

Dendritic cell responses to *Plasmodium falciparum* in a malaria-endemic setting

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Research

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Abstract

Background: *Plasmodium falciparum* causes the majority of malaria cases world-wide, mostly affecting children in sub-Saharan Africa. Non-sterile clinical immunity that protects from symptoms develops slowly and is relatively short-lived. Moreover, current malaria vaccine candidates fail to induce durable high-level protection in endemic settings, possibly due to the immunomodulatory effects of the malaria parasite itself. Because dendritic cells play a crucial role in initiating immune responses, here we sought to better understand the impact of cumulative malaria exposure as well as concurrent *P. falciparum* infection on dendritic cell phenotype and function.

Methods: In this cross-sectional study we assessed the phenotype and function of dendritic cells freshly isolated from peripheral blood samples of Malian adults with a lifelong history of malaria exposure who were either uninfected (n=27) or asymptotically infected with *P. falciparum* (n=8). Additionally, we measured plasma cytokine and chemokine levels in these adults and in Malian children (n=19) with acute symptomatic malaria.

Results: With the exception of lower plasmacytoid dendritic cell frequencies in asymptotically infected Malian adults, peripheral blood dendritic cell subset frequencies and HLA-DR surface expression did not differ by infection status. Peripheral blood myeloid dendritic cells of uninfected Malian adults responded to *in vitro* stimulation with *P. falciparum* blood-stage parasites by up-regulating the costimulatory molecules HLA-DR, CD80, CD86 and CD40 and secreting IL-10, CXCL9 and CXCL10. In contrast, myeloid dendritic cells of asymptotically infected Malian adults exhibited no significant responses above the uninfected red blood cell control. IL-10 and CXCL9 plasma levels were elevated in both asymptomatic adults and children with acute malaria.

Conclusions: Our findings indicate that myeloid dendritic cells of uninfected adults with a lifelong history of malaria exposure are able to up-regulate co-stimulatory molecules and produce cytokines in a manner that is comparable to mDCs of malaria-naïve individuals. Whether mDCs of malaria-exposed individuals are efficient antigen-presenting cells capable of mounting an appropriate immune response remains to be determined. The data also highlights IL-10 and CXCL9 as important factors in both asymptomatic and acute malaria and add to our understanding of asymptomatic *P. falciparum* infections in malaria-endemic areas.

Background

Malaria caused by *Plasmodium falciparum* remains a major public health threat. An estimated 3.4 billion people in 92 countries are at risk of malaria. In 2018, *P. falciparum* caused approximately 228 million malaria cases and 405,000 deaths worldwide, the majority among children in sub-Saharan Africa [1]. The recent scale-up of artemisinin-based combination therapy and insecticide-treated bed nets has reduced the malaria burden in Africa [2]. Worryingly, the trend toward declining malaria cases and deaths observed over the last decade appears to have stalled even before the global COVID-19 pandemic

emerged [1]. Now, a recent study suggests that malaria-related deaths in 2020 could increase to more than double those of 2019 if malaria-prevention activities are interrupted due to COVID-19 [3].

Ultimately, a key tool for the control, elimination, and eventual eradication of malaria is an effective vaccine, yet the leading vaccine candidate RTS,S confers only partial, short-lived protection in African children [4]. A consistent finding across several human malaria vaccine trials is a lower immunogenicity in malaria-exposed individuals relative to malaria-naïve individuals [5]. The development of highly effective malaria vaccines has been hindered in part by a poor understanding of the interaction between *P. falciparum* and the human immune system [6], particularly in malaria-endemic areas, where immunoregulatory networks are induced by repeated *P. falciparum* infections [7–12].

In malaria-naïve individuals, *P. falciparum* infections lead to high parasitemia and a strong pro-inflammatory cytokine and chemokine response, referred to as “cytokine storm”, causing fever and other malaria symptoms [13, 14]. However, in endemic areas with repeated exposure, parasitemia can often be controlled and asymptomatic infections are the rule rather than the exception [15–17]. Although sterile immunity does not appear to be reliably achieved, protective immunity to malaria can be acquired through natural *P. falciparum* infection, but only after years of repeated exposures [18]. Once acquired, this immunity appears to wane rapidly in the absence of ongoing exposure [19].

A crucial part of this process are dendritic cells (DCs). As sentinels of the immune system, DCs are not only important for early cytokine responses but are also essential for bridging and regulating the innate and adaptive immune responses to pathogenic infections and vaccines [20]. DCs reside throughout the body and sample their surroundings for pathogens. Once they phagocytose or encounter pathogenic material they undergo a rapid maturation process and migrate to secondary lymphoid organs to present antigen through MHC molecules to naïve T cells and thus initiate the adaptive immune response [20, 21]. DC maturation is typically characterized by up-regulation of MHC class II (HLA-DR), co-stimulatory markers, and secretion of chemokines and immunomodulatory cytokines such as IL-6, IL-1 β , TNF and IL-10. Depending on the nature of their activation, DCs can prime naïve CD4 T cells to become Th1, Th2, or other T helper cell subsets [22] or induce tolerance [23].

The two major human DC subsets, plasmacytoid dendritic cells (pDCs) and myeloid DCs (mDCs), have different roles in initiating and coordinating the immune response. While pDCs secrete high levels of IFN α and TNF upon activation, mDCs up-regulate costimulatory molecules and are highly efficient antigen presenting cells [24].

Despite their essential role in initiating adaptive immune responses to natural infections as well as vaccines, little is known about the role DCs play in the immune response to *Plasmodium*, especially in human malaria infections. Studies both in humans and mouse models have been contradictory [25]. The seminal report on human DCs and *P. falciparum* suggested that DC maturation was blocked by the parasite leading to dysfunction and suboptimal allogenic T cell activation [26]. Conclusions drawn from this report were later challenged by Elliot et al. showing the blocking effect was parasite dose dependent

[27]. The experiments in these studies were conducted with monocyte-derived DCs and therefore may not be fully representative of human primary DC functions.

Very few studies have addressed DC function using purified primary DCs. One study analyzed primary DC functions *ex vivo* from individuals with acute *P. falciparum* malaria from an endemic area in Papua, Indonesia [28]. The authors analyzed DCs within PBMCs (not isolated DCs) and showed that acute infection increased spontaneous apoptosis and dysfunction characterized by decreased costimulatory marker up-regulation, antigen uptake and allogenic T cell activation. Increased spontaneous apoptosis correlated with increased IL-10 levels in the plasma of study subjects with acute malaria. A study investigating DCs from children and adults with asymptomatic malaria from the same cohort did not analyze DC viability but showed unchanged numbers and HLA-DR expression suggesting functional DCs in these asymptotically infected individuals [29]. We recently showed that, in malaria-naïve US individuals, enriched primary blood DCs undergo an atypical maturation process characterized by up-regulation of costimulatory molecules and chemokines without significant secretion of cytokines. Despite the lack of inflammatory cytokine secretion, these DCs were capable of inducing a robust Th1-like CD4 T cell response *in vitro* [30]. During symptomatic infection, this response is likely enhanced by host factors such as reactive oxygen species [31].

