Asymptomatic Plasmodial Infection Is Associated With Increased Tumor Necrosis Factor Receptor II–Expressing Regulatory T Cells and Suppressed Type 2 Immune Responses

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Background. In malaria-endemic areas, a proportion of individuals 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, tumor necrosis factor receptor type II (TNFRII) expression on Tregs, and cytokine responses induced by Plasmodium falciparum–infected red blood cells (RBCs) were measured, and values for asymptomatic infected subjects were compared to those for uninfected controls. To ascertain that alterations found were due to the presence of malaria parasites, the immune responses were analyzed in 16 children before and 1 month after antimalarial treatment.

Results. TNFRII expression, a marker of activation on Tregs, was higher during infection but decreased upon treatment. GATA3-positive cells and the level of interleukin 13 secretion in response to P. falciparum–infected RBCs appeared to be suppressed by plasmodial infection, as both increased after antimalarial treatment. TNFRII expression on Tregs correlated positively with TNF in response to P. falciparum–infected RBCs, 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.

Keywords. malaria; Treg; Th1; Th2; TNFRII.

In malaria-endemic areas, immunity is gradually acquired, leading to a 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]. The study of immune responses during asymptomatic carriage of parasites is expected to provide insight into mechanisms that allow parasite survival, on one hand, and restrict the development of clinical symptoms, on the other [4].

Immunological studies have focused mainly on the characterization of interferon γ (IFN-γ) and tumor necrosis factor (TNF), as these cytokines are considered to be important for destruction of the parasites [1].
The responses of T-helper 2 (Th2) cells, which can interact with B cells and induce antibody class switching, during malaria have not been characterized extensively. However, recently, attention has been given to the role of regulatory T cells (Tregs) in malaria, as reviewed by Scholzen et al [5]. Although definitions may vary, an expansion of CD4+ Tregs is consistently reported in human experimental [6] and natural infection with Plasmodium falciparum and with Plasmodium vivax [7–10]. The proportion of Tregs has been reported to be positively correlated with parasite growth [7, 10, 11], which may suggest that induction of Tregs leads to parasite expansion or that blood-stage parasites recruit natural Tregs and/or directly induce de novo Tregs. In addition to their quantity, the quality of Tregs in terms of their activation status might be an important determining factor in disease progression [5].

One of the activation markers of Tregs that may be important during malarial infections is TNF receptor type II (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: although TNFRII may limit TNF-induced fever and inflammation, it may also impair TNF bioactivity, which could favor parasite growth. A study involving adults from Papua, Indonesia, concluded that TNFRII expression on Tregs in peripheral blood and soluble TNFRII and TNF levels in plasma were higher in patients with severe malaria, compared with patients with uncomplicated malaria [10]. Furthermore, immune responses induced by P. falciparum–parasitized red blood cells (RBCs) 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 P. falciparum–infected RBC stimulation of peripheral blood mononuclear cells (PBMCs) induces TNFRII expression on Tregs [13].

To assess the immune regulatory network during asymptomatic parasitemia, we investigated the presence of TNFRII-expressing Tregs and other T-cell subsets in a group of schoolchildren on Flores Island, Indonesia, where malaria is endemic, by examining ex vivo T-cell subsets and in vitro cytokine responses to P. falciparum–infected RBCs. To determine whether observed differences were caused by malaria parasites, cells from a group of schoolchildren were analyzed before and after antimalarial treatment.

METHODS

Study Population

In a cross-sectional study, Plasmodium-infected and -uninfected children were compared, and in a longitudinal study, infected children before and after treatment of plasmodial infection were compared. Participants resided in an area on Flores Island, where P. falciparum, P. vivax, and P. malariae are endemic [14, 15]. The cross-sectional study aimed to recruit 100 children aged 5–15 years, as the pilot study microscopy data indicated that the prevalence of plasmodial infection was 20% in the area. Children were randomly selected from schools, and 84 were willing to donate blood. A total of 80 subjects donated sufficient blood for PBMC isolation and polymerase chain reaction (PCR) analysis, and sufficient cells were available from 58 individuals for culture to assess cytokine responses. These 58 subjects had baseline characteristics similar to those of the total group. For the treatment study, 20 Plasmodium-infected children who had no clinical symptoms and were from the same age group were selected and treated on the basis of the blood slide result. Sixteen treated subjects donated sufficient blood for PBMC isolation after treatment. According to the 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 participants’ parents or guardians gave informed consent.

