantly lower levels observed in the *Pf*[+] group (*P*=0.018, *P*=0.016, and *P*=0.003, respectively). IFN-γ levels (across-group *P*<0.001, Figure 5D) mimicked the patterns seen in IL-12 and IL-15, with the G[-]/*Pf*[+] group

having the highest levels, relative to the moderate levels in the G[+]/*Pf*[+] group (P=0.006), and negligible levels in the *Pf*[+] children (P<0.001) and healthy controls (P=0.023). The G[+]/*Pf*[+] co-infected group also has higher levels of IFN-γ relative to the malaria-only group (P=0.008). Unexpectedly, TNF-α levels (inter-group P=0.003, Figure 5E) were highest in the healthy control group, followed by the *Pf*[+] children and the two co-infected groups, while the G[-]/*Pf*[+] (P=0.030) and G[+]/*Pf*[+] (P=0.004) co-infected groups' TNF-α concentrations were significantly lower than the *Pf*[+] group. The *Pf*[+] group had significantly depressed levels of IFN-α (P<0.001 inter-group, Figure 5F) compared to the G[-]/*Pf*[+] (P<0.001) and G[+]/*Pf*[+] (P<0.001) co-infected groups (Figure 5F). Thus, while the co-infected children have higher pro-inflammatory cytokine levels, these levels do not appear to contribute to exacerbating SMA, but may assist in controlling parasitemia.

5.5.6 Anti-inflammatory cytokine production

The anti-inflammatory cytokines, particularly IL-10, are known to play a key role in abrogating a robust initial pro-inflammatory response to malaria, as well as recovery from anemia (Couper, Blount *et al.* 2008; Scholzen, Mittag *et al.* 2009). However, we have previously shown that phagocytized *Pf*Hz promotes over-production of IL-10, which in turn suppresses IL-12, thereby exacerbating anemia and slowing parasite clearance (Keller, Yamo *et al.* 2006). Upon examination of the anti-inflammatory cytokines, we observed similar patterns to those seen in the pro-inflammatory molecules, with the co-infected children showing the greatest levels relative to the

mono-infected *Pf*[+] group, with a few important exceptions. Circulating concentrations of IL-4, IL-5, and IL-7 differed across-groups (Kruskal-Wallis [K-W] $P<0.001$, Figure 6A) and were significantly elevated in the G[+]/*Pf*[+] and G[-]/*Pf*[+] co-infected children relative to children with malaria co-infection ($P<0.01$) and healthy controls ($P<0.05$), respectively. Consistent with our previous findings, circulating IL-10 concentrations differed across the groups (K-W $P=0.042$, Figure 6D) and were higher in the *Pf*[+], G[-]/*Pf*[+], and G[+]/*Pf*[+] groups relative to healthy controls ($P<0.001$, $P=0.038$, $P=0.035$, respectively). However, the G[-]/*Pf*[+] group had lower levels compared to *Pf*[+] group ($P=0.016$). Although no significant differences were noted in circulating IL-13 levels across the three infected groups after exclusion of the healthy controls from the initial omnibus Kruskal-Wallis test ($P=0.366$), IL-13 levels were generally suppressed in the infected children compared to the healthy controls, with the lowest levels being found in the G[+]/*Pf*[+] group (Figure 6E). Although the TNF-α:IL-10 ratio was not significantly different across the groups ($P=0.329$, Figure 6F), there was a general skewing towards a type 2 millieu in the infected children relative to the healthy controls. Overall, we noted a greater cytokine response in the co-infected children, with higher concentrations of both pro- and anti-inflammatory cytokines. However, lower levels in a few key cytokines, such as TNF-α and IL-10, may be central to controlling parasitemia without exacerbating anemia.

5.5.7 Multiple mediation model

Since the primary finding of this study was a decreased level of parasitemia in the absence of exacerbated anemia in the co-infected children, we determined the combination of IMs responsible for this outcome. We first performed a Pearson correlation between the IMs and the dependent variable (i.e., parasitemia, Figure 7, *P*-values right of IMs) to select variables with significant correlations for populating the indirect.sbs SPSS macro. We also determined the correlation between the independent variable (Infection status: 0, *Pf*[+] only; 1, Bacteremia co-infected) and the IMs (Figure 7, *P*-values left of IMs). These analyses revealed that 13 of the IMs were correlated with the independent variable (i.e., infection status), with the following presented in order of strength of significance: IL-4; IL-5; IL-7; IL-15; IFN-α; IL-12; IFN-γ; IL-17; IL-1Ra; TNF-α; IL-1β; IL-10; and IL-2. Of these, only TNF-α and IL-10 had negative associations with infection status, while the others were positively associated with infection status, indicating that levels were greater in the bacteremia co-infected children than the *Pf*[+] mono-infected children. The dependent variable, parasitemia, was correlated with the following IMs, in order of strength of association: IL-10; IL-12; TNF-α; IFN-γ; IL-1β; IL-6; IL-5; IL-2; IL-4; and IL-7. Most of the IMs were negatively associated with parasitemia, except IL-10, TNF-α, and IL-6. The multiple mediation model was processed with 10,000 bootstrap samples and yielded four significant IMs: IL-4; IL-10; IL-12; and IFN-γ (Figure 7, significant IMs, black text; non-significant IMs, white text). The bootstrapped paired contrasts subsequently indicated that IL-4 had the strongest strength of association with infection status and parasitemia.

5.6 DISCUSSION

We have characterized the inflammatory milieu in children mono-infected with malaria, or co-infected with either G[+] bacteremia or G[-] bacteremia and *Pf*[+] residing in a holoendemic *P. falciparum* region. Following exclusion of HIV-1 seropositive and pro-viral DNA positive children, G[-]/*Pf*[+] and G[+]/*Pf*[+] co-infected children presented with reduced parasitemia and similar hemoglobin levels. Although most inflammatory molecules are associated with control of parasitemia and exacerbation of anemia in malaria, a multiple mediation model identified unique inflammatory mediators associated with decreased parasitemia in bacteremia co-infected children.

Consistent with previous studies (Graham, Molyneux *et al.* 2000; Morpeth, Ramadhani *et al.* 2009), NTS were the most prevalent bacterial isolates in *Pf*[+]-co-infected children in the current study. Although the risk factors for this phenomenon include increased malnutrition, malarial anemia, unclean food and water, HIV infection, contact with animals, age <3 years, excessive iron availability from hemolysis and anemia, and sickle cell allele carriage [reviewed in (Morpeth, Ramadhani *et al.* 2009)], co-existence of these factors in differing proportions appear the most plausible explanation of increased prevalence of NTS bacteremia in African children. There was a notable absence of typical childhood pathogens such as *S. pneumoniae* and *H. influenzae*, which may be partially explained by the fact that the enrollment criteria excluded children who presented with symptoms indicative of respiratory tract infections, most of which result from infection with these pathogens. In addition, the recent vaccination efforts in Kenya (HiB conjugate vaccination began in November

2001; pneumococcal vaccine expected in 2010), the fastidiousness of these organisms, and unprescribed antimicrobial treatment may lead to reduced pathogen recovery, particularly in individuals with low bioburdens. Our results showing a higher rate of *S. aureus* are consistent with studies elsewhere in Africa (Sigauque, Roca *et al.* 2009).