These previous studies provide valuable insight into the effect of highly inflammatory conditions during malaria on circulating DCs and early processes during primary DC activation by the parasite in malaria-naïve individuals. However, they do not reveal how isolated DCs from malaria exposed or asymptotically infected individuals in malaria-endemic areas respond to the parasite. Addressing this question will help us understand whether initial immunological responses to vaccines or natural infections are affected by regulatory networks in individuals living in endemic areas.

Therefore, we set out to address whether intense, lifelong malaria exposure or concurrent asymptomatic *P. falciparum*-infections in malaria-endemic Mali have an effect on DC frequency, viability and response to the parasite.

Methods

Mali study site, participants and sample collection

Both study sites, Kambila (ClinicalTrials.gov NCT00471302) and Kalifabougou (ClinicalTrials.gov NCT01322581) in Mali, have been described in detail before [32, 33]. Both are rural villages approximately 20 km north (Kambila) and 48 km northwest (Kalifabougou) of Bamako in a region that typically experiences intense, seasonal *P. falciparum* transmission from July through December each year [32]. For both study sites the enrollment exclusion criteria included anemia (hemoglobin < 11 g/dL), current use of anti-malarials, corticosteroids or other immunosuppressants, fever > 37.5 °C or evidence of an acute infection, and current pregnancy. US plasma was obtained from adult donors between the ages of 35 and 70 enrolled in a healthy donor protocol at the NIH Department of Transfusion Medicine, Clinical Center (ClinicalTrials.gov NCT00001846). Eligibility criteria for these donors included no history of malaria in the

past 12 months. Further demographic and travel history data were not available from these anonymous donors, but prior *P. falciparum* exposure was unlikely.

The cross-sectional study in Kambila included 35 adults (Table 1) who exhibited no symptoms of malaria and were enrolled between November and December 2016. Asymptomatic *P. falciparum* infections in these individuals were detected by PCR analysis of blood spots at the NIH after *in vitro* assays had been conducted in Mali. Detailed methods for the detection of *P. falciparum* blood-stage infection by PCR have been described before [33]. Plasma samples from 19 children (Table 1) residing in Kalifabougou were obtained in May 2013 for the healthy baseline time point and during the following transmission season for the acute malaria and 7 days post treatment convalescence time points. Acute malaria was defined as $\geq 2,500$ asexual parasites/ μL , an axillary temperature of ≥ 37.5 °C or self-reported fever within 24 hours, and no other cause of fever discernible by physical exam. Acute malaria was treated according to the Malian National Malaria Control Program guidelines.

Peripheral blood was collected by venipuncture into 8 mL sodium citrate-containing cell preparation tubes (BD, Vacutainer CPT Tubes) and transported to the laboratory in Bamako where PBMCs were isolated according to the manufacturer's instructions. The blood volume was 48 mL for adults and 8 mL for children. PBMCs and dendritic cells were analyzed or isolated immediately. Plasma samples were cryopreserved at -80 °C and later shipped to the NIH. The same sample collection and processing was conducted for the US donors.

***P. falciparum* culture and lysate preparation**

Asexual blood stage cultures of the *P. falciparum* strain 3D7 were maintained at 5% hematocrit in RPMI 1640, 25 mM HEPES supplemented with 10 $\mu\text{g}/\text{mL}$ gentamicin, 250 μM hypoxanthine, 25 mM sodium bicarbonate, and 0.5% Albumax II under atmospheric conditions of 5% oxygen, 5% carbon dioxide, and 90% nitrogen. Late-stage *P. falciparum*-iRBCs (trophozoites and schizonts) were isolated using MACS Cell Separation LS Columns (Miltenyi Biotec). *P. falciparum*-iRBCs were washed, resuspended at $1 \times 10^6/\mu\text{L}$ and lysed by three consecutive freeze/thaw cycles. Uninfected RBC lysates were prepared accordingly and used as negative control. Lysates were stored at -80 °C until use.

Dendritic cell enrichment and culture

Myeloid Dendritic Cell Isolation Kits (Miltenyi Biotec) were used to negatively enrich primary peripheral blood mDCs from PBMCs, following the manufacturer's instructions. Primary mDCs were cultured with RPMI 1640 supplemented with 10% heat-inactivated human AB serum (Valley Biomedicals) at 37 °C and 5% CO_2 . Enriched mDCs were seeded at 1.5×10^5 per well in 96-well tissue culture-treated plates and cultured with *P. falciparum*-iRBC lysates at a ratio of 1:3 (DC/iRBC) for 24 h. Uninfected RBC lysates were used as a negative control. mDCs were then harvested for FACS analysis and supernatants were stored at -80 °C until shipment to the NIH and cytokine/chemokine analysis.

Flow cytometry

Flow cytometry was performed using a BD LSR II cytometer (BD Biosciences), and data were analyzed with FlowJo 10 (Tree Star). Primary DC subsets were identified using the following labeled monoclonal antibodies: a lineage cocktail (CD3 UCHT1, CD14 HCD14, CD19 HIB19, CD20 2H7 and CD56 HCD56), HLA-DR (L243), CD303 (201A), CD1c (L161), CD141 (M80) and CD16 (3G8). DC apoptosis was quantified by staining a separate PBMC aliquot with monoclonal antibodies against lineage, HLA-DR (L243) and Annexin V as well as 7-AAD. mDC maturation after culture with *P. falciparum*-iRBC lysate was analyzed using the following antibodies: HLA-DR (L243), CD80 (2D10), CD86 (IT2.2) and CD40 (5C3). All antibodies, Annexin V and 7-AAD were purchased from BioLegend or BD Biosciences.

Cytokine and chemokine analysis

To analyze cytokine and chemokine levels in plasma and culture supernatants, the following kits were used: LEGENDplex Human Inflammation Panel 1 (13-plex) (Biolegend), Cytometric Bead Array Human Inflammatory Cytokine Kit (BD Biosciences) and Cytometric Bead Array Human Chemokine Kit (BD Biosciences).

