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RELATIONSHIP BETWEEN INFLAMMATORY MEDIATOR PATTERNS AND ANEMIA IN HIV-1 POSITIVE AND EXPOSED CHILDREN WITH PLASMODIUM FALCIPARUM MALARIA

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Abstract

Anemia is the primary hematological manifestation of both *Plasmodium falciparum* malaria and HIV-1 in pediatric populations in sub-Saharan Africa. We have previously shown that HIV-1 positive and exposed children have greater risk of developing severe anemia (hemoglobin, Hb<6.0 g/dL) during acute malaria. However, enhanced severity of anemia was unrelated to either erythropoietic suppression or parasite-driven red blood cell hemolysis. To further explore mechanisms of anemia, circulating inflammatory mediators (IMs) were determined using a 25-plex bead array in *P. falciparum*-infected (*Pf*[+]) children (3-36 mos., n=194) stratified into three groups: HIV-1 negative (HIV-1[−]/*Pf*[+]); HIV-1 exposed (HIV-1[exp]/*Pf*[+]); and HIV-1 infected (HIV-1[+]/*Pf*[+]). IL-12, MIG/CXCL9, eotaxin/CCL11, and GM-CSF differed significantly and progressively increased across the groups (HIV-1[−]→HIV-1[exp]→HIV-1[+]). To further explore the relationship between the inflammatory milieu (i.e., cytokines, chemokines, and growth factors) and HIV-1 status, the large panel of IMs was reduced into discrete groups by principal component factor analysis. Of the six principal components that emerged, three components were significantly higher in the HIV-1 positive and exposed groups, demonstrating that inflammatory profiles differ according to HIV-1 status. Additional analyses exploring the relationship between the components and anemia revealed significant positive correlations between Hb and component 3 (IL-1Ra, IL-7, IL-17, IFN-α, IFN-γ, MIG/CXCL9) in the (HIV-1[−]/*Pf*[+]) group, and component 4 (IL-4, IL-5, IL-12, Eotaxin/CCL11) in HIV-1[+]/*Pf*[+]

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children. Further analyses of the HIV-1[+]/*Pf*[+] group revealed that IL-12 had the strongest association with anemia. Results presented here demonstrate that there are unique relationships between the inflammatory environment and anemia in HIV-1 positive and exposed children with malaria.

Keywords

pediatric; Africa; anemia; falciparum; HIV-1; inflammation

INTRODUCTION

Currently, sub-Saharan Africa is home to 90% of the world's *Plasmodium falciparum* infections [1] and 64% of the HIV-1 infections [2]. Children are the most affected group in this region, with malaria and HIV-1 being responsible for 18% and 6%, respectively, of deaths in children under the age of five [3] with the primary manifestation of each disease being anemia.

One important mechanism common to both diseases, which may contribute to development of severe anemia (SA), is perturbation in inflammatory mediator (IM) production [4-6]. Results from our laboratory and others 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 was associated with enhanced immuno-pathogenesis of severe malarial anemia (SMA) [4, 7-9]. We also showed that children with malaria had 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) [10, 11]. Changes in (relative) IM levels may promote SMA pathogenesis by altering the erythropoietic cascade. For example, TNF-α rapidly and irreversibly inhibits human erythroid progenitors [12], while IFN-γ directly suppresses human erythroid colony-forming units (CFU-E) [12]. IFN-γ also up-regulates IL-15, which in turn, inhibits erythropoiesis at the CFU-E stage [13]. In addition, our previous results showed that pediatric malarial anemia is characterized by suppression of IL-12 [4].

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 [14], which potentially depletes CD4+ T-cells required to mount an effective immune response [15-17]. The chronic immune activation phase is associated with elevated levels of IL-1β [18, 19], IL-6 [20, 21], IL-10 [22], IFN-γ [22], TNF-α [18, 19], MIP-1α/CCL3 [23, 24], MIP-1β/CCL4 [24], and RANTES/CCL5 [23-26], and decreased IL-12 levels [27-30]. Thus, changes in IM profiles 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 IM profile(s) associated with worsening anemia in co-infected children.

MATERIALS

Study participants

Children aged 3-36 months (n=194) 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 [31]. None of the children included in the study had cerebral malaria, non-falciparum malarial infections, bacteremia, or hookworm infections. A detailed description of the study area and pediatric anemia can be found in our previous publication [32].

