**Research Article**

*Plasmodium falciparum* malaria drives epigenetic reprogramming of human monocytes toward a regulatory phenotype

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**Abstract**

In malaria-naïve children and adults, *Plasmodium falciparum*-infected red blood cells (*Pf*-iRBCs) trigger fever and other symptoms of systemic inflammation. However, in endemic areas where individuals experience repeated *Pf* infections over many years, the risk of *Pf*-iRBC-triggered inflammatory symptoms decreases with cumulative *Pf* exposure. The molecular mechanisms underlying these clinical observations remain unclear. Age-stratified analyses of uninfected, asymptomatic Malian individuals before the malaria season revealed that monocytes of adults produced lower levels of inflammatory cytokines (IL-1β, IL-6 and TNF) in response to *Pf*-iRBC stimulation compared to monocytes of Malian children and malaria-naïve U.S. adults. Moreover, monocytes of Malian children produced lower levels of IL-1β and IL-6 following *Pf*-iRBC stimulation compared to 4–6-month-old infants. Accordingly, monocytes of Malian adults produced more IL-10 and expressed higher levels of the regulatory molecules CD163, CD206, Arginase-1 and TGM2. These observations were recapitulated in an *in vitro* system of monocyte to macrophage differentiation wherein macrophages re-exposed to *Pf*-iRBCs exhibited attenuated inflammatory cytokine responses and a corresponding decrease in the epigenetic marker of active gene transcription, H3K4me3, at inflammatory cytokine gene loci. Together these data indicate that *Pf* induces epigenetic reprogramming of monocytes/macrophages toward a regulatory phenotype that attenuates inflammatory responses during subsequent *Pf* exposure.

**Trial Registration**: ClinicalTrials.gov NCT01322581.
Author summary

The malaria parasite is mosquito-transmitted and causes fever and other inflammatory symptoms while circulating in the bloodstream. However, in regions of high malaria transmission the parasite is less likely to cause fever as children age and enter adulthood, even though adults commonly have parasites detected in their blood by microscopy. Monocytes are cells of the innate immune system that secrete molecules that cause fever and inflammation when encountering microorganisms like malaria. Although inflammation is critical to initiating normal immune responses, excessive inflammation can cause disease in infected individuals. In Mali, we conducted a study of a malaria-exposed population from infants to adults and found that monocytes of Malian adults produced less inflammation than monocytes of Malian children and U.S. adults who had never been exposed to malaria. Moreover, monocytes of Malian children tended to produce less inflammation than monocytes of Malian infants. Accordingly, monocytes exposed to malaria parasites in the laboratory became less inflammatory when re-exposed to malaria again later, and these monocytes ‘turned down’ their inflammatory genes. This study helps us understand how people become resistant to fever and other inflammatory symptoms caused by malaria.

Introduction

*Plasmodium falciparum* infection in non-immune individuals can result in severe, life-threatening malaria when *P. falciparum*-infected red blood cells (*Pf*iRBCs) trigger systemic inflammation [1,2], and sequester in blood vessels of vital organs [3]. Conversely, in areas of intense *P. falciparum* transmission, infected individuals are commonly asymptomatic [4], even when parasitemia exceeds that which predictably induces fever and other inflammatory symptoms in non-immune individuals. Non-sterilizing, clinical immunity to blood-stage malaria can be acquired with repeated infections over years and is associated with the acquisition of *P. falciparum*-specific humoral and cellular adaptive immune responses [5,6]. The relatively inefficient acquisition of adaptive immunity that protects from malaria has been ascribed to the extensive genetic diversity of *P. falciparum* parasites [7], the clonal variation in proteins the parasite exports to the erythrocyte surface [8], and dysregulation of B and T cell responses [9,10].

Less is known about the impact of cumulative *P. falciparum* exposure on cells of the innate immune system, such as monocytes and macrophages, and how this may relate to the acquisition of clinical immunity. During *Plasmodium* blood-stage infection, circulating blood monocytes and tissue macrophages perform crucial effector functions that contribute to host defense against malaria including cytokine production, phagocytosis of infected erythrocytes, and antigen presentation [11]. However, excessive production of pro-inflammatory cytokines such IL-1β, IL-6 and TNF by monocytes/macrophages can result in systemic inflammation that causes fever and other disease manifestations of malaria [11,12].

Recent studies have shown that various immune perturbations can epigenetically and metabolically reprogram monocytes/macrophages, such that after cells return to a non-activated state, their response to subsequent challenges is altered [13]. Depending on the nature of the initial immune perturbation, the subsequent response of monocytes/macrophages may be diminished (tolerance) or enhanced (trained immunity) relative to the primary response [13]. It has been shown that immune training of monocytes can be generated at the level of myeloid progenitors in the bone marrow [14], which could explain how monocytes, which survive in...
circulation for only 1–7 days [15], could exhibit a ‘memory’ phenotype for 3–12 months after the primary stimulus [13].

Therefore, we hypothesized that *P. falciparum* exposure is associated with functional changes in circulating monocytes that persist in the absence of ongoing malaria exposure when monocytes have returned to a non-activated steady state. More specifically, given the long-standing clinical observation that individuals become ‘tolerant’ to the inflammatory effects of blood-stage *P. falciparum* infection [16], we hypothesized that an inverse relationship exists between age (a surrogate for cumulative *P. falciparum* exposure in endemic areas) and *Pf*-iRBC-inducible inflammatory cytokine production from monocytes at their non-activated steady state.

To test this hypothesis, we analyzed the phenotype and function of monocytes obtained from an age-stratified cohort study in Mali that spans infancy to adulthood. Monocytes were collected cross-sectionally from asymptomatic, uninfected study volunteers at the end of the 6-month dry season, which is a period of negligible *P. falciparum* transmission. In addition, we adapted an in vitro system of monocyte to macrophage differentiation to directly investigate the impact of *P. falciparum* blood-stage parasites on monocytes/macrophages at the molecular level.