Statistical analysis

Statistical analyses were performed using Prism 8 (GraphPad Software) and JMP (SAS Institute). Depending on the experimental design, a paired or unpaired t-test with Welch's correction was performed. For data in Fig. 6 that were mixed paired and unpaired, a linear mixed model ANOVA with Tukey post hoc tests was performed. The statistical test and p values are described in the figure legends, with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Results

Study cohort

To address whether lifelong exposure to intense *P. falciparum* transmission or concurrent asymptomatic *P. falciparum*-infections lead to altered frequency or function of DCs, we analyzed freshly isolated, primary myeloid DC activation by *P. falciparum*-iRBC lysate *in vitro* in a malaria-endemic setting in a cross-sectional study. In the rural village of Kambila, Mali, where malaria transmission is intense and seasonal [32], we enrolled 35 asymptomatic adults in the study. Peripheral venous blood was drawn at the peak of the malaria transmission season and transported to a laboratory at the Malaria Research and Training Centre in Bamako, Mali, where *in vitro* experiments were conducted immediately upon sample arrival. Plasma samples were cryopreserved and shipped to the NIH for cytokine and chemokine analysis. Asymptomatic infection was determined using PCR at the NIH, Rockville. Of the 35 enrolled Malian individuals, 8 were PCR-positive for peripheral *P. falciparum* blood-stage infection (Table 1). We observed a significant difference in age between the two groups: asymptotically *P. falciparum*-infected adults were on average 9 years older than the uninfected group (uninfected: 42 ± 8 ; infected: 51 ± 5). Due to naturally acquired clinical immunity to the parasite, adults in this cohort rarely develop symptomatic infections [18]. To be able to characterize cytokine and chemokine plasma levels of symptomatic *P. falciparum*-infections along with asymptomatic infections, we added plasma samples from 19 children

between the ages of 6 and 9 years into the study (Table 1). These children resided in a different village than the adult cohort. Malaria endemicity in this village was comparable to the study site of the adult cohort [33]. The groups were mostly sex balanced (Table 1).

Table 1. Study cohort.

	Subjects	Age	Sex
		median (IQR)	M/F
Adults uninfected	27	42 (37-49)	15/12
Adults infected	8	51 (46-55)	5/3
Children	19	8 (6-9)	11/8

Subset distribution and apoptosis of DCs in malaria-exposed and asymptotically-infected adults

First, enumeration of peripheral blood DC subsets was conducted to identify possible differences in DC numbers between malaria-exposed but uninfected and asymptotically *P. falciparum*-infected individuals. The human peripheral blood DC compartment can be identified by flow cytometry as lineage (CD3, CD14, CD19, CD20 and CD56) negative and HLA-DR positive cells [34]. This population can be further divided into myeloid (mDC) and plasmacytoid DC (pDC) subsets. pDCs can be classified by their expression of CD303. mDCs are composed of two classical subsets, CD1c⁺ and CD141⁺ DCs, and a third subset characterized by the expression of CD16 (Figure 1A) [35, 36]. We used this classification to assess the frequency of these DC subsets in malaria-exposed but uninfected and asymptotically *P. falciparum*-infected Malian adults (Figure 1). No significant difference in peripheral numbers of total DCs or mDC subsets was observed. pDC numbers in the circulation of asymptotically *P. falciparum*-infected individuals, however, were found to be on average approximately half of the frequency of pDCs in uninfected individuals (Figure 1B).

A decrease in HLA-DR surface expression, which might lead to suboptimal T cell priming, has been described in acute malaria patients [28, 37-42]. Therefore, we asked whether altered HLA-DR expression was associated with asymptomatic *P. falciparum*-infection. We found that HLA-DR surface levels in uninfected and asymptotically-infected Malians were comparable (Figure 1C). As expected, CD1c⁺ mDCs expressed the highest levels of HLA-DR on their surface, while CD16⁺ exhibited low surface HLA-DR (Figure 1C) [36, 43].

Figure 1. Frequencies and HLA-DR expression of primary blood DC subsets. Cells were gated on viable mononuclear cells (PBMCs) and then further gated on singlets. Blood DCs were identified as Lin⁻ HLA-DR⁺ cells (DCs) and further divided into CD16⁺ and CD16⁻, which were gated on CD1c⁺ and CD141⁺ DCs. CD1c/CD141 double-negative DCs were gated on CD303 to identify pDCs (A). Blood DC counts (B) and HLA-DR expression as MFI (C) were quantified comparing uninfected ($n = 27$) and asymptomatic *P. falciparum*-infected ($n = 8$) Malian adults. Each circle represents one individual donor at one time point; bars represent mean with standard deviation. * $P < 0.05$ by unpaired t-test. MFI – median fluorescence intensity.

Figure 2. Analysis of primary blood DC apoptosis in uninfected versus asymptotically infected adults. Cells were gated on viable mononuclear cells (PBMCs), then further gated on singlets and 7-AAD negative (live) cells. Blood DCs were identified as Lin⁻ HLA-DR⁺ cells (DCs) and apoptosis determined by Annexin-V binding assays. Representative dot plots and a histogram are shown (A). Percentage of Annexin-V positive DCs in uninfected ($n = 27$) and asymptotically infected ($n = 8$) Malian adults (B). Each circle represents one individual donor at one time point; bars represent mean with standard deviation. Non-significant by unpaired t-test (B).

In patients with acute malaria, it has been reported that peripheral blood DCs undergo apoptosis [28]. In this study, no significant difference of total DC apoptosis was observed between the two groups (Figure 2). The infected group exhibited a rather large variability of DC apoptosis percentages, ranging from 2.75% to 69.5%.

These data suggest that DC subset numbers in circulation remain largely unchanged in asymptotically *P. falciparum*-infected adults compared to uninfected individuals.

mDCs up-regulate costimulatory molecules and secrete IL-10, CXCL9 and CXCL10 in response to *P. falciparum* in malaria-exposed adults

Although it would be informative to analyze pDCs and mDCs from individuals living in an endemic area, we decided to focus only on mDCs for this study to enable a more detailed assessment and their ability to efficiently prime naïve T cells. A limitation of commonly conducted activation analyses using whole PBMCs are the various, possibly regulatory, cell types present in PBMCs. To avoid uncontrollable effects of other cell types, we enriched mDCs by negative selection depleting cells bound to magnetic beads with lineage-specific antibodies. Enriched mDCs were comprised of CD1c⁺ and CD141⁺ mDC subsets. Enrichment and *in vitro* incubation with *P. falciparum* blood-stage lysates were undertaken with freshly isolated cells to minimize the effects of handling, freezing, and thawing the cells. DC surface marker expression as well as cytokine and chemokine secretion commonly associated with mDC activation were analyzed after stimulation with *P. falciparum*-iRBC lysate for 24h. Uninfected RBC lysate was used as a negative control. To minimize batch effects we assessed mDC responses to the parasite for all 35 enrolled uninfected and asymptotically *P. falciparum*-infected adults simultaneously. However, due to generally low numbers of DCs in circulation, some samples had too few DCs to successfully execute the assay. Out of the 27 uninfected adults, 21 had sufficient mDC numbers to conduct the experiments. Of the 8 asymptotically infected individuals only 4 had sufficient mDC numbers for analysis. In these 4, we were unable to identify a trend or significant differences, likely due to low sample numbers (Figure 3).

