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

[1]Rajan Guha*, [2]Anna Mathioudaki, [5]Safiatou Doumbo, [5]Didier Doumtabe, [1]Jeff Skinner, [3]Gunjan Arora, [4]Shafiuddin Siddiqui, [1]Shanping Li, [5]Kassoum Kayentao, [5]Aissata Ongoiba, [2]Judith Zaugg, [5]Boubacar Traore, [1]Peter D. Crompton*

[1]Malaria Infection Biology and Immunity Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland, United States

[2]Structural and Computational Biology Unit, The European Molecular Biology Laboratory, Heidelberg, Germany

[3]Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut, United States

[4]Laboratory of Genome Integrity, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, United States

[5]Malaria Research and Training Centre, Department of Epidemiology of Parasitic Diseases, International Center of Excellence in Research, University of Sciences, Technique and Technology of Bamako, Bamako, Mali

*Correspondence: Peter D. Crompton(pcrompton@niaid.nih.gov),

Rajan Guha (rajan.guha@nih.gov)

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 monocytes collected from uninfected, asymptomatic Malian individuals before the malaria season revealed an inverse relationship between age and *Pf*-iRBC-inducible inflammatory cytokine (IL-1β, IL-6 and TNF) production, whereas Malian infants and malaria-naïve U.S. adults produced similarly high levels of inflammatory cytokines. 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. These findings also suggest that past malaria exposure could mitigate monocyte-associated immunopathology induced by other pathogens such as SARS-CoV-2.

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 malaria parasites in their blood. 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, too much inflammation can harm infected individuals. In Mali, we conducted a study of a malaria-exposed population from infants to adults and found that participants' monocytes produced less inflammation as age increases, whereas monocytes of Malian infants and U.S. adults, who had never been exposed to malaria, both produced high levels of inflammatory molecules. Accordingly, monocytes exposed to malaria 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 immune to inflammatory symptoms of malaria and may also help explain why people in malaria-endemic areas appear to be less susceptible to the harmful effects of inflammation caused by other pathogens such as SARS-CoV-2.

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 malaria transmission, *P. falciparum*-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 malaria [16], we hypothesized that an inverse relationship exists between age (a surrogate for cumulative malaria 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

101 adapted an in vitro system of monocyte to macrophage differentiation to directly investigate
102 the impact of *P. falciparum* blood-stage parasites on monocytes/macrophages at the molecular
103 level.
104
105 **Results**
106 **Malaria exposure associates with reduced production of *Pf*-inducible inflammatory cytokines**
107 **and increased production of IL-10 from monocytes**
108 To analyze the relationship between malaria exposure and monocyte function we isolated
109 monocytes from subjects enrolled in an age-stratified cohort in Mali and also from healthy
110 malaria-naïve U.S. adults as controls. A detailed description of the Kalifabougou cohort has
111 been published [17]. Malian subjects ranged from 4-6-month-old infants born during the six-
112 month dry season, when malaria transmission is negligible [4], to adults exposed to a lifetime of
113 repeated *P. falciparum* infections. All Malian subjects in this cross-sectional analysis were
114 negative for *P. falciparum* infection by PCR at the time of blood collection, which occurred just
115 before the 6-month malaria season. To simulate re-exposure to *P. falciparum* blood-stage
116 parasites in vitro, isolated monocytes were co-cultured with *P. falciparum*-infected red blood
117 cells (*Pf*-iRBCs) at a ratio of 1:30 (monocytes:*Pf*-iRBCs) for 24 hours [18]. Secreted cytokines and
118 chemokines were measured in supernatants by bead-based multiplex arrays. Among the Malian
119 subjects we observed an inverse relationship between age and the production of the
120 inflammatory cytokines TNF, IL-1β and IL-6, whereas Malian infants and malaria-naïve U.S.
121 adults produced similarly high levels of inflammatory cytokines (Figure 1 A-C). In contrast,
122 production of the anti-inflammatory cytokine IL-10 increased with age among Malian subjects,

123 whereas Malian infants and malaria-naïve U.S. adults produced similarly low levels of IL-10
124 (Figure 1D). Taken together, these data suggest that cumulative malaria exposure, or exposure
125 to other factors associated with malaria transmission, skews monocytes toward a regulatory
126 phenotype characterized by decreased *P. falciparum*-inducible production of inflammatory,
127 pyrogenic cytokines and increased production of the anti-inflammatory cytokine IL-10,
128 consistent with the epidemiological observation that febrile malaria risk decreases with
129 cumulative malaria exposure in this cohort [17], and in areas of intense malaria transmission
130 more generally [19].
131
132 **Monocytes of malaria-exposed adults skew toward an M2 regulatory profile phenotypically**
133 **and transcriptionally**
134 Next, we examined the molecular basis of malaria-associated skewing of monocytes toward a
135 regulatory phenotype by comparing the phenotypic and transcriptional profiles of monocytes
136 collected before the malaria season from Malian children and adults, as well as malaria-naïve
137 U.S. adults. PBMCs of Malian children (aged 4-6 years; n=9) and adults (n=9), as well as U.S.
138 adults (n=7) were analyzed *ex vivo* by flow cytometry for surface expression of the myeloid cell
139 markers CD14, CD16, CD86, CD163, CD206 and HLA-DR [20] gated on live monocytes.
140 Visualization of the flow cytometry data by distributed stochastic neighbor embedding (t-SNE)
141 analysis [21] revealed that monocytes of Malian adults expressed higher levels of the
142 alternatively activated (M2) monocytes/macrophage markers CD163 and CD206 compared to
143 Malian children and U.S. adults (Figure 2A); and CD163 and CD206 expression was largely
144 confined to the CD14+ and CD14+CD16+ monocyte clusters (Figure 2B). Consistent with the t-

145 SNE visualization, MFI values of CD163 and CD206 were significantly higher on monocytes of
146 Malian adults compared to Malian children and U.S. adults (Figure 2C and D).
147 M2 cells are known to produce high levels of arginase 1 [22]. Therefore we further tested the
148 hypothesis that cumulative malaria exposure is associated with skewing of monocytes towards
149 an M2 phenotype by stimulating PBMCs of Malian children (aged 4-6 years; n=9) and adults
150 (n=9), as well as U.S. adults (n=9) with *Pf*-iRBCs for 18 hours and quantifying intracellular
151 arginase-1 in monocytes by flow cytometry. Visualization of the flow cytometry data by t-SNE
152 analysis showed that monocytes of Malian adults produced higher levels of arginase 1 in
153 response to *Pf*-iRBC stimulation compared to Malian children and U.S. adults (Figure 2E).
154 Consistent with the t-SNE plots, MFI values of arginase 1 were significantly higher within
155 monocytes of Malian adults compared to Malian children and U.S. adults (Figure 2F).
156
157 To further investigate the molecular basis of malaria-associated skewing of monocytes toward
158 an M2 regulatory type, we conducted whole genome RNA-seq analysis of monocytes isolated
159 from Malian children and adults. RNA-seq was performed on unstimulated monocytes (n=4
160 from each age group) as well as monocytes that had been stimulated with *Pf*-iRBCs for 24 hours
161 (n=8 from each age group) to simulate re-exposure to *P. falciparum* blood-stage parasites.
162 Monocytes were stimulated with *Pf*-iRBCs at a ratio of 1:5 (monocytes:*Pf*-iRBCs) to reduce *P.*
163 *falciparum* nucleic acid during sequencing. Principal components analysis of the RNA-seq data
164 showed segregation of transcription profiles based on age—an effect that became more
165 pronounced following stimulation with *Pf*-iRBCs (Figure 3A). Here we focused on differential
166 gene expression between monocytes of children versus adults following *Pf*-iRBC stimulation.