**Results**

**Malaria exposure associates with reduced production of *Pf*-inducible inflammatory cytokines and increased production of IL-10 from monocytes**

To analyze the relationship between malaria exposure and monocyte function we isolated monocytes from PBMC samples of subjects enrolled in an age-stratified cohort in Mali and also from healthy malaria-naïve U.S. adults as controls. A detailed description of the Kalifabougou cohort has been published [17]. Malian subjects ranged from 4–6-month-old infants born during the six-month dry season, when malaria transmission is negligible [18], to adults exposed to a lifetime of repeated *P. falciparum* infections. All Malian subjects in this cross-sectional analysis were negative for *P. falciparum* infection by PCR at the time of blood collection, which occurred just before the 6-month malaria season. To simulate re-exposure to *P. falciparum* blood-stage parasites in vitro, isolated monocytes were co-cultured with *P. falciparum*-infected red blood cell lysate (*Pf*-iRBCs) at a ratio of 1:30 (monocytes:*Pf*-iRBCs) for 24 hours [19]. Secreted cytokines were measured in supernatants by bead-based multiplex arrays. We found that monocytes of Malian adults produced lower levels of the inflammatory cytokines IL-1β, IL-6 and TNF in response to *Pf*-iRBC stimulation compared to monocytes of Malian infants and children or malaria-naïve U.S. adults (Fig 1A–1C). Moreover, monocytes of Malian children aged 1–2 years produced lower levels of IL-1β following *Pf*-iRBC stimulation compared to 4–6-month-old infants (Fig 1B), and monocytes of Malian children aged 1–2 years and 4–6 years produced lower levels of IL-6 following *Pf*-iRBC stimulation compared to 4–6-month-old infants (Fig 1C). In contrast, monocytes of Malian adults produced higher levels of the anti-inflammatory cytokine IL-10 in response to *Pf*-iRBC stimulation compared to monocytes of Malian children and U.S. adults (Fig 1D), whereas Malian children and U.S. adults produced similarly low levels of IL-10 (Fig 1D). Taken together, these data suggest that cumulative malaria exposure, or exposure to other factors associated with malaria transmission (e.g. co-infection history, microbiota composition, nutritional status) skew monocytes toward a regulatory phenotype characterized by decreased *P. falciparum*-inducible production of inflammatory, pyrogenic cytokines and increased production of the anti-inflammatory cytokine IL-10, consistent with the epidemiological observation that febrile malaria risk
decreases with cumulative malaria exposure in this cohort [17], and in areas of intense malaria transmission more generally [20].

**Monocytes of malaria-exposed adults skew toward a regulatory profile phenotypically and transcriptionally**

Next, we examined the molecular basis of malaria-associated skewing of monocytes toward a regulatory phenotype by comparing the phenotypic and transcriptional profiles of monocytes collected before the malaria season from Malian children and adults, as well as malaria-naïve U.S. adults. PBMCs of Malian children (aged 4–6 years; n = 9) and adults (n = 9), as well as U.S. adults (n = 7) were analyzed ex vivo by flow cytometry for surface expression of the myeloid cell markers CD14, CD16, CD86, CD163, CD206 and HLA-DR [21] gated on live monocytes. Visualization of the flow cytometry data by distributed stochastic neighbor embedding (t-SNE) analysis [22] revealed that monocytes of Malian adults expressed higher levels of the regulatory or alternatively activated (M2) monocytes/macrophage markers CD163 and CD206 compared to Malian children and U.S. adults (Fig 2A); and elevated expression of CD163 and CD206 was largely confined to the CD14+ and CD14+CD16+ monocyte clusters (Fig 2A–2C).
Fig 2. Monocytes of Malian adults upregulate markers of M2 regulatory monocytes/macrophages. (A-E) PBMCs from Malian children (aged 4–6 years; n = 9) and adults (n = 9) before the malaria season, as well as healthy malaria-naive U.S. adults (n = 7) were analyzed ex vivo by flow cytometry with gating on live monocytes for surface expression of CD14, CD16, CD163 and CD206. (A, B) t-SNE analysis of monocytes for all subjects in each group. Expression levels of each marker are indicated by color scales. (C) t-SNE analyzed data (shown in A and B) subjected to gating for monocyte subsets based on CD14 and CD16 expression using concatenated data from all subjects. MFI of (D) CD163 and (E) CD206 surface expression on manually gated live monocytes. (F, G) PBMCs were stimulated with Pf-iRBCs and analyzed for arginase-1 expression in monocytes intracellularly. (F) Representative t-SNE plots showing expression of Arginase-1 in the clusters in three different groups of monocytes. Expression of Arginase-1 is indicated
Moreover, HLA-DR and CD86 expression tended to be higher on CD14+ and CD14+CD16+ monocyte clusters (S3 Fig). Consistent with the t-SNE visualization, the geometric mean fluorescence intensity (MFI) values of CD163 and CD206 were significantly higher on monocytes of Malian adults compared to Malian children and U.S. adults (Fig 2D and 2E). Of note, there were no significant differences in the proportions of monocyte subsets between children and adults.

M2 regulatory monocytes/macrophages are known to produce high levels of arginase 1 [23]. Therefore we further tested the hypothesis that cumulative malaria exposure is associated with skewing of monocytes towards an M2 regulatory phenotype by stimulating PBMCs of Malian children (aged 4–6 years; n = 9) and adults (n = 9), as well as U.S. adults (n = 9) with Pf-iRBCs for 18 hours and quantifying intracellular arginase 1 in monocytes by flow cytometry. Visualization of the flow cytometry data by t-SNE analysis showed that monocytes of Malian adults produced higher levels of arginase 1 in response to Pf-iRBC stimulation compared to Malian children and U.S. adults (Fig 2F). Consistent with the t-SNE plots, MFI values of arginase 1 were significantly higher within monocytes of Malian adults compared to Malian children and U.S. adults (Fig 2G).

