CHAPTER 3

Asymptomatic plasmodial infection is associated with increased TNFRII-expressing Tregs and suppressed type 2 immune responses

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Abstract

Background In malaria-endemic areas, a proportion of the population becomes chronic carriers of parasites with few or no clinical signs. There is little information on cellular immune responses in asymptomatic parasite carriers.
Methods In 80 schoolchildren residing in a malaria-endemic area of Flores Island, Indonesia, T-helper subsets, regulatory T-cell (Treg) frequencies, TNFRII expression on Treg and *P. falciparum*-infected red blood cell (PfRBC)-induced cytokine responses were measured and asymptomatic infected subjects were compared to uninfected controls. To ascertain that alterations found was due to the presence of malaria parasites, the immune responses were analyzed in 16 children before and one month after anti-malarial treatment.
Results TNFRII expression, a marker of activation on Treg, was higher during infection, but decreased upon treatment. GATA3-positive cells as well as level of IL-13 secretion in response to PfRBC appeared to be suppressed by plasmodial infection as both increased after anti-malarial treatment. TNFRII expression on Treg correlated positively with TNF in response to PfRBC, but this association disappeared following treatment.
Conclusions Malaria parasites associated with asymptomatic infections seem to result in increased TNFRII expression on Tregs as well as suppressed Th2 cytokine responses, features that might be important for survival of the parasites in asymptomatic carriers.

Introduction

In malaria-endemic areas, immunity is gradually acquired, leading to lower malaria incidence and more frequent asymptomatic parasitemia with increasing age[1,2]. The presence of malarial parasites at subclinical levels is thought to be relevant for development and maintenance of protective immune responses associated with prevention of malaria attacks[3]. Studying immune responses during asymptomatic carriage of parasites, is expected to provide insight into mechanisms that allow parasite survival on the one hand and restrict the development of clinical symptoms on the other[4].

Immunological studies have focused mainly on the characterization of IFN-γ and TNF as these are considered to be important for destruction of the parasites[1]. Type 2 responses, which can interact with B-cells and induce antibody class switching, have not been characterized extensively during malaria infection. However, recently, attention has been given to the role of regulatory T-cells (Treg) in malaria as reviewed by Scholzen *et al.*[5]. Although definitions may vary, an expansion of $CD4^+$ Treg is consistently reported in human experimental[6] and natural infection with *Plasmodium falciparum* (*P. falciparum*) as well as *P. vivax*[7-10]. The proportion of Treg has been reported to be positively correlated with parasite growth[7,10,11], which may suggest that either induction of Treg leads to parasite expansion, or blood-stage parasites recruit natural Treg and/or directly induce *de novo* Treg. In addition to their quantity, the quality of Treg in terms of their activation status might be an important determining factor in disease progression[5].

One of the activation markers of Treg that may be important during malarial infections is TNF-receptor type 2 (TNFRII). TNF(R) family members are implicated in parasite elimination as well as in the development of fever and other clinical symptoms[12]. Interestingly, TNFRII may have dual effects; while limiting TNF-induced fever and inflammation, it may also impair TNF bioactivity, which could favor parasite growth. A study in adults from Papua, Indonesia, concluded that TNFRII expression on Treg in peripheral blood and soluble TNFRII and TNF levels in plasma were higher in patients with severe versus uncomplicated malaria[10]. Furthermore, *P. falciparum*-parasitized (Pf)RBC-induced immune responses in malaria-naive donors were more strongly inhibited by $CD25^+TNFRII^+$ Tregs than by their $TNFRII^-$ counterparts[10]. In addition, in malaria-naive subjects, in vitro PfRBC stimulation of PBMC induces TNFRII expression on Treg[13].

To assess the immune regulatory network during asymptomatic parasitemia, we investigated the presence of TNFRII-expressing Treg and other T-cell subsets in a group of school children on Flores Island where malaria is endemic, by examining *ex vivo* T-cell subsets and *in vitro* cytokine responses to PfRBC. To determine whether observed differences were caused by malaria parasites, cells from a group of school children were analyzed before and after anti-malarial treatment.

Methods

Study population

In a cross-sectional study, *Plasmodium*-infected versus -uninfected children, and in a longitudinal study, infected children before and after treatment of plasmodial infection were compared. Participants resided in an area where *P. falciparum*, *P. vivax* and *P. malariae* are endemic on Flores island, Indonesia[14,15]. The cross-sectional study, aimed to recruit 100 children between 5 and 15 years, as the pilot study data from the area indicated that the prevalence of plasmodial infection by microscopy was 20%. Children were randomly selected from schools, of which 84 were willing to donate blood. A total of 80 subjects donated sufficient blood for peripheral blood mononuclear cell (PBMC) isolation as well as PCR and sufficient cells were available from 58 individuals for culture to assess cytokine responses. These 58 were similar to the total group in their baseline characteristics. For the treatment study, 20 *Plasmodium*-infected children with no clinical symptoms, in the same age group, were selected and were treated based on the blood slide result. Sixteen treated subjects donated sufficient blood for PBMC isolation after treatment. According to availability of cells, *ex vivo* phenotype and cytokine responses were measured.

The Committee of Medical Research Ethics of the University of Indonesia approved the study and the participant's parents or guardians gave informed consent.