Although gender did not differ in our clinical categories, prevalence of female gender was higher in both co-infected groups relative to the malaria-only group. This observation may be related to the fact that the caregiver may view girls as less resilient and seek care earlier or more frequently than with co-infected males (Schlagenhauf, Chen *et al.* ; Lallement, Teyssier *et al.* 1986), or the result of more frequent urinary tract infections in females, which have been left untreated (Hoberman, Chao *et al.* 1993). We also noted fewer WBCs, specifically granulocytes, in the G[+]/*Pf*[+] co-infected group relative to the *Pf*[+]-alone and G[-]/*Pf*[+] groups. The decrease in leukocyte lineages in the infected children potentially indicate increased migration to lymphoid tissues and/or apoptosis, which are known to follow acute malaria in African children (Toure-Balde, Sarthou *et al.* 1996; Hviid, Kurtzhals *et al.* 1997). As such, it will be important to explore the chemokine milieu in co-infected children and its association with the leukocytic indices. Additional hematological findings presented here showing decreased MCHC in the co-infected children relative to the mono-infected children appear to indicate a microcytic hypochromic anemia. Although Hb levels were similar across the groups and appear to suggest iron deficiency anemia based on the low MCHC, reduced Hb concentrations may also result from the increased levels of pro-

inflammatory cytokines, particularly in the co-infected groups (Anderson, Aronson *et al.* 2000; Bwibo and Neumann 2003; Doherty 2007; Prentice, Ghattas *et al.* 2007).

In addition, our findings indicating lower parasite burdens in co-infected children in the absence of worsening anemia are similar to previous studies in Mozambique, showing lower parasite densities in the absence of changes in hematocrit in children with severe malaria and bacteremia (Bassat, Guinovart *et al.* 2009). Consistent with lower parasite densities, co-infected children had almost half the prevalence of HDP as the mono-infected group, with the G[-]/*Pf*[+] co-infected children having had nearly five-fold lower median parasitemia. Moreover, parasite reduction in the G[+]/*Pf*[+] co-infected children was approximately one-third of that observed in the mono-infected group, likely due to the superior inflammatogenic potential of lipopolysaccharide (LPS) versus the G[+] cell wall constituents previously demonstrated in *in vitro* experiments (Hessle, Andersson *et al.* 2005). Though slightly elevated, changes in axillary temperature cannot explain the decreases in parasitemia observed in the G[-]/*Pf*[+] and G[+]/*Pf*[+] co-infected groups, suggesting mechanisms other than fever in controlling parasitemia.

Although our previous studies revealed intraleukocytic acquisition of *Pf*Hz contributes to suppression of erythropoiesis and dysregulation in the inflammatory milieu (Were, Hittner *et al.* 2006; Keller, Ouma *et al.* 2009; Ouma, Keller *et al.* 2010) PCM and PCN were not associated with co-infection status in the current study, suggesting that *Pf*Hz may not be involved in promoting IM production in bacteremic children exposed to holoendemic malaria. Additional results presented here illustrating

similarities in Hb levels and prevalence of SMA and RPI between co-infected and mono-infected children suggest that the cytokine profile may contribute to parasitemia control without impacting negatively on Hb and erythropoietic responses given the negative influence of type 1 cytokines on erythropoiesis (Bertero and Caligaris-Cappio 1997; Ershler 2003; Means 2004).

The inflammatory mediator profiles indicated there were elevated pro-inflammatory cytokine levels, which decreased in magnitude from the G[-]/*Pf*[+] co-infected group, to the G[+]/*Pf*[+] group, and finally, the malaria mono-infected group. This regressive inflammatory mediator profile paralleled the parasitemia pattern indicating the potential induction of anti-parasite inhibitory and elimination events. This premise is supported by results showing elevations in IL-1β, IL-1Ra, IL-12, IL-15, IL-17, IFN-α, and IFN-γ in G[-]/*Pf*[+] co-infected children compared to malaria mono-infection. However, a net increase in IL-1β in G[-]/*Pf*[+] co-infected children appears to be counter-balanced by a similar increase in IL-1Ra, as evidenced by similarities in the IL-1β:IL-1Ra ratio across the groups. An increase in IL-12 in the co-infected groups and a decrease in malaria mono-infection is consistent with previous studies indicating suppression of IL-12 in children with malaria (Luty, Perkins *et al.* 2000; Perkins, Weinberg *et al.* 2000; Keller, Yamo *et al.* 2006). The increase in IL-12 in the co-infected groups, in the absence of marked Hb reductions, may be associated with increased parasitemia control as previously shown in human and murine studies (Normaznah, Halim *et al.* 1999; Luty, Perkins *et al.* 2000; Perkins, Weinberg *et al.* 2000; Su and Stevenson 2002; Boutlis, Lagog *et al.* 2003). The upregulation in IL-15 in co-infected

children compared to malaria mono-infection and healthy control may be related to the potent type 1 immune activities of IL-15, which include parasite and bacterial clearance, and induction of humoral responses (Hirose, Suzuki *et al.* 1998; Ing, Gros *et al.* 2005). However, previous studies demonstrating that IL-15 enhances the erythropoiesis-stunting effect of IFN-γ (Zoumbos, Gascon *et al.* 1984; Means, Krantz *et al.* 1994; Mullarky, Szaba *et al.* 2007) may be related to increases in both cytokines in the current study for maintaining the balance involved in controlling anemia development. Although IL-15 is known to induce production of IL-17 from CD4+ T cells (Ziolkowska, Koc *et al.* 2000), plasma IL-17 was suppressed in malaria mono-infected children with similar levels in the other groups. This finding is similar to previous studies in Indian malaria patients showing reduced plasma IL-17 in mild malaria relative to cerebral malaria and healthy controls (Jain, Armah *et al.* 2008). Additional pro-inflammatory cytokine results showing elevated IFN-γ and IFN-α in co-infected children relative to the mono-infected and healthy controls is consistent with reduced parasite densities in these individuals. This premise is supported by previous malaria studies demonstrating that IFN-γ mediates clearance of plasmodial parasites, while IFN-α may contribute to parasite clearance through induction of effector molecules such as nitric oxide (NO) (Kremsner, Winkler *et al.* 1996; Perkins, Kremsner *et al.* 1999). In contrast, reduced TNF-α levels in the co-infected children relative to the malaria mono-infected children may be related to the counter-regulatory effects of IFN-γ-induced NO, which downregulates nitric oxide synthase (NOS)-inducing cytokines, such as TNF-α, through a feedback mechanism (Florquin, Amraoui *et al.* 1994). Taken together, these results

indicate upregulation of pro-inflammatory cytokine production in co-infected children, with the exception of TNF-α, a cytokine that if overproduced may lead to increased malarial anemia through its ability to enhance both erythrophagocytosis and dyserythropoiesis (Clark and Chaudhri 1988).

The profile of anti-inflammatory cytokine response was similar to the pattern observed for the pro-inflammatory mediators. Plasma IL-4, IL-5, and IL-7 levels were elevated in co-infected children, while IL-10 levels were reduced in co-infected children. Our findings of elevated IL-4 in co-infected children differs from recent studies by Cabantous *et al.* (Cabantous, Poudiougou *et al.* 2009) in Malian children showing elevation of IL-4 in SMA compared to uncomplicated malaria. While it appears that down-regulation of the type 1 immune response may have exacerbated malaria in the Malian children, elevated IL-4 levels in co-infected children points to counter-regulatory activity of IL-4 on the potentially-damaging type 1 response, as well as the synergistic effect of IL-4 on EPO and IL-12 (Peschel, Paul *et al.* 1987), and IL-6 (Rennick, Jackson *et al.* 1989; Dybedal, Larsen *et al.* 1995) in promoting erythropoiesis. This hypothesis appears plausible since the type 1 response was too potent to be attenuated by IL-4. Elevated IL-7 levels in co-infected children may be related to its activity in supporting erythropoiesis (Aiello, Keller *et al.* 2007), and hence protecting against reductions in Hb levels in the markedly, potent pro-inflammatory environment observed in these children. Additional findings showing reduced IL-10 levels in co-infected children that were associated with increased parasitemia control parallels previous studies showing that the degree of parasitemia is closely correlated with the pattern of IL-10 production

(Jason, Archibald *et al.* 2001; Hugosson, Montgomery *et al.* 2004). Our findings are also supported by previous studies showing that increased IL-10 secretion by CD4+ T-cells inhibits parasite killing in malaria infection (Kobayashi, Morii *et al.* 1996; Couper, Blount *et al.* 2008). In addition, our previous investigations and those of others demonstrated poor recovery from malarial anemia in children with elevated IL-10 levels (Ong'echa, Remo *et al.* 2007), largely through reduced IL-12 production (Mohan and Stevenson 1998; Keller, Yamo *et al.* 2006).