To further investigate the molecular basis of malaria-associated skewing of monocytes toward an M2 regulatory type, we conducted whole genome RNA-seq analysis of monocytes isolated from PBMCs of Malian children and adults. RNA-seq was performed on unstimulated monocytes (n = 4 from each age group) as well as monocytes that had been stimulated with Pf-iRBCs for 24 hours (n = 8 from each age group) to simulate re-exposure to *P. falciparum* blood-stage parasites. Monocytes were stimulated with Pf-iRBCs at a ratio of 1:5 (monocytes: Pf-iRBCs) to reduce *P. falciparum* nucleic acid during RNA sequencing. Principal components analysis of the RNA-seq data showed segregation of transcription profiles based on age—an effect that became more pronounced following stimulation with Pf-iRBCs (Fig 3A). Here we focused on differential gene expression between monocytes of children versus adults following Pf-iRBC stimulation. Consistent with the analysis of secreted cytokines (Fig 1), Malian children had significantly higher expression of the genes encoding TNF [log2 fold change (FC) 1.8, Benjamini-Hochberg (BH) adjusted p value = 0.0001] and IL6 (FC 2.3, BH p = 0.007) compared to Malian adults (Fig 3B and 3C and S1 Table). In addition, the expression of TLR5, TLR7, CXCL9, CXCL10, NLRP1, NLRP3, FCGR3A, PTX3 and various HLA molecules was significantly upregulated in children relative to adults (Fig 3B–3E and S1 Table for FC and adjusted p values). Consistent with the blunted pro-inflammatory cytokine responses that we observed from monocytes of Malian adults in response to Pf-iRBC stimulation (Fig 1), expression of NFKB1, a positive regulator of inflammation, was downregulated (FC -3.8, BH p = 4.6 E-26) in monocytes of adults relative to children (Fig 3C and S1 Table), while expression of the multifunctional enzyme transglutaminase-2 (TGM2), a known marker of the M2 regulatory phenotype, was significantly higher (FC 7.5, BH p = 1.7 E-79) in monocytes of adults versus children (Fig 3B and 3D and S1 Table). Accordingly, expression of the chemokines CCL22 (FC 5.0, BH p = 9.2 E-21) and CCL24 (FC 9.6, BH p = 3.8 E-21) were upregulated in adult monocytes, consistent with the chemokine repertoire of M2 regulatory monocytes/macrophages [24]. ARG1 that encodes arginase 1 was not differentially expressed in monocytes of Malian children versus adults, in contrast to flow cytometry data showing higher arginase 1 in stimulated monocytes of Malian adults (Fig 2F and 2G)—incongruent findings that may be due to differences in experimental conditions or expression kinetics of RNA versus protein.
RNA-seq analysis of monocytes after Pf-iRBC stimulation reveals a regulatory signature in Malian adults that is distinct from children. Monocytes isolated from PBMCs of Malian children (n = 8) and adults (n = 8) were stimulated \textit{in vitro} with Pf-iRBC lysate at a monocyte:Pf-iRBC ratio of 1:5 for 24 hours and then total RNA was isolated for sequencing. From separate subjects (n = 4 subjects in each age group), total RNA from unstimulated monocytes was also sequenced. (A) Principal-component analysis of log2-normalized gene counts across all samples. (B-E) Heatmaps representing log2-normalized gene counts of Pf-iRBC stimulated monocytes from Malian children and adults. Each column represents one individual sample. Heatmaps represent gene sets with pre-specified functions: (B) M1/M2 monocyte/macrophage signature, (C) inflammation, (D) phagocytosis, and (E) antigen processing and presentation.

https://doi.org/10.1371/journal.ppat.1009430.g003
Together these data indicate that monocytes of Malian adults are skewed toward a regulatory phenotype, whereas monocytes of Malian children more closely resemble those of malaria-naïve U.S. adults, suggesting that cumulative malaria exposure, or other factors associated with malaria transmission (e.g. co-infection history, microbiota composition, nutritional status), and not age per se, drives functional changes in monocytes that persist in uninfected individuals.

**Pre-exposure of monocytes to *P. falciparum* blunts subsequent inflammatory responses to *P. falciparum* and LPS**

The Malian adults in this study whose monocytes skew toward a regulatory phenotype have been exposed to a lifetime of repeated *P. falciparum* infections, but whether *P. falciparum* per se can drive this phenotype remains unclear. To more directly test this hypothesis, and to further dissect the molecular mechanisms underlying our ex vivo observations, we adapted an in vitro model of monocyte to macrophage differentiation [25] to incorporate exposure to *P. falciparum* blood-stage parasites (Fig 4). Briefly, elutriated monocytes from healthy U.S. adults were incubated for 24 hours with medium alone, uninfected red blood cells (RBC) or Pf-iRBC. At 24 hours, supernatants and cells were collected from some replicate wells for cytokine analysis, while monocytes in other replicate wells were washed and incubated for 3 additional days in human serum and medium to allow monocytes to differentiate into macrophages (Mf). On day 5, the three populations of macrophages were either harvested for ChIP and cytokine gene expression analysis or re-stimulated with Pf-iRBCs or lipopolysaccharide for 24 hours prior to measuring cytokines in supernatants. Of note, on day 5, cell frequency and viability did not differ significantly between macrophages in medium alone (Mf) or following co-culture with RBCs (RBC-Mf) or Pf-iRBCs (Pf-iRBC-Mf) (S2 Fig). Also of note, titration experiments determined that a monocyte:Pf-iRBC ratio of 1:15 optimally balanced cytokine production and monocyte viability in this model (S1 Fig).