Malaria diagnostics and treatment

Asymptomatic malaria was defined as a positive thick or thin blood smear, with no signs of fever or chills at consultation or in the last 48 hours and no other clinical complaint. Exclusion criteria were clinical symptoms in the last 48h or treatment for malaria in the preceding 7 days. Blood was drawn on the day of clinical examination. The 80 children who provided blood for the cross-sectional study were examined for malarial parasitemia by microscopy and later by real-time PCR for *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*[16] in the central laboratory. The infected children for the treatment study were selected by microscopy in the field with subsequent examination by PCR. Children in the longitudinal study were treated according to the current guidelines at the local health center, at the time comprising single-dose sulfadoxine-pyrimethamine (SP; 25 mg/kg bodyweight sulfadoxine and 1,25 mg/kg pyrimethamine) for *P. falciparum* and three days of chloroquine (total 25 mg/kg) combined with fourteen days of primaquine (0,25 mg/kg per day) for *P. vivax*. The WHO recommended artemisinin-based combination therapy (ACT) was not fully operational everywhere in Indonesia[17], including our study area. Treatment efficacy was assessed by microscopic detection of parasites at post-treatment blood sampling, 28–32 days post-treatment.

Hematological assessments
Blood was collected into sodium heparin-vacutainers (BD Biosciences, Franklin Lakes, USA) and complete blood counts were determined (Coulter® ACT Diff Hematology Blood Analyzer; Beckman Coulter, Brea, CA, USA). WHO reference values for anemia in school-age children were used (11.5 and 12 g/dl for hemoglobin in children <11 years and between 12 and 14 years of age respectively)[18].

Intestinal parasites
Geohelminth infections were determined by microscopic examination of formalin-preserved stool samples after applying the formol-ether-acetate concentration method[16].

Cell isolation and stimulation
PBMC were obtained by gradient centrifugation over Ficoll. After isolation, a small number of cells were fixed with the FOXP3 Staining set (eBioscience Inc., San Diego, USA) and cryopreserved until further analysis. Freshly isolated PBMC were cultured in RPMI 1640 (Gibco, Invitrogen, Carlsbad, USA) with 10% FCS (Greiner Bio-One GmbH, Frickenhausen, Germany). *P. falciparum*-infected and uninfected RBC (PfRBC, uRBC; kindly provided by the department of Microbiology, Radboud University Medical Centre Nijmegen) were used for stimulation. Information on preparation of PfRBC is provided in the supplementary material. After 96h supernatants were harvested and preserved at -20°C.

Flow cytometry
Fixed PBMC were thawed and permeabilized with FOXP3 Staining set (eBioscience). PBMC were stained with two panels of antibodies, details of which are shown in table S1. Extra information on our gating strategy is given in the supplementary material. PBMC from the two study groups were stained and acquired at different time points dictated by the study timelines and therefore the absolute values can not be compared between study groups; however the pre- and post-treatment samples were measured simultaneously within one experiment on the same day. Flow cytometry data were acquired on a FACSCanto machine (BD Biosciences) and analyzed with FlowJo software (Treestar Inc., Ashland, USA).

Cytokine multiplex analysis
Cytokines (IFN-γ, TNF, IL-10 and IL-13) were measured by Multiplex Bead Immunoassay, using Luminex 100™ xMAP (Luminex Corp., Austin, TX, USA), according to supplier's protocol (Biosource, Invitrogen). Half the detection limit indicated by manufacturer was used for values below detection limit and one

outlying data point was excluded. The background cytokine levels of cells stimulated with uRBC were not subtracted, but analyzed separately. Samples from the two study groups were measured at different times, precluding direct comparison of cytokine levels between the studies. Pre- and post-treatment samples were measured simultaneously within one experiment on the same day.

Data analysis

Analysis was performed in SPSS 18.0. Cross-sectional comparisons between groups were tested with Student's t-test or Mann-Whitney test for data not normally distributed. For data before and after treatment, paired analysis was done using paired t-test or Wilcoxon Signed Ranks Test. Correlations were analyzed using Spearman's test. In the multiplex cytokine analysis Bonferroni correction was applied by multiplying the p-values by the number of non-correlated measurements.

Results

Study population

In the cross-sectional study, T cell subsets and cytokine responses were compared in 80 schoolchildren (26 infected and 54 uninfected). To verify that differences found were due to malarial parasites, we designed a second study where we looked at the effect of malarial treatment on the same parameters. From a thick blood smear survey, 20 asymptomatic children infected with malarial parasites were identified and treated for their infection, 16 of whom also provided blood samples post-treatment. Characteristics of the children in the three groups, cross-sectional uninfected, cross-sectional infected and longitudinal infected before treatment are shown in Table 1.