Multiple mediation modeling identified IL-4, IL-10, IL-12, and IFN-γ as the most prominent mediators that influenced parasitemia. In this analysis, IL-4 emerged as the most closely associated with both infection status and parasitemia. In addition, Pearson correlations, used to load the mediation model, showed inverse associations between IL-4, IL-12, and IFN-γ, and parasitemia, and a positive correlation between IL-10 and parasitemia. This finding suggests that a combination of type 1 and type 2 responses is required to control parasitemia. Furthermore, the modeling revealed that there are the key differences in cytokine production that controls parasitemia in malaria mono-infection versus co-infection, and that co-infection appears to promote an optimal response for controlling parasitemia with minimal impact on hemoglobin levels.

While it is not unexpected that the G[-]/*Pf*[+] co-infected children had the lowest parasitemia, given the potent immune response generated by LPS, it is interesting that these children did not have more anemia. One possible explanation for this phenomenon would be the temporal effects of cytokine production. For example, the pro-inflammatory cytokine response acts rapidly to control parasitemia, but requires a

sustained type 1 inflammatory environment response for an extended period to impact the erythropoietic pathway and thereby decrease the Hb. In addition, absolute cytokine levels at any given point in time may not comprehensively represent the overall inflammatory response since the timing and magnitude of inflammatory mediators are critical in determining the outcome of infection. Previous studies by Kobayashi *et al.* (Kobayashi, Ishida *et al.* 2000) support this notion by demonstrating that administration of IL-10 in a lethal *P. yoelii* infection promotes prolonged survival, but has no impact on parasitemia levels. Moreover, IL-10 secretion too early in infection allows unfettered parasite replication, while a late IL-10 response may allow type 1 cytokines to have a detrimental effect on erythropoiesis by either delaying Hb recovery or leading to severe anemia and death. Although results from the mediation model suggest that both type 1 and 2 responses are important for controlling parasitemia, we postulate that the significant association with type 2 mediators (IL-4 and IL-10) may be critical for preserving erythropoiesis by attenuating the considerable type 1 environment elicited by co-infection.

Results presented here demonstrate the cytokine profile that predominates in malaria and bacteremic co-infected children living in a holoendemic *P. falciparum* transmission area. In addition, we show the specific IMs that are responsible for improved control over malaria parasitemia without worsening anemia. While there remains a rudimentary understanding of the cytokine pathways involved in control and

recovery from various infections, novel findings presented here suggest an important role for cytokines that were previously largely unexplored in human malaria, such as IL-4.

5.7 ACKNOWLEDGMENTS

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Gregory C. Davenport collected and analyzed the data and prepared the manuscript. Tom Were conducted the microbiological testing. James B. Hittner designed the data analysis strategy and provided statistical input. Collins Ouma conducted the HIV testing. Yamo Ouma analyzed the blood smears for parasitologic indices. John M. Vulule aided in the study design and supervision of the work in Kenya. John M. Ong'echa aided in the study design and interpretation of the results. Douglas J. Perkins designed the study, supervised the activities in New Mexico, Pennsylvania, and Kenya, and co-wrote the manuscript with GCD.

5.8 TABLES AND FIGURES

Table 8. *Bacterial isolate distribution.*

Organism	Isolates	Percent of Isolates
Gram negative organisms		
Acinetobacter sp.	1	2.8
Escherichia coli	1	2.8
Pseudomonas aeruginosa	1	2.8
Salmonella enterica ssp. arizonae	1	2.8
Salmonella enterica ssp. enteritidis	2	5.6
Salmonella enterica ssp. typhimurium	16	44.4
Gram positive organisms		
Enterococcus sp.	1	2.8
Staphylococcus aureus	13	36.1

Organisms isolated from pediatric blood culture according to Gram reaction category that were included in these analyses. Blood was drawn into pediatric Isostat tubes, plated onto Chocolate agar plates, and incubated for 18 hrs at 37°C with 5% CO2. Subcultured colonies were incubated 18-24 hrs on inverted specialty media (Chocolate Agar, MacConkey's Agar, Mannitol Salt Agar, and Blood Agar Plates). Plates were incubated for an additional four days if no growth was obtained in the first 24 hrs. Plates were inspected daily, and unique colonies identified through standard microbiological and biochemical techniques (i.e., Gram stain, colony characteristics, API biochemical panels, and agglutination tests).

Table 9. *Clinical, demographic, and hematological characteristics of the study participants.*

Characteristic	Healthy Controls	*Pf*[+]	G[-]/*Pf*[+]	G[+]/*Pf*[+]	*P* *
Number of subjects	14	156	22	14	N / A
Age; months	7.07 (11.39)	9.08 (8.70)	7.38 (7.50)	12.11 (9.94)	0.161
Gender; male/female **	8/6	88/68	6/16	5/9	**0.041**
Axillary temperature (°C)	36.4 (0.4)	37.5 (1.7)	38.0 (1.5)	37.1 (1.6)	0.141
Glucose (mMol/L)	4.89 (0.59)	4.90 (1.48)	5.59 (1.70)	5.10 (4.94)	0.067
WBC ($x10^9$/µL)	10.50 (3.95)	11.75 (6.95)	13.35 (9.15)	9.10 (4.60)	**0.047**
Lymphocytes ($x10^3$/µL)	6.70 (2.05)	5.60 (3.87)	6.65 (4.20)	4.80 (4.95)	0.431
Monocytes ($x10^3$/µL)	0.80 (0.55)	1.10 (0.90)	1.15 (0.67)	0.60 (0.90)	0.161
Granulocytes ($x10^3$/µL)	3.10 (2.35)	4.75 (4.17)	4.75 (5.80)	2.60 (2.30)	**0.010**
Hemoglobin (Hgb, g/dL)	11.7 (0.8)	5.9 (3.7)	5.8 (1.8)	6.0 (2.8)	0.594
Hematocrit (Hct, %)	35.2 (1.9)	18.8 (11.7)	19.1 (4.7)	19.7 (7.4)	0.792
RBC ($x10^6$/µL)	4.82 (0.31)	2.85 (1.90)	2.69 (1.27)	2.93 (1.09)	0.642
MCHC (g/dL)	33.4 (0.9)	32.5 (2.5)	30.9 (4.2)	30.9 (2.8)	**0.003**
RDW	16.4 (4.3)	21.2 (4.8)	22.4 (3.9)	22.7 (5.8)	0.053
Platelets ($x10^3$/µL)	355 (219)	153 (111)	159 (107)	160 (98)	0.720
Mortality; n (%) ***	1 (7.1)	6 (3.8)	2 (9.1)	0 (0)	0.537

HIV-1 negative children (aged <3 yrs) infected with *P. falciparum* malaria were divided into parasitemic (*Pf*[+]), G[-]/*Pf*[+] co-infected, and G[+]/*Pf*[+] bacteremia co-infected groups. In addition, a cohort of afebrile healthy children without parasitemia and hemoglobin (Hb) >11.0g/dL were included as controls to detect depressed cytokine levels associated with infection. WBC (white blood cells); RBC (red blood cells); MCHC (mean corpuscular hemoglobin concentration); RDW (red cell distribution width). * Data are presented as median (interquartile range) and compared using the Kruskal-Wallis test unless stated otherwise. ** Differences in categorical variables were compared using Pearson's χ^2 test. *** Pair-wise contrasts among independent proportions were chosen as the statistical method to evaluate the categorical mortality data. The *P*-value for a chi square test would be uninterprettable since the cell sum was less than 20. Complete blood counts were determined in venous blood using a Coulter® AcT diff2™ (Beckman Coulter Corp.).

