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Introduction

The clinical spectrum of *P. falciparum* infection ranges from asymptomatic parasite carriage to a febrile disease that may develop into a severe, life-threatening illness. The factors that determine disease severity are not completely understood but are likely to include both parasite and host components [1–3]. Ultimately, the interplay between the parasite and the immune response likely determines the outcome of the infection [4]. Although sterile immunity - completely preventing re-infection - is hardly ever seen and protection against clinical symptoms of uncomplicated disease is only acquired after repeated infections [5], immunity to severe disease and death may be acquired after as few as one or two infections [6] suggesting that different immune mechanisms underlie these different levels of immunity.

While there is a growing consensus that killing of malaria parasites or malaria-infected red blood cells requires the synergistic action of antibodies and cell-mediated immune responses [5,7], the mechanisms conferring protection against severe disease are less clear. Given that pathology of severe disease has repeatedly been linked to sustained and/or excessive inflammatory responses [4], acquiring the ability to regulate these responses adequately may be a key determinant of immunity that protects against severe disease [8]. Thus, while an early inflammatory response is needed to control parasite replication in human *P. falciparum* malaria [9–11], excessive levels of pro-inflammatory cytokines such as TNF-α [12–15], IFN-γ [16,17], IL-1β and IL-6 [14,18,19] are associated with severe pathology. Conversely, low levels of regulatory cytokines such as TGF-β have been associated with acute [20] and severe malaria [21,22], a relative deficiency in IL-10 was seen in those who succumbed to severe malaria [23], significantly lower ratios of IL-10 to TNF-α were found in patients with severe malarial anaemia [24,25], and high ratios of IFN-γ, TNF-α and IL-12 to TGF-β or IL-10 were associated with decreased risk of malaria but increased risk of clinical disease in those who became infected [26]. In summary, therefore, immunity against severe malaria may depend upon the host’s ability to regulate the magnitude and timing of the cellular immune response, allowing the sequential induction of appropriate levels of inflammatory- and anti-inflammatory cytokines at key stages of the infection.

Given these associations between severe disease and exacerbating immune pathology, a number of studies have explored the role of CD4+CD25+FOXP3+CD127−/low regulatory T cells (Tregs) in determining the outcome of malaria infection. Induced and/or activated in response to malaria infection [27], Tregs may be beneficial to the host in the later part of the infection - when parasitaemia is being cleared - by down-regulating the inflammatory response and thereby preventing immune-mediated pathology. On the other hand, if Tregs mediate their suppressive effects too early, this could hamper the responses required for initial
control of parasitaemia, permitting unbridled parasite growth which may also lead to severe disease. Malaria-specific induction of Tregs has been observed in a variety of experimental malaria infections in mice [28–31], but their role in preventing severe malarial pathology is unclear. Thus, in BALB/c mice infected with a lethal strain of P. yoelii, ablation of Treg activity by depletion of CD25+ cells either allowed mice to control parasitaemia and survive [32] or had no impact on the course of disease [33]. Depleting CD25+ cells of BALB/c mice infected with either P. berghei ANKA [34] or P. berghei ANK1 [35] reduced neither parasitaemia nor mortality, but increased the severity of symptoms in the diseased mice, suggesting at least some benefit from Tregs in this model. Rather oddly, injection of CD25+ T cell-depleted BALB/c mice with P. chabaudi adami DS led to increased parasitaemia and more severe anaemia [30]. Finally, CD25+ T cell depletion around the time of parasite inoculation reduced the incidence of experimental cerebral malaria in C57BL/6 mice infected with P. berghei ANK1 in two independent studies [29,31], but not when CD25+ T cells were depleted 30 days prior to infection [31]. Whilst various explanations have been offered for these discrepant results, including differences in the various strains of mice and parasites employed, the microbial microenvironment in which the mice are kept, and the precise CD25 depletion protocols employed, these studies are currently not very helpful when trying to understand the role of Tregs in human malaria infections.

Malaria naive individuals undergoing experimental P. falciparum sporozoite infection showed an increase in FOXP3 mRNA expression and expansion of Tregs 10 days after infection; Treg induction correlated with high circulating levels of TGF-β, low levels of pro-inflammatory cytokines and rapid parasite growth [27] suggesting - but not proving - that Treg activation early in infection may inhibit the development of effective cellular immunity. More recently, we have observed that Treg populations appear to be transiently expanded and activated during the malaria transmission season in individuals from a malaria endemic community [36], again suggesting that naturally acquired malaria infection can drive the expansion and activation of Tregs. However, although Tregs have been implicated in IL-10-mediated down-regulation of Th1-like responses in the placenta of malaria-infected women [37] and reduced Treg frequencies and function have been linked to enhanced anti-parasite immunity in certain ethnic groups in West Africa [38] the potential for Tregs to influence the clinical outcome of malaria infections is still unclear.