Figure 3. Analysis of *P. falciparum*-induced activation of mDCs from asymptotically infected Malian adults. mDCs were enriched from peripheral blood and incubated with *P. falciparum*-iRBC or uninfected RBC lysate at a ratio of 1:3 [DC:(i)RBC] for 24 h and analyzed for surface marker expression (A), cytokine (B) and chemokine (C) secretion. All non-significant by paired t-test.

mDCs isolated from uninfected Malian adults were able to up-regulate the activation markers HLA-DR, CD80, CD86 and CD40 upon stimulation with parasite lysate (Figure 4A). Malian mDCs did not secrete significant amounts of the inflammatory cytokines IL-1 β , IL-6 or TNF, but we measured a slight but significant increase of IL-10 in the culture supernatant when mDCs were stimulated with *P. falciparum*-iRBC lysate (Figure 4B). mDCs from malaria-exposed adults also secreted the Th1-associated chemokines CXCL9 (MIG) and CXCL10 (IP-10) as well as CCL2 but no CCL5 (Figure 4C).

Figure 4. Analysis of *P. falciparum*-induced activation of mDCs from uninfected Malian adults. mDCs were enriched from peripheral blood and incubated with *P. falciparum*-iRBC or uninfected RBC lysate at a ratio of 1:3 [DC:(i)RBC] for 24 h and analyzed for surface marker expression (A), cytokine (B) and chemokine (C) secretion ($n = 21$). * $P < 0.05$ and *** $P < 0.001$ by paired t-test.

Plasma cytokine and chemokine levels in asymptotically-infected adults and children with acute malaria

Next, plasma cytokine and chemokine levels in the described cohort of asymptotically *P. falciparum*-infected and uninfected Malian adults were analyzed by bead-based immunoassays. In addition to the cytokines and chemokines tested in DC supernatants, cytokines commonly associated with T cell responses were tested.

We detected slightly elevated levels of IL-10 (Figure 5A) and CXCL9 (Figure 5B) in plasma of asymptotically infected compared to uninfected adults. This observation corroborates our finding that DCs in these individuals respond to the parasite with IL-10 and CXCL9 *in vitro* (Figure 4). All other analyzed cytokines and chemokines, namely IL-6, TNF, IL-1 β , IL-18, IFN γ , IFN α , CCL2, CCL5 and CXCL10, were comparable between the two groups (Figure 5), suggesting they were at baseline levels in both groups.

Figure 5. Plasma cytokine and chemokine analysis in uninfected and asymptotically *P. falciparum*-infected adults. Cytokine (A) and chemokine (B) concentrations in plasma were analyzed in asymptomatic uninfected ($n = 27$) or *P. falciparum*-infected Malian adults ($n = 8$). Each circle represents one individual donor at one time point; bars represent mean with standard deviation. * $P < 0.05$ and ** $P < 0.01$ by unpaired t-test.

Finally, to compare these responses to symptomatic malaria, plasma cytokine and chemokine levels were measured longitudinally in Malian children before (healthy baseline, HB), during (malaria, Mal) and after (convalescence, Conv) an acute symptomatic malaria episode. US plasma was used as a control. As expected, most inflammatory cytokine and chemokine levels tested increased significantly during acute malaria and decreased to baseline levels at the convalescent time point 7 days post treatment. Similar to asymptotically infected adults (Figure 5A), plasma IL-10 levels exhibited the largest increase with a 150-fold increase during acute malaria compared to baseline (Figure 6A). Mean IL-6 and IFN γ levels increased roughly 30- and 3.5-fold, respectively. Surprisingly, the inflammatory cytokines IL-1 β and TNF did not show significantly higher plasma levels during acute malaria. Overall IFN α levels in plasma were

low, but we measured a significantly higher amount of this cytokine during acute malaria compared to convalescence (Figure 6A). The Th1-associated chemokines CXCL9 and CXCL10 exhibited a robust increase in plasma levels during acute malaria (Figure 6B).

Figure 6. Plasma cytokine and chemokine analysis before, during and after symptomatic *P. falciparum* infection in children. Cytokine (A) and chemokine (B) concentrations in plasma were analyzed at healthy baseline (HB), during symptomatic acute infection (Mal) and 7 days post treatment (Conv) in Malian children ($n = 19$) and US adults ($n = 5-6$). Each circle represents one individual donor and time point; bars represent mean. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ by linear mixed model ANOVA.

Most plasma cytokine and chemokine levels returned to baseline by the convalescent timepoint, with the exception of IL-18, CCL5 and CXCL9 (Figure 6).

Taken together, these data indicate that IL-10 plays a prominent role in the cytokine response to *P. falciparum* in malaria-endemic settings.

Discussion

The biology of primary human DCs is understudied due in part to their low frequencies in peripheral blood and the technical difficulties of relevant experimental assays. Our understanding of primary human DC responses to *P. falciparum* in particular remains very limited (reviewed in [44]). In light of evidence that the leading malaria vaccine candidate RTS,S confers only partial, short-lived protection in African children [4], it is of great importance to gain a better understanding of DC responses, particularly in malaria-endemic settings. Here, we attempted to address this knowledge gap by analyzing DC frequency, viability and responsiveness to *P. falciparum* in a malaria-endemic setting.

Our findings suggest that mDC numbers in circulation remain stable during asymptomatic malaria in Malian adults. pDC frequencies, however, were lower in individuals with asymptomatic *P. falciparum* infections compared to uninfected individuals. Several other studies have reported lower numbers of circulating DCs in acute malaria patients in both children and adults [28, 37, 45–47]. In asymptomatic *P. falciparum* infections in children and adults in Papua, Indonesia, DC numbers were found to be unchanged, including pDC frequencies [29]. This discrepancy could be explained by the distinct study sites. Malaria generally presents differently in Asia and in West Africa, likely due to a number of differences including transmission intensity and availability of resources [48, 49](cite). We also observed that individuals with asymptomatic infections in our cohort were significantly older than the uninfected controls. Since pDC, but not mDC, numbers in circulation decline with age [50–52], lower pDC frequencies in this group could be attributed to age rather than asymptomatic malaria. It is also unclear whether decreased numbers of DCs in circulation is due to DC apoptosis [28], or migration of activated DCs out of circulation to target tissues. In this study, we did not observe a higher percentage of apoptotic total DCs in asymptotically infected compared to uninfected adults. Since the percentage of apoptotic pDCs was not assessed, however, the total DC data could mask a difference between groups in pDC apoptosis. The limited number of infected individuals and rather large variability of DC apoptosis limits the

interpretability of these data. Additional experiments will be needed to address whether peripheral blood pDCs undergo apoptosis in asymptotically infected individuals.