Malaria Diagnostic Tests and Treatment

Asymptomatic malaria was defined as a Plasmodium-positive thick or thin blood smear or PCR analysis, 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 48 hours 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 Plasmodium ovale [16] in the central laboratory. The infected children in 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 consisting of single-dose sulfadoxine-pyrimethamine (sulfadoxine dose, 25 mg/kg body weight; pyrimethamine dose, 1.25 mg/kg) for P. falciparum and of chloroquine (total dose, 25 mg/kg) for 3 days combined with primaquine (0.25 mg/kg per day) for 14 days for P. vivax. The World Health Organization–recommended artemisinin-based combination therapy was not fully available everywhere in Indonesia [17], including our study area. Treatment efficacy was assessed by microscopic detection of parasites at posttreatment blood sampling, 28–32 days after treatment.

Hematological Assessments

Blood was collected into sodium heparin vacutainers (BD Biosciences, Franklin Lakes, NJ), and complete blood counts were determined (Coulter ACT Diff Hematology Blood Analyzer; Beckman Coulter, Brea, CA). World Health Organization reference values for anemia in school-aged children were used.
levels (hemoglobin, 11.5 g/dL for children aged <11 years and 12 g/dL for children aged 12 and 14 years) [18].

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

Cell Isolation and Stimulation
PBMCs were obtained by gradient centrifugation over Ficoll. After isolation, a small number of cells were fixed with the FOXP3 staining set (eBioscience, San Diego, CA) and cryopreserved until further analysis. freshly isolated PBMCs were cultured in Roswell Park Memorial Institute 1640 medium (Gibco, Invitrogen, Carlsbad, CA) with 10% fetal calf serum (Greiner Bio-One Frickenhausen, Germany). *P. falciparum*-infected RBCs and -uninfected RBCs (kindly provided by the Department of Microbiology, Radboud University Medical Center Nijmegen) were used for stimulation. Information on the preparation of *P. falciparum*-infected RBCs is provided in the Supplementary Materials. After 96 hours, supernatants were harvested and preserved at −20°C.

Flow Cytometry
Fixed PBMCs were thawed and permeabilized with a FOXP3 staining set (eBioscience). PBMCs were stained with 2 panels of antibodies, details of which are shown in Table S1. Extra information on our gating strategy is given in the Supplementary Materials. PBMCs from the 2 study groups were stained and acquired at different time points dictated by the study time lines, and therefore the absolute values could not be compared between study groups. However, the pre- and post-treatment samples were measured simultaneously in 1 experiment on the same day. Flow cytometry data were acquired on a FACSCanto machine (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR).

Cytokine Multiplex Analysis
Levels of cytokines (IFN-γ, TNF, interleukin 10 [IL-10], and interleukin 13 [IL-13]) were measured by a multiplex bead immunoassay, using LumineX 100 ×MAP (LumineX, Austin, TX), according to the supplier’s protocol (Biosource, Invitrogen). Half the detection limit indicated by the manufacturer was used for values below the detection limit, and 1 outlying data point was excluded. The background cytokine levels for cells stimulated with uninfected RBCs were not subtracted but were analyzed separately. Samples from the 2 study groups were measured at different times, precluding direct comparison of cytokine levels between the studies. Pre- and posttreatment samples were measured simultaneously in 1 experiment on the same day.

Data Analysis
Analysis was performed in SPSS 18.0. Cross-sectional comparisons between groups were performed with the Student t test or, for nonnormally distributed data, the Mann-Whitney test. For data obtained before and after treatment, paired analysis was done using a paired t test or the Wilcoxon signed ranks test. Correlations were analyzed using the Spearman test. In the multiplex cytokine analysis, Bonferroni correction was used by multiplying the P values by the number of noncorrelated 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, in which 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, of whom 16 also provided blood samples after treatment. Characteristics of the children in the 3 groups—cross-sectional uninfected, cross-sectional infected, and longitudinal infected before treatment, are shown in Table 1.