167 Consistent with the analysis of secreted cytokines (Figure 1), Malian children had significantly
168 higher expression of the genes encoding TNF [log2 fold change (FC) 1.8, Benjamini-Hochberg
169 (BH) adjusted p value = 0.0001] and IL6 (FC 2.3, BH p = 0.007) compared to Malian adults
170 (Figure 3B and C; Supplemental Table). In addition, the expression of *TLR5*, *TLR7*, *CXCL9*,
171 *CXCL10*, *NLRP1*, *NLRP3*, *FCGR3A*, *PTX3* and various HLA molecules were significantly
172 upregulated in children relative to adults (Figures 3B-E; Supplemental Table for FC and adjusted
173 p values). Consistent with the blunted pro-inflammatory cytokine responses that we observed
174 from monocytes of Malian adults in response to *Pf*-iRBC stimulation (Figure 1), expression of
175 *NFKB1*, a positive regulator of inflammation, was downregulated (FC -3.8, BH p = 4.6 E-26) in
176 monocytes of adults relative to children (Figure 3C; Supplemental Table), while expression of
177 the multifunctional enzyme transglutaminase-2 (*TGM2*), a known marker of the M2 regulatory
178 phenotype, was significantly higher (FC 7.5, BH p = 1.7 E-79) in monocytes of adults versus
179 children (Figures 3B and D; Supplemental Table). Accordingly, expression of the chemokines
180 *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
181 monocytes, consistent with the chemokine repertoire of M2 cells [23].
182
183 Together these data indicate that monocytes of Malian adults are skewed toward a regulatory
184 M2 phenotype, whereas monocytes of Malian children more closely resemble those of malaria-
185 naïve U.S. adults, suggesting that cumulative malaria exposure, or other factors associated with
186 malaria transmission, and not age *per se*, drives functional changes in monocytes that persist in
187 uninfected individuals.
188

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

191 The Malian adults in this study whose monocytes skew toward an M2 phenotype have been
192 exposed to a lifetime of repeated *P. falciparum* infections, but whether *P. falciparum per se* can
193 drive this phenotype remains unclear. To more directly test this hypothesis, and to further
194 dissect the molecular mechanisms underlying our ex vivo observations, we adapted an in vitro
195 model of monocyte to macrophage differentiation [24] to incorporate exposure to *P.*
196 *falciparum* blood-stage parasites (Figure 4). Briefly, elutriated monocytes from healthy U.S.
197 adults were incubated for 24 hours with medium alone, uninfected red blood cells (RBC) or *Pf*-
198 iRBC. At 24 hours, supernatants and cells were collected from some replicate wells for cytokine
199 analysis, while monocytes in other replicate wells were washed and incubated for 3 additional
200 days in human serum and medium to allow monocytes to differentiate into macrophages (Mf).
201 On day 5, the three populations of macrophages were either harvested for ChIP and cytokine
202 gene expression analysis or re-stimulated with *Pf*-iRBCs or lipopolysaccharide for 24 hours prior
203 to measuring cytokines in supernatants. Of note, on day 5, cell frequency and viability did not
204 differ significantly between macrophages in medium alone (Mf) or following co-culture with
205 RBCs (RBC-Mf) or Pf-iRBCs (*Pf*-iRBC-Mf) (Supplementary Figure 2). Also of note, titration
206 experiments determined that a monocyte:*Pf*-iRBC ratio of 1:15 optimally balanced cytokine
207 production and monocyte viability in this model (Supplementary Figure 1).

208

209 As expected, monocytes of U.S. adults (n=3) co-cultured for 24 hours with *Pf*-iRBCs upregulated
210 the expression of genes encoding several pro-inflammatory cytokines including TNF, IL-6 and IL-

211 1β (Figure 5A), which was confirmed at the protein level in an independent experiment of U.S.
212 adults (n=9) by cytokine analysis of supernatants (Figure 5D-F). On day 5, after monocytes had
213 matured into macrophages, pro-inflammatory cytokine gene expression in *Pf*-iRBC-Mfs
214 decreased relative to the 24-hour timepoint but remained higher than pro-inflammatory
215 cytokine gene expression in the Mf and RBC-Mf controls (Figure 5B), consistent with the
216 removal of *Pf*-iRBC by washing at the 24-hour timepoint. On day 5, Mf, RBC-Mf and *Pf*-iRBC-Mf
217 were co-cultured with *Pf*-iRBC lysate. After 24 hours, gene expression analysis showed
218 upregulation of several pro-inflammatory cytokines in Mf and RBC-Mf relative to the 5-day
219 timepoint that was not apparent in *Pf*-iRBC-Mf (Figure 5C). This pattern was confirmed at the
220 protein level for TNF, IL-6 and IL-1β in an independent experiment of U.S. adults (n=9) by
221 cytokine analysis of supernatants (Figure 5G-I). Plots of ΔCt values (mean ±SE of the 3 subjects
222 in Figure 5A-C) for TNF and IL-6 at the three timepoints (Figure 5J and K) further illustrates how
223 pre-exposure of monocytes to *P. falciparum* blunts the subsequent inflammatory response of
224 newly differentiated macrophages upon restimulation with *P. falciparum*. We observed a
225 similar dampening of TNF, IL-6 and IL-1β responses to LPS in *Pf*-iRBC-Mf (Figure 5L-N).
226

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

229 Epigenetic modifications in monocytes/macrophages underpin the immunological imprinting of
230 tolerance or trained immunity following exposure to LPS or β-glucan, respectively [24]. Here we
231 hypothesized that *P. falciparum* induces epigenetic modifications in the regulatory regions of
232 pro-inflammatory genes such that inflammatory responses are dampened upon re-exposure to

233 *P. falciparum* (i.e. tolerance). To test this hypothesis, we performed Chromatin
234 Immunoprecipitation (ChIP) on monocytes/macrophages collected at each of the 3 timepoints
235 shown in Figure 4 using an antibody specific for H3K4me3, an epigenetic modification
236 of Histone H3 enriched at active promoters that positively correlates with transcription [25].
237
238 After 24-hours, monocytes stimulated with *Pf*-iRBC were enriched for the active H3K4me3
239 histone mark at the TNF and IL-6 promoter regions relative to monocytes incubated with RBCs
240 or medium alone (Figure 6A, D), consistent with cytokine data at the same timepoint (Figure
241 5D-F). On day 5, after maturation of monocytes into macrophages (Mf), the active H3K4me3
242 histone mark remained enriched at the TNF and IL-6 promoter regions of cells that had been
243 stimulated with *Pf*-iRBC (Pf-iRBC-Mf) relative to macrophages initially incubated with RBCs
244 (RBC-Mf) or medium alone (Mf) (Figure 6B, E). After the three cell populations (Mf, RBC-Mf and
245 Pf-iRBC-Mf) were stimulated with Pf-iRBC for 24 hours, the active H3K4me3 histone mark
246 became enriched in the TNF and IL-6 promoter regions of the Mf and RBC-Mf populations,
247 whereas the active H3K4me3 histone mark decreased in the TNF and IL-6 promoter regions in
248 the Pf-iRBC-Mf population (Figure 6C, F). Plots of the H3K4me3 histone mark at the TNF and IL-
249 6 promoter regions at all three timepoints further illustrate how pre-exposure to *P. falciparum*
250 diminishes H3K4me3 histone mark enrichment of newly differentiated macrophages upon
251 restimulation with *P. falciparum* (Figure 6G and H)—an epigenetic pattern of TNF and IL-6
252 regulation consistent with the pattern of TNF and IL-6 expression in the same model (Figure 5J
253 and K).
254

255 **Discussion**

256 In areas of intense malaria transmission the risk of febrile malaria decreases with age as
257 individuals are exposed to repeated *P. falciparum* infections over many years [17]. The
258 relatively slow development of clinical immunity to malaria is associated with the gradual
259 acquisition of *P. falciparum*-specific adaptive immune responses [26]. Here we sought to
260 understand the impact of *P. falciparum* exposure on the phenotype and function of innate
261 immune cells, namely, the monocyte/macrophage lineage—an important source of fever-
262 inducing pro-inflammatory cytokines during blood-stage malaria infection [11].

263

264 In the Mali cohort we observed an inverse relationship between age and *Pf*-iRBC-inducible
265 production of the inflammatory cytokines IL-1β, IL-6 and TNF from monocytes; whereas
266 monocytes of Malian infants born during the dry season (negligible *P. falciparum* transmission)
267 and malaria-naïve U.S. adults produced similarly high levels of inflammatory cytokines,
268 indicating that age alone is not responsible for the functional changes in monocytes observed in
269 the Mali cohort, but rather malaria exposure itself and/or other factors associated with malaria
270 transmission. In addition, monocytes of Malian adults produced higher levels of the anti-
271 inflammatory cytokine IL-10 in response to *Pf*-iRBC stimulation. Consistent with these
272 functional data, monocytes of Malian adults expressed higher levels of the regulatory
273 molecules CD163, CD206, Arginase-1 and TGM2 [27-31].