Table 1. Demographic and infection characteristics of the study population. The study population is divided in three groups: cross-sectional uninfected, cross-sectional infected and longitudinal pre-treatment.

	cross-sectional study		longitudinal study
	uninfected	asymptomatic infected	asymptomatic infected
N	54	26	16
age (median; range)	8.8 (6 - 15)	9.3 (6 - 13)	8.7 (4 - 16)
sex (M / F)	25 / 29	12 / 14	5 / 11
BMI (mean ±SEM)	14.6 (±0.23)	14.7 (±0.33)	15.5 (±0.71)
%CD4+ (mean ±SEM)[a]	34.5 (±0.92)	32.3 (±2.19)	34.2 (±1.58)
***Plasmodium* species (n of Pf / Pv / Pm)[b]**	- / - / -	17 [c,d] / 9 [c] / 2 [d]	12 [c,d] / 5 [c] / 1 [d]
CT value of positive PCR (mean ±SEM)	N.A.	34.83 (±0.86)[e]	31.78 (±0.98)[e]
geohelminth prevalence[f]	80.4 % (37 of 46)	68.2 % (15 of 22)	58.3 % (7 of 12)

[a] percentage of total lymphocytes
[b] assessed by PCR and if no PCR data by microscopy
[c] 1 mixed Pf Pv infection
[d] 1 mixed Pf Pm infection
[e] p=0.046 in t-test
[f] assessed by microscopy after formol-ether concentration

In the cross-sectional group, microscopy identified 7 infected (8.8% of total) children; clearly much lower than the 18% prevalence found in pilot studies (data

not shown), however PCR revealed that 26 (32.5%) of the children were infected. The longitudinal study used microscopy, the method available in the field, for selection of study subjects. Examination of blood samples by PCR showed that 2 children in both groups (respectively 7.7% and 12.5% of infected) were infected with 2 *Plasmodium* spp., but excluding these children did not change the results, therefore they were retained in the analysis. Although children with microscopically detectable parasitemia in the longitudinal study did not report clinical symptoms or visit the health clinic in the week prior to inclusion, 41% had leukocytosis and 40% were anemic according to WHO guidelines[18]. There were no differences in immunological outcomes between children with or without either of these hematological alterations.

Frequency of and TNFRII expression by Treg decrease after anti-malarial treatment

To test the hypothesis that TNFRII-positive Tregs are present in asymptomatic parasitemia, we assessed the Treg compartment and its activation status by analyzing TNFRII expression on $CD25^{hi}FOXP3^{+}$ $CD4^{+}$ T-cells; the gating strategy is shown in Figure 1A. In the cross-sectional study, mean fluorescent intensity (MFI) of TNFRII expression on Treg as well as the proportion of $TNFRII^{+}$ Treg were significantly higher in the infected compared to the uninfected group (Figure 1B; mean MFI 702 in infected versus 610 in uninfected; p=0.008 and Figure 1C; 12.8% versus 8.5% respectively; p=0.007). This was not the case for whole $CD25^{hi}FOXP3^{+}$ Treg, which was lower in infected children, although the difference did not reach statistical significance (Figure 1D, mean 0.60% vs. 0.75% respectively; p=0.082). In the longitudinal study, TNFRII expression on Treg decreased significantly after treatment (Figure 1E, mean MFI of TNFRII 1526 to 1410; p=0.034), but $TNFRII^{+}$ Treg proportions did not change (Figure 1F, mean 10.3% to 8.9%; p=0.35). The $CD25^{hi}FOXP3^{+}$ Treg frequency also decreased, however this was not statistically significant (Figure 1G; mean 0.79% to 0.65% Treg of $CD4^{+}$ T-cells; p=0.091). The intensity of TNFRII expression on the total $CD4^{+}$ T-cell population was markedly lower compared to that of Treg and was similar in infected and uninfected individuals, but also decreased after anti-malarial treatment (mean MFI of TNFRII 911 to 808; p=0.0001, data not shown).