In the cross-sectional group, microscopy identified infection in 7 children, leading to a prevalence of 8.8%. This value was clearly much lower than the 18% prevalence found in pilot studies (data not shown), but PCR revealed that 26 of the children (32.5%) 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 (7.7%) in the cross-sectional infected group and 2 children (12.5%) from the longitudinal infected group were infected with 2 *Plasmodium* species; exclusion of these children did not change the results, so 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 and children without either of these hematological alterations.

Frequency of and TNFRII Expression by Tregs Decrease After Antimalarial Treatment
To test the hypothesis that TNFRII-positive Tregs are present during asymptomatic parasitemia, we assessed the Treg compartment and its activation status by analyzing TNFRII expression on CD25<sup>hi</sup>FOXP3<sup>+</sup> CD4<sup>+</sup> T cells; the gating strategy is shown in Figure 1A. In the cross-sectional study, the
Table 1. Demographic and Infection Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cross-sectional Study</th>
<th>Longitudinal Study</th>
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<tbody>
<tr>
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<td>Uninfected (n=54)</td>
<td>Asymptomatic Infected (n=26)</td>
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<tr>
<td>Age, y, median (range)</td>
<td>8.8 (6–15)</td>
<td>9.3 (6–13)</td>
</tr>
<tr>
<td>Sex</td>
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<td></td>
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<td>Male</td>
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<tr>
<td>BMI</td>
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<td>14.7±0.33</td>
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<tr>
<td>CD4+ T-cells, %a</td>
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<td>32.3±2.19</td>
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<tr>
<td>P. falciparum</td>
<td>NA</td>
<td>17c,d</td>
</tr>
<tr>
<td>P. vivax</td>
<td>NA</td>
<td>9c</td>
</tr>
<tr>
<td>P. malariae</td>
<td>NA</td>
<td>2d</td>
</tr>
<tr>
<td>Ct value of positive PCR result</td>
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<td>34.83±0.86a</td>
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<tr>
<td>Geohelminth prevalence, % (proportion)f</td>
<td>80.4 (37/46)</td>
<td>68.2 (15/22)</td>
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Data are no. of subjects or mean ± standard error of the mean, unless otherwise indicated. The study population was divided in 3 groups: cross-sectional uninfected, cross-sectional infected, and longitudinal pretreatment. Abbreviations: BMI, body mass index; Ct, threshold cycle; NA, not applicable; PCR, polymerase chain reaction.

a Percentage of total lymphocytes.

b Assessed by PCR and, if no PCR data, by microscopy.

c 1 mixed P. falciparum and P. vivax infection.

d 1 mixed P. falciparum and P. malariae infection.

* P = .046, by the t test.

f Assessed by microscopy after formol-ether concentration.

infected group, compared with the uninfected group, had a greater mean fluorescence intensity (MFI) of TNFRII expression on Tregs (702 vs 610; P = .008; Figure 1B) and a greater proportion of TNFRII+ Tregs (12.8% vs 8.5%; P = .007; Figure 1C). This was not the case for CD25hiFOXP3+ Tregs, for which the mean frequency in the total CD4+ T-cell population was lower in infected children, compared with uninfected children, although the difference was not statistically significant (0.60% vs 0.75%; P = .082; Figure 1D). In the longitudinal study, the MFI of TNFRII expression on Tregs before treatment was significantly greater than that after treatment (1526 vs 1410; P = .034; Figure 1E), but the mean TNFRII+ Treg proportions before and after treatment were not significantly different (10.3% and 8.9%, respectively; P = .35; Figure 1F). The mean CD25hiFOXP3+ Treg frequency in the total CD4+ T-cell population after treatment was also less than that before treatment, but the difference was not statistically significant (0.79% vs 0.65%; P = .091; Figure 1G). The MFI of TNFRII expression on the total CD4+ T-cell population was markedly lower than that among Tregs and was similar in infected and uninfected individuals; it was also significantly less after treatment, compared with before treatment (911 vs 808; P = .0001; data not shown).