274

275 Importantly, the monocytes analyzed in this study were collected from uninfected,
276 asymptomatic individuals at the end of the 6-month dry season when the immune system is in

277 a relatively non-activated state. Since monocytes survive in circulation for only 1-7 days [15],
278 we postulate that the observed skewing of circulating monocytes toward a tolerant phenotype
279 with increasing age reflects the 'reprogramming' of bone marrow progenitor cells by past
280 malaria exposure and/or other immune perturbations associated with malaria transmission.
281 This hypothesis is consistent with recent studies in mice showing that BCG or β-glucan can
282 epigenetically and metabolically reprogram myeloid progenitors in the bone marrow [32, 33]—
283 a mechanism that may be particularly relevant to malaria as the bone marrow is a major site of
284 growth and sexual development for *Plasmodium* parasites [34].
285
286 We previously found in longitudinal analyses of Malian children that *Pf*-iRBC-inducible
287 production of IL-1β and IL-6 by monocytes was lower 7 days after treatment of febrile malaria
288 relative to that induced at the pre-infection baseline before the six-month malaria season [18].
289 However, the skewing of monocytes toward a tolerant phenotype in children after febrile
290 malaria seems to depend on ongoing *P. falciparum* exposure, as children's monocytes appear to
291 return to a 'non-tolerant' steady state baseline after the subsequent 6-month dry season.
292 Interestingly, a study conducted in a region of Uganda where *P. falciparum* transmission occurs
293 year-round found that older children had a dampened pro-inflammatory serum cytokine
294 response during acute malaria compared to younger children [35], consistent with the notion
295 that ongoing exposure may be required to maintain a tolerant state in children.
296
297 That innate immune 'memory' may be relatively short-lived after a single immune perturbation
298 is consistent with other studies that show the trained immunity phenotype persists 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 tolerant phenotype such that adults maintain a tolerant 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 [36].

To more directly assess the impact *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 [24, 37]. With this model we found at both the mRNA and protein levels that macrophages derived from monocytes previously exposed to *Pf*-iRBCs 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 tolerant phenotype.

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 PfSPZ malaria vaccine in Mali and Tanzania reported an inverse

relationship between cumulative *P. falciparum* exposure or transmission intensity and vaccine-specific antibody responses [38, 39]. 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 play a role in vaccine hypo-responsiveness in malaria-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 [40]. In much of Africa to date, the COVID-19 pandemic has not been as severe as predicted [41]. Although many environmental, genetic, sociocultural and institutional factors could contribute to lower COVID-19 morbidity and mortality in Africa compared to other regions [42], we speculate that COVID-19 severity in Africa may be mitigated 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 [37, 43, 44] 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. These findings may differ from the present study for several reasons. In

343 contrast to purified monocytes used in the present study, Schrum et al. performed *in vitro*
344 studies with adherent PBMCs, which may contain cells other than monocyte/macrophages. In
345 addition, our in vitro studies used a higher cell to Pf-iRBC ratio (1:15 vs. 1:0.5) that more closely
346 approximates in vivo parasitemia during febrile malaria. Finally, we restimulated
347 monocytes/macrophages with Pf-iRBCs rather than TLR agonists alone.
348
349 Taken together, our findings offer mechanistic insight into the long-standing clinical observation
350 that individuals exposed to intense malaria transmission can tolerate malaria parasites in their
351 blood at levels that would predictably produce fever in previously unexposed individuals [16].
352 In future studies it will be of interest to determine the generalizability of these findings in other
353 malaria-exposed populations and to assess the extent to which variation in transmission
354 intensity influence monocyte phenotype and function. In addition, it will be of interest to track
355 *ex vivo* monocyte epigenetic profiles within individuals over time and with repeated infections
356 to better understand the quality and kinetics of malaria-induced tolerance.
357
358 **Materials and Methods**
359 **Study subjects**
360 The field study was conducted in the rural village of Kalifabougou, Mali where intense *P.*
361 *falciparum* transmission occurs from June through December each year. The cohort study has
362 been described in detail elsewhere [17]. Briefly, 695 healthy children and adults aged 3 months
363 to 25 years were enrolled in an ongoing cohort study in May 2011. Exclusion criteria at
364 enrollment included a hemoglobin level <7 g/dL, axillary temperature ≥37.5°C, acute systemic

365 illness, underlying chronic disease, or use of antimalarial or immunosuppressive medications in
366 the past 30 days. The present study focused on children aged 6-months to 8 years and adults.
367 For this study venous blood samples were collected from study subjects at their healthy
368 uninfected baseline before the malaria season.
369
370 **PBMC processing**
371 Blood samples (8 ml) were drawn by venipuncture into sodium citrate-containing cell
372 preparation tubes (BD, Vacutainer CPT Tubes) and transported 20 km to the laboratory where
373 PBMCs were isolated and frozen within three hours according to the manufacturer's
374 instructions. PBMCs were frozen in human AB serum (Sigma) containing 10% dimethyl sulfoxide
375 (DMSO; Sigma-Aldrich, St. Louis, MO), kept at -80°C for 24 hours, and then stored in liquid
376 nitrogen.
377
378 **Isolation of monocytes**
379 PBMCs and elutriated monocytes were obtained from healthy U.S. volunteers by counterflow
380 centrifugal elutriation at the National Institutes of Health (NIH) Blood Bank under Institutional
381 Review Board approved protocols of both the National Institute of Allergy and Infectious
382 Diseases and the Department of Transfusion Medicine. Elutriated monocytes were further
383 purified with a monocyte isolation kit (Stem Cell technologies) to minimize donor-to-donor
384 variability in contaminating cell populations. Monocyte purity was routinely >98% as assessed
385 by flow cytometry. Similarly, monocytes were isolated from PBMCs of Malian donors by a
386 negative selection monocyte isolation kit (Stem Cell Technologies) without depleting CD16 such

387 that monocytes were isolated 'untouched'. Non-monocyte/macrophage cells were directly
388 depleted with a cocktail of biotin-conjugated antibodies followed by magnetic removal of
389 labeled cells.
390
391 **Preparation of *P. falciparum*-infected red blood cell lysate for in vitro stimulation**
392 3D7 *P. falciparum* parasites were maintained in fresh human ORh$^+$ erythrocytes at 3%
393 hematocrit in RPMI 1640 medium (KD Medical) supplemented with 10% heat-inactivated ORh$^+$
394 human serum (Interstate Blood Bank, Memphis, Tennessee), 7.4% Sodium Bicarbonate (GIBCO,
395 Invitrogen) and 25 mg/ml of gentamycin (GIBCO, invitrogen), at 37°C in the presence of a gas
396 mixture containing 5% O2, 5% CO2 and 90% N2. Parasite cultures were confirmed to be free of
397 mycoplasma and acholeplasma using an ELISA-based Mycoplasma Detection Kit (Roche) which
398 contains polyclonal antibodies specific for *M. arginini*, *M. hyorhinis*, *A. laidlawii* and *M. orale*. *P.*
399 *falciparum*-infected red blood cells (Pf-iRBCs) were enriched for knobs using Zeptogel (contains
400 gelatin) sedimentation. Pf-iRBCs were enriched at the schizont stage with percoll-sorbitol
401 gradient and centrifugation, washed, and resuspended in complete medium in the absence of
402 human serum or Pf-iRBCs schizonts were isolated in RPMI 1640 medium supplemented with
403 0.25% Albumax (GIBCO, Invitrogen) and 7.4% Sodium Bicarbonate (GIBCO, Invitrogen) using
404 magnetic columns (LD MACS Separation Columns, Miltenyi Biotec). Control preparations of
405 uninfected red blood cells (RBCs) from the same blood donor were obtained and tested in all
406 experiments. Lysates of Pf-iRBCs and RBCs were obtained by three freeze-thaw cycles using
407 liquid nitrogen and a 37°C water bath.
408

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

410 Monocytes or PBMCs were cultured with RBCs or Pf-iRBCs. Cells were cultured in complete
411 RPMI (RPMI 1640 plus 10% human AB serum, 1% penicillin/streptomycin), at 37°C in a 5% CO_2
412 atmosphere. PBMCs were stimulated with RBC or Pf-iRBC lystate at a ratio of 3 RBCs or 3 Pf-
413 iRBCs per PBMC, whereas monocytes were stimulated at a ratio of 5-30 RBCs or 5-30 Pf-iRBCs
414 per monocyte. For *in vitro* experiments with elutriated monocytes, cells were first allowed to
415 adhere in monocyte attachment medium (Promocell) for 1.5 hr before culturing as described
416 above.