To investigate the role of Tregs during clinical malaria infection, we have compared cellular immune responses of children with either severe or uncomplicated malaria. Interestingly, although we did not observe any significant differences in Treg numbers or function between severe and uncomplicated malaria cases, our data do indicate that malaria-induced Tregs may limit the magnitude of malaria-specific Th1 memory responses and thus moderate pro-inflammatory responses to subsequent infections, providing a possible explanation for the very rapid acquisition of immunity to severe malaria. Moreover, we have identified a population of FOXP3+, CD45RO+, CD4+ T cells which co-produce IL-10 and IFN-γ and which are more prevalent in children with uncomplicated malaria than in those with severe disease. We suggest that these IL-10 producing effector T cells may contribute to clearing malaria infection without-inducing immune-mediated pathology.

Results

Immune responses of 59 Gambian children with severe P. falciparum malaria were compared with those of 65 children with uncomplicated clinical malaria and with 20 healthy (control) children of similar age and recruited from the same study area at the same time (Table 1). On admission, only 12 (9.4%) patients had a white blood cell count (WBC) above the age-specific norm and there was no significant difference in median WBC count between uncomplicated and severe cases, suggesting that few if any children had a concomitant systemic bacterial infection. No difference was observed in the differential WBC between the two groups. As expected, [39–41] numbers of lymphocytes, CD3+ and CD4+ T cells were significantly lower during the acute disease than during convalescence in both the severe and the uncomplicated groups (Table 1A). Parasite density on admission was two-fold higher in patients with severe malaria than in those with uncomplicated malaria; severely ill children also had significantly lower hemoglobin levels and were on average 2.4 years younger than children with uncomplicated disease. The number of P. falciparum clones per clinical isolate ranged from 1 to 4 (with an overall mean of 2 (CI95%: 1.8–2.1)), and – as has been observed previously [42] - did not differ significantly between the three groups (p = 0.3, Table 1B); Other factors potentially confounding immune responses, such as the degree of malnutrition or intestinal helminth infections were of similarly low prevalence in both severe and uncomplicated malaria cases and were not associated with severity of disease (Table 1B).

For statistical analysis, patients were classified as uncomplicated or severe cases, with the latter being further subdivided into those patients suffering from cerebral malaria (CM), severe anaemia (SA) or severe respiratory distress (SRD) (grouped together as Sx) and those suffering only from severe prostration (grouped as Sp). Data were analysed using linear regression, with a random effect to allow for the within subject measurements over time, adjusting for age, sex, duration of prior symptoms and numbers of clones causing the infection. Due to the multiplicity of comparisons that were made within the model, resulting from multiple responses and multiple comparisons within response, hypotheses rejected with a probability of less than 0.012 have a false discovery rate of 5% [43].
Similar numbers and proportions of Tregs among uncomplicated and severe malaria cases, increasing during convalescence

We hypothesized that children with severe malaria would have fewer circulating Tregs than children with uncomplicated malaria, or that Tregs of severely ill children would be less active than those of children with uncomplicated disease. However, the proportion of cells expressing a Treg phenotype (defined by flow cytometry as CD3+CD4+ lymphocytes being FOXP3+ and CD127low; Figure 1A, 1B, and 1C) was similar in the acute (D0) and the convalescent phase (D28) for both uncomplicated and severe cases, increasing significantly and similarly elevated in both severe and uncomplicated malaria cases during convalescence (Figure 3D). Taken together, these data indicate that in both uncomplicated and severe cases of malaria infections - a significant positive correlation between parasite density and the frequency of Tregs within the CD4+ T cell population (p = 0.002, Figure 2).

Tregs display an activated memory phenotype during acute disease

Since fully differentiated Tregs predominantly express an activated/memory phenotype [44] T cells from children with severe and uncomplicated malaria were analysed for expression of CD45RO (Figure 3A and 3B). In both uncomplicated and severe cases, the proportion of all T cells expressing CD45RO was significantly higher (p < 0.001) during acute disease than during convalescence (data not shown). Irrespective of disease severity, more than 90% of Tregs expressed CD45RO (Figure 3A and 3B). In both uncomplicated and severe cases, the proportion of all T cells expressing CD45RO was significantly higher (p < 0.001) during acute disease than during convalescence (data not shown). Irrespective of disease severity, more than 90% of Tregs expressed CD45RO during the early stages of blood stage infection [27], we observe here in - children with either severe or uncomplicated malaria infections - a significant positive correlation between parasite density and the frequency of Tregs within the CD4+ T cell population (p = 0.002, Figure 2).
Tregs are predominantly of a memory phenotype and are activated during acute malaria infection. Similar Treg function in uncomplicated and severe malaria patients