![Diagram of monocyte to macrophage differentiation](https://doi.org/10.1371/journal.ppat.1009430.g004)
As expected, monocytes of U.S. adults (n = 3) co-cultured for 24 hours with *Pf*-iRBCs upregulated the expression of genes encoding several pro-inflammatory cytokines including TNF, IL-6 and IL-1β (Fig 5A), which was confirmed at the protein level in an independent experiment of U.S. adults (n = 9) by cytokine analysis of supernatants (Fig 5D–5F). On day 5, after monocytes had matured into macrophages, pro-inflammatory cytokine gene expression by *Pf*-iRBC-Mfs decreased relative to the 24-hour timepoint but remained higher than pro-inflammatory cytokine gene expression in the Mf and RBC-Mf controls (Fig 5B), consistent with the removal of *Pf*-iRBC by washing at the 24-hour timepoint. On day 5, Mf, RBC-Mf and *Pf*-iRBC-Mf were co-cultured with *Pf*-iRBC lysate. After 24 hours, gene expression analysis showed upregulation of several pro-inflammatory cytokines in Mf and RBC-Mf relative to the 5-day timepoint that was not apparent in *Pf*-iRBC-Mf (Fig 5C). This pattern was confirmed at the protein level for TNF, IL-6 and IL-1β in an independent experiment of U.S. adults (n = 9) by cytokine analysis of supernatants (Fig 5G–5I). Plots of ΔCt values (mean ± SE of the 3 subjects in Fig 5A–5C) for TNF and IL-6 at the three timepoints (Fig 5J and 5K) further illustrates how pre-exposure of monocytes to *P. falciparum* blunts the subsequent inflammatory response of newly differentiated macrophages upon restimulation with *P. falciparum*. We observed a similar dampening of TNF, IL-6 and IL-1β responses to LPS in *Pf*-iRBC-Mf (Fig 5L–5N).

*P. falciparum* exposure induces epigenetic changes in monocytes consistent with regulation of inflammation

Epigenetic modifications in monocytes/macrophages underpin the immunological imprinting of tolerance or trained immunity following exposure to LPS or β-glucan, respectively [25]. Here we hypothesized that *P. falciparum* induces epigenetic modifications in the regulatory regions of pro-inflammatory genes such that inflammatory responses are dampened upon re-exposure to *P. falciparum* (i.e. tolerance). To test this hypothesis, we performed Chromatin Immunoprecipitation (ChIP) on monocytes/macrophages collected at each of the 3 timepoints shown in Fig 4 using an antibody specific for H3K4me3, an epigenetic modification of Histone H3 enriched at active promoters that positively correlates with transcription [26].

After 24 hours, monocytes stimulated with *Pf*-iRBC were enriched for the active H3K4me3 histone mark at the TNF and IL-6 promoter regions relative to monocytes incubated with RBCs or medium alone (Fig 6A and 6D), consistent with cytokine data at the same timepoint (Fig 5D–5F). On day 5, after maturation of monocytes into macrophages (Mf), the active H3K4me3 histone mark remained enriched at the TNF and IL-6 promoter regions of cells that had been stimulated with *Pf*-iRBC (*Pf*-iRBC-Mf) relative to macrophages initially incubated with RBCs (RBC-Mf) or medium alone (Mf) (Fig 6B and 6E). After the three cell populations (Mf, RBC-Mf and *Pf*-iRBC-Mf) were stimulated with *Pf*-iRBC for 24 hours, the active H3K4me3 histone mark became enriched in the TNF and IL-6 promoter regions of the Mf and RBC-Mf populations, whereas the active H3K4me3 histone mark decreased in the TNF and IL-6 promoter regions in the *Pf*-iRBC-Mf population (Fig 6C and 6F). Plots of the H3K4me3 histone mark at the TNF and IL-6 promoter regions at all three timepoints further illustrate how pre-exposure to *P. falciparum* diminishes H3K4me3 histone mark enrichment of newly differentiated macrophages upon restimulation with *P. falciparum* (Fig 6G and 6H)—an epigenetic pattern of TNF and IL-6 regulation consistent with the pattern of TNF and IL-6 expression in the same model (Fig 5J and 5K).

Discussion

In areas of intense malaria transmission the risk of febrile malaria decreases with age as individuals are exposed to repeated *P. falciparum* infections over many years [17]. The relatively
Fig 5. Pre-exposure of monocytes to *P. falciparum* dampens subsequent inflammatory responses to *P. falciparum* and LPS. Monocytes of U.S. adults were incubated with medium, RBC or Pf-iRBC. At 24 hours, cells were analyzed by (A) cytokine gene expression Taqman arrays (n = 3 subjects), and supernatants were analyzed by a bead-multiplex assay (n = 9 subjects) to quantify (D) TNF, (E) IL-6 and (F) IL-1β. In the same experiment, replicate monocytes were incubated with medium, RBC or Pf-iRBC, washed at 24 hours and incubated for 3 additional days in human serum to permit macrophage (Mf) differentiation. On day 5, the three populations of macrophages were analyzed by (B) cytokine gene expression arrays (n = 3 subjects). Finally, in the same experiment, replicate monocytes were incubated with medium, RBC or Pf-iRBC, washed at 24 hours, incubated for 3 additional days in human serum to permit Mf differentiation, and then co-
slow development of clinical immunity to malaria is associated with the gradual acquisition of
*P. falciparum*-specific adaptive immune responses [27]. Here we sought to understand the
impact of *P. falciparum* exposure on the phenotype and function of innate immune cells,

![Heatmaps](https://doi.org/10.1371/journal.ppat.1009430.g005)

Fig 6. *P. falciparum* exposure drives epigenetic reprogramming of monocyte/macrophages toward a regulatory
phenotype. Monocytes of U.S. adults (n = 4) were incubated with medium, RBC or *Pf*-iRBC for 24 hours and then
analyzed by chromatin immunoprecipitation (ChIP) and RT-PCR to quantify H3K4me3 enrichment at (A) TNF and
(D) IL-6 promoter sites. Replicate monocytes were incubated with medium, RBC or *Pf*-iRBC, washed at 24 hours, and
incubated for 3 days in human serum to permit Mf differentiation. On day 5, cells were analyzed by ChIP and RT-PCR
to quantify H3K4me3 enrichment at (B) TNF and (E) IL-6 promoter sites. Finally, replicate monocytes were incubated
with medium, RBC or *Pf*-iRBC, washed at 24 hours, incubated for 3 days in human serum, co-cultured with *Pf*-iRBC
for 24 hours, and analyzed by ChIP and RT-PCR to quantify H3K4me3 enrichment at (C) TNF and (F) IL-6 promoter
sites. Kinetics of H3K4me3 enrichment at (G) TNF and (H) IL-6 promoter sites across indicated timepoints. Results
are shown as means ±SEM fold enrichment of H3K4me3 antibody as percent of input. (A-F) One-way ANOVA with
Tukey’s adjustment for multiple comparisons. P values indicate level of significance. (G, H) Two-way ANOVA with
Tukey’s adjustment for multiple comparisons. P values indicate level of significance between Pf-iRBC vs. medium and
Pf-iRBC vs. RBC, respectively.

https://doi.org/10.1371/journal.ppat.1009430.g006
namely, the monocyte/macrophage lineage—an important source of fever-inducing pro-inflammatory cytokines during blood-stage malaria infection [11].