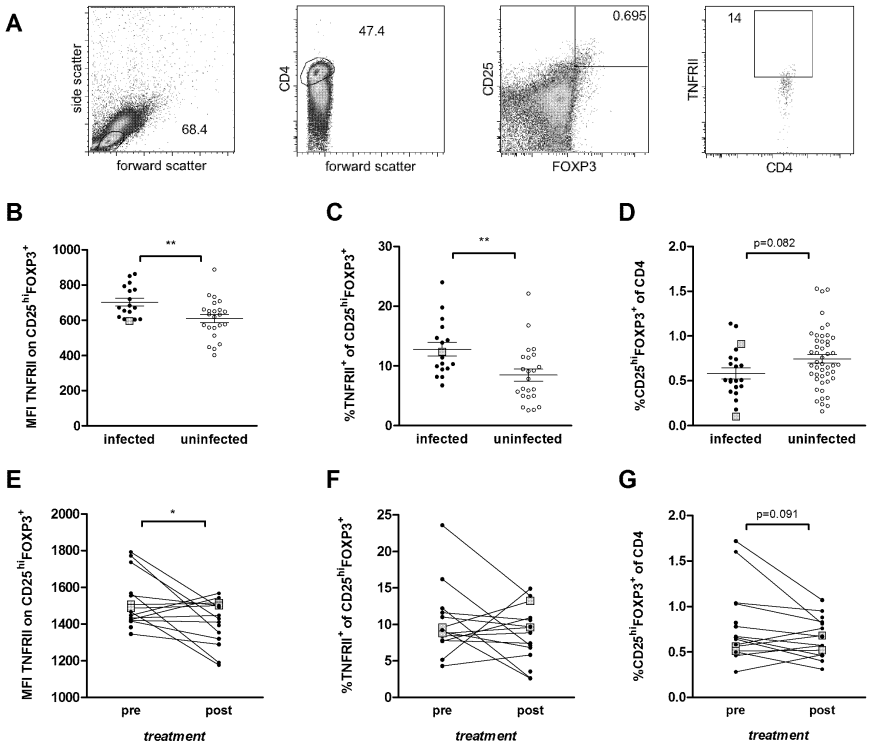

Figure 1. Increased TNFRII expression on Treg during malaria infection which decreases after treatment. PBMC were isolated and fixed and after preservation, cells were stained for flow cytometry to detect Treg and their TNFRII expression. For the cross-sectional study, TNFRII expression was measured on a subset of the samples. (A) A representative example illustrating the gating strategy for Treg as $CD25^{hi}FOXP3^{+}$ subset of $CD4^{+}$ T cells and TNFRII expression within the $CD25^{hi}FOXP3^{+}$ subset. The gate for TNFRII expression on Treg was derived from a fluorescence-minus-one control (FMO). MFI of TNFRII expression on Treg (B, E), fraction of $TNFRII^{+}$ Treg (C, F) and mean Treg frequencies (D, G) were compared between *Plasmodium*-infected (closed symbols) and -uninfected (open symbols) individuals (B-D), as well as before and after treatment (E-G). Squares represent individuals infected with 2 species of *Plasmodium*. Lines connect data points of the same individuals. Note that the fluorescent intensities and cell percentages are not comparable between the two study groups, as flow cytometric assays were performed on different days. *p<0.05, **p<0.01; p-values between 0.05 and 0.10 are indicated.

Th1 subset is not altered while Th2 cells increase after treatment

To assess whether circulating T-helper cells are polarized towards Th1 and/or Th2 during plasmodial infection, the transcription factors for Th1 (Tbet) and Th2 (GATA3) were analyzed in $CD4^{+}$ T-cells. In the cross-sectional study, we found a higher percentage of $Tbet^{+}$ cells in the infected group (Figure 2A; geometric means

infected 3.45% vs. uninfected 2.32%, p=0.046), while the GATA3$^+$ subset was similar (Figure 2B; geomeans 0.92% in infected vs. 1.02% in uninfected). When we analyzed the subsets before and after treatment, the frequency of the Th1 cell subset was unaltered (Figure 2C; geomeans Tbet 3.08% pre- compared to 2.69% post-treatment), whereas elimination of parasites led to an increase in the frequency of Th2 (GATA3$^+$) cells from 1.95% to 2.37% (p=0.021; Figure 2D).

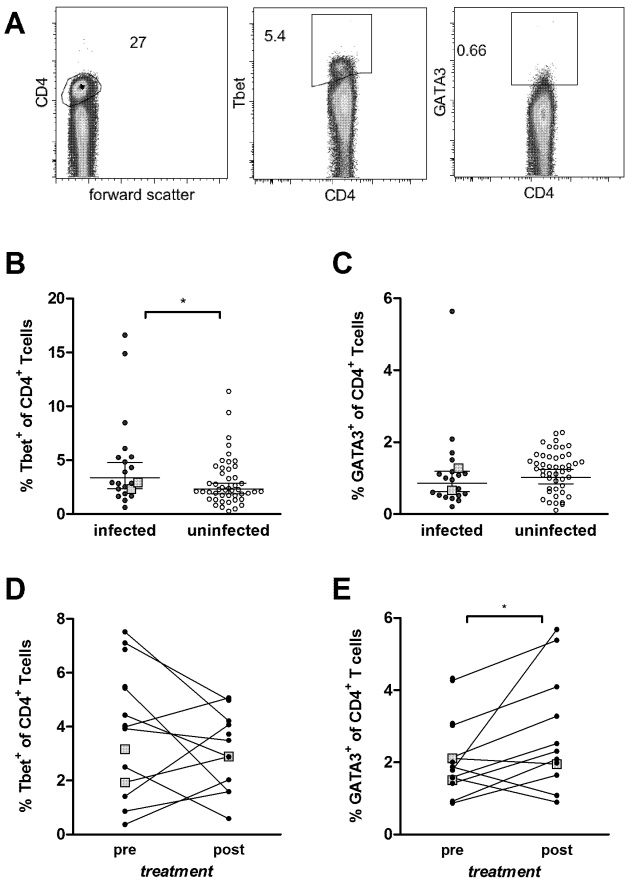

Figure 2. The proportion of Th2 cells in peripheral blood decreases after anti-malarial treatment. Isolated PBMC were stained and measured by flow cytometry and a representative example of the gating on CD4$^+$ T cells is shown in (A). Percentages of Tbet$^+$ (B, D) and GATA3$^+$ (C, E) CD4$^+$ T cells are shown for the cross-sectional subset (B-C; infected vs. uninfected n=22 vs. n=47) and the longitudinal study subjects (D-E; n=11). Closed symbols represent *Plasmodium*-infected individuals; open symbols indicate uninfected subjects, squares represent individuals infected with 2 species of *Plasmodium*. For the cross-sectional comparison, geometric means are depicted. Lines connect data points of the same individuals. *p<0.05

Parasite-specific cytokine responses before and after malarial treatment

We had sufficient cells in 58 children in the cross-sectional study and 15 subjects at both time points in the longitudinal study to be able to measure PfRBC-induced cytokine responses. Interestingly, we found no differences in TNF and IFN-γ production but lower IL-13 production in response to PfRBC in infected versus uninfected children (Figure 3A; geomeans of TNF 85 pg/ml vs. 98 pg/ml, IFN-γ 375 pg/ml vs. 398 pg/ml and IL-13 120 pg/ml vs. 349 pg/ml in infected and uninfected respectively; for IL-13 p=0.010). When we assessed the effect of anti-malarial treatment in the longitudinal study, IFN-γ production did not change while both TNF and IL-13 responses increased after treatment (Figure 3b; geomeans TNF from 364 to 745 pg/ml, p=0.041; IFN-γ from 1723 to 1960 pg/ml; IL-13 from 237 to 327 pg/ml, p=0.023). IL-10 responses to PfRBC did not differ between groups or after treatment (data not shown). After Bonferroni correction only the effects on IL-13 production remained significant. Cytokine responses to uninfected RBC did not differ in either the cross-sectional or the treatment study (data not shown).