Three different indicators were used to assess the regulatory potential of Tregs during acute malaria infection. Firstly, using a classical anti-CD25 depletion assay, we assessed the ability of Tregs to suppress *P. falciparum* shizont extract (PfSE)-driven lymphocyte proliferation. Anti CD25 treatment removed approximately half (geometric mean 48.8%; CI95%: 41–58%) of the CD4^+^ T cells that were FOXP3^+^CD127^2^low and this was associated with a 1.76 fold and 1.57-fold (geometric means) increase in PfSE-induced lymphoproliferation in severe and uncomplicated cases (U) for days 0 (D0) and 28 (D28) and compared to healthy controls of a similar age range recruited from the study area at the same time. (E) absolute numbers of cells expressing a Treg phenotype. (F) FOXP3 mRNA levels expressed as a ratio to the house keeping gene 18s rRNA. Dots represent individual data; bars represent the median. P-values are derived from the random effects linear regression model, adjusting for age, sex, duration of symptoms and numbers of clones isolated.

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**Figure 1. Similar number and proportion of Tregs in uncomplicated and severe disease, increasing during convalescence.** Tregs were defined as CD3^+^CD4^+^PBMC being FOXP3^+^and CD127^low^: (A) CD3^+^ viable PBMC were displayed according to CD4 and FOXP3 and FOXP3 positive cells were defined on contour display as cells above the 90% contour level. (B) CD3^+^CD4^+^FOXP3^+^ PBMC were displayed according to CD127 expression to define the CD127 low cut off. (C) both gates were applied to CD3^+^CD4^+^ PBMC. (D) The percentage of Tregs for severe cases is shown for the subgroup (S_a) comprising cases with CM (triangles), SA (squares) and SRD (circles), for the subgroup with severe prostration (S_p) and for uncomplicated cases (U) for days 0 (D0) and 28 (D28) and compared to healthy controls of a similar age range recruited from the study area at the same time. (E) absolute numbers of cells expressing a Treg phenotype. (F) FOXP3 mRNA levels expressed as a ratio to the house keeping gene 18s rRNA. Dots represent individual data; bars represent the median. P-values are derived from the random effects linear regression model, adjusting for age, sex, duration of symptoms and numbers of clones isolated.

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**Similar Treg function in uncomplicated and severe malaria patients**

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uncomplicated cases, respectively, with no significant difference between the groups (p = 0.343, Figure 4A).

Next, since reduced expression of SOCS-2, a member of the suppressors of cytokine signaling family confined to Tregs [45], has been linked to impaired Treg function in Africans [38] we compared SOCS-2 mRNA levels among severe and uncomplicated cases. While SOCS-2 levels were found to be significantly reduced during acute disease compared to convalescence (p = 0.0076), no difference was observed between those with severe and those with uncomplicated malaria (data not shown).

Finally, since high concentrations of TNF-α have been reported to impair Treg activity [upregulating and then signaling via TNFR2, leading to decreased SOCS-2 mRNA levels among severe and uncomplicated cases. While SOCS-2 levels were found to be significantly reduced during acute disease compared to convalescence (p = 0.0076), no difference was observed between those with severe and those with uncomplicated malaria (data not shown).

These data confirm previous studies indicating a shift towards a more inflammatory response during acute and severe malaria but, significantly, our data extend the previous observations by revealing that this inflammation is not balanced by a commensurate increase in Treg function. Indeed, our data strongly suggest that the potent inflammation induced during an acute malaria infection overwhelms the normal homeostatic capacity of the immune system and, in particular, that the Treg response in children with severe malaria is insufficient to balance a much stronger Th1 effector response.

To investigate further the dynamics of pro-inflammatory/regulated responses during clinical malaria, plasma concentrations and mRNA transcripts of inflammatory (IFN-γ, TNF-α) and regulatory cytokines (IL-10) were assayed. In accordance with previous observations, plasma concentrations of IFN-γ, TNF-α and IL-10 were all significantly higher during acute disease than during convalescence, with significantly higher levels in severely ill children compared to uncomplicated cases (Figure 5B, 5C, and 5D). Levels of mRNA transcripts for IL-10 and IFN-γ were also significantly elevated in all groups during acute disease, but there was no significant difference between severity groups (Figure S1A and S1B). For both severe and uncomplicated cases, levels of IFN-γ mRNA were highly correlated with levels of IL-10 mRNA during the acute phase (severe: r = 0.833 p<0.001, uncomplicated: r = 0.693 p<0.001), suggesting that IFN-γ production is being balanced by IL-10 production. Interestingly, IFN-γ mRNA levels on day 0 correlated with FOXP3 mRNA on day 0 for both severe (r = 0.39 p = 0.003) and uncomplicated cases (r = 0.44 p = 0.0001), suggesting that IFN-γ may also be driving FOXP3 expression.