In the Mali cohort we found that monocytes of adults produced lower levels of the inflammatory cytokines IL-1β, IL-6 and TNF in response to Pf-iRBC stimulation compared to monocytes of infants and children, or malaria-naïve U.S. adults. Furthermore, monocytes of Malian children produced lower levels of IL-1β and IL-6 following Pf-iRBC stimulation compared to 4–6-month-old infants who were born during the dry season (negligible *P. falciparum* transmission). These data indicate that age alone is not responsible for the functional changes in monocytes observed in the Mali cohort, but rather malaria exposure itself and/or other factors associated with malaria transmission (e.g. co-infection history, microbiota composition, nutritional status). In contrast, monocytes of Malian adults produced higher levels of the anti-inflammatory cytokine IL-10 in response to *Pf*-iRBC stimulation. Consistent with these functional data, monocytes of Malian adults expressed higher levels of the regulatory molecules CD163, CD206, Arginase-1 and TGM2 [28–32].

Importantly, the monocytes analyzed in this study were collected from uninfected, asymptomatic individuals at the end of the 6-month dry season when the immune system is in a relatively non-activated state. Since monocytes survive in circulation for only 1–7 days [15], we postulate that the skewing of circulating monocytes toward a regulatory phenotype with increasing age reflects the ‘reprogramming’ of bone marrow progenitor cells by past malaria exposure and/or other immune perturbations associated with malaria transmission. This hypothesis is consistent with recent studies in mice showing that Bacillus-Calmette Guérin (BCG) or β-glucan can epigenetically and metabolically reprogram myeloid progenitors in the bone marrow [33,34]—a mechanism that may be particularly relevant to malaria as the bone marrow is a major site of growth and sexual development for *Plasmodium* parasites [35]. Alternatively, a recent study using the rodent malaria model by Nahrendorf *et al*. found that epigenetic reprogramming of monocytes in tolerized hosts occurs within the spleen [36].

We previously found in longitudinal analyses of Malian children that Pf-iRBC-inducible production of IL-1β and IL-6 by monocytes was lower 7 days after treatment of febrile malaria relative to that induced at the pre-infection baseline before the six-month malaria season [19]. However, the skewing of monocytes toward a regulatory phenotype in children after febrile malaria seems to depend on ongoing *P. falciparum* exposure, as children’s monocytes generally appear to return to a ‘non-tolerant’ steady-state baseline after the subsequent 6-month dry season [19]. However, a study in the same cohort found that children who are resistant to febrile malaria begin the malaria season with evidence of tolerized monocytes that upregulate p53, which is associated with attenuation of *Plasmodium*-induced inflammation [37]. Interestingly, a study conducted in a region of Uganda where *P. falciparum* transmission occurs year-round found that older children had a dampened pro-inflammatory serum cytokine response during acute malaria compared to younger children [38], consistent with the notion that ongoing exposure may be required to maintain a tolerant state in most children.

That innate immune ‘memory’ may be relatively short-lived after a single immune perturbation is consistent with other studies showing that functional reprogramming of innate immune cells can persist for at least 3 months and up to 1 year [13]. Therefore, it seems plausible that with each 6-month malaria season, the homeostatic setpoint of monocytes gradually shifts toward a regulatory phenotype such that adults maintain a regulatory phenotype even through the 6-month dry season. This is consistent with whole blood transcriptome analysis of Malian adults who exhibited a blunted inflammatory response during *P. falciparum* infection relative to Dutch adults who were experimentally infected with *P. falciparum* for the first time [39]. Interestingly, a recent clinical trial of malaria-naïve Dutch adults showed that volunteers vaccinated with BCG presented with earlier and more severe clinical symptoms following
controlled *P. falciparum* infection, compared to unvaccinated volunteers. Moreover, parasite-mia in BCG-vaccinated volunteers correlated inversely with the degree of NK cell and monocyte activation [40]. Therefore, it may be of interest in future studies to determine whether revaccinating individuals with BCG in malaria-endemic areas modifies the regulatory monocyte phenotype we observed in malaria-experienced adults, as well as their clinical response to natural *P. falciparum* infection.

To more directly assess the impact of *P. falciparum* blood-stage parasites on monocytes/macrophages and to gain insight into potential molecular mechanisms of *Plasmodium*-induced tolerance, we employed an *in vitro* system of monocyte to macrophage differentiation and *P. falciparum* co-culture using monocytes collected from malaria-naïve U.S. adults [25,41]. With this model we found at both the mRNA and protein levels that macrophages derived from monocytes previously exposed to *Pf*-iRBCs *in vitro* had an attenuated inflammatory cytokine response upon re-exposure to *Pf*-iRBCs or exposure to the TLR4 agonist LPS. This corresponded to a decrease in the epigenetic marker of active gene transcription, H3K4me3, at inflammatory cytokine gene loci. These findings are consistent with the hypothesis that malaria contributes directly to the reprogramming of monocytes/macrophages toward a regulatory phenotype. It will be of interest in future studies to apply this *in vitro* model to monocytes collected from malaria-exposed individuals. Going forward it will also be important to determine whether these *in vitro* epigenetic findings are recapitulated by *ex vivo* analysis of monocytes of malaria-exposed children and adults.