All children in the study had *P. falciparum* infections (*Pf*[+]) and were divided into three groups: HIV-1 negative (HIV-1[−]/*Pf*[+], negative HIV-1 serology, n=148); HIV-1 exposed (HIV-1[exp]/*Pf*[+], at least one (of two) positive serological tests 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 maternal HIV-1 status was unknown, HIV-1[exp]/*Pf*[+] children presumably acquired HIV-1 antibodies from their mothers during gestation and/or through breastfeeding since none of the children in the cohort had received prior blood transfusions. Children with HIV-1[+]/*Pf*[+] co-infection were *not further* stratified according to disease stages due to 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. 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) residing in western Kenya [33]. Children were treated according to Ministry of Health, Kenya, guidelines and written informed consent was obtained from the participants' parents/guardians. Human subjects approval for the study was granted by the University of New Mexico, University of Pittsburgh, and Kenya Medical Research Institute (KEMRI).

Laboratory measurements

For results presented here, none of the children had been initiated on antiretroviral therapy and/or received any other treatment interventions prior to sample acquisition. Asexual malaria trophozoites in thick and thin peripheral blood smears, and reticulocyte count were determined according to previous methods [32, 34]. 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. 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 [34].

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).

Statistical analyses

Data were analyzed using SPSS (version 15.0). Clinical, demographic, hematological, and immunological measures were compared between groups by either analysis of variance (ANOVA) or Kruskal-Wallis tests, followed by pairwise post-hoc comparisons with Student's t-tests or Mann-Whitney U tests, respectively. Pearson's Chi-Square (χ^2) or Fisher's exact test were used for comparing proportions. Statistical significance was determined at $P \leq 0.05$. To reduce and synthesize the large number of IMs from the multiplex assay into discrete groups, the IMs were subjected to a principal components factor analysis (PCA), and the resulting components were then correlated with Hb for the three HIV-1 status groups to determine the relationship between the components and outcome variables. 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.

RESULTS

Demographic, clinical, and hematologic characteristics

Children with falciparum malaria were stratified into three categories: malaria alone (HIV-1[−]/*Pf*[+], n=148); HIV-1 exposed (HIV-1[exp]/*Pf*[+], n=30); and co-infected (HIV-1[+]/*Pf*[+], n=16). Study participant demographics and clinical characteristics are listed in Table 1. Age (P=0.592) and gender proportions (P=0.921) were similar across the groups. Hb concentrations progressively declined across the groups (P=0.017) with more SA cases (Hb <6.0g/dL) in the HIV-1[+]/*Pf*[+] group (P=0.023). RPI was comparable across the groups (P=0.554). White blood cell and granulocyte counts were also similar, although, there was a distinct monocytosis in the HIV-1[+]/*Pf*[+] group (P=0.033). Parasitemia and the geometric mean parasitemia in the HIV-1[+]/*Pf*[+] group were less than half that of the other two groups, but differences were not significant (P=0.112 and P=0.086, respectively). Consistent with our previous results [34], worsening anemia in co-infected children was not associated with either reduced erythropoietic responses (RPI<2.0) or red blood cell (RBC) hemolysis due to elevated parasitemia.

Pro- and anti-inflammatory cytokine production

Since dysregulation in IM production plays an important role in promoting anemia [5, 11, 35-38], circulating levels of cytokines, chemokines, and growth factors were determined with a human 25-plex bead array assay. Our previous investigations [4, 38, 39], and those of others [5], 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 IM production in HIV-1[exp] and HIV-1[+] children with falciparum malaria is largely unreported. Analyses of circulating cytokines revealed that IL-12 differed across the groups (P=0.002, Table 2). IL-12 was increased in the HIV-1[exp]/*Pf*[+] group (P=0.055) and highest in the HIV-1[+]/*Pf*[+] group (P=0.003, Table 2). 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 2). None of the other pro- or anti-inflammatory cytokines showed significant differences (Table 2).

Chemokine and growth factor production

We previously demonstrated that β-chemokines were dysregulated in children with malaria [10, 32]. Although altered chemokines are well-documented in HIV-1[+] children [40, 41], chemokine patterns have not been reported in malaria/HIV-1 co-infected children. Results presented here demonstrate that eotaxin/CCL11 levels differed across the three groups (P=0.003, Table 2). 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 2). MIG/CXCL9 also showed a significant across group difference (P=0.027) and was increased in the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups (P=0.038 and P=0.058, Table 2). Circulating concentrations of the hematopoietic and immune-regulatory cytokine, GM-CSF, also differed across the groups (P=0.011, Table 2), 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 2). No significant between across differences were observed for the other chemokines.