The balance of pro-and anti-inflammatory cytokine responses clearly changed with time, but somewhat surprisingly, there were no marked differences in cytokine ratios between children with differing levels of disease severity. Thus, the ratios of TNF-α or IFN-γ to IL-10 on day 0 were similar in all disease severity groups (Figure 5E and 5F) and ratios of IFN-γ to IL-10 mRNA were similar in all disease severity groups both on day 0 and day 28 (Figure S1F). However, IFN-γ mRNA levels were on average only 3.2-fold higher on day 0 than day 28 but IL-10 mRNA levels were

Stronger Th-1 responses observed in severe compared to uncomplicated cases are balanced by IL-10

Since the balance of T-effector to Treg responses is likely to be as important, or more important, than the absolute levels of either [26], we compared the ratio of the levels of mRNA for the Th1 transcription factor T-BET with those for FOXP3, currently considered the best marker for Tregs, and the ratio of T-effector cells (defined as CD3+CD4+CD25−FOXP3−, T-effector) over Tregs among the various groups. As shown in Figure 5A, in all groups the T-BET/FOXP3 ratio was significantly higher during acute disease than during convalescence and a similar, albeit not significant, trend was observed for the ratio of T-effector/Tregs (data not shown). Moreover, since the absolute number of circulating T-effector cells was significantly higher in severe cases than in uncomplicated cases (p = 0.01, data not shown), the T-effector/Treg ratio tended to be higher among severe cases than in uncomplicated cases on day 0 (p = 0.039) and a similar trend was seen for the T-BET/FOXP3 ratio (p = 0.058). The ratio of FOXP3 to GATA-3 (Th2 lineage factor) mRNA was similar for both time points in all groups (data not shown) but the Th1/Th2 ratio (T-BET/GATA-3 mRNA) was significantly higher during acute disease in children with CM, SA or SRD compared to those suffering from severe prostration (p = 0.0075), indicating that the expansion of the T-effector population is biased towards Th1 responses.
29-fold higher on day 0, resulting in a significantly lower IFN-γ/IL-10 mRNA ratio on day 0 than day 28 (Figure S1F).

CD25−FOXP3− CD45RO+ T cells but not Tregs are the major source of IL-10 during acute malaria

IL-10 is a crucial immunoregulatory cytokine in both human [23] and murine [33,49] malaria; we have recently identified CD4+ T effector cells as a major source of IL-10 [33], but the source of IL-10 in human malaria infection is unknown. In other protozoal infections of mice CD4+ effector T cells that co-produce IFN-γ and IL-10 have been identified [50–52]. We therefore cultured freshly isolated PBMCs from 30 children with acute malaria (17 severe, 13 uncomplicated) and 20 healthy control children, with or without PMA and Ionomycin (PI), for 5 hours and analyzed them for the presence of intracellular IL-10 and IFN-γ by flow cytometry (Figure 6A). No cytokine production was observed in unstimulated cells (data not shown), and PBMC from healthy children failed to produce any IL-10 in response to PI (data not shown), indicating that stimulation with PI predominantly induces cytokine production from recently activated cells.

By contrast, distinct populations of IL-10+ and IFN-γ+ cells were seen among the PI-stimulated cells from children with acute severe or uncomplicated malaria, with a small but easily distinguishable population of cells (approx 1% of all PBMC) producing both cytokines simultaneously (Figure 6A, right plot). In both severe and uncomplicated cases, IL-10 producing cells were predominantly CD45RO+ CD4+ T cells (Figure 6B and 6C) and were almost exclusively FOXP3− and CD25− (Figure 6D). Moreover, although a transient increase of FOXP3 in activated human T-effector cells has been reported [53], in our hands less than 1% (median 0.97%, CI95%: 0.67–1.27%) of IFN-γ producing cells were FOXP3+ (Figure 6E).

Overall, among children with acute malaria, approx 4% of PI-stimulated CD4+ T cells produced IL-10 and approx 8% produced IFN-γ and neither the proportions of cells producing one or the other cytokine (Figure 6F) nor the ratio of IFN-γ/IL-10 producing cells (data not shown) differed significantly between severe and uncomplicated cases. However, intriguingly, the proportion of IFN-γ+ CD4+ T cells that also produce IL-10 was almost twice as high among uncomplicated cases as among severe cases (p = 0.045, Figure 6G).