Given the non-specific nature of monocyte tolerance, the findings of this study may have implications for vaccine responsiveness and the clinical course of non-malaria infections in malaria-exposed populations, even when individuals are uninfected and asymptomatic. For example, studies of the PiSPZ malaria vaccine in Mali and Tanzania reported an inverse relationship between cumulative *P. falciparum* exposure or transmission intensity and vaccine-specific antibody responses [42,43]. In particular, the PiSPZ vaccine was less immunogenic in malaria-exposed African adults compared to malaria-naïve U.S. adults [43]. Given that monocytes can help initiate vaccine-specific adaptive immunity through cytokine production, and to a lesser extent antigen processing and presentation, it is plausible that tolerized monocytes could play a role in vaccine hypo-responsiveness in malaria-exposed adults in endemic areas. It is also conceivable that tolerized monocytes/macrophages could modify the clinical manifestations of non-malaria infections, particularly those linked to monocyte/macrophage-associated pathological inflammation. For example, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the coronavirus disease 2019 (COVID-19) pandemic, has been associated with excessive inflammation, including high levels of circulating IL-6 and TNF, that is thought to be a major cause of disease severity and death in patients with COVID-19 [44]. In some areas of Africa to date, the COVID-19 pandemic has not been as severe as predicted [45]. Although many environmental, genetic, sociocultural and institutional factors could contribute to lower COVID-19 morbidity and mortality in Africa (actual or reported) compared to other regions [46], we speculate that COVID-19 severity in Africa could be mitigated in part by pre-existing differences in the immune system, including tolerized monocytes that produce lower levels of pro-inflammatory cytokines when activated.

In contrast to our findings here, recent studies have reported that malaria induces a state of hyper-responsiveness [41,47,48] that is functionally similar to the trained immunity induced by BCG vaccination [13]. For example, Schrum et al. found that initial stimulation with Pf-iRBCs induced human adherent PBMCs to hyper-respond to subsequent stimulation with the TLR2 agonist Pam3CSK4, and led to increased H3K4me3 at the *TNF, PTGS2*, and *IL-6* promoters [41]. These findings may differ from the present study for several reasons. In contrast to purified monocytes used in the present study, Schrum et al. performed *in vitro* studies with adherent PBMCs, which may contain cells other than monocyte/macrophages, raising the
possibility that Pf-iRBC-induced cytokine production from non-monocyte/macrophage cell populations could drive differential epigenetic and functional profiles. In addition, our in vitro studies used a higher cell to Pf-iRBC ratio (1:15 vs. 1:0.5) that more closely approximates in vivo parasitemia during febrile malaria. Finally, we restimulated monocytes/macrophages with Pf-iRBCs rather than TLR agonists alone. Nonetheless, it remains an intriguing possibility that Pf-iRBCs render monocytes/macrophages hypo-responsive to Pf-iRBC re-stimulation, while inducing 'training' or hyper-responsiveness to re-stimulation with TLR2 agonists.

This study has limitations. The study does not include children and adolescents aged 7–17 years who are developing clinical immunity to malaria; therefore, we cannot determine if there is a direct link between the expansion of regulatory monocytes and clinical immunity—a question that can be addressed in future longitudinal studies with more age groups and a larger sample size. It will also be important to adequately power future studies to investigate sex-specific differences in innate immune function [49] in the context of malaria. In addition, it will be of interest to determine whether the in vitro epigenetic findings are recapitulated by ex vivo analysis of monocytes of malaria-exposed children and adults. Finally, although age is a reasonable surrogate for cumulative malaria exposure among non-migratory populations in high transmission settings, age may not directly represent malaria exposure.

Taken together, our findings offer mechanistic insight into the long-standing clinical observation that individuals exposed to intense malaria transmission can tolerate malaria parasites in their blood at levels that would predictably produce fever in previously unexposed individuals [16]. In future studies it will be of interest to determine the generalizability of these findings in other malaria-exposed populations and to assess the extent to which variation in transmission intensity influence monocyte phenotype and function. In addition, it will be of interest to track ex vivo monocyte epigenetic profiles within individuals over time and with repeated infections to better understand the quality and kinetics of malaria-induced tolerance.

Materials and methods

Ethics statement

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 the study (ClinicalTrials.gov NCT01322581). Written, informed consent was obtained from the parents or guardians of participating children or from adult participants.

Study subjects

The field study was conducted in the rural village of Kalifabougou, Mali where intense P. falciparum transmission occurs from June through December each year. The cohort study has been described in detail elsewhere [17]. Briefly, 695 healthy children and adults aged 3 months to 25 years were enrolled in an ongoing cohort study in May 2011. Exclusion criteria at enrollment included a hemoglobin level <7 g/dL, axillary temperature ≥37.5°C, acute systemic illness, underlying chronic disease, or use of antimalarial or immunosuppressive medications in the past 30 days. The present study focused on children aged 6 months to 8 years and adults. For this study venous blood samples were collected from study subjects at their healthy uninfected baseline before the malaria season.

PBMC processing

Blood samples (8 ml) were drawn by venipuncture into sodium citrate-containing cell preparation tubes (BD, Vacutainer CPT Tubes) and transported 20 km to the laboratory where
PBMCs were isolated and frozen within three hours according to the manufacturer’s instructions. PBMCs were frozen in human AB serum (Sigma) containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), kept at -80˚C for 24 hours, and then stored in liquid nitrogen.

**Isolation of monocytes**

PBMCs and elutriated monocytes were obtained from healthy U.S. volunteers by counterflow centrifugal elutriation at the National Institutes of Health (NIH) Blood Bank under Institutional Review Board approved protocols of both the National Institute of Allergy and Infectious Diseases and the Department of Transfusion Medicine. Monocytes of PBMCs and elutriated samples were isolated using a negative selection kit (EasySep Human Monocyte Enrichment Kit without CD16 Depletion; STEMCELL technologies). The same negative selection kit was used to isolate monocytes from thawed PBMCs of Malian donors. Non-monocyte/macrophage cells were directly depleted with a cocktail of biotin-conjugated antibodies followed by magnetic removal of labeled cells. Monocyte purity was routinely >98% as assessed by flow cytometry.