Inflammatory mediator ratios

We [4, 11, 39, 42] and others [43, 44] showed that ratios of pro- and anti-inflammatory mediators (e.g., TNF-α:IL-10, IL-1β:IL-1Ra, and IL-2:IL-2R) were more biologically informative than their absolute (individual) values. Of the five biologically relevant ratios examined, only the IL-2:IL-2R ratio approached significance (P=0.063, Table 2). However, there was a trend towards a more pro-inflammatory environment in the HIV-1[exp]/*Pf*[+] and HIV-1[+]/*Pf*[+] groups (Table 2).

Principal component analysis of inflammatory mediators

To identify unique profiles of IMs associated with anemia, twenty-five IMs and five IM ratios were subjected to 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. As shown in Table 3, PCA revealed six principal 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). The six principal components provided an excellent fit to 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 3.

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 groups, indicating that the IM levels in these three components are influenced by HIV status. Post hoc pair-wise comparisons, using Fisher's Least Significant Difference test, indicated that IM levels in component 2 were significantly higher in the HIV-1[exp]/*Pf*[+] group (M=3.217) relative to the HIV-1[−]/*Pf*[+] group (M=1.092, *P*=0.001; Table 4). In addition, the HIV-1[exp]/*Pf*[+] group (M=6.137) also had significantly higher IM levels in component 3 than the HIV-1[−]/*Pf*[+] group (M=5.265, *Pf*=0.006; Table 4). Additional analyses revealed that the HIV-1[+]/*Pf*[+] group (M=4.008) had higher levels of IMs for component 4 than the HIV-1[−]/*Pf*[+] group (M=3.461, *P*=0.006; Table 4). Thus, there are unique profiles of IM production that differ according to HIV-1 status.

Relationship between inflammatory mediator profiles and anemia

Since there were differing patterns of IM production that distinctly emerged among the groups, correlation analyses were then performed to examine the relationship between the components and anemia (i.e., Hb). The first series of analyses in the HIV-1[−]/*Pf*[+] group revealed a positive correlation between component 3 and Hb (r=0.170, *P*=0.040, Table 4). Although no significant relationships emerged between the components and Hb for the HIV-1[exp]/*Pf*[+] group, there was a marginally significant association between component 4 and Hb for the HIV-1[+]/*Pf*[+] group (r=0.490, *P*=0.054, Table 4).

To further probe the correlation in the primary group of interest (i.e., HIV-1[+]/*Pf*[+]), a leave-one-out-method was adopted whereby, for component 4, each IM was systematically removed and the correlation between the component and Hb was re-calculated. The purpose of these post hoc analyses was to ascertain which IMs exert the greatest influence on the Hb-component correlation. Of the four IMs that comprise component 4, IL-12 exerted the greatest influence on the correlation with Hb, illustrated by the fact that IL-12 exclusion decreased the magnitude of the correlation from 0.490 to 0.340, a 30.6% reduction.

DISCUSSION

Our previous investigations in Kenyan children demonstrated that co-infection with malaria and HIV-1 was associated with increased susceptibility to SA [32, 34]. However, the underlying mechanisms of worsening anemia in co-infected children are largely undetermined. Since perturbations in the inflammatory milieu can promote enhanced anemia through a number of different mechanisms [13, 45, 46], we performed comprehensive measurement of IMs followed by statistical modeling to determine the relationship between IM profiles and anemia in the different HIV-1 status groups.

Recent literature outlining the causes of 'anemia of inflammation' suggests that immune activation has a significant and lasting effect on Hb concentrations (reviewed in [47, 48]). Although studies by our group [4, 6, 10, 11, 49-51] and others [8, 41, 52-54] demonstrated that both malaria and HIV-1 cause significant changes in IM production, a comprehensive evaluation of the inflammatory milieu has not been reported in co-infected children. Technologies, such as the micro bead assays, which concomitantly measure an inclusive panel of IMs in the small volumes of blood available from anemic children, allowed us to determine the inflammatory profile in malaria-infected children with differing HIV-1 status.