Table 10. *Erythropoietic indices and intraleukocytic hemozoin.*

Attribute	*Pf*[+]	G[-]/*Pf*[+]	G[+]/*Pf*[+]	*P* *
Number of subjects	153	22	14	N / A
SMA, Hb <6.0g/dL; n (%) [a]	81 (52.9)	14 (63.6)	8 (57.1)	0.628
SMA, Hb <5.0g/dL; n (%) [a]	42 (27.5)	6 (27.3)	2 (14.3)	0.562
RPI; median (IQR) [b]	0.98 (1.44)	0.86 (1.00)	0.92 (2.57)	0.837
RPI <2; n (%) [a]	36 (23.8)	3 (13.6)	4 (30.8)	0.453
PCM; n (%) [a]	83 (53.2)	12 (54.5)	8 (57.1)	0.957
PCN; n (%) [a]	22 (14.1)	1 (4.5)	2 (14.3)	0.455

SMA (severe malarial anemia); RPI (reticulocyte production index); PCM (pigment-containing monocytes); PCN (pigment-containing neutrophils). A total of 30 monocytes and 100 neutrophils were examined per slide, and the number of PCN and PCM were expressed as a percentage of the total number of neutrophils and monocytes, respectively. [a] Differences in categorical variables were compared using Pearson's χ^2 test. [b] Data are presented as median (interquartile range) and differences between the three groups were compared using Kruskal-Wallis test.

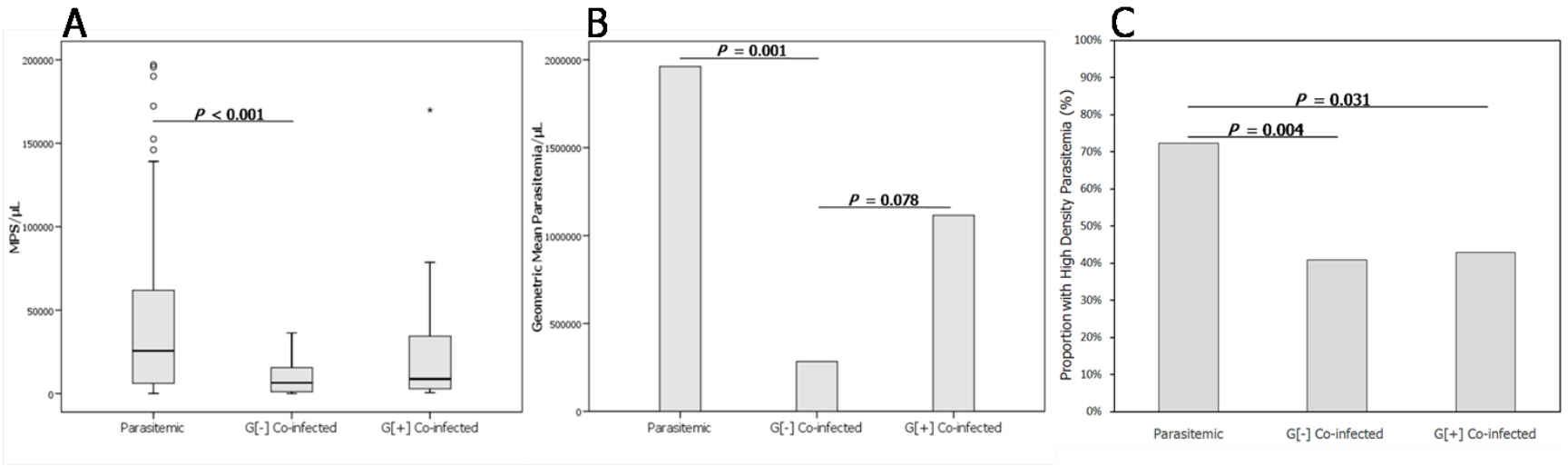

Figure 3. ***Parasitologic indices.*** Data are presented as box plots with whiskers and outliers. The box represents the interquartile range, while the whiskers represent the 10th and 90th percentiles. The line across the box indicates the median value, open circles (°) represent extremes, and asterisks (*) depict outliers. Differences between groups were determined using Mann-Whitney U test. (A) MPS [Malaria Parasites] (Kruskal-Wallis test P=0.001). (B) Geometric Mean parasitemia (ANOVA P=0.011). (C) Proportion of High Density Parasitemia (%) (Kruskal-Wallis test P=0.002).