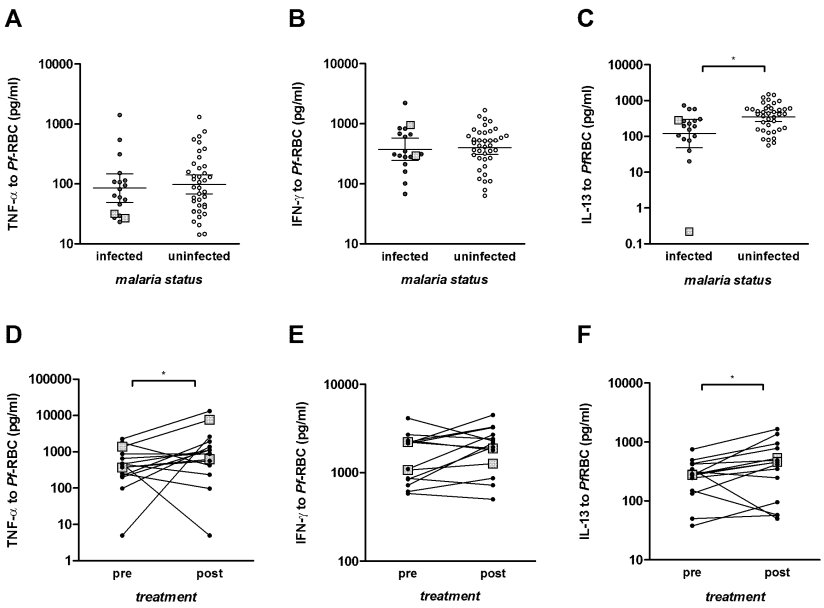

Figure 3. Suppression of TNF and IL-13 but not IFN-γ production to PfRBC. PBMC from Indonesian children were cultured with PfRBC for 4 days. Culture supernatants were analyzed for levels of TNF (A, D), IFN-γ (B, E) and IL-13 (C, F). Geometric means of cytokine production were compared between *Plasmodium*-infected and -uninfected children (A-C; n=19 vs. n=39 respectively). Squares represent individuals infected with 2 species of *Plasmodium*. Treatment effects on cytokine production were tested by paired analysis (D-F; n=15); lines connect data points of the same individuals. Cytokine levels are not comparable between the two study subsets, as multiplex assays were performed on different days. *p<0.05

Correlation of TNFRII expression on Treg and cytokine production to PfRBC
After observing higher TNFRII expression and lower cytokine production to PfRBC during asymptomatic parasitemia, we hypothesized that high TNFRII expression on Treg might be inversely correlated with PfRBC-specific cytokine production. We found a positive correlation between TNFRII expression on Treg and TNF production to PfRBC (Figure 4A; Spearman rho=0.66, p=0.002). A positive correlation was also observed between TNFRII expression on Treg and PfRBC-induced IL-13 (Figure 4B; rho=0.46, p=0.047), but the levels of TNF and IL-13 were themselves not correlated. After treatment these correlations were no longer evident (Figure 4C for TNF, rho=0.17, p=0.55; Figure 4D for IL-13, rho= -0.31, p=0.27).