Taken together, these data indicate that during acute, uncomplicated or severe, malaria infections IL-10 producing cells are overwhelmingly T effector cells and that Th1 effector cells that also produce IL-10 are more prevalent in children with uncomplicated malaria than in children with severe malaria.
The frequency of Tregs during acute disease is negatively associated with the magnitude of subsequent malaria-specific IFN-γ memory responses. It has been reported that Tregs present at the time of infection [54] or vaccination [55] may restrict the development of subsequent Th1 memory responses. To determine whether Tregs present during acute malaria infection might similarly affect the induction of immunological memory, we compared FOXP3 mRNA levels on day 0 with malaria-specific IFN-γ memory responses (as assessed by PfSE-specific cultured ELISPOT) among PBMC collected from 34 of our convalescent malaria patients (19 severe and 15 uncomplicated) on Day 28.

As shown in Figure 7A, cells from uncomplicated and severe cases mounted similarly strong IFN-γ memory responses following culture with PfSE. When plotted against FOXP3 mRNA levels measured on day 0, a linear by linear hyperbolic fit revealed that higher levels of FOXP3 mRNA on day 0 were highly significantly associated with lower malaria-specific IFN-γ memory responses on Day 28, suggesting that Tregs induced during the acute infection may limit the magnitude of subsequent Th1 responses (Figure 7B). For neither group could a significant effect of parasitaemia on the memory response be observed (r = 0.12, p = 0.962 for severe and r = 0.015, p = 0.957 for uncomplicated cases).

Discussion

We hypothesized that the balance of inflammatory to regulatory immune responses would be biased towards a more inflammatory response in children with severe malaria than in children with uncomplicated malaria, that this balance would be restored during convalescence and – crucially – that this would be associated with differences in the proportion, absolute number or function of circulating classical (CD4+ CD127⁻/lo FOXP3+) regulatory T cells. In partial support of these hypotheses, the number of cells expressing a Treg phenotype and FOXP3- mRNA levels were both significantly higher during convalescence than during the acute clinical episode and the ratio of the Th1 transcription factor T-BET to the Treg transcription factor FOXP3 was significantly higher during acute disease than during convalescence in both severe and uncomplicated cases, compatible with the notion that Tregs fail to sufficiently regulate pro-inflammatory responses which might contribute to the onset of symptomatic malaria infection.

Given our previous observation of Treg expansion during the pre-patent phase of malaria infection [27], we suggest that Tregs are induced/activated shortly after parasite emergence from the liver, that their numbers in peripheral blood then decline as a result of sequestration of CD4⁺ T cells during acute disease [40,41,56] and then, as has been described for other T cell subsets [39,57], Tregs regain access to the circulation after malaria is cured. The significant positive correlation of Treg numbers with parasitaemia, as well as the correlation between FOXP3 mRNA and IFN-γ mRNA levels in acute samples, further supports the notion that the initial infection induces a proportional increase in
Tregs, attempting to balance the effector T cell response, and is in line with the recently proposed concept that antigenic challenge will give rise to an antigen specific Treg response, proportional in size to the inflammatory response [58]. Moreover, the Tregs circulating during acute malaria infections almost exclusively expressed an activated memory phenotype suggesting that they have expanded from a pre-existing pool of memory T-cells. This interpretation would be in line with recent elegant work in humans demonstrating that Tregs are derived by rapid turnover of memory populations in vivo [59], and with data from murine studies where, after CD25-depletion, malaria infection very rapidly drives differentiation of Tregs from circulating mature CD4\(^+\) T cells [60]. Obviously, it would be of interest to study the relationship between Tregs and effector T cell kinetics and parasite biomass, which is not readily measurable. Future studies may explore the usefulness of P. falciparum Histidine Rich Protein 2 in this context, which has recently been suggested as a surrogate marker for parasite biomass [61].

However, despite clear evidence of Treg induction and reallocation during acute malaria infection, we could not find any robust differences in Treg parameters between children with severe and uncomplicated disease. Thus, neither Treg numbers nor FOXP3 mRNA levels differed significantly between children with uncomplicated malaria and those with severe malaria, and three different indicators of Treg function - their capacity to suppress lymphoproliferation, their expression of SOCS-2 [45] and TNFR2 [46,48] were all similar in severely ill children and children with uncomplicated disease. Furthermore, the similar distortion in the T-BET/FOX3 mRNA ratio during acute disease and the lack of any marked differences between the two groups in ratios of inflammatory to anti-inflammatory cytokines, as well as the close correlation between IFN-\(\gamma\) and IL-10 in both groups which is in line with previous observations in experimental human malaria infections [62], suggests that the systemic shift towards a pro-inflammatory immune response is similar in children with either severe or uncomplicated disease.