**Preparation of *P. falciparum*-infected red blood cell lysate for in vitro stimulation**

3D7 *P. falciparum* parasites were maintained in fresh human ORh+ erythrocytes at 3% hematocrit in RPMI 1640 medium (KD Medical) supplemented with 10% heat-inactivated ORh+ human serum (Interstate Blood Bank, Memphis, Tennessee), 7.4% Sodium Bicarbonate (GIBCO, Invitrogen) and 25 mg/ml of gentamycin (GIBCO, invitrogen), at 37˚C in the presence of a gas mixture containing 5% O2, 5% CO2 and 90% N2. Parasite cultures were confirmed to be free of mycoplasma and acholeplasma using an ELISA-based Mycoplasma Detection Kit (Roche) which contains polyclonal antibodies specific for *M. arginini*, *M. hyorhinis*, *A. laidlawii* and *M. orale*. *P. falciparum*-infected red blood cells (Pf-iRBCs) were enriched for knobs using Zeptogel (contains gelatin) sedimentation. Pf-iRBCs were enriched at the schizont stage with percoll-sorbitol gradient and centrifugation, washed, and resuspended in complete medium in the absence of human serum or Pf-iRBCs schizonts were isolated in RPMI 1640 medium supplemented with 0.25% Albumax (GIBCO, Invitrogen) and 7.4% Sodium Bicarbonate (GIBCO, Invitrogen) using magnetic columns (LD MACS Separation Columns, Miltenyi Biotec). Control preparations of uninfected red blood cells (RBCs) from the same blood donor were obtained and tested in all experiments. Lysates of Pf-iRBCs and RBCs were obtained by three freeze-thaw cycles using liquid nitrogen and a 37˚C water bath.

**In vitro stimulation of PBMCs and monocytes with *P. falciparum*-infected red blood cell lysate**

Monocytes or PBMCs were cultured with RBCs or Pf-iRBC lysate. Cells were cultured in complete RPMI (RPMI 1640 plus 10% human AB serum, 1% penicillin/streptomyccin) at 37˚C in a 5% CO2 atmosphere. PBMCs were stimulated with RBC or Pf-iRBC lysate at a ratio of 3 RBCs or 3 Pf-iRBCs per PBMC (Fig 2F and 2G). For experiments in which secreted cytokines from monocytes were measured in supernatants after 24 hours of stimulation (Fig 1) we used a monocyte:Pf-iRBC ratio of 1:30 based on previously published methods [19]. For experiments involving whole genome RNA-seq analysis of monocytes that had been stimulated for 24 hours (Fig 3) we used a monocyte:Pf-iRBC ratio of 1:5 to reduce *P. falciparum* nucleic acid...
during RNA sequencing. For the *in vitro* model of monocyte to macrophage differentiation that spanned 5 days (Figs 4 and 5) we determined through titration experiments that a monocyte:*Pf*-iRBC ratio of 1:15 optimally balanced cytokine production and monocyte viability in this model (S1 and S2 Figs). For *in vitro* experiments with elutriated monocytes, cells were first allowed to adhere in monocyte attachment medium (Promocell) for 1.5 hr before culturing as described above.

**Flow cytometry**

PBMCs were washed in PBS with 4% heat-inactivated FCS, incubated for 15 min on ice with a live-dead dye in PBS, washed, and then surface stained with lymphocyte lineage dump-APC (CD3, CD19, CD56, CD20), CD14-BUV805, CD206-BV421, CD163-FITC, CD16-BUV395, HLA-DR-APC-R700 and CD86-BV650. For intracellular staining, following surface staining cells were fixed and permeabilized using a Foxp3 staining kit (e-biosciences). Cells were then stained with Arginase1-PE-Cy7 in permeabilization buffer. After washing, cells were resuspended in 4% heat-inactivated FCS containing FACS buffer and data were acquired by a Symphony Flow Cytometer (BD Biosciences). Flow cytometry and t-SNE analyses were performed with FlowJo software (FlowJo10.5.3). For t-SNE analysis, down sampling was done on live monocytes which were devoid of aggregates and dead cells, followed by concatenating the samples according to biological replicates and sample group. Finally, the default t-SNE algorithm was run with 1000 iterations, perplexity 30 and a learning rate of 200.

**Cytokine measurements in supernatants**

 Supernatants were thawed and immediately analyzed with Bio-plex human cytokine assays (Bio-Rad Laboratories, Inc.) following the manufacturer’s instructions. The following cytokines were measured: IL-1β, IL-6, IL-10 and TNF. Briefly, 50 uL of supernatant was incubated with anti-cytokine antibody-coupled magnetic beads for 30 min at room temperature with shaking at 300 RPM in the dark. Between each step the complexes were washed three times in wash buffer using a vacuum manifold. The beads were then incubated with a biotinylated detector antibody for 30 min before incubation with streptavidin-phycoerythrin for 30 minutes. Finally, the complexes were resuspended in 125 mL of detection buffer and 100 beads were counted with a Luminex 200 device (Bio-Rad Laboratories, Inc.). Final concentrations were calculated from the mean fluorescence intensity and expressed in pg/mL using standard curves with known concentrations of each cytokine.