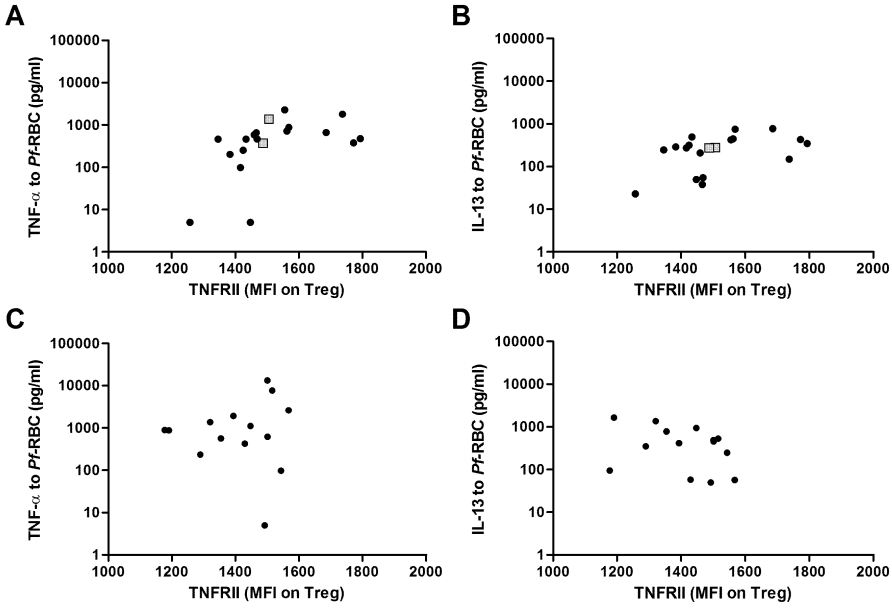

Figure 4. TNFRII expression on Treg is associated with cytokine production to PfRBC during plasmodial infection and stabilizes after treatment. PBMC were assessed for TNFRII expression on $CD25^{hi}FOXP3^{+}$ Treg and stimulated with PfRBC to detect cytokine production in culture supernatants. MFI of TNFRII expression on Treg is depicted on the x-axis and TNF (A, C) or IL-13 (B, D) production to PfRBC is depicted on the y-axis, at pre-treatment (A-B; n=19) and post-treatment (C-D; n=14) time points. Squares represent individuals infected with 2 species of *Plasmodium*. Spearman's correlation coefficients (rho) and p-values are indicated.

Discussion

We report increased TNFRII-expression on Treg during asymptomatic plasmodial infection, which decreases after anti-malarial treatment, suggesting that plasmodial parasites in children lead to activation of these cells even at a subclinical level. We also show that IL-13 responses to *P. falciparum* antigens (PfRBC) are downregulated during asymptomatic parasitemia, and restored after treatment, without changes in type 1 responses.

Very few studies have focused on asymptomatic infections, which from an immunological perspective are interesting, since in malaria-endemic areas large proportion of the population may harbor chronic, clinically silent infections[1]. We studied two groups of schoolchildren with plasmodial infections, confirmed by microscopy and/or PCR, but who were asymptomatic. Whether they were truly asymptomatic, cannot be concluded unequivocally, since we relied on symptoms assessed at the time of examination and on self-reported history of clinical symptoms in the previous 48h. However, we may conclude that in apparently healthy children, malaria parasites induce clear immunological changes.

An increased frequency of TNFRII-expressing Treg has been reported in adults with severe malaria in Papua, Indonesia, compared to uncomplicated malaria cases and asymptomatic controls[10]. Interestingly, Treg numbers and soluble TNFRII plasma concentrations decreased significantly in those with uncomplicated malaria when given artemisinin combination therapy. Another study in children in the Gambia, found that in contrast to the Papua study, both MFI and percentage of TNFRII expression by Treg and also $FOXP3^{+}CD127^{-/low}$ Treg frequency were not different between severe and uncomplicated acute *P. falciparum* malaria infections[11]. However, again after treatment lower TNFRII expression and $TNFRII^{+}$ Treg were found. These two studies suggest that *P. falciparum* is associated with higher levels of TNFRII-expressing Treg. Our findings are in line, showing that even in school-age children with asymptomatic parasitemia, malarial parasites are associated with increased TNFRII expression on Treg.

When total Treg rather than TNFRII-expressing Treg were analyzed in the Gambian study, Treg frequencies were increased at convalescence, suggesting they were lost or sequestered during severe or uncomplicated clinical malaria[11]. We indeed found a tendency for lower frequencies of total Treg in peripheral blood in infected subjects in our cross-sectional study, yet after treatment, Treg numbers decreased, albeit non-significantly. Discrepancies found in studies of Treg, are likely to reflect differences in study population, presence of other co-infections and the Treg phenotypes studied. For example, in our study, malaria infection intensity was different in the groups participating in the cross sectional and the longitudinal studies. In the cross-sectional study, infection was detected by the more sensitive PCR, while in the longitudinal study, this was based on microscopy, which will only

detect higher intensity infections. It is possible that when assessing CD25 and FOXP3 expression, we look at a mixture of activated effector T cells and suppressive Tregs[19]. In higher intensity infections of our longitudinal study group, more activated T cells could have been included in the Treg gate. This is partly why we preferred to focus on the expression of TNFRII on Treg, as a more specific marker of suppressive function of Treg[10]. Moreover our study population consists of children exposed to both *P. falciparum* and *P. vivax*, which potentially lead to altered immunological outcomes compared with those exposed to *P. falciparum* alone[8,20].

Analysis of Th1 and Th2 cell subsets suggested a lower frequency of circulating Th2 cells and a lower in vitro IL-13 response to parasite antigens during asymptomatic plasmodial infection, which increased post-treatment. So far, few immunological studies of malaria have considered type 2 responses in any detail. In a study in Papua New Guinea, IL-4 responses to PfRBC did not differ between infected and uninfected children[21]. IL-4 production to *P. falciparum* schizont lysates was also unaffected by intermittent preventive treatment with SP of infants in Mozambique[22]. However, in the same study, IL-13, the Th2 cytokine examined in our study, was elevated in the plasma of children treated with sulfadoxine-pyrimethamine (SP)[22]. Interestingly, *P. falciparum*-lysate-specific IgE antibodies, dependent on Th2 cell activity, were associated with a reduced risk of malaria episodes regardless of age in a Tanzanian population[23]. Although not specifically addressed, this protective effect might have been due to the better control of malarial parasites. Moreover in the Fulani, an ethnic group in West Africa that is resistant to clinical malaria episodes and plasmodial parasitemia, GATA3 and IL-4 genes are increased, in parallel with the downregulation of FOXP3 and CTLA4 genes[24]. The Fulani have also been shown to have higher percentages of IL-4 producing cells in response to *P. falciparum* antigens compared to the sympatric malaria-susceptible Dogon tribe[25]. Taken together, it is tempting to speculate that, along with Th1-type IFN-γ and TNF responses, there may be a protective role for Th2-associated immune responses in plasmodial infections. Our observed lower frequency and cytokine responses of Th2 cells in subjects with plasmodial infection compared to uninfected and treated individuals may be in line with the notion that plasmodial parasites' survival is dependent on suppression of parasite-specific Th2 responses, which are known to be involved in promoting B cell survival and antibody switching.