At first glance, these data do not appear to support the hypothesis that deficiencies in Treg function - their capacity to suppress lymphoproliferation, their expression of SOCS-2 [45] and TNFR2 [46,48] - were all similar in severely ill children and children with uncomplicated disease. However, we did observe significantly higher Th1 effector responses (more T-effector cells, higher concentrations of IFN-\(\gamma\) and TNF-\(\alpha\) in children with severe malaria than in children with uncomplicated disease, suggesting that the classical FOXP3\(^+\) Treg response that develops during acute malaria infection may be insufficient to balance the florid effector T cell response that develops particularly in children with severe disease. This would be in line with evidence showing that as the strength of the

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**Figure 5. Ratios of Th1 over Treg cells and pro-inflammatory over regulatory cytokines.** (A) Ratio of the mRNA level of the Th1 transcription factor T-BET over FOX3 for uncomplicated and severe cases during the acute phase (D0) and convalescence (D28) as determined by RT-PCR. Plasma levels for (B) IFN-\(\gamma\), (C) TNF-\(\alpha\), and (D) IL-10 measured with a bioplex system and the ratios of (E) IFN-\(\gamma\)/IL-10 protein as well as (F) TNF-\(\alpha\)/IL-10 protein on day 0. (NB: Protein ratios could not be calculated for Day 28, since cytokine concentrations were rarely above the detection limit of the assay. In Figure 5E and 5F only ratios for samples that had detectable responses for both cytokines on Day 0 are shown.) Symbols, bars and p-values as defined in Figure 1.

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Figure 6. Phenotypic characterization of cytokine-producing cells. PBMC from 30 children with acute *P. falciparum* infection were cultured with growth medium (GM) or pulsed with PMA and ionomycin (P/I) for 5 hours and assessed for the production of IL-10 and IFN-γ by flow cytometry. (A) Representative example of PBMC stained with isotype control (left panel) or anti-IL-10 and anti-IFN-γ (right panel). (B) Representative example of the phenotype of IL-10 producing cells. The geometric mean with CI95% is shown. (C) Proportion of IL-10 producing cells. (D) Representative example of co-staining for IL-10 and FOXP3 (left panel) or CD25 (right panel). Across all samples tested, 0.13% (median; CI95%: 0.09–0.16%) of PBMC stained positively for both IL-10 and FOXP3, corresponding to 2.4% (median; CI95%: 1.7–4%) of IL-10 producing cells being FOXP3+ . Approx 0.79% (median; CI95%: 0.69–1.1%) of CD3+ CD4+ PBMC were IL-10+ and CD25+, corresponding to a 8.68% (median; CI95%: 4.9–9.8%) of IL-10+ T cells being CD25+. (E) Representative example of co-staining for IFN-γ and FOXP3. Across all samples tested, 0.29% (median; CI95%: 0.21–0.37%) of PBMC stained positively for both IFN-γ and FOXP3, corresponding to 0.97% (median; CI95%: 0.67–1.27%) of IFN-γ producing cells being FOXP3+. (F) Percentage of IL-10 single
inflammatory stimulus increases, the suppressive capacity of human Tregs declines and the resistance of T-effector cells to regulation increases [63]. The situation observed during an acute, clinical malaria infection is thus in clear contrast to the situation in healthy, malaria-exposed individuals where Treg numbers closely track numbers of T-effectors, precisely maintaining an apparently optimal T-effector:Treg ratio [36].

IL-10 is well-established as a vital homeostatic regulator of malaria-induced inflammation that prevents immune-pathology in mice [49,64], promotes the necessary switch from early Th1 to subsequent Th2 responses [65,66], and has been linked to protection from severe malaria anaemia [24,25], and death [23] in humans. However, the cellular source of IL-10 in human malaria cases was, until now, ill defined. Contrary to our expectations, but in striking agreement with observations in P. yoelii-infected mice [33], CD45RO+ CD4+ T cells (that are CD25+ and FOXP3+) and not classical Tregs are the only substantial source of IL-10 during acute malaria infection. This observation is reminiscent of that made by Nylen [67] in patients with acute visceral leishmaniasis. Moreover, in our patients, a significant proportion of IL-10 producing CD4+ T cells were simultaneously producing IFN-γ, identifying them as Th1 cells. Although IL-10 secreting Th1 cells have been described recently in two murine models of toxoplasmosis [50], and cutaneous leishmaniasis [51], as far as we are aware, this is the first demonstration of IL-10 producing Th1 cells during human infections. Intriguingly, the proportion of these cells within the total CD4+ T cell population was significantly higher in children with uncomplicated malaria than in children with severe malaria suggesting that in human P. falciparum infection, as in murine T.gondii infections [50], IL-10 producing Th1 cells, activated by a strong inflammatory stimulus, may act as anti-parasitic effector cells with a “built in” control mechanism to prevent the onset of immune pathology. If so, then the ability of these self-regulating effector cells to localize to sites of parasite sequestration in tissues, where they mediate parasite killing whilst simultaneously blocking tissue damage, may be key to clinical immunity to malaria. Thus, our data strongly suggest that the percentage of IL-10-producing Th1 effector cells, rather than IL-10/IFN-γ double positive CD4+ T cells co-producing IL-10, as well as the percentage of IL-10 producing producing IFN-γ is shown for severe (open bars) and uncomplicated (shaded bars) cases. Bars in (F) and (G) represent the geometric mean with 95% CI.