**RNA isolation, RNA-Seq and cytokine gene expression array**

Cells were kept in RNAProtect buffer (Qiagen) at -80°C until RNA was isolated. RNA was isolated using the RNAeasy kit according to the manufacturer’s instructions. The quality and quantity of isolated RNA was determined with the Agilent Bioanalyzer. Only RNA with RIN values greater than 7 were used for analyses. cDNA was prepared from 10 ng of total RNA using the Ovation RNA-Seq System V2 (Tecan) according to manufacturer’s instructions. This method employs both poly-T and random primers so that both poly-adenylated and non-poly-adenylated RNA is included. The cDNA product was end-repaired using the NEBNext End Repair Module (New England Biolabs). RNA-Seq libraries were prepared using 1 μg of end-repaired cDNA using the TruSeq Stranded RNA Kit (Illumina), however due to the method of amplification the libraries were not stranded. Unique dual-indexed barcode adapters were applied to each library. Libraries were pooled in an equimolar ratio for sequencing. The pooled libraries were sequenced on one lane of an S4 flow cell on a NovaSeq 6000 using version 1 chemistry to achieve a minimum of 49 million 150 base pair reads. The data was
processed using RTA version 3.4.4 and BWA-0.7.12. For the RNA-seq analysis, quality control and adapter trimming were performed using FASTQC and cutadapt, respectively (Martin, 2013). Then, the reads were aligned to the hg19 reference genome using STAR aligner (Dobin et al., 2013). Reads were counted using featureCounts (Liao, Smyth, & Shi, 2014). For the identification of differentially expressed genes among the different groups, we used DESeq2 with the design formula “~ condition” (Love, Huber, & Anders, 2014). Benjamini-Hochberg (BH) correction was performed with an adjusted p-value threshold set to 0.01. For the gene expression array, we used TaqMan Array, Human Cytokine Network, fast 96-well plate, and real time PCR was performed from isolated cDNA according to the manufacturer’s instructions. cDNA was isolated from RNA using the Superscript-VILO cDNA synthesis kit followed by Real Time RT-PCR using TaqMan Fast Advanced Master Mix using the Quant Studio 6 instrument.

**Cell culture**

For ChIP analysis and Taqman RNA Array analysis, 10x10^6 elutriated and purified monocytes were plated on 100 mm dishes. Monocytes were pre-incubated with cell culture medium (RPMI), Pf-iRBCs or RBCs for 24 hours in a total volume of 10 mL. After wash-out, cells were cultured in RPMI supplemented with 10% human pooled AB serum containing homeostatic levels of M-CSF that induces macrophage differentiation. Cells were collected at 24 hours and on day 5 were counted prior to chromatin immunoprecipitation. After wash-out, cells were cultured in RPMI supplemented with 10% human pooled AB serum. For cytokines production, 2.5x10^4 to 5x10^4 purified or elutriated monocytes were plated in a 96 well flat bottom plate. Monocytes were pre-incubated as above for 24 hours in a total volume of 100–200 μL. After a wash-out, cells were cultured in RPMI supplemented with 10% human pooled serum and supernatants were collected for analysis.

**ChIP analysis**

Briefly, cells were fixed in 1% formaldehyde for 10 min and quenched with glycine. Chromatin was sonicated from these cells using a Bioruptor Pico (Diagenode) for four cycles of 10× (30 s ON, 30 s OFF) on the HIGH setting. Chromatin precipitation was performed using rabbit anti-human H3K4me3 IgG Ab (Active Motif) as described previously [50]. DNA was then quantified using qPCR with the following primer pairs: IL-6, FW 5’-AGCTCTATCTCCCTTCAG245 G-3’; RV 5’-ACACCCCTCCCTCAACAG-3’; TNF, FW 5’-CAGCCAGTTCTCCTCCCT T-3’; RV 5’-GCTTTTCAGTGCTCATGGTGT-3’[41]. For all ChIP experiments, qPCR values were normalized as percent recovery of the input DNA.

**Statistical analysis**

Most continuous data were compared using the unpaired Mann-Whitney test or paired Wilcoxon sign rank test, as appropriate. Bonferroni adjustments were applied to correct for multiple comparisons where needed. One-way or two-way ANOVA with Tukey post hoc tests were used to compare continuous variables in situations where data was assumed to be normal or where the two-way experimental design precluded a nonparametric test. All statistical tests are specified in the figure legends. Statistical significances were defined using 2-tailed p-values or adjusted p-values of 0.05 or less. Most statistical tests were computed using GraphPad Prism version 8 (http://www.graphpad.com/scientific-software/prism/). Some heatmaps, principal components analysis (PCA) plots and t-SNE plots were produced using R 3.6.1 or FlowJo (version 10.5).
Supporting information

S1 Fig. Titration of monocyte:Pf-iRBC ratio for vitro model of monocyte to macrophage differentiation. Elutriated monocytes from healthy U.S. adults (n = 3) were co-cultured with increasing concentrations of Pf-iRBCs. After 24 hours, IL-1β (A), TNF (B) and IL-6 (C) were measured in supernatants, and (D) cell viability was determined by trypan blue dye exclusion and expressed as percent viability.

(TIF)

S2 Fig. Cell viability at day 5 in the vitro model of monocyte to macrophage differentiation. Elutriated monocytes from healthy U.S. adults (n = 9) were incubated for 24 hours with medium alone, uninfected red blood cells (RBC) or Pf-iRBC (monocyte:Pf-iRBC ratio 1:15). At 24 hours, cells were washed and incubated for 3 additional days in human serum plus medium to allow monocytes to differentiate into macrophages (Mf). To quantify cell viability on day five, 10% v/v alamarBlue HS was added to the culture medium of the three populations of macrophages (Mf, RBC-Mf and Pf-iRBC-Mf) for 5 hours and fluorescence intensity (FI) was measured according to the manufacturer’s instructions. FI was normalized to the fluorescence signal in media without cells. Data were analyzed by the Wilcoxon test with Bonferroni adjustment, and levels of significance between the groups are indicated.

(TIF)

S3 Fig. Expression of HLA-DR and CD86 on monocytes of Malian children and adults, and U.S. adults. PBMCs from Malian children (aged 4–6 years; n = 9) and adults (n = 9) before the malaria season, as well as healthy malaria-naïve U.S. adults (n = 7) were gated for live monocytes and analyzed ex vivo by flow cytometry for surface expression of (A) HLA-DR and (B) CD86. t-SNE analysis of monocytes for all subjects in each group. Expression of each marker is indicated by a color scale.

(TIF)

S1 Table. Table of differentially expressed genes of Pf-iRBC-stimulated PBMCs of Malian adults versus children. This table shows statistically significant (p<0.01) differentially expressed genes in monocytes from Malian adults stimulated with Pf-iRBC relative to Pf-iRBC stimulated monocytes from Malian children. Adjusted P values were calculated with the Benjamini-Hochberg method.

(XLSX)

Acknowledgments

We thank the residents of Kalifabougou, Mali, for participating in this study. We also thank Dr. Alice Young and other members of the National Human Genome Research Institute for the RNA sequencing.

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