Along with IL-13, TNF production induced by PfRBC in vitro was suppressed during asymptomatic infection. Although we hypothesized that TNFRII$^+$ Treg would suppress cytokine production to PfRBC, we found a positive association between TNFRII expression levels on Treg with both TNF and IL-13 levels stimulated by PfRBC. It is known that TNF induces TNFRII expression and expansion of Treg populations[12], presumably to control TNF-associated

inflammatory responses and tissue damage. Moreover, in rheumatoid arthritis (RA), some studies have suggested that shedding of TNFRII may be a mechanism whereby Treg can prevent TNF action[26]. The positive correlation between TNFRII expressing Treg and TNF as well as our observations that TNFRII expression decreased after parasite elimination and that the correlation of TNFRII-expression on Treg with PfRBC-induced cytokines waned supports the notion that there is a dynamic interaction between parasites, TNF and TNFRII expressing Tregs.

The positive association of TNFRII expressing Treg with IL-13 production during plasmodial infection might seem more difficult to reconcile with the hypothesis that Th2 responses may be suppressed by malaria parasites to enhance their survival. However, it is possible that IL-13 is correlated with TNF, which can induce TNFRII expression, and therefore an indirect causal relationship is found. The numbers in the separate studies were too small but when data from all malaria-infected individuals in the two study groups were combined, indeed TNF and IL-13 were significantly correlated (not shown). It is also known that Th2 cells can have a more pro-inflammatory character when co-expressing cytokines such as TNF[27], whilst modified Th2 cells co-expressing anti-inflammatory cytokines[28] could play a role in controlling pro-inflammatory responses. In addition, a recent paper has shown a tight co-regulation and cooperation of FOXP3 and GATA3 transcription[29]. Therefore, our data could suggest that the pro-inflammatory Th2 cells are suppressed during infection while the anti-inflammatory Th2 cells are correlated with TNFRII expression on Treg. Studies are needed to better characterize cytokine co-expression by single cells to determine the contribution of different cell subsets to malarial immunology.

In conclusion, since a considerable proportion of endemic populations may be asymptomatic parasite carriers, this group needs to be studied more intensively. Based on our data, we propose that in vivo malaria-induced TNF upregulates TNFRII on Treg, which might increase their activity and therefore more effectively prevent inflammation. Moreover we show that Th2 responses, which are often ignored, might be an important component of immunity to malaria parasites that are targeted by Tregs.

References

1. Langhorne, J., Ndungu, F.M., Sponaas, A.M. & Marsh, K. Immunity to malaria: more questions than answers. *Nature immunology* 9, 725-732 (2008).
2. Marsh, K. & Kinyanjui, S. Immune effector mechanisms in malaria. *Parasite immunology* 28, 51-60 (2006).
3. Males, S., Gaye, O. & Garcia, A. Long-term asymptomatic carriage of Plasmodium falciparum protects from malaria attacks: a prospective study among Senegalese children. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 46, 516-522 (2008).
4. Riley, E.M., Wahl, S., Perkins, D.J. & Schofield, L. Regulating immunity to malaria. *Parasite immunology* 28, 35-49 (2006).
5. Scholzen, A., Minigo, G. & Plebanski, M. Heroes or villains? T regulatory cells in malaria infection. *Trends in parasitology* 26, 16-25 (2010).
6. Walther, M.*, et al.* Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23, 287-296 (2005).
7. Bueno, L.L.*, et al.* Plasmodium vivax: induction of CD4+CD25+FoxP3+ regulatory T cells during infection are directly associated with level of circulating parasites. *PloS one* 5, e9623 (2010).
8. Goncalves, R.M.*, et al.* CD4+ CD25+ Foxp3+ regulatory T cells, dendritic cells, and circulating cytokines in uncomplicated malaria: do different parasite species elicit similar host responses? *Infection and immunity* 78, 4763-4772 (2010).
9. Jangpatarapongsa, K.*, et al.* Plasmodium vivax parasites alter the balance of myeloid and plasmacytoid dendritic cells and the induction of regulatory T cells. *European journal of immunology* 38, 2697-2705 (2008).
10. Minigo, G.*, et al.* Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. *PLoS pathogens* 5, e1000402 (2009).
11. Walther, M.*, et al.* Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe Plasmodium falciparum malaria. *PLoS pathogens* 5, e1000364 (2009).
12. Randall, L.M. & Engwerda, C.R. TNF family members and malaria: old observations, new insights and future directions. *Experimental parasitology* 126, 326-331 (2010).
13. Scholzen, A., Mittag, D., Rogerson, S.J., Cooke, B.M. & Plebanski, M. Plasmodium falciparum-mediated induction of human CD25Foxp3 CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta. *PLoS pathogens* 5, e1000543 (2009).
14. Elyazar, I.R., Hay, S.I. & Baird, J.K. Malaria distribution, prevalence, drug resistance and control in Indonesia. *Advances in parasitology* 74, 41-175 (2011).
15. Ende, H.D.A. Health profile of Ende District. (Ministry of Health Indonesia, 2009).
16. Wiria, A.E.*, et al.* Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* 10, 77 (2010).
17. Harijanto, P.N. Malaria treatment by using artemisinin in Indonesia. *Acta medica Indonesiana* 42, 51-56 (2010).
18. WHO. Nutritional anaemias. Report of a WHO scientific group. (1968).
19. Finney, O.C., Riley, E.M. & Walther, M. Phenotypic analysis of human peripheral blood regulatory T cells (CD4+FOXP3+CD127lo/-) ex vivo and after in vitro