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Cell preparation

Blood samples were processed within 2 hours of collection. Plasma was removed, stored at $-80\degree C$ and replaced by an equal volume of RPMI 1640 (Sigma-Aldrich). PBMC were isolated after density centrifugation over a 1.077 Nycoprep (Nycomed, Sweden) gradient (800 g, 30 min) and washed twice in RPMI 1640. Cells were either stained for flow cytometry directly ex-vivo, or cultured in RPMI 1640 containing 10% human AB+ serum, 100 mg/ml streptomycin, 100 U/ml penicillin (all Sigma-Aldrich), and 2 mM L-glutamine (Invitrogen Life Technologies), referred to as complete growth medium (GM).

Flow cytometry

Fresh PBMC were stained using the following fluorochrome labeled mouse or rat anti-human antibodies: FITC anti-TNF-receptor II (R&D), PE anti-FOXP3 (clone PCH101), Pacific Blue anti CD3, APC-Alexa Fluor 750 anti-CD127 (all Ebioscience), APC anti-CD25, PerCP anti CD4 (BD systems), ECD anti-CD45R0 (Beckman-Coulter), and appropriate isotype controls.

IL-10 and IFN-γ production by PBMC from 30 children with acute P. falciparum (D0) was assessed after 5 hours stimulation in GM containing PMA (50 ng/ml) and Ionomycin (1000 ng/ml) or GM alone.

Plasmodium falciparum parasites (3D7 strain) were cultured in vitro as described [73] and were routinely shown to be mycoplasma free by PCR (Bio Whittaker). Schizont-infected erythrocytes were harvested from synchronized cultures by centrifugation through a Percoll gradient (Sigma-Aldrich). PfSE was prepared by two rapid freeze-thaw cycles in liquid nitrogen and a 37$\degree C$ water bath. Extracts of uninfected erythrocytes (uRBC) were prepared in the same way.

Multiplex analysis of plasma cytokine concentration

Plasma concentrations of IFN-γ, TNF-α and IL-10 were determined for each subject and time point on the Bio-Plex H200 system, using X-Plex TM assays (both Bio-Rad Laboratories), according to the manufacturer’s instructions. Data were analysed using the Bio-Plex® Manager software. The detection limit was defined as the concentration corresponding to a fluorescence value above the mean background fluorescence in control wells plus 3 SD, being 8.76 pg/ml for IFN-γ, 5 pg/ml for TNF-α and 0.57 pg/ml for IL-10. Values below this threshold were set to these levels.

Multiplex analysis of plasma cytokine concentration

Plasma concentrations of IFN-γ, TNF-α and IL-10 were determined for each subject and time point on the Bio-Plex® 200 system, using X-Plex™ assays (both Bio-Rad Laboratories), according to the manufacturer’s instructions. Data were analysed using the Bio-Plex® Manager software. The detection limit was defined as the concentration corresponding to a fluorescence value above the mean background fluorescence in control wells plus 3 SD, being 8.76 pg/ml for IFN-γ, 5 pg/ml for TNF-α and 0.57 pg/ml for IL-10. Values below this threshold were set to these levels.

**Plasmodium falciparum** culture and schizont antigens

*P. falciparum* parasites (3D7 strain) were cultured in vitro as described [73] and were routinely shown to be mycoplasma free by PCR (Bio Whitaker). Schizont-infected erythrocytes were harvested from synchronized cultures by centrifugation through a Percoll gradient (Sigma-Aldrich). PfSE was prepared by two rapid freeze-thaw cycles in liquid nitrogen and a 37$\degree C$ water bath. Extracts of uninfected erythrocytes (uRBC) were prepared in the same way.

**Proliferation assay**

PBMC from 10 severe and 10 uncomplicated malaria cases collected on day 0 were depleted of CD25$^+$ cells or mock depleted using magnetic beads (Dynal Biotech, UK), at a bead to PBMC ratio of 7:1, and cell proliferation was determined by $[^{3}H]$-thymidine (Amersham, UK) incorporation after 6 days in culture.
with PISE, uRBC (RBC:PBMC ratio equivalent to 2:1), GM, or 2 days culture with PMA (10 ng/ml)+ Ionomycin (100 ng/ml), as described [27].