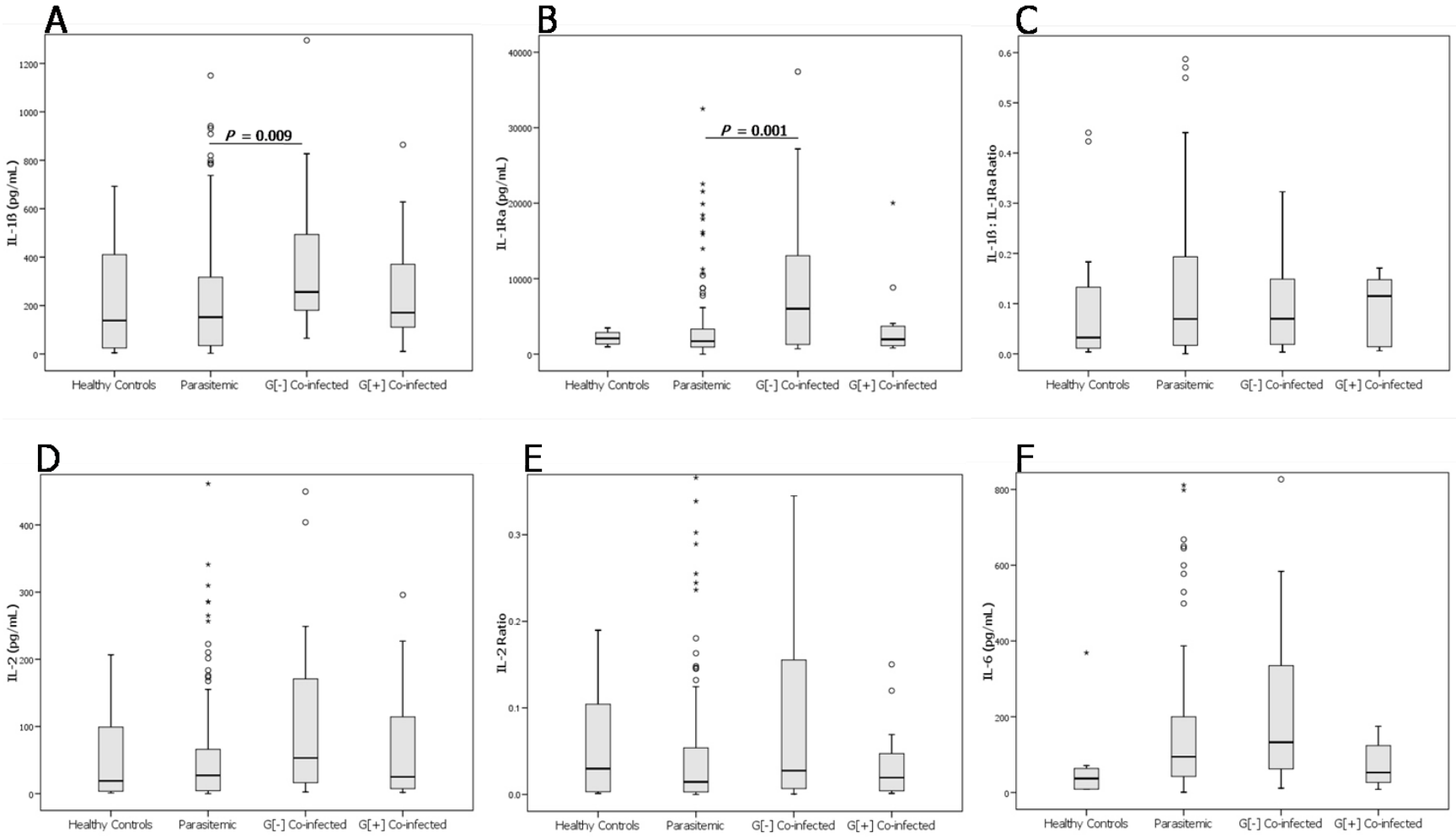

Figure 4. ***Pro-inflammatory mediator profiles.*** Data are presented as box plots with whiskers and outliers. The box represents the interquartile range, while the whiskers represent the 10th and 90th percentiles. The line across the box indicates the median value, open circles (°) represent extremes, and asterisks (*) depict outliers. Pair wise comparisons between groups were conducted with Mann-Whitney U test. All units are in pg/mL. (A) IL-1β (Kruskal-Wallis test *P*=0.027). (B) IL-1Ra (Kruskal-Wallis test *P*=0.005). (C) IL-1 Ratio (Kruskal-Wallis test *P*=0.854). (D) IL-2 (Kruskal-Wallis test *P*=0.068). (E) IL-2 Ratio (Kruskal-Wallis test *P*=0.575). (F) IL-6 (Kruskal-Wallis test *P*=0.115).

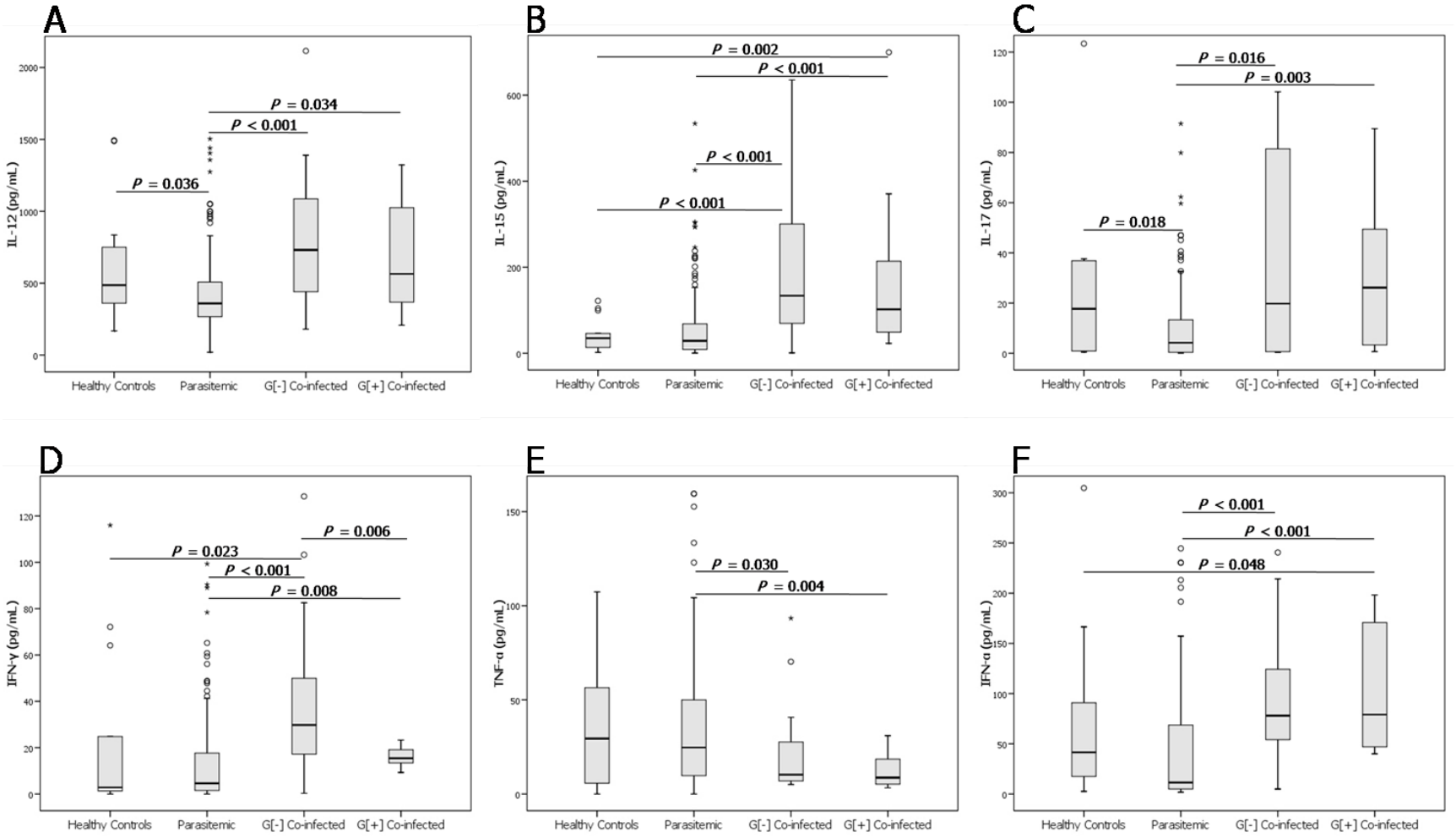

Figure 5. ***Pro-inflammatory mediator profiles.*** Data are presented as box plots with whiskers and outliers. The box represents the interquartile range, while the whiskers represent the 10th and 90th percentiles. The line across the box indicates the median value, open circles (°) represent extremes, and asterisks (*) depict outliers. Pair wise comparisons between groups were conducted with Mann-Whitney U test. All units are in pg/mL. (A) IL-12 (Kruskal-Wallis test $P<0.001$). (B) IL-15 (Kruskal-Wallis test $P<0.001$). (C) IL-17 (Kruskal-Wallis test $P=0.002$). (D) IFN-γ (Kruskal-Wallis test $P<0.001$). (E) TNF-α (Kruskal-Wallis test $P=0.003$). (F) IFN-α (Kruskal-Wallis test $P<0.001$).