417

418 **Flow cytometry**

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

431

432 **Cytokine measurements in supernatants**

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

444

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

446 Cells were kept in RNAProtect buffer (Qiagen) at -80°C until RNA was isolated. RNA was isolated
447 using the RNAeasy kit according to the manufacturer's instructions. The quality and quantity of
448 isolated RNA was determined with the Agilent Bioanalyzer. Only RNA with RIN values greater
449 than 7 were used for analyses. cDNA was prepared from 10 ng of total RNA using the Ovation®
450 RNA-Seq System V2 (Tecan) according to manufacturer's instructions. This method employs
451 both poly-T and random primers so that both poly-adenylated and non-poly-adenylated RNA is
452 included. The cDNA product was end-repaired using the NEBNext End Repair Module (New

453 England Biolabs). RNA-Seq libraries were prepared using 1 µg of end-repaired cDNA using the
454 TruSeq Stranded RNA Kit (Illumina), however due to the method of amplification the libraries
455 were not stranded. Unique dual-indexed barcode adapters were applied to each library.
456 Libraries were pooled in an equimolar ratio for sequencing. The pooled libraries were
457 sequenced on one lane of an S4 flow cell on a NovaSeq 6000 using version 1 chemistry to
458 achieve a minimum of 49 million 150 base pair reads. The data was processed using RTA
459 version 3.4.4 and BWA-0.7.12. For the RNA-seq analysis, quality control and adapter trimming
460 were performed using FASTQC and cutadapt, respectively (Martin, 2013). Then, the reads were
461 aligned to the hg19 reference genome using STAR aligner (Dobin et al., 2013). Reads were
462 counted using featureCounts (Liao, Smyth, & Shi, 2014). For the identification of differentially
463 expressed genes among the different groups, we used DESeq2 with the design formula "~
464 condition" (Love, Huber, & Anders, 2014). Benjamini-Hochberg (BH) correction was performed
465 with an adjusted p-value threshold set to 0.01. For the gene expression array, we used
466 TaqMan™ Array, Human Cytokine Network, fast 96-well plate, and real time PCR was
467 performed from isolated cDNA according to the manufacturer's instructions. cDNA was isolated
468 from RNA using the Superscript-VILO cDNA synthesis kit followed by Real Time RTPCR using
469 TaqMan™ Fast Advanced Master Mix using the Quant Studio™ 6 instrument.
470
471 **Cell culture**
472 For ChIP analysis and Taqman RNA Array analysis, $10x10^6$ elutriated and purified monocytes
473 were plated on 100 mm dishes. Monocytes were pre-incubated with cell culture medium
474 (RPMI), Pf-iRBCs or RBCs for 24 hours in a total volume of 10 mL. After wash-out, cells were

475 cultured in RPMI supplemented with 10% human pooled AB serum containing homeostatic
476 levels of M-CSF that induces macrophage differentiation. Cells were collected at 24 hours and
477 on day 5 were counted prior to chromatin immunoprecipitation. After wash-out, cells were
478 cultured in RPMI supplemented with 10% human pooled AB serum. For cytokines production,
479 $2.5x10^4$ to $5x10^4$ purified or elutriated monocytes were plated in a 96 well flat bottom plate.
480 Monocytes were pre-incubated as above for 24 hours in a total volume of 100-200 µL. After a
481 wash-out, cells were cultured in RPMI supplemented with 10% human pooled serum and
482 supernatants were collected for analysis.
483
484 **ChIP analysis**
485 Briefly, cells were fixed in 1% formaldehyde for 10 min and quenched with glycine. Chromatin
486 was sonicated from these cells using a Bioruptor Pico (Diagenode) for four cycles of 10× (30 s
487 ON, 30s OFF) on the HIGH setting. Chromatin precipitation was performed using rabbit anti-
488 human H3K4me3 IgG Ab (Active Motif) as described previously [45]. DNA was then quantified
489 using qPCR with the following primer pairs: IL-6, FW 5′-AGCTCTATCTCCCCTCCAGG-3′, RV 5′-
490 ACACCCCTCCCTCACACAG-3′; TNF, FW 5′-CAGGCAGGTTCTCTTCCTCT-3′, RV 5′-
491 GCTTTCAGTGCTCATGGTGT-3′[37]. For all ChIP experiments, qPCR values were normalized as
492 percent recovery of the input DNA.
493
494 **Statistical analysis**
495 Most continuous data were compared using the unpaired Mann-Whitney test or paired
496 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)

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Figure Legends

710 **Figure 1. Monocytes of Malian adults exhibit reduced *P. falciparum*-inducible inflammatory**
711 **cytokine production and increased IL-10 production.** PBMCs were collected cross-sectionally
712 from an age-stratified cohort in Mali before the malaria season when all subjects were negative
713 for *Pf* infection by PCR; and also, from malaria-naïve U.S. adults. Monocytes were isolated from
714 thawed PBMCs and stimulated with iRBC lysate at a ratio of 1 monocyte to 30 iRBCs. After 24
715 hours, cell culture supernatants were analyzed to quantify secreted (**A**) TNF, (**B**) IL-1β, (**C**) IL-6
716 and (**D**) IL-10. Means ± SEM are shown. Data were analyzed by the Brown Forsythe and Welch
717 ANOVA test followed by Dunnett's T3 multiple comparison test. Level of significance between
718 groups are indicated by P values.
719
720 **Figure 2. Monocytes of Malian adults upregulate markers of M2 monocytes/macrophages.** (**A-**
721 **D**) Monocytes from Malian children (aged 4-6 years; n=9) and adults (n=9) before the malaria
722 season, as well as healthy malaria-naïve U.S. adults (n=7) were analyzed *ex vivo* by flow
723 cytometry for surface expression of CD14, CD16, CD163 and CD206. (**A, B**) t-SNE analysis of
724 monocytes for all subjects in each group. Expression of each marker is indicated by a color
725 scale. MFI of (**C**) CD163 and (**D**) CD206 surface expression on manually gated live monocytes.
726 (**E,F**) PBMCs were stimulated with *Pf*-iRBCs and analyzed for arginase-1 expression in
727 monocytes intracellularly. (**E**) Representative t-SNE plots showing expression of Arginase-1 in
728 the clusters in three different groups of monocytes. Expression of Arginase-1 is indicated by a
729 color scale. **(F)**. Expression MFI level of intracellular Arginase-1 on manually gated live
730 monocytes comparing three different groups. Data were analyzed by the Mann-Whitney test

731 with Bonferroni adjustment, and the level of significance between groups is indicated by P
732 values.
733
734 **Figure 3. RNA-seq analysis of monocytes after *Pf*-iRBC stimulation reveals an M2 regulatory**
735 **signature in Malian adults that is distinct from children.** Isolated monocytes from Malian
736 children (n=8) and adults (n=8) were stimulated *in vitro* with *Pf*-iRBC lysate at a monocyte: *Pf*-
737 iRBC ratio of 1:5 for 24 hours and then total RNA was isolated for sequencing. From separate
738 subjects (n=4 subjects in each age group), total RNA from unstimulated monocytes was also
739 sequenced. **(A)** Principal-component analysis of log2-normalized gene counts across all
740 samples. (**B-E)** Heatmaps representing log2-normalized gene counts of *Pf*-iRBC stimulated
741 monocytes from Malian children and adults. Each column represents one individual sample.
742 Heatmaps represent gene sets with pre-specified functions: (**4B**) M1/M2
743 monocyte/macrophage signature, (**4C**) inflammation, (**4D**) phagocytosis, and (**4E**) antigen
744 processing and presentation.
745
746 **Figure 4. In vitro model of monocyte to macrophage differentiation during exposure to *P.***
747 ***falciparum* blood-stage parasites.** Elutriated monocytes from healthy U.S. adults were
748 incubated for 24 hours with medium alone, uninfected red blood cells (RBC) or *Pf*-iRBC
749 (monocyte:*Pf*-iRBC ratio 1:15). At 24 hours, supernatants and cells were collected from some
750 replicate wells for cytokine analysis, and the ChIP assay, while monocytes in other replicate
751 wells were washed and incubated for 3 additional days in human serum plus medium to allow
752 monocytes to differentiate into macrophages (Mf). On day 5, the three populations of