Cultured ELISPOT

Cultured ELISPOTs were performed to assess malaria specific IFN-γ memory responses, adapting an established method [74]. Up to 1 million PBMCs collected on day 28 were cultured in 24 well plates for 6 days in 1 ml GM and stimulated with either PISE, uRBC (RBC:PBMC ratio equivalent to 2:1), or GM respectively. At day 3, half the medium was exchanged and rIL-2 (final concentration 20 IU/well) was added. On day 6 cells were harvested, washed three times, and 1.5×10^5 cells seeded into duplicate wells onto Millipore MAIP S45 plates and restimulated overnight with PISE, uRBC (concentrations as above), GM or PHA-L (5 μg/ml). IFN-γ ELISPOT was performed using MabTech antibodies according to the manufacturer’s instructions. Spot forming cell numbers were counted using an ELISPOT plate reader (AutoImmuneDiagnostica, Vers. 3.2). Results are expressed as spot forming units (SFU) per million PBMC after subtraction of individual background values (GM for PHA-L, uRBC for PISE) being deducted. Assays were discounted if the positive control (PHA-L) was <50 SFU, or the negative control was >30 SFU.

RT-PCR

For quantitative reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from PAX tubes following the manufacturer’s instructions and reverse transcribed into cDNA using TaqMan® reagents for reverse transcription (Applied Biosystems), following the manufacturer’s protocol. Gene expression profiles for FOXP3, IL-10, SOCS-2 and IFN-γ were measured by RT-PCR on a DNA Engine Opticon® (MJ Research) with QuantiTect SYBR Green PCR kits (Qiagen Ltd) using primers (all Sigma Genosys) previously described: IFN-γ, IL-10, FOXP3 designed by [75], and SOCS-2 designed by [76].

T-BET and GATA-3 gene expression was determined using the TaqMan® Probe kit using the primers (all Metabion) designed by [77]. 18S rRNA, amplified using a commercially available kit (rRNA primers and VIC labeled probe, Applied Biosystems), was used as an internal control. Data were analysed using Opticon Monitor 3™ analysis software (BioRad) and are expressed as the ratio of the transcript number of the gene of interest over the endogenous control, 18S rRNA.

Parasite genotyping

Genomic DNA from each parasite isolate was genotyped by sequencing the highly polymorphic block 2 region of the msp1 gene to assess the number of clones infecting each patient [78].

Statistical analysis

Analysis was performed using linear regression, with a random effect to allow for the within subject measurements over time, where the response variables were log transformed to improve the normality and constant variance assumptions. Significance (measured at the 5% level) tests for the effects of malaria group (uncomplicated, S_A or S_B), time (day 0 and day 28) and their interaction were adjusted for the possible confounding effects of age, sex, duration of prior symptoms and numbers of clones causing the infection. Where there was no significant malaria group and time interaction, p-values for the overall comparison of day 0 vs. day 28 were given. Within day 0, comparisons of severe vs. uncomplicated and the two groups of severely ill patients (S_A vs S_B) were adjusted for any malaria group and time interactions. To allow for the multiplicity of tests resulting from multiple responses and multiple comparisons within a response performed in the model, a false discovery rate (FDR) of 5% was assumed. Using the Benjamini and Hochberg approach [43] only tests with a p-value below 0.012 have an FDR of ≤5%. Due to the large number of tests family-wise error rate correction methods were too conservative. Analyses were performed using Stata version 9 and Matlab version R2008a.

Supporting Information

Figure S1 mRNA levels for IFN-γ, IL-10 and Th-1 as well as Th-2 lineage transcription factors in severe and mild disease. Found at: doi:10.1371/journal.ppat.1000364.s001 (1.99 MB TIF)

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Author Contributions

Conceived and designed the experiments: MW SD EMR. Performed the experiments: MW OGF MN EJL. Ana SJ HHC NE. Analyzed the data: MW DJJ. Contributed reagents/materials/analysis tools: MW NGE DJC. Wrote the paper: MW EMR. Responsible for the statistical model applied to the data: DJJ. Contributed to FACS work: OCF. Contributed substantially to various aspects of the lab work: MN. Responsible for the recruitment of the study patients: AE. Designed the RT-PCR experiments: SD. Did most of the ELISPOTs: EJL. Performed the sequencing of the parasites: ANA. Contributed to flow cytometry and ELISPOT experiments: SJ. Contributed to parasite sequencing and prepared malaria antigens: HHC. Provided expertise on designing the RT-PCR assays and helped with parasite sequencing: NE. Recruited patients into the study and responsible for the conduct according to GCP guidelines: JO. Provided expertise in the design and analysis: DJC. Contributed to the overall design and concept of the study as well as to the analysis and write up: EMR.

References