restimulation with malaria antigens. *European journal of immunology* 40, 47-60 (2010).
20. Hemmer, C.J.*, et al.* Stronger host response per parasitized erythrocyte in Plasmodium vivax or ovale than in Plasmodium falciparum malaria. *Tropical medicine & international health : TM & IH* 11, 817-823 (2006).
21. Robinson, L.J.*, et al.* Cellular tumor necrosis factor, gamma interferon, and interleukin-6 responses as correlates of immunity and risk of clinical Plasmodium falciparum malaria in children from Papua New Guinea. *Infection and immunity* 77, 3033-3043 (2009).
22. Quelhas, D.*, et al.* Intermittent preventive treatment with sulfadoxine-pyrimethamine does not modify plasma cytokines and chemokines or intracellular cytokine responses to Plasmodium falciparum in Mozambican children. *BMC immunology* 13, 5 (2012).
23. Bereczky, S.*, et al.* Elevated anti-malarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria. *International journal for parasitology* 34, 935-942 (2004).
24. Torcia, M.G.*, et al.* Functional deficit of T regulatory cells in Fulani, an ethnic group with low susceptibility to Plasmodium falciparum malaria. *Proceedings of the National Academy of Sciences of the United States of America* 105, 646-651 (2008).
25. Farouk, S.E.*, et al.* Different antibody- and cytokine-mediated responses to Plasmodium falciparum parasite in two sympatric ethnic tribes living in Mali. *Microbes and infection / Institut Pasteur* 7, 110-117 (2005).
26. van Mierlo, G.J.*, et al.* Cutting edge: TNFR-shedding by CD4+CD25+ regulatory T cells inhibits the induction of inflammatory mediators. *J Immunol* 180, 2747-2751 (2008).
27. Ito, T.*, et al.* TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *The Journal of experimental medicine* 202, 1213-1223 (2005).
28. Maizels, R.M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature reviews. Immunology* 3, 733-744 (2003).
29. Rudra, D.*, et al.* Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nature immunology* 13, 1010-1019 (2012).

Supplementary methods

Specific gating of regulatory T cells by flow cytometry

Monocytes were excluded based on the forward scatter / side scatter plot, but since CD3 staining was not included, some monocytes could have been included in the CD4-positive T cell gate. However, by selecting CD25- and FOXP3-positive cells for assessing TNFRII expression on Treg, we have excluded all monocytes in the Treg analysis.

Since both CD25 and FOXP3 are also markers of T cell activation, we defined Treg cells as FOXP3-positive cells with high CD25 expression, to include more pure Treg cells with low contamination of activated (effector) T cells.

Preparation of *Plasmodium falciparum* -infected and uninfected RBC

Cryopreserved *P. falciparum*-infected (PfRBC) and uninfected RBC (uRBC) were kindly provided by Professor Sauerwein at Radboud University Medical Centre Nijmegen). Mature asexual stages of the *P. falciparum* NF54 strain were purified by Percoll gradient centrifugation[1], resulting in 80-90% parasitemia in the obtained RBC, consisting of >95% schizonts / mature trophozoite[2,3]. RBC were used for stimulation in a PBMC:RBC ratio of 1:2.5; pilot experiments had indicated optimal dose and time for these experiments.

References

1. Rivadeneira, E.M., Wasserman, M. & Espinal, C.T. Separation and concentration of schizonts of Plasmodium falciparum by Percoll gradients. *The Journal of protozoology* **30**, 367-370 (1983).
2. Hartgers, F.C.*, et al.* Responses to malarial antigens are altered in helminth-infected children. *The Journal of infectious diseases* **199**, 1528-1535 (2009).
3. McCall, M.B.*, et al.* Plasmodium falciparum infection causes proinflammatory priming of human TLR responses. *J Immunol* **179**, 162-171 (2007).

Table S1. Details of the panel of antibodies used for flow cytometry.

Marker	Clone	Fluorochrome	Company information
CD4	SK3 RPA-T4	PE-Cy7 APC-eFluor780	BD Biosciences, Franklin Lakes, USA eBioscience Inc., San Diego, USA
CD25	2A3	PECy7	BD Biosciences
FOXP3	PCH101	APC or eFluor450	eBioscience
TNFRII	MR2-1	Biotin + streptavidin-Qdot525	Hycult Biotech Inc., Plymouth Meeting, USA Invitrogen, Carlsbad, USA
Tbet	4B10	PerCP-Cy5.5	eBioscience
GATA3	TWAJ	eFluor660	eBioscience