753 macrophages (Mf, RBC-Mf and *Pf*-iRBC-Mf) were either harvested for ChIP and cytokine gene
754 expression analysis; or re-stimulated with *Pf*-iRBCs or LPS for 24 hours prior to supernatants
755 and cells being collected for cytokine analysis.
756
757 **Figure 5. Pre-exposure of monocytes to *P. falciparum* dampens subsequent inflammatory**
758 **responses to *P. falciparum* and LPS.** Monocytes of U.S. adults were incubated with medium,
759 RBC or *Pf*-iRBC. At 24 hours, cells were analyzed by (**A**) cytokine gene expression Taqman arrays
760 (n=3 subjects), and supernatants were analyzed by a bead-multiplexed assay (n=9 subjects) to
761 quantify (**D**) TNF, (**E**) IL-6 and (**F**) IL-1β. In the same experiment, replicate monocytes were
762 incubated with medium, RBC or *Pf*-iRBC, washed at 24 hours and incubated for 3 additional
763 days in human serum to permit macrophage (Mf) differentiation. On day 5, the three
764 populations of macrophages were analyzed by (**B**) cytokine gene expression arrays (n=3
765 subjects). Finally, in the same experiment, replicate monocytes were incubated with medium,
766 RBC or *Pf*-iRBC, washed at 24 hours, incubated for 3 additional days in human serum to permit
767 Mf differentiation, and then co-cultured with *Pf*-iRBC or LPS for 24 hours. Cells were harvested
768 for (**C**) cytokine gene expression arrays (n=3 subjects), and supernatants were analyzed (n=9
769 subjects) to quantify TNF, IL-6 and IL-1β induced by (**G-I**) *Pf*-iRBC or (**L-N**) LPS. ΔCt values (mean
770 ±SE) for (**J**) TNF and (**K**) IL-6 at the indicated timepoints for the 3 subjects shown in A-C. (A-C)
771 Heatmaps were generated from ΔCt values, with lower ΔCt values corresponding to higher gene
772 expression. ΔCt values were normalized to 18S rRNA expression. (D-I and L-N) Lines represent
773 median values. Data were analyzed by the Wilcoxon test with Bonferroni adjustment, and
774 significance levels between the groups are indicated by P values. (J and K) Two-way ANOVA was

775 performed followed by Tukey's multiple comparisons test. Significance level between
776 conditions (Pf-iRBC stim vs. Control Medium and Pf-iRBC stim vs. RBC stim, respectively) are
777 indicated by P values at each timepoint.
778
779 **Figure 6. *P. falciparum* exposure drives epigenetic reprogramming of monocyte/macrophages**
780 **toward a tolerant phenotype.** Monocytes of U.S. adults (n=4) were incubated with medium,
781 RBC or *Pf*-iRBC for 24 hours and then analyzed by chromatin immunoprecipitation (ChIP) and
782 RT-PCR to quantify H3K4me3 enrichment at (**A**) TNF and (**D**) IL-6 promoter sites. Replicate
783 monocytes were incubated with medium, RBC or *Pf*-iRBC, washed at 24 hours, and incubated
784 for 3 days in human serum to permit Mf differentiation. On day 5, cells were analyzed by ChIP
785 and RT-PCR to quantify H3K4me3 enrichment at (**B**) TNF and (**E**) IL-6 promoter sites. Finally,
786 replicate monocytes were incubated with medium, RBC or *Pf*-iRBC, washed at 24 hours,
787 incubated for 3 days in human serum, co-cultured with *Pf*-iRBC for 24 hours, and analyzed by
788 ChIP and RT-PCR to quantify H3K4me3 enrichment at (**C**) TNF and (**F**) IL-6 promoter sites.
789 Kinetics of H3K4me3 enrichment at (**G**) TNF and (**H**) IL-6 promoter sites across indicated
790 timepoints. Results are shown as means ±SEM fold enrichment of H3K4me3 antibody as
791 percent of input. (A-F) One-way ANOVA with Tukey's adjustment for multiple comparisons. P
792 values indicate level of significance. (G, H) Two-way ANOVA with Tukey's adjustment for
793 multiple comparisons. P values indicate level of significance between Pf-iRBC vs. medium and
794 Pf-iRBC vs. RBC, respectively.
795

796 **Supplementary Table**: Table of differentially expressed genes of Pf-iRBC-stimulated PBMCs of
797 Malian adults versus children.
798 This table shows statistically significant (padj<0.01) differentially expressed genes in monocytes
799 from Malian adults stimulated with Pf-iRBC relative to Pf-iRBC stimulated monocytes from
800 Malian children. Adjusted P values were calculated with the Benjamini-Hochberg method.
801
802 **Supplementary Figure 1.**
803 **Titration of monocyte:*Pf*-iRBC ratio for vitro model of monocyte to macrophage**
804 **differentiation.** Elutriated monocytes from healthy U.S. adults (n=3) were co-cultured with
805 increasing concentrations of *Pf*-iRBCs. After 24 hours, IL-1β (**A**), TNF (**B**) and IL-6 (**C**) were
806 measured in supernatants, and (**D**) cell viability was determined by trypan blue dye exclusion
807 and expressed as percent viability.
808
809 **Supplementary Figure 2.**
810 **Cell viability at day 5 in the vitro model of monocyte to macrophage differentiation.**
811 Elutriated monocytes from healthy U.S. adults (n=9) were incubated for 24 hours with medium
812 alone, uninfected red blood cells (RBC) or *Pf*-iRBC (monocyte:*Pf*-iRBC ratio 1:15). At 24 hours,
813 cells were washed and incubated for 3 additional days in human serum plus medium to allow
814 monocytes to differentiate into macrophages (Mf). To quantify cell viability on day five, 10% v/v
815 alamarBlue HS was added to the culture medium of the three populations of macrophages (Mf,
816 RBC-Mf and *Pf*-iRBC-Mf) for 5 hours and fluorescence intensity (FI) was measured according to
817 the manufacturer's instructions. FI was normalized to the fluorescence signal in media without

818 cells. Data were analyzed by the Wilcoxon test with Bonferroni adjustment, and levels of

819 significance between the groups are indicated.