Of the 16 pro- and anti-inflammatory cytokines investigated, only IL-12 and IFN-γ emerged as markedly different across the groups. Although we previously showed that children with malarial anemia had progressively declining IL-12 with increasing anemia severity [4, 39], findings here revealed increasing IL-12 across the groups, despite worsening anemia. An investigation in HIV/tuberculosis co-infected patients from Zimbabwe reported similar findings in which IL-12 increased with the acquisition of serological HIV positivity [55]. Since HIV-1(+) children in the current study are presumably in the earlier phases of HIV infection (e.g., median age of 12.1 mos.) in which non-specific immune activation is ongoing, this may explain their elevated IL-12 levels. For example, Byrnes *et al.* [56] found significantly elevated IL-12p70 levels in acutely (<2 months post-infection) and early (2-12 months)-infected HIV patients compared to healthy controls, with IL-12 levels remaining significantly elevated six months after diagnosis, regardless of whether the patient received highly active anti-retroviral therapy (HAART) treatment. Thus, although IL-12 appears important for promoting and maintaining Hb levels in the context of malaria mono-infection, results presented here in co-infected children highlight the fact that elevated IL-12 levels alone cannot salvage a child from worsening anemia.

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 decreases with HIV disease progression [22]. Previous studies also illustrated that IFN-γ suppresses erythropoiesis through up-regulation of IL-15 [13]. 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 [37]. However, results presented here showing that IFN-γ was elevated in the HIV-1[+]/*Pf*[+] group and highest in HIV-1[exp]/*Pf*[+], 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 worsening anemia in co-

infected children. One explanation for these findings may be related to the fact that IFN-γ alters erythropoiesis via an indirect fashion through perturbations in a number of other cytokines, *all* of which were *not* significantly different between the groups (see Table 2).

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 were reported in children with *P. falciparum*, suggesting that tissue penetration is presumably directed by a chemotactic agent, such as eotaxin/CCL11 [57]. In addition, eosinophilia during the convalescent phase of disease is associated with a favorable recovery from malarial anemia [58]. The potential 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 design. The finding 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 [59]. Increased eotaxin/CCL11, MIG/CXCL9, and GM-CSF levels may also play an important role in promoting enhanced HIV-1 pathogenesis [60, 61]. 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 PCA mathematically determined six principal components from the 25 IMs and five inflammatory ratios examined here. 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 (IL-1β, IL-2, GM-CSF, IL-1β:IL-10, IL-1β:IL-1Ra, and IL-2:IL-2R) and 3 (IL-1Ra, IL-7, IL-17, IFN-α, IFN-γ, and MIG/CXCL9) than the HIV-1[−]/*Pf*[+] children. Since components 2 and 3 are predominantly pro-inflammatory, elevation 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 individual IMs comprising these two components, the HIV-1[exp]/*Pf*[+] group had five of the greatest individual values (i.e., IL-1β:IL-10, IL-2:IL-2R, IL-7, IL-17, and IFN-γ), further supporting the notion that presence of virus is not necessary for promoting a pro-inflammatory milieu. Comparison of mean levels for the components also showed that the HIV-1[+]/*Pf*[+] group had significantly higher levels of component 4 (IL-4, IL-5, IL-12, and Eotaxin/CCL11) than the HIV-1[−]/*Pf*[+] children. Component 4 was 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 as reported previously [62, 63].

Once unique patterns of IM-derived components were identified for the different groups, we then examined the relationship between the components and anemia. These analyses showed a significant positive association between Hb and component 3 (IL-1Ra, IL-7, IL-17, IFN-α,

IFN-γ, MIG/CXCL9) in HIV-1[−]/*Pf*[+] children. The positive direction of the correlation was unexpected since component 3 contains several factors typically associated with inhibition of the erythropoietic response (IL-17, IFN-α, and IFN-γ) [13, 64, 65]. The mathematically unbiased approach utilized here, however, underscores the fact that our understanding of the IMs making up this particular component, and their influence on anemia, requires further exploration.

Exploration of the association between the components and Hb also revealed a positive significant relationship between Hb and component 4 (IL-4, IL-5, IL-12, Eotaxin/CCL11) in the HIV-1[+]/*Pf*[+] group. The relationship is particularly impressive given the large magnitude (r=0.490) and the small number of HIV-1[+]/*Pf*[+] children in the analysis (n=16). Further analyses using the 'leave-one-out-method' showed that IL-12 exerted the greatest influence on the relationship, such that removal of IL-12 from component 4 yielded a 30.6% reduction in the magnitude of the correlation. This finding is consistent with the pattern seen for IL-12 in the three groups in which IL-12 progressively increased, and was highest in HIV-1[−]/*Pf*[+] children. The presence, or even exposure, to HIV-1 appears to impact on IL-12 levels in these children, and while elevated IL-12 is typically associated with favorable hematological outcomes in malaria (reviewed in [66]), increased IL-12, along with IL-1, IL-6, TNF-α, and IFN-γ, are linked to 'poor responses' to erythropoietin in dialysis patients (reviewed in [46]). The mechanism(s) associated with elevated levels of IL-12 in the context of worsening anemia in co-infected children is currently unknown and requires further exploration.