A few studies have reported an increase in CD141⁺ mDCs in children with acute malaria [38, 39, 53]. This increase was shown to be induced by Flt3 ligand which was in turn induced by a signaling cascade starting with type I interferon. Confirming previous findings in asymptomatic adults with malaria [29], no such increase was observed in the present study, suggesting that a threshold inflammatory response that includes type I interferon may be necessary to trigger expansion of CD141⁺ mDCs through Flt3 ligand.

A number of studies have assessed cytokines in the plasma of acute malaria patients, concluding that the so called “cytokine storm”, characteristic for *P. falciparum* infections, contributes to pathology [54]. Here, we also report an increase of most inflammatory cytokines tested during a first febrile malaria episode of the season in Malian children compared to their baseline and 7 days post treatment (convalescence). Our data corroborates previous observations for IL-18, IFN γ and IL-10 in the same cohort and adds IL-6 and IFN α as being up-regulated while IL-1 β , IL-17A and TNF remained unchanged [55]. TNF levels were previously shown to be elevated during malaria as well. This discrepancy is surprising and might be due to a difference in age, the present study assessed children between the ages of 6 and 9 while the previous study focused on children between the ages of 3 to 12. Another difference was the year of the blood draw, 2011 vs 2013, and kit used to analyze cytokine levels in plasma. This highlights the importance of repeated assessment of parameters at different time points to be able to draw accurate conclusions.

Plasma chemokine levels during human malaria are less well documented. Similar to other studies in Africa [56, 57], we found high levels of CXCL9 and CXCL10 in the plasma of children with acute malaria and significantly higher CXCL9 levels in asymptotically-infected compared to uninfected adults. CXCL10 has been shown to have a negative effect on the development of immunity and promotes severe outcomes in murine [58, 59] and human [60] malaria. Similar to our findings with mDCs stimulated with *P. falciparum*-iRBC lysates, CXCL9 and CXCL10 have been reported to exhibit increased expression in murine splenic DCs during *Plasmodium chabaudi* infection, with no significant increase in inflammatory cytokine gene expression [61]. Comparable to our findings, mDCs from malaria-naïve individuals secrete high levels of CXCL9 and CXCL10 in response to the parasite *in vitro*. Interestingly, these chemokines are induced to a greater extent by the parasite than by LPS, the gold standard DC activator [30], suggesting that these chemokines may be characteristic of malaria infection. Similar to the “cytokine storm”, inflammatory chemokines like CXCL9 and CXCL10 are likely important to mount an effective immune response to the parasite early during the infection, but may be detrimental under certain circumstances.

In addition to CXCL9 and CXCL10, we observed that low levels of IL-10 were secreted by mDCs in Malian adults upon stimulation with the parasite. That IL-10 was detectable in the plasma of asymptotically infected adults and at high levels in children with acute malaria is consistent with an important role for this regulatory cytokine in malaria. Indeed, IL-10 has been extensively described in murine malaria models and human studies as an important regulatory factor (reviewed in [62]). Although commonly believed to

be produced mainly by Tr1 cells, our data suggests that mDCs may be a significant contributor to the overall IL-10 response during *Plasmodium* infection.

Studies that assess plasma cytokine or chemokine levels in asymptomatic malaria infections are scarce. We found that IL-10 and CXCL9 are significantly elevated in asymptomatic *P. falciparum*-infected Malian adults. While children with asymptomatic malaria have increased IFN γ , TNF and IL-4 levels [63], IL-10 and CXCL10 were elevated in asymptotically infected pregnant women [64–66]. Our data suggest that asymptotically infected adults in endemic areas exhibit elevated levels of IL-10 as well. CXCL9 was not assessed by these studies and, together with IL-10, might represent a potential biomarker to detect asymptomatic infections.

As observed before with a subset of the data [30], DCs isolated from uninfected Malian adults were able to up-regulate the activation markers HLA-DR and CD86 upon stimulation with parasite lysate. Likely due to larger sample size, here we demonstrate that CD80 and CD40 were significantly up-regulated as well. Similar to responses seen with malaria-naïve US DCs [30], Malian mDCs did not secrete significant amounts of the inflammatory cytokines IL-1 β , IL-6 or TNF. Unlike the cytokine responses exhibited by US DCs however, we measured a slight but significant increase in IL-10 when Malian DCs were stimulated with *P. falciparum*-iRBC lysate. Whether mDCs from malaria-naïve US individuals are also capable of responding to the parasite with IL-10 secretion will have to be addressed in future experiments. The absence of a significant increase in the previous study could be due to a lower sample size and does not permit further conclusions at this time. Comparable to the US DC response to the parasite, DCs from Malians secreted the Th1-associated chemokines CXCL9 and CXCL10 as well as CCL2 but no CCL5. Except for IL-10 secretion, *in vitro* DC responses to the parasite in malaria-exposed uninfected Malians seem, at least qualitatively, comparable to malaria-naïve US DCs. Future studies will have to address whether up-regulation of costimulatory molecules and chemokines in these DCs translates into effective T cell priming in malaria-endemic areas.

Limitations of our study include the low number of infected individuals enrolled, the cross-sectional design and the lack of data on DC-T cell interactions. Future studies should include a larger sample size, and longitudinal analysis of DC responses and functionality that include T cell activation. The Malian study site is particularly well suited for such studies due to the sharply demarcated and predictable malaria transmission season which enables the analysis of DCs from uninfected individuals before the malaria season and the same individuals as they become infected during the ensuing malaria season. Despite these limitations, the present study provides valuable insight into baselines DC frequencies and responses as well as *in vivo* cytokine and chemokine responses to the parasite in a malaria-endemic setting.

Conclusions

Although far from complete, together with the report on peripheral blood DC frequencies in asymptomatic malaria in Papua, Indonesia [29], our study begins to address if mDC responses are altered by malaria-

exposure. Our data suggest, that the mDC response to *P. falciparum* in malaria-endemic Mali is comparable to DC responses in malaria-naïve individuals. However, if these responses translate to efficient T cell activation remains to be addressed. This report also adds to the importance of significant immunological changes such as up-regulation of IL-10 and CXCL9 in asymptomatic malaria, which often is overlooked despite its importance as a reservoir [67, 68].