FIGURE-1

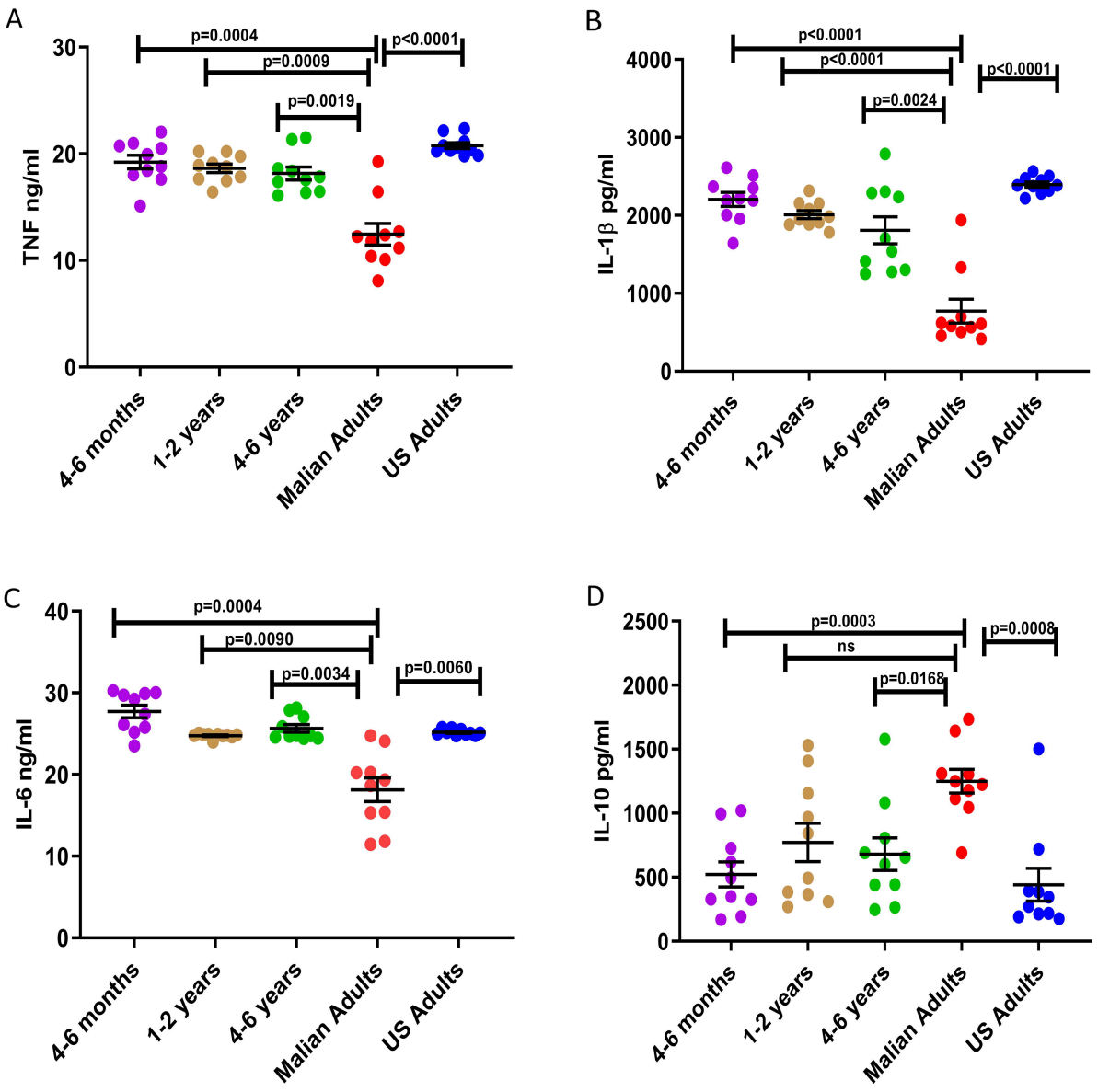

A
TNF ng/ml
p=0.0004
p=0.0009
p<0.0001
p=0.0019
4-6 months
1-2 years
4-6 years
Malian Adults
US Adults
B
IL-1β pg/ml
p<0.0001
p<0.0001
p<0.0001
p=0.0024
C
IL-6 ng/ml
p=0.0004
p=0.0090
p=0.0034
p=0.0060
D
IL-10 pg/ml
p=0.0003
ns
p=0.0168
p=0.0008

FIGURE-2

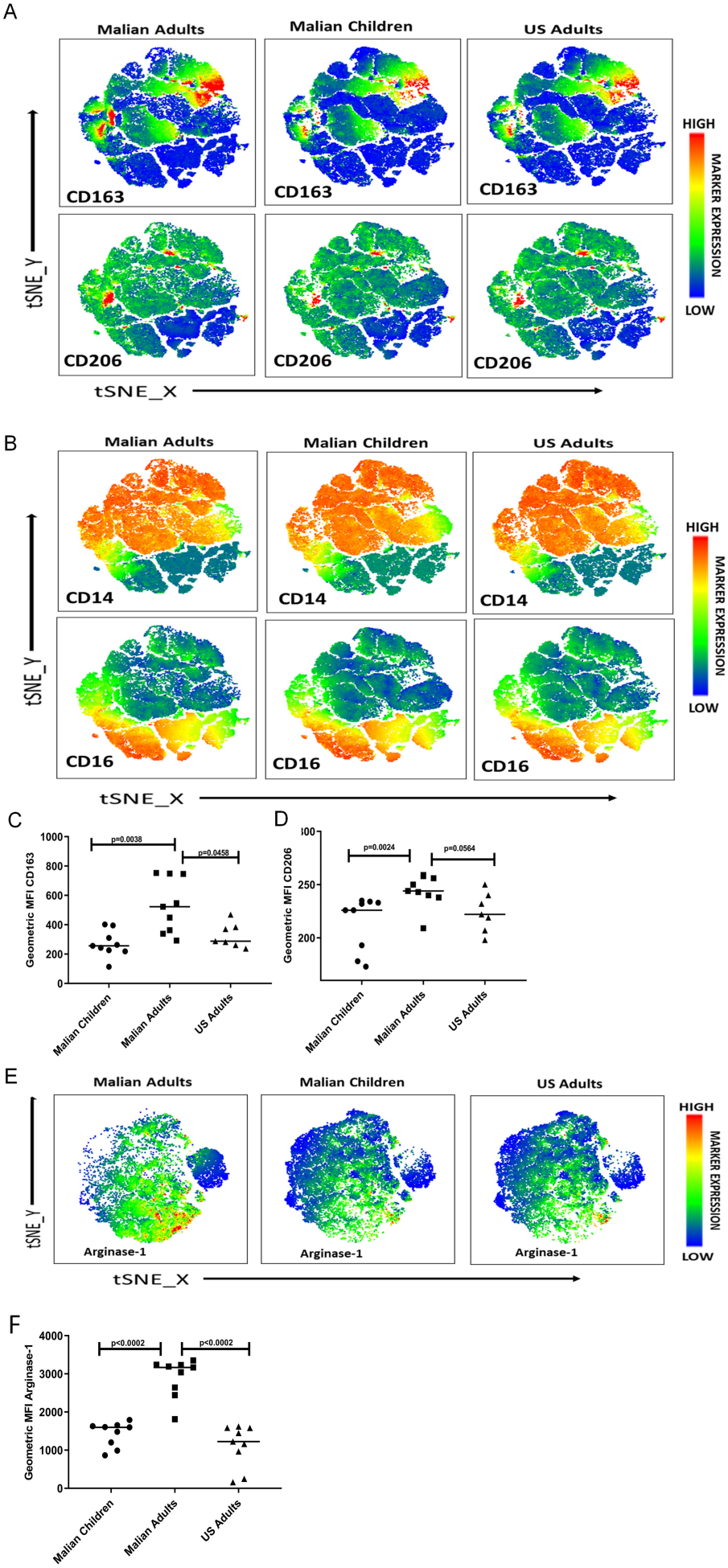

A
Malian Adults
Malian Children
US Adults
CD163
CD206
HIGH
LOW
MARKER EXPRESSION
tSNE_Y
tSNE_X
B
CD14
CD16
C
Geometric MFI CD163
p=0.0038
p=0.0458
Malian Children
Malian Adults
US Adults
D
Geometric MFI CD206
p=0.0024
p=0.0564
E
Arginase-1
F
Geometric MFI Arginase-1
p<0.0002
p<0.0002