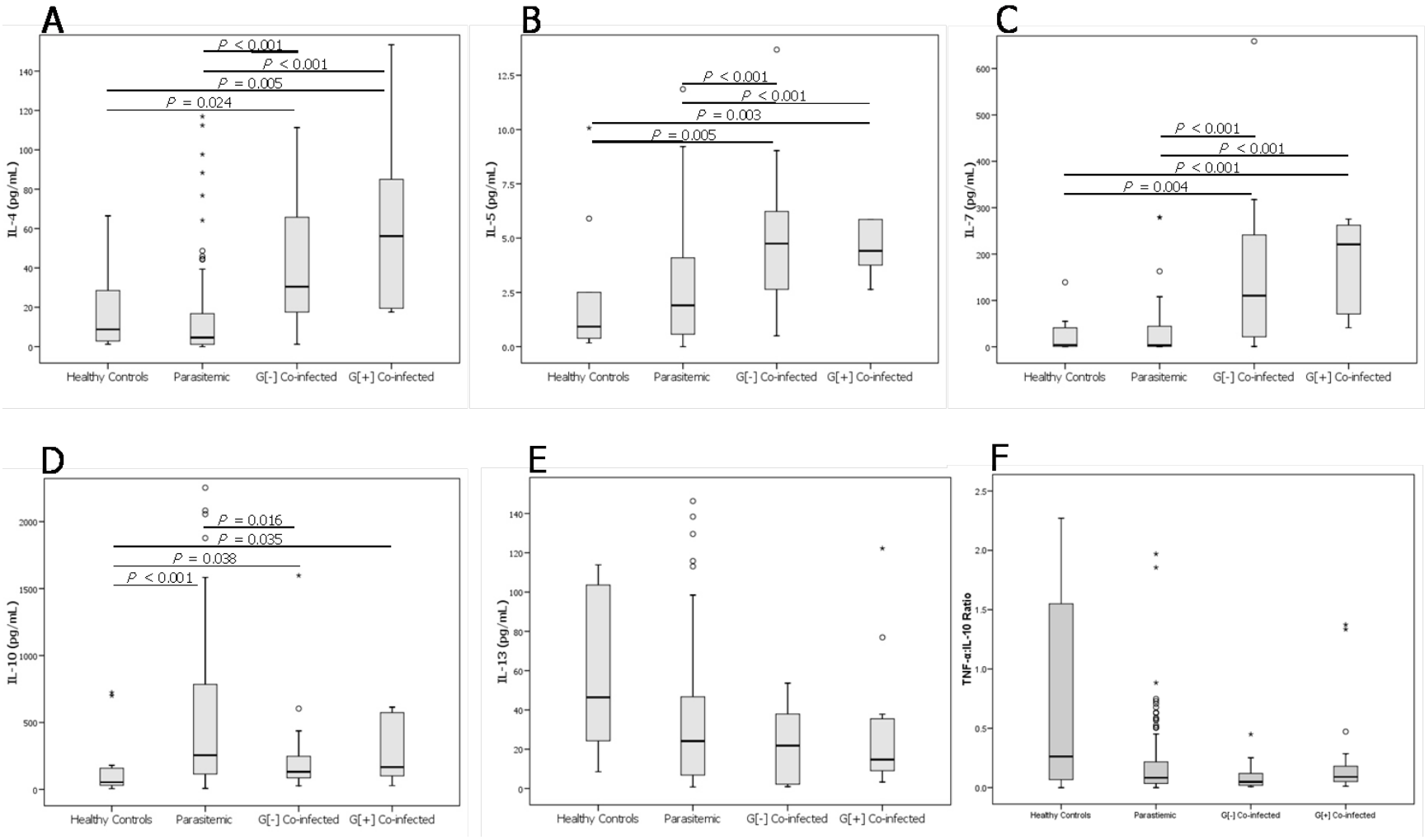

Figure 6. ***Anti-inflammatory mediator profiles.*** Data are presented as box plots with whiskers and outliers. The box represents the interquartile range, while the whiskers represent the 10th and 90th percentiles. The line across the box indicates the median value, open circles (°) represent extremes, and asterisks (*) depict outliers. Pair wise comparisons between groups were conducted with Mann-Whitney U test. All units are in pg/mL. (A) IL-4 (Kruskal-Wallis test $P<0.001$). (B) IL-5 (Kruskal-Wallis test $P<0.001$). (C) IL-7 (Kruskal-Wallis test $P<0.001$). (D) IL-10 (Kruskal-Wallis test $P=0.042$). (E) IL-13 (Kruskal-Wallis test $P=0.366$). (F) TNF-α:IL-10 ratio (Kruskal-Wallis test $P=0.329$).

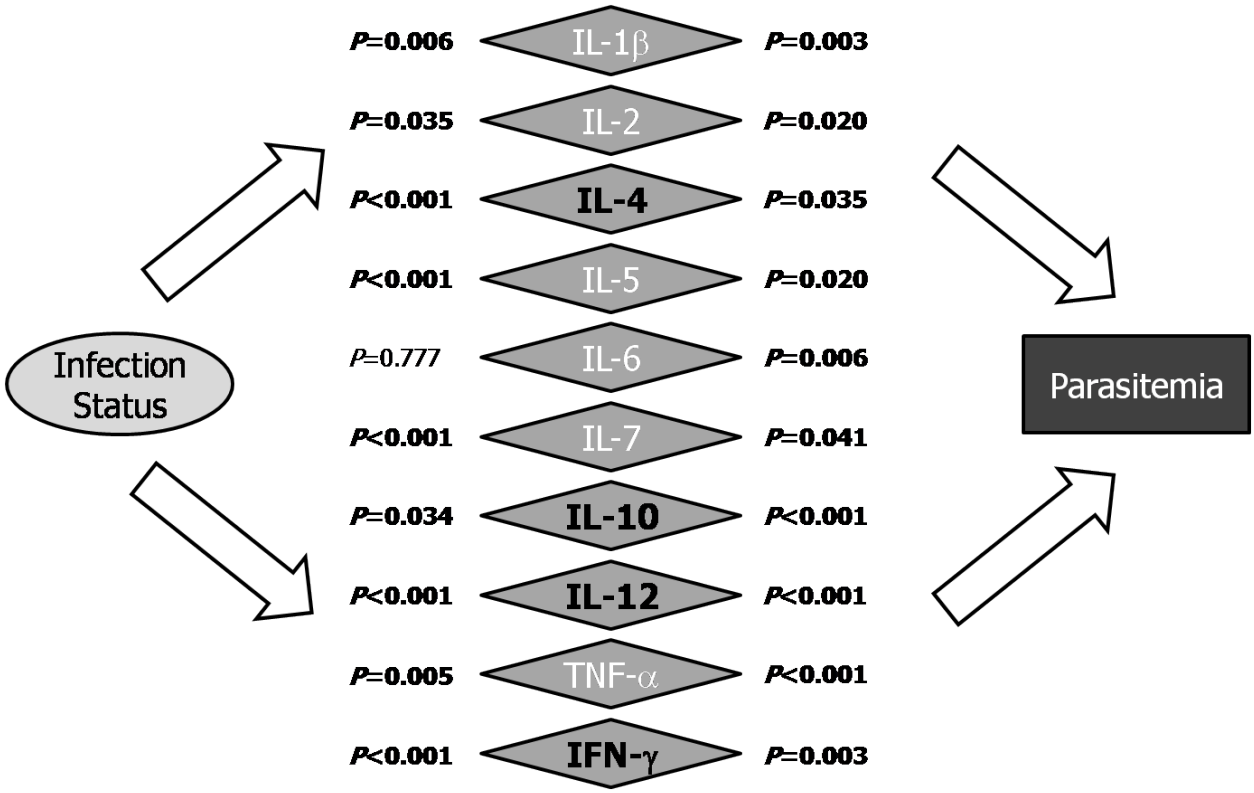

Figure 7. ***Multiple mediation model.*** Inflammatory mediators were correlated with the independent variable (Infection Status, *P*-values to left of mediators) and Parasitemia (*P*-values to the right of mediators) individually, and those with significant correlations with the dependent variable (Parasitemia) were used to populate the multiple mediation model. All nonparametric variables were log transformed. "Infection Status" was dummy coded as follows: 0, *Pf*[+] mono-infected; 1, bacteremia co-infected. The multiple mediation model was populated with 10 inflammatory mediators that showed significant correlation with Parasitemia, with age used as a covariate, and processed using 10,000 bootstrap samples. Post-hoc pair wise contrasts for the four significant mediators (black type; IL-4, IL-10, IL-12, IFN-γ) were evaluated by examining the bootstrapped confidence intervals, with those intervals not including zero being significant.