List Of Abbreviations

DC	Dendritic cell
iRBC	Infected red blood cell
mDC	Myeloid dendritic cell
pDC	Plasmacytoid dendritic cell
RBC	Red blood cell

Declarations

Ethics approval and consent to participate

The ethics committee of the Faculty of Medicine, Pharmacy and Dentistry at the University of Sciences, Techniques and Technology of Bamako, and the Institutional Review Board of NIAID NIH approved this study (ClinicalTrials.gov NCT01322581 and NCT00471302). Written, informed consent was obtained from the parents or guardians of participating children or from adult participants.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

TCT, SL and AG performed experiments and analyzed data. AO, SD, DD, KK and BT designed and coordinated the study in Mali. AG and JS performed the statistical analyses. PDC and AG conceived of and designed the study, participated in its design and coordination in Mali, and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

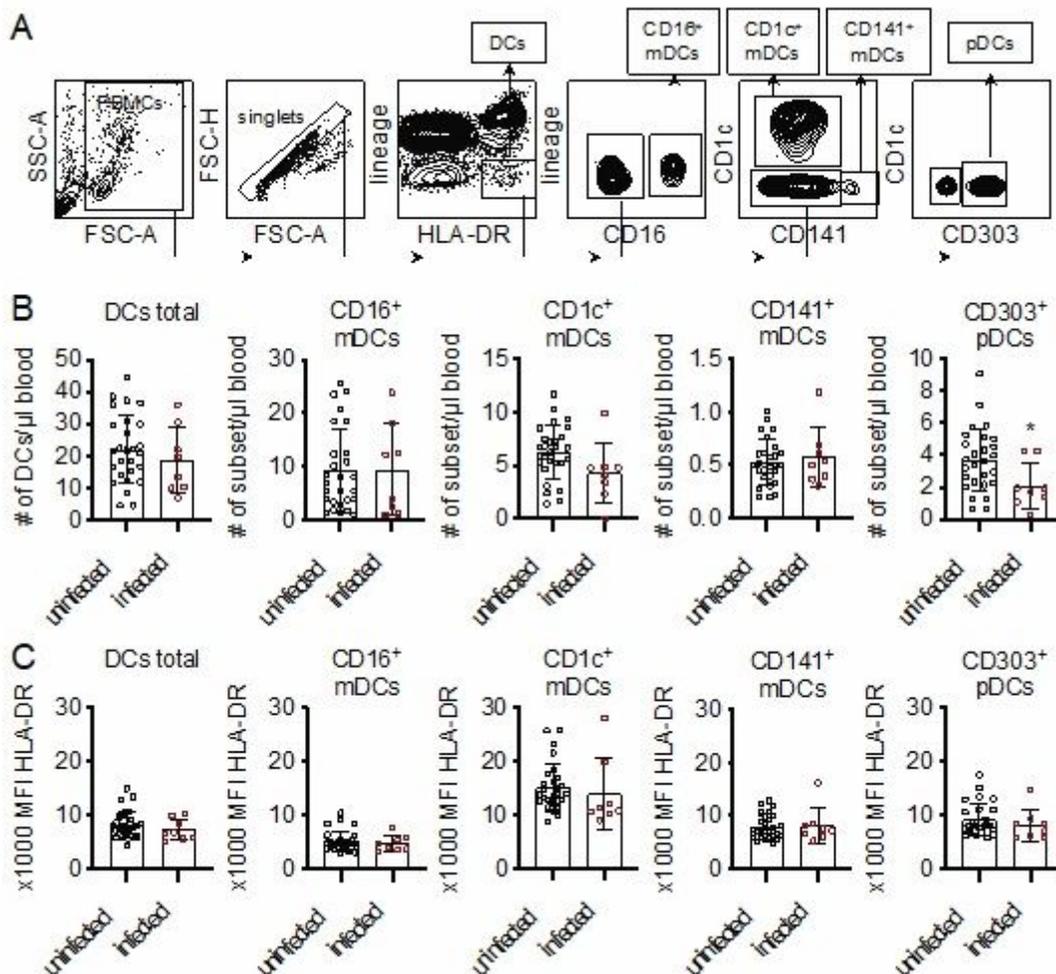


Figure 1

Frequencies and HLA-DR expression of primary blood DC subsets. Cells were gated on viable mononuclear cells (PBMCs) and then further gated on singlets. Blood DCs were identified as Lin⁻ HLA-DR⁺ cells (DCs) and further divided into CD16⁺ and CD16⁻, which were gated on CD1c⁺ and CD141⁺ DCs. CD1c/CD141 double-negative DCs were gated on CD303 to identify pDCs (A). Blood DC counts (B) and HLA-DR expression as MFI (C) were quantified comparing uninfected (n = 27) and asymptomatic *P. falciparum*-infected (n = 8) Malian adults. Each circle represents one individual donor at one time point; bars represent mean with standard deviation. *P<0.05 by unpaired t-test. MFI – median fluorescence intensity.

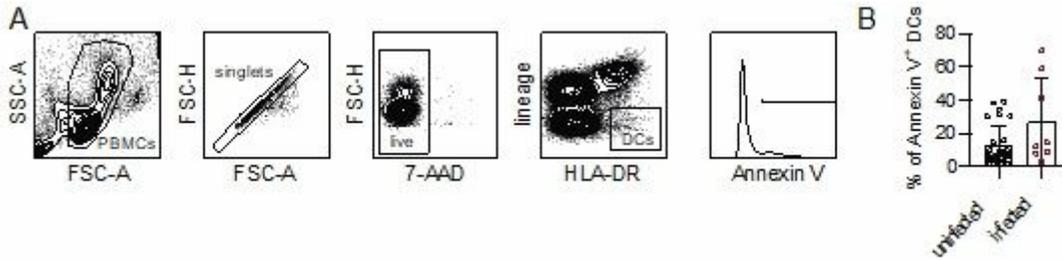


Figure 2

Analysis of primary blood DC apoptosis in uninfected versus asymptotically infected adults. Cells were gated on viable mononuclear cells (PBMCs), then further gated on singlets and 7-AAD negative (live) cells. Blood DCs were identified as Lin⁻ HLA-DR⁺ cells (DCs) and apoptosis determined by Annexin-V binding assays. Representative dot plots and a histogram are shown (A). Percentage of Annexin-V positive DCs in uninfected (n = 27) and asymptotically infected (n = 8) Malian adults (B). Each circle represents one individual donor at one time point; bars represent mean with standard deviation. Non-significant by unpaired t-test (B).