FIGURE-3

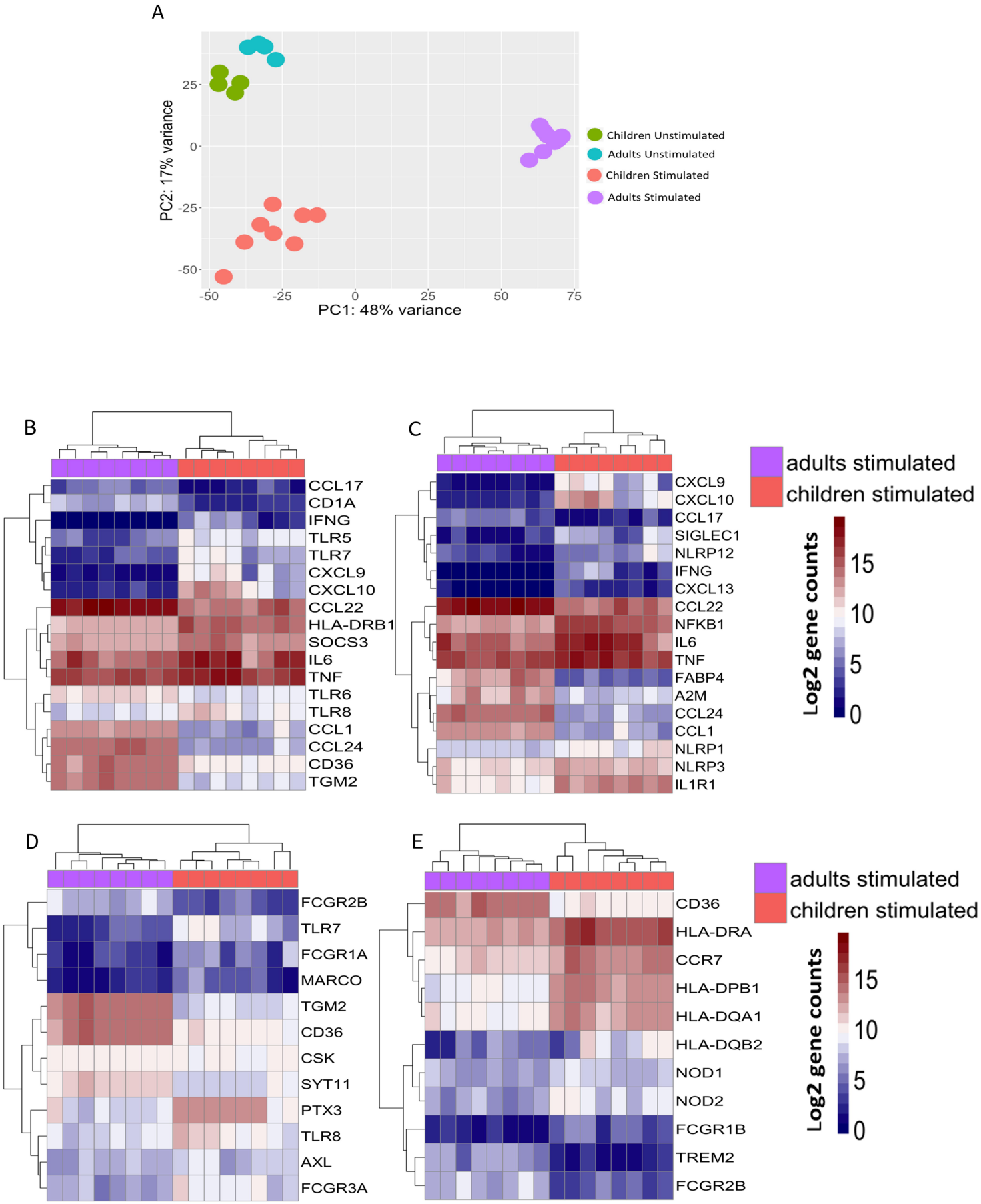

A
PC2: 17% variance
PC1: 48% variance
25
0
-25
-50
-50
-25
0
25
50
75
Children Unstimulated
Adults Unstimulated
Children Stimulated
Adults Stimulated
B
CCL17
CD1A
IFNG
TLR5
TLR7
CXCL9
CXCL10
CCL22
HLA-DRB1
SOCS3
IL6
TNF
TLR6
TLR8
CCL1
CCL24
CD36
TGM2
C
CXCL9
CXCL10
CCL17
SIGLEC1
NLRP12
IFNG
CXCL13
CCL22
NFKB1
IL6
TNF
FABP4
A2M
CCL24
CCL1
NLRP1
NLRP3
IL1R1
adults stimulated
children stimulated
Log2 gene counts
15
10
5
0
D
FCGR2B
TLR7
FCGR1A
MARCO
TGM2
CD36
CSK
SYT11
PTX3
TLR8
AXL
FCGR3A
E
CD36
HLA-DRA
CCR7
HLA-DPB1
HLA-DQA1
HLA-DQB2
NOD1
NOD2
FCGR1B
TREM2
FCGR2B
adults stimulated
children stimulated
Log2 gene counts
15
10
5
0

FIGURE-4

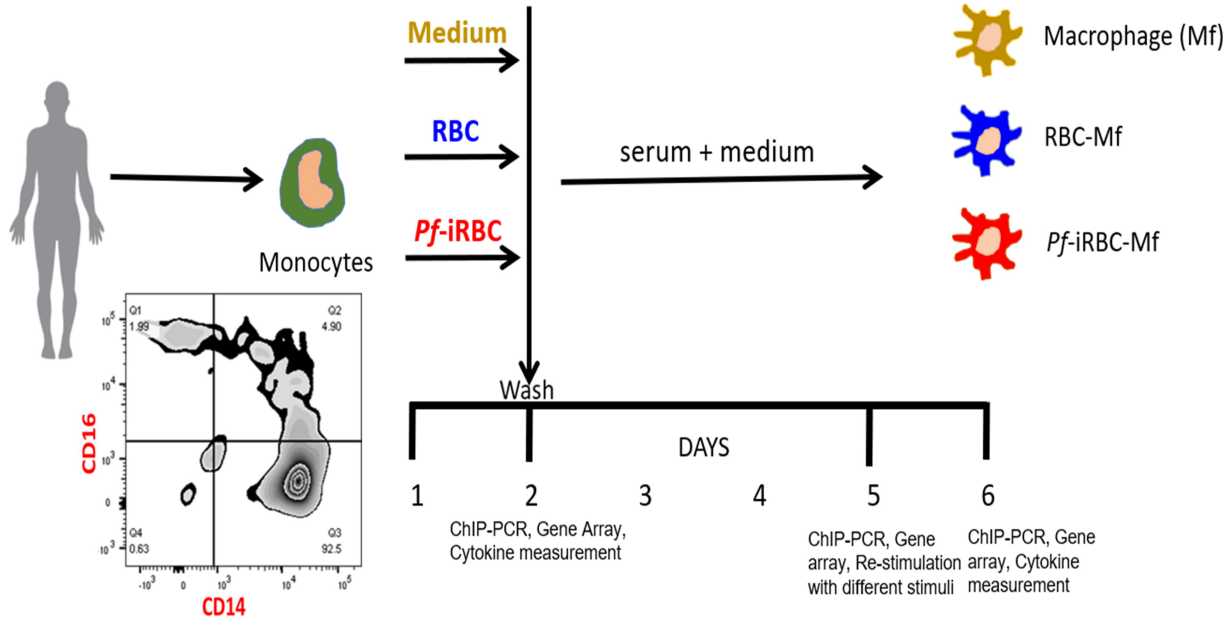

Medium
RBC
Pf-iRBC
Monocytes
serum + medium
Macrophage (Mf)
RBC-Mf
Pf-iRBC-Mf
Wash
DAYS
1
2
3
4
5
6
ChIP-PCR, Gene Array, Cytokine measurement
ChIP-PCR, Gene array, Re-stimulation with different stimuli
ChIP-PCR, Gene array, Cytokine measurement
CD16
CD14
Q1
1.99
Q2
4.90
Q4
0.63
Q3
92.5