6.0 DISCUSSION

Children residing in holoendemic regions of sub-Saharan Africa endure a near-constant assault from *P. falciparum*-infected mosquitoes. Mosquito net use for children is sporadic at best, especially in the poorer, rural regions of western Kenya. The result of these chronic bites from infected Anopheles mosquitoes is repeated infection throughout childhood. For those fortunate enough to emerge from their early childhood alive and without permanent sequelae, they enjoy life-long protection from severe disease as long as they continue to inhabit that region. Unfortunately, the majority of children endure at least transient anemia from their chronic infections, and many develop severe disease in the form of SMA, which carries a substantial risk of death. While CM carries a significantly higher mortality rate, and spans more years at risk, SMA affects far more children, resulting in many more hospital visits and significantly greater childhood mortality. If malaria was the only serious risk in these *P. falciparum* regions it would garner significant attention due to the sheer numbers of people affected; however, these same regions have been impacted the greatest by the HIV epidemic as well. In addition, whether it be due to a genetic predisposition or lack of clean water and food supplies, invasive Salmonellosis, as well as other bacterial pathogens, are more frequently isolated from the blood cultures of children residing in sub-Saharan Africa, particularly those regions hardest hit by malaria. This body of work attempts to

elucidate the cytokine profiles associated with these infections and co-infections, as well as the predictors of anemia and hematologic outcomes resulting from these infections, on Kenyan children.

Initially, a small number of hastily assembled studies, which were poorly controlled and underpowered, failed to find an interaction between malaria and HIV-1 (Nguyen-Dinh, Greenberg *et al.* 1987; Colebunders, Bahwe *et al.* 1990; Muller, Musoke *et al.* 1990; Greenberg, Nsa *et al.* 1991; Kalyesubula, Musoke-Mudido *et al.* 1997). Now we know that HIV viral load increases substantially during malaria infection (Hoffman, Jere *et al.* 1999; Ma, Sun *et al.* 2000; Mwapasa, Rogerson *et al.* 2004; Kublin, Patnaik *et al.* 2005), as do malaria episodes (Whitworth, Morgan *et al.* 2000) and anemia (van Eijk, Ayisi *et al.* 2002; Grimwade, French *et al.* 2004; Cohen, Karstaedt *et al.* 2005) during HIV infection. Since the primary manifestation of HIV-1 in children is anemia, it stands to reason that having two anemia-inducing infections will result in a net decrease in Hb greater than from either infection alone. We have previously shown significantly more anemia in HIV-1[+] children co-infected with malaria (Otieno, Ouma *et al.* 2006). Surprisingly, we also discovered significantly more anemia in antibody positive and virus negative children, i.e. HIV-1[exp], born to HIV-1[+] mothers (Otieno, Ouma *et al.* 2006; Davenport, Ouma *et al.* 2010). This is a striking finding being that their only infection was malaria, suggesting that their exacerbated anemia may be the result of gestation in an HIV-1[+] mother. We have also determined that this additional anemia is not the result of discrepancy in care, as the HIV-1[+]/*Pf*[+] and HIV-1[exp]/*Pf*[+] children do not have more wasting or stunting that the HIV-1[-]/*Pf*[+]

children (Davenport *et al.*, unpublished data). Interestingly, the two major contributors to anemia in malaria, parasitemia and dyserythropoiesis, were not significantly different across the groups. The caveat to this finding is that erythropoiesis should be proportional to the degree of anemia, so by the mere fact that the Hb values were different, the erythropoietic indices should differ as well. As for parasitemia, both of the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups had lower parasitemia, presumably due to a more enhanced pro-inflammatory milieu, which may also explain the blunted erythropoietic response in the children with worsening anemia.

The primary finding of the first study in Aim 1 resulted from the hierarchical multiple regression model, which demonstrated that PCM, age, and HIV-1 status were the three significant predictors of Hb, in that order. Pearson correlation indicated that Hb increased with age, while advancing HIV-1 status and increasing *Pf*Hz burdens were associated with lower Hb values. When G6PD and sickle cell status were added to the model, which each showed interesting but non-significant trends across the three HIV-1 categories, neither of these genetic traits were significant predictors of Hb. In addition to the effect *Pf*Hz has on pro-inflammatory cytokine production (Sherry, Alava *et al.* 1995; Jaramillo, Gowda *et al.* 2003; Keller, Davenport *et al.* 2006; Ong'echa, Remo *et al.* 2008), and hence, erythropoiesis (Mayani, Little *et al.* 1995; Tarumi, Sawada *et al.* 1995; Cashman, Clark-Lewis *et al.* 1999; Dufour, Corcione *et al.* 2003), a recent report found a direct effect on erythropoietic precursors in the bone marrow (Lamikanra, Theron *et al.* 2009). In addition to finding that *Pf*Hz directly induced caspases, and therefore apoptosis, in erythroid precursors without any inflammatory mediator input,

they also reported that macrophage phagocytosis of *Pf*Hz actually protected erythroid precursor cells from the harmful effect of *Pf*Hz (Lamikanra, Theron *et al.* 2009).

We followed up this initial determination of severe anemia predictors in malaria and HIV-1 co-infected children by analyzing the circulating inflammatory mediators in the same groups. As we observed in the first aim, both the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] co-infected groups had more profound anemia than the HIV-1[-]/*Pf*[+] children. The across-group comparisons of the inflammatory mediators revealed that IL-12 and IFN-γ were increased in the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups. This finding was unexpected since IL-12 has been found to support erythropoiesis (Bellone, Ostlie *et al.* 1993; Jacobsen, Veiby *et al.* 1996) and low levels of this important cytokine has been implicated in children suffering from severe anemia (Aste-Amezaga, Ma *et al.* 1998; Luty, Perkins *et al.* 2000; Perkins, Weinberg *et al.* 2000; Casals-Pascual, Kai *et al.* 2006; Keller, Yamo *et al.* 2006). Given that HIV-1 infection is known to cause immune activation resulting in a pro-inflamamtory environment [reviewed in (Ford, Puronen *et al.* 2009)], it is not surprising that this type 1 cytokine is elevated. Similarly, elevation of IFN-γ, as we observed here, would be expected in an immune activation scenario, but unlike IL-12, has been directly implicated in causing impaired erythropoiesis through an IL-15 mechanism (Mullarky, Szaba *et al.* 2007). GM-CSF was also substantially increased in the the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] co-infected groups, which, like IL-12, should support erythropoiesis (Liehl, Hildebrandt *et al.* 1994) as well as parasite clearance in concert with TNF-α (Kumaratilake, Ferrante *et al.* 1996). Interestingly, IL-12 was also identified as the most influential molecule in component 4,

which was the only component significantly and positively associated with Hb. Given the elevated levels of IFN-γ, IL-12 may be driving production of this erythropoiesis-inhibiting cytokine, and may be an exacerbation of the immune activation syndrome known to be elicited by HIV-1 (Martinez-Maza, Crabb *et al.* 1987; Ascher and Sheppard 1988; Ascher and Sheppard 1990; Sheppard, Ascher *et al.* 1991; Hazenberg, Otto *et al.* 2003). These findings highlight that malaria, and other cytokine-mediated diseases, are not necessarily the result of the particular inflammatory mediators produced, but rather the timing and concentration of these cytokines. While IFN-γ, and, more importantly, IL-12 and GM-CSF would be expected to be a positive response to these two infections, and resulted in lower parasitemia levels in the HIV-1[+]/*Pf*[+] co-infected children, it appears their high concentrations have deleterious effects on the erythropoietic response to the degree of anemia. Moreover, given what is known about these significant cytokines and growth factors, one would expect a positive hematologic and parasitologic outcome in the HIV-1[+]/*Pf*[+] children, but the worsening anemia in this group indicates that soluble mediators alone, or the mediators measured in this study, cannot account for the effect on erythropoiesis or RBC destruction and sequestration.