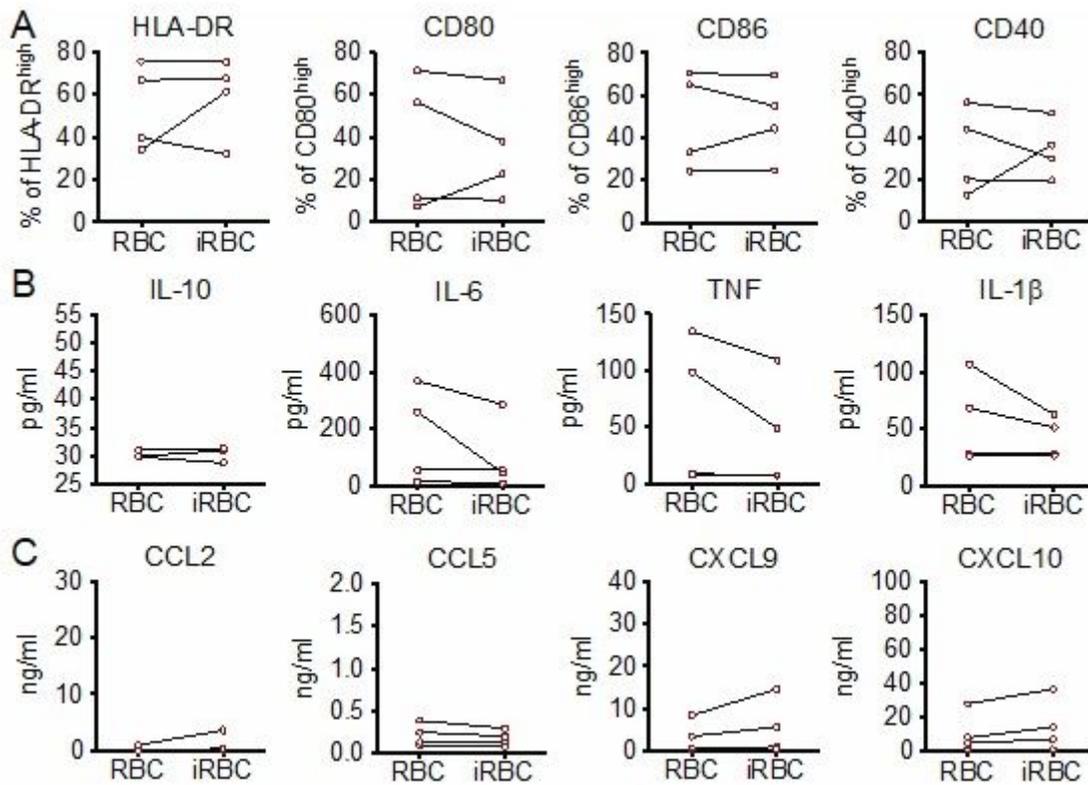


Figure 3

Analysis of *P. falciparum*-induced activation of mDCs from asymptotically infected Malian adults. mDCs were enriched from peripheral blood and incubated with *P. falciparum*-iRBC or uninfected RBC lysate at a ratio of 1:3 [DC:(i)RBC] for 24 h and analyzed for surface marker expression (A), cytokine (B) and chemokine (C) secretion. All non-significant by paired t-test.

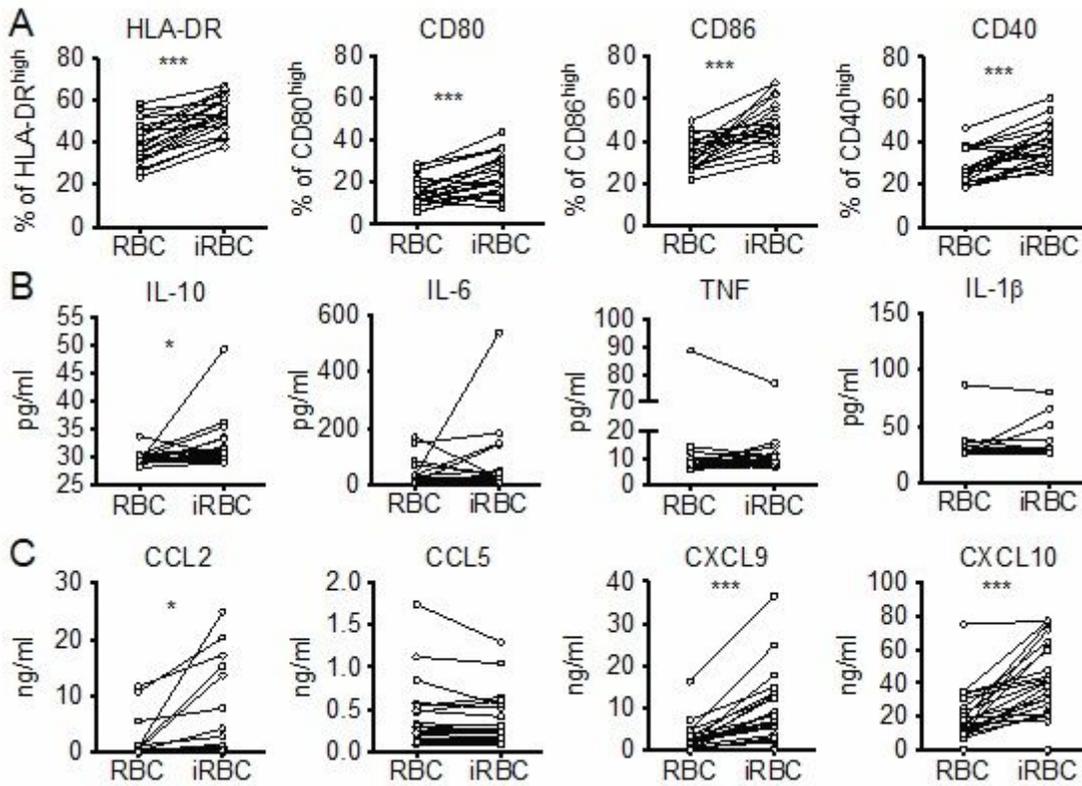


Figure 4

Analysis of *P. falciparum*-induced activation of mDCs from uninfected Malian adults. mDCs were enriched from peripheral blood and incubated with *P. falciparum*-iRBC or uninfected RBC lysate at a ratio of 1:3 [DC:(i)RBC] for 24 h and analyzed for surface marker expression (A), cytokine (B) and chemokine (C) secretion (n = 21). *P<0.05 and ***P < 0.001 by paired t-test.