Taken together, results presented here demonstrate that the pattern (i.e., magnitude and directionality) of IMs associated with anemia (Hb) differed according to HIV-1 status, demonstrating that the inflammatory milieu in malaria-infected children is influenced by both HIV-1 exposure and infection. Although not explored here, more profound anemia in HIV-1 exposed and positive children may be related to additional inflammatory processes that promote splenic and capillary sequestration of RBCs [67-70] and/or the potential for poor nutritional status. Future investigations focusing on the significantly dysregulated IMs examined here may offer novel insight into the molecular mechanisms responsible for enhanced anemia in co-infected children.

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GCD collected and analyzed the data and prepared the manuscript. JBH designed the data analysis strategy, helped prepare the manuscript, and provided statistical support. TW assisted in generation of the data and manuscript preparation. JMO assisted in overall study design, manuscript preparation, and supervision of the experiments in Kenya. DJP designed the study, supervised the activities in New Mexico, Pennsylvania, and Kenya, and assisted in the writing of the manuscript.

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Table 1

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 ($\times 10^9$/μL)[a]	11.7 (6.2)	11.1 (6.2)	12.0 (6.4)	0.784
Granulocytes ($\times 10^3$/μL)[a]	42.3 (23.0)	40.3 (15.0)	37.4 (19.0)	0.436
Monocytes ($\times 10^3$/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

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 were compared using ANOVA.

Table 2

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, 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).

Table 3

Rotated component matrix for the principal component factor analysis.

	Inflammatory Mediators	Factor Loading	Eigenevalue	Percent Variance	Cumulative Percent Variance
Comp 1	IL-6	0.835			
	IL-8	0.605			
	IL-15	0.507			
	TNF-α	0.790			
	MIP-1β/CCL4	0.843			
	MCP-1/CCL2	0.696	7.702	25.673	25.673
Comp 2	IL-1β	0.902			
	IL-2	0.871			
	GM-CSF	0.669			
	IL-1β:IL-10	0.783			
	IL-1β:IL-1Ra	0.769			
	IL-2:IL-2R	0.852	4.999	16.664	42.337
Comp 3	IL-1Ra	0.712			
	IL-7	0.617			
	IL-17	0.558			
	IFN-α	0.406			
	IFN-γ	0.654			
	MIG/CXCL9	0.604	2.769	9.229	51.565
Comp 4	IL-4	0.517			
	IL-5	0.642			
	IL-12	0.684			
	Eotaxin/CCL11	0.769	2.012	6.708	58.273
Comp 5	IP-10/CXCL10	0.488			
	IL-10	0.642			
	TNF-α:IL-10	0.854	1.944	6.479	64.752
Comp 6	IL-2R	0.583			
	IL-13	0.742			
	MIP-1α/CCL3	0.555			
	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 (comp 1 to 6) based on visual inspection of the scree plot and the Eigenvalue >1 rule (range of eigenvalues: 1.283-7.702). 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.

Table 4

Association between the principal components, disease status, and anemia.

Component / Variable		1	2	3	4	5	6
HIV-1[−]/*Pf*[+] Group							
Comp	Mean	7.875	1.092	5.265	3.461	3.620	3.645
	Std Dev	1.685	3.507	1.726	0.775	0.987	0.722
	P-value	N/A	N/A	N/A	N/A	N/A	N/A
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*[+] Group							
Comp	Mean	8.043	3.217	6.137	3.610	3.700	3.753
	Std Dev	1.851	3.117	1.783	0.685	0.841	0.836
	P, vs. HIV-1[−]	0.581	**0.001**	**0.006**	0.261	0.633	0.410
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*[+] Group							
Comp	Mean	8.633	1.545	6.052	4.008	3.451	3.824
	Std Dev	1.597	3.506	1.048	0.603	0.588	0.687
	P, vs. HIV-1[−]	0.103	0.616	0.100	**0.006**	0.505	0.360
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 two 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-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. There were no significant differences between the HIV-1[exp]/*Pf*[+] and the HIV-1[+]/*Pf*[+] groups. N/A, not applicable.