In the final aim, we investigated HIV-1[-] children to determine the effect of malaria and bacteremia co-infection on the inflammatory milieu. Interestingly, and unexpectedly, we found that co-infected children did not have more severe anemia, even though parasitemia levels and proportion of children with HDP were significantly lower. Beyond these two striking findings, the three groups of children were nearly identical from a clinical and hematologic standpoint, including their erythropoietic

indices and numbers of pigment-containing leukocytes. From an immunologic perspective, the malaria and bacteremia co-infected children had higher levels of IMs compared to children with malaria-alone and healthy controls. Specifically, there were higher levels of the pro-inflammatory cytokines IL-12, IL-15, IL-17, IFN-γ, and IFN-α in the bacteremic children. As we noted in the previous study, IL-12 alone is not capable of rescuing falling Hb concentrations, perhaps due to increased production of IFN-γ, IL-15, and IFN-α are known to directly inhibit erythropoiesis (Roodman, Bird *et al.* 1987; Fandrey and Jelkmann 1991; Means, Dessypris *et al.* 1992; Shami and Weinberg 1996; Zhu, Ye *et al.* 2000; Mullarky, Szaba *et al.* 2007). Similar elevations were noted in the anti-inflammatory molecules as well, with IL-4, IL-5, and IL-7 showing significantly greater concentrations in the bacteremia co-infected children, while IL-10 and TNF-α levels were slightly higher in the malaria mono-infected children. The difference between these groups may be what was not elevated, i.e., TNF-α and IL-10, rather than those IMs that were. TNF-α is also a proven inhibitor of erythropoiesis (Roodman, Bird *et al.* 1987; Fandrey and Jelkmann 1991; Shami and Weinberg 1996; Allen, Breen *et al.* 1999), and the molecule most often cited as being the precipitator of poor outcomes in malaria [reviewed in (Clark and Cowden 2003)] as well as bacteremia [reviewed in (Zanotti and Kumar 2002)], and IL-10 has been shown to reduce IL-12 levels with negative consequences on recovery from anemia (Keller, Yamo *et al.* 2006).

In the series of studies presented here, we observed two different co-infections with malaria having significantly different impacts on anemia. While the Hb values were nearly identical in the malaria infected and bacteremia co-infected children, there was

increased RDW, suggesting the presence of younger RBCs and a sign of recent erythropoiesis. This erythropoiesis was taking place in the bacteremia co-infected children despite erythrocyte indices suggesting an iron-deficient state was present, which is not unexpected in a systemic bacterial illness consuming the available iron. In addition to the iron-deficiency, there was greater production of inflammatory mediators in the bacteremia co-infected versus the HIV-1 co-infected children, which should have resulted in a more inhibited erythropoiesis and more profound anemia. This observation was supported by the lower parasitemias observed in the bacteremia co-infected, also suggesting the presence of a more pro-inflammatory environment.

The IM commonalities between the two co-infections were increases in IL-4, IL-7, IL-12, IFN-α, and IFN-γ. To some extent, these commonalities rule out these IMs as being major influences on erythropoiesis, and hence, the cause of the exacerbated anemia observed in the HIV-1/*Pf*[+] co-infected, but absent in the bacteremia co-infected. While IFN-α (Zhou, Chen *et al.* 1995) and IFN-γ (Musso, Calosso *et al.* 1999; Gysemans, Ladriere *et al.* 2005; Wenxin, Jinxiang *et al.* 2005) each elicit molecules capable of inhibiting erythropoiesis, i.e., nitric oxide and IL-15, respectively (Mullarky, Szaba *et al.* 2007; Cokic and Schechter 2008), the surprising common finding was increased IL-12, which has been shown in a number of studies to be important in boosting erythropoietic responses (Mohan and Stevenson 1998; Mohan and Stevenson 1998; Zhang, Prather *et al.* 2010). While seemingly important in supporting the Hb levels in the bacteremia co-infected children, this important cytokine was not protective in the HIV-1 co-infected group, despite a positive correlation with Hb in the principal

component factor analysis. Furthermore, studies in renal disease patients resistant to EPO therapy have found they had higher circulating IL-12 levels, as well as greater IL-10, TNF-α, and IFN-γ production by PBMCs (Macdougall and Cooper 2002; Macdougall and Cooper 2002), suggesting higher IL-12 levels may not always be beneficial in anemia.

The cytokines that differed in significance and directionality between the bacteremic and HIV co-infected groups were increases in IL-5, IL-15, and IL-17 noted in the bacteremia co-infections but not the HIV-1 coinfected group. Out of those three cytokines, only IL-15 (Mullarky, Szaba *et al.* 2007) has been reported to affect erythropoiesis, therefore this explanation would only be plausible if the increase were in the HIV-1[+]/*Pf*[+] group. However, decreases in two key molecules, IL-10 and TNF-α, noted in the bacteremic children but not in the HIV-1[+]/*Pf*[+] children, may hold the key to the differences in anemia severity. IL-10 has been reported to contribute to anemia by reducing IL-12 concentrations (Keller, Yamo *et al.* 2006; Cambos, Bazinet *et al.* 2010), which does not seem to be a factor in these two co-infections given the increased IL-12 levels. However, as mentioned above, increased secretion of IL-10 from the PBMCs of poor EPO responders has been noted (Macdougall and Cooper 2002; Macdougall and Cooper 2002). In addition, TNF-α is a well-known inhibitor of erythropoiesis (Roodman, Bird *et al.* 1987; Johnson, Waddelow *et al.* 1989; Rusten and Jacobsen 1995). Decreases in these two potent molecules may be the difference

between the exacerbated anemia observed in the HIV-1/*Pf*[+] co-infected children and the comparable anemia seen in the malaria mono-infected versus bacteremia co-infected children.

The work presented here helps elucidate the complex cytokine cascade that accompanies infection with malaria, HIV-1, and bacteremia in a sub-Saharan African population, and the potential effects on parasite clearance and recovery from anemia. One caveat to these studies is the cytokine measurements, as well as the parasitologic and hematologic values, are from a single point in time, and need to be confirmed using a longitudinal approach that will provide an extended picture of an extremely dynamic and complex set of infectious diseases and the immune response to those diseases. Studies using an *in vitro* model of erythropoiesis, as we have previously developed with CD34+ cells (Awandare *et al.*, 54th Annual Meeting of the American Society of Tropical Medicine and Hygiene, 2005, Washington DC, USA), may also help elucidate the role of each cytokine, or combinations of cytokines, critical or detrimental to erythrocyte production.

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