FIGURE-5

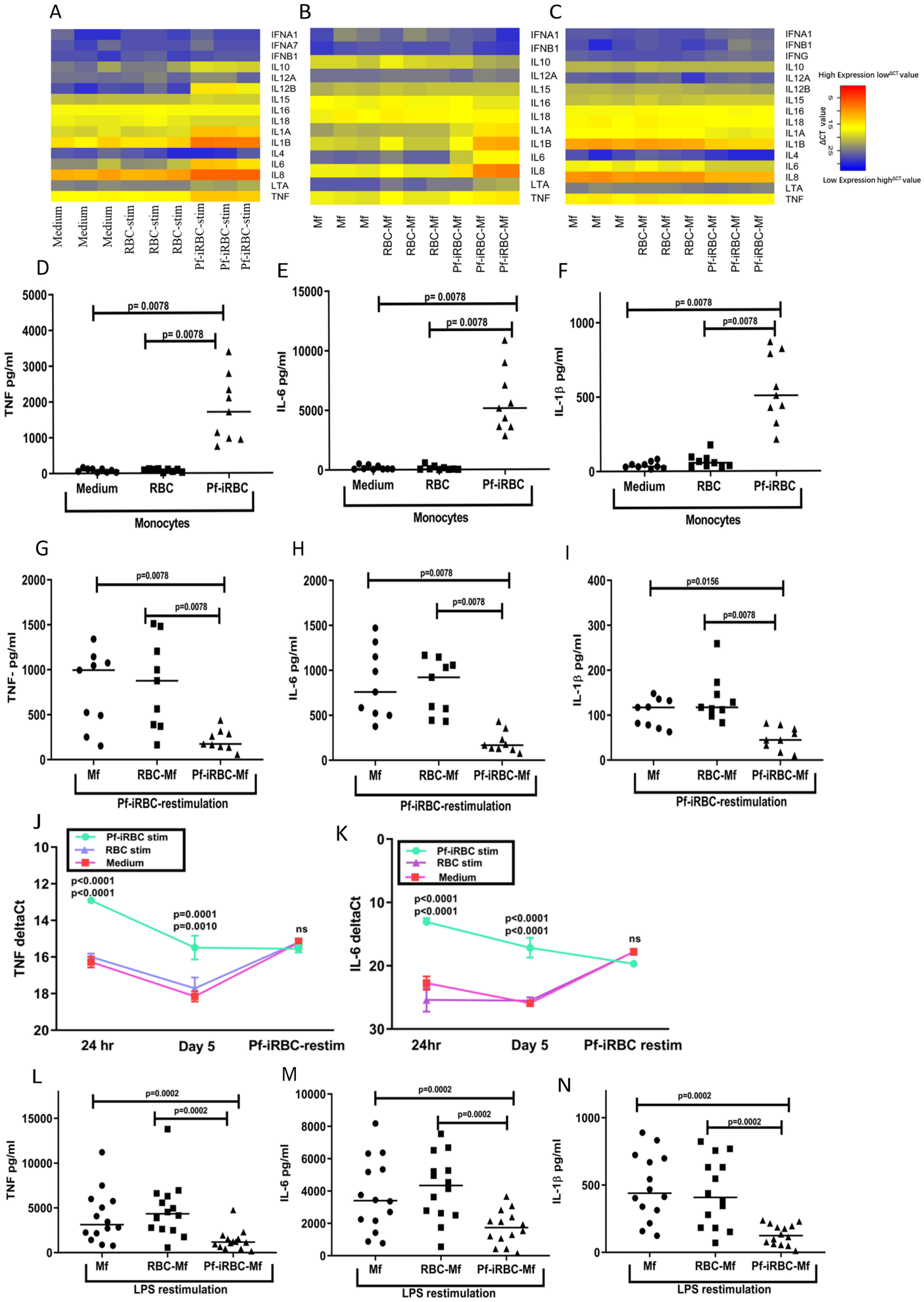
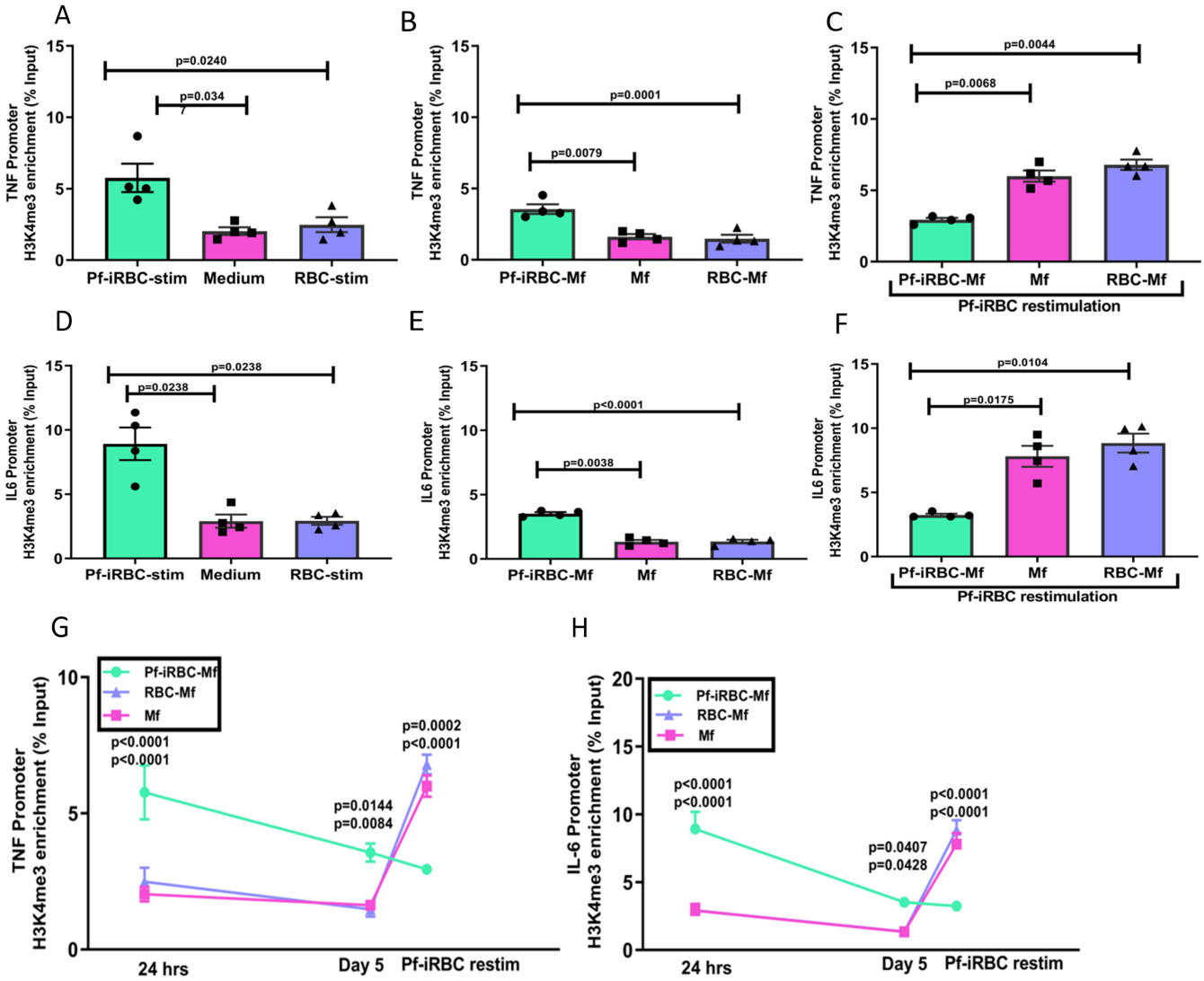

A
B
C
IFNA1
IFNA7
IFNB1
IL10
IL12A
IL12B
IL15
IL16
IL18
IL1A
IL1B
IL4
IL6
IL8
LTA
TNF
Medium
RBC-stim
Pf-iRBC-stim
Mf
RBC-Mf
Pf-iRBC-Mf
IFNG
High Expression low^ΔCT value
Low Expression high^ΔCT value
ΔCT value
D
E
F
TNF pg/ml
IL-6 pg/ml
IL-1β pg/ml
p= 0.0078
p=0.0078
Medium
RBC
Pf-iRBC
Monocytes
G
H
I
TNF- pg/ml
p=0.0156
Mf
RBC-Mf
Pf-iRBC-Mf
Pf-iRBC-restimulation
J
K
Pf-iRBC stim
RBC stim
Medium
TNF deltaCt
IL-6 deltaCt
p<0.0001
p=0.0001
p=0.0010
ns
24 hr
Day 5
Pf-iRBC-restim
24hr
Pf-iRBC restim
L
M
N
LPS restimulation

FIGURE-6


A
TNF Promoter
H3K4me3 enrichment (% Input)
p=0.0240
p=0.034
Pf-iRBC-stim
Medium
RBC-stim
B
p=0.0001
p=0.0079
Pf-iRBC-Mf
Mf
RBC-Mf
C
p=0.0044
p=0.0068
Pf-iRBC restimulation
D
IL6 Promoter
p=0.0238
p=0.0238
E
p<0.0001
p=0.0038
F
p=0.0104
p=0.0175
G
p<0.0001
p<0.0001
p=0.0144
p=0.0084
p=0.0002
p<0.0001
24 hrs
Day 5
Pf-iRBC restim
H
IL-6 Promoter
p<0.0001
p<0.0001
p=0.0407
p=0.0428
p<0.0001
p<0.0001