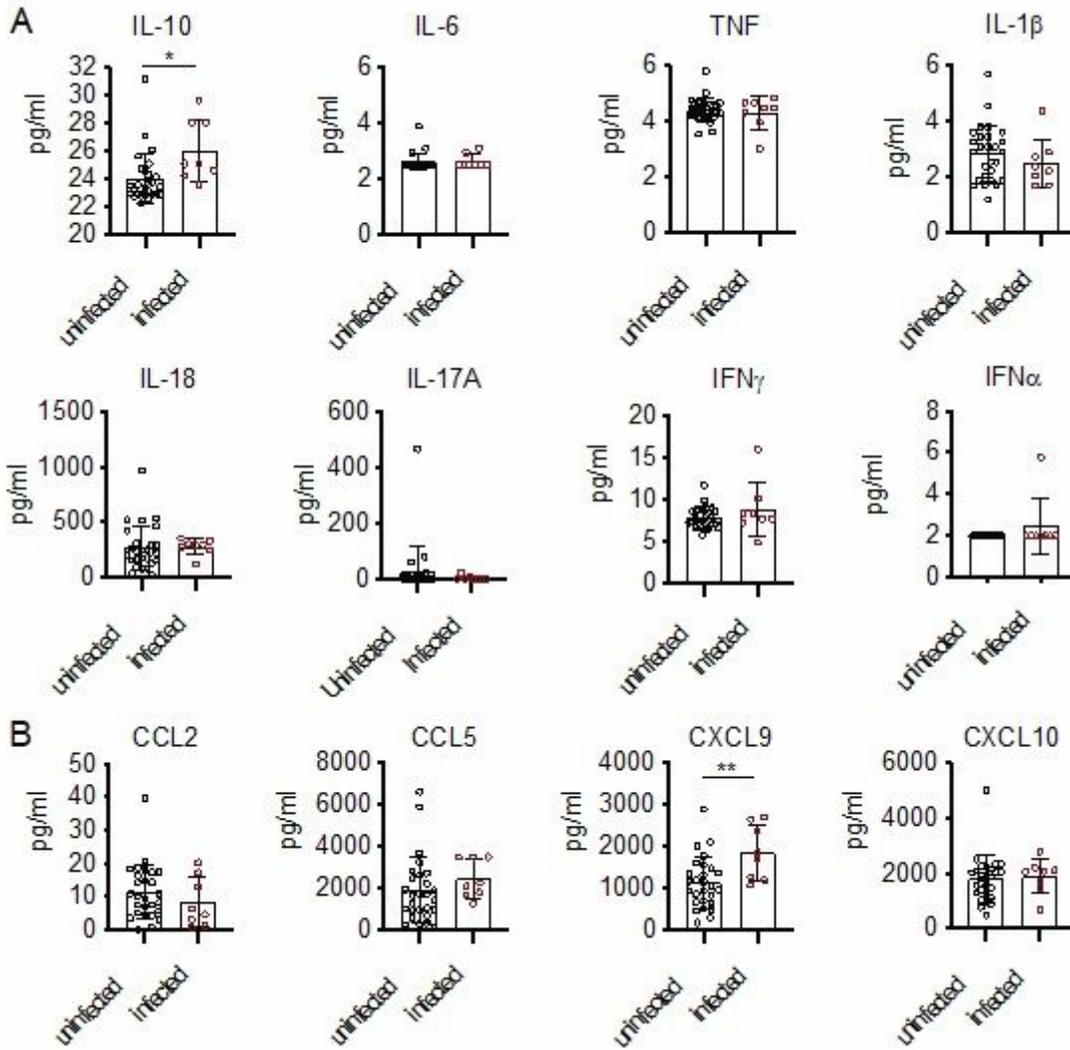


Figure 5

Plasma cytokine and chemokine analysis in uninfected and asymptotically *P. falciparum*-infected adults. Cytokine (A) and chemokine (B) concentrations in plasma were analyzed in asymptomatic uninfected (n = 27) or *P. falciparum*-infected Malian adults (n = 8). Each circle represents one individual donor at one time point; bars represent mean with standard deviation. *P<0.05 and **P<0.01 by unpaired t-test.

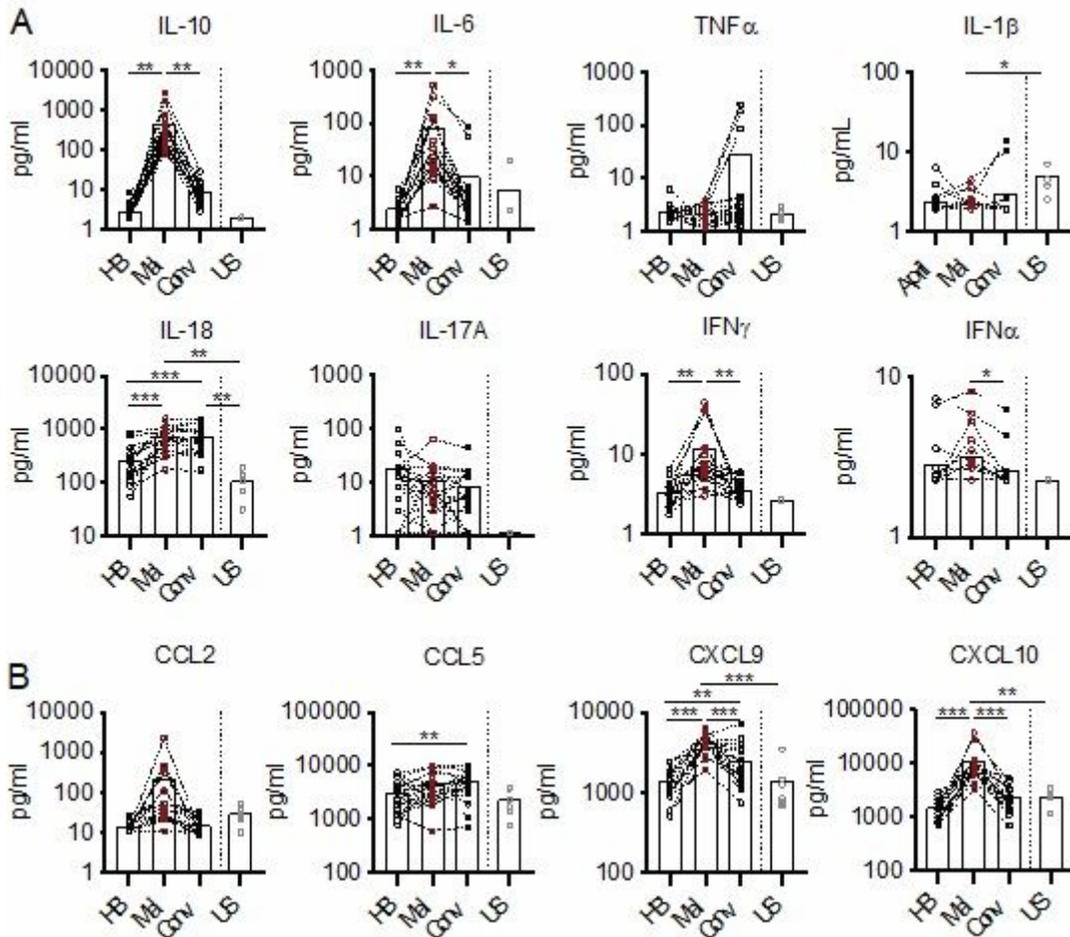


Figure 6

Plasma cytokine and chemokine analysis before, during and after symptomatic *P. falciparum* infection in children. Cytokine (A) and chemokine (B) concentrations in plasma were analyzed at healthy baseline (HB), during symptomatic acute infection (Mal) and 7 days post treatment (Conv) in Malian children (n = 19) and US adults (n = 5-6). Each circle represents one individual donor and time point; bars represent mean. *P<0.05, **P<0.01 and ***P<0.001 by linear mixed model ANOVA.