The T-Helper 1 (Th1) Cell Subset Is Not Altered, Whereas the Frequency of Th2 Cells Increases After Treatment

To assess whether circulating T-helper cells are polarized toward the Th1 and/or Th2 subsets 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 geometric mean percentage of Tbet+ cells in the infected group, compared with the uninfected group (3.45% vs 2.32%; P = .046; Figure 2A), whereas the values for the GATA3+ subset were similar (0.92% and 1.02%, respectively; Figure 2B). When we analyzed the subsets before and after treatment, the frequency of the Th1 cell subset was unaltered (3.08% and 2.69%, respectively; Figure 2C), whereas elimination of parasites led to an increase in the frequency of the Th2 cell subset (1.95% and 2.37%, respectively; P = .021; Figure 2D).

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 measure cytokine responses induced by P. falciparum–infected RBCs. Interestingly, we found no significant differences between infected and uninfected children in geometric mean levels of TNF (85 pg/mL and 98 pg/mL, respectively) and IFN-γ (375 pg/mL and 398 pg/mL, respectively) in response to P. falciparum–infected RBCs; however, infected children had a significantly lower geometric mean level of IL-13 (120 pg/mL vs 349 pg/mL; P = .010; Figure 3A). When we compared geometric mean cytokine production before treatment to that after treatment in the longitudinal study, IFN-γ production did not change significantly (from 1723 pg/mL to 1960 pg/mL), whereas significant increases were observed for both TNF (from 364 pg/mL to 745 pg/mL; P = .041) and IL-13 (from 237 pg/mL to 327 pg/mL; P = .023; Figure 3B). IL-10 responses to P. falciparum–infected RBCs 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 RBCs did not differ in either the cross-sectional or treatment studies (data not shown).

Correlation of TNFRII Expression on Tregs and Cytokine Production to P. falciparum–Infected RBCs

After observing higher TNFRII expression and lower cytokine production in response to P. falciparum–infected RBCs during
asymptomatic parasitemia, we hypothesized that high TNFRII expression on Tregs might be inversely correlated with production of cytokines in response to *P. falciparum*-infected RBCs. We found a positive correlation between TNFRII expression on Tregs and TNF production in response to *P. falciparum*-infected RBCs (Spearman rho = 0.66; *P* = .002; Figure 4A). A positive correlation was also observed between TNFRII expression on Tregs and IL-13 production induced by *P. falciparum*-infected RBCs (Spearman rho = 0.46; *P* = .047; Figure 4B), but the levels of TNF and IL-13 were themselves not correlated. After treatment, these correlations were no longer evident for TNF (Spearman rho = 0.17; *P* = .55; Figure 4C) and IL-13 (Spearman rho = −0.31; *P* = .27; Figure 4D).

**DISCUSSION**

We report that TNFRII expression on Tregs is increased during asymptomatic plasmodial infection and that expression decreases after antimalarial treatment, suggesting that even a subclinical level of plasmodial parasites leads to activation of these cells in children. We also show that IL-13 responses to *P. falciparum* antigens (*P. falciparum*-infected RBCs) are downregulated during asymptomatic parasitemia and restored after treatment, without changes in Th1 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 2 groups of schoolchildren who had plasmodial infections confirmed by microscopy and/or PCR but 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 48 hours. However, we may conclude that in apparently healthy children, malaria parasites induce clear immunological changes.

An increased frequency of TNFRII-expressing Tregs has been reported in adults with severe malaria in Papua, Indonesia, compared with the frequencies in patients with uncomplicated malaria and in asymptomatic controls [10]. Interestingly, Treg numbers and soluble TNFRII plasma concentrations decreased significantly in individuals with uncomplicated malaria after they were 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 Tregs and also FOXP3+/CD127−/low Treg frequency were not different between patients with severe and those with uncomplicated acute *P. falciparum* infections [11]. However, again after treatment lower TNFRII expression and TNFRII+ Treg numbers were found. These 2 studies suggest that *P. falciparum* is associated with higher levels of TNFRII-expressing Tregs. Our findings are in line, showing that even in school-aged children, malaria parasites are associated with increased TNFRII expression on Tregs.

When total Tregs rather than TNFRII-expressing Tregs were analyzed in the Gambian study, Treg frequencies were

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**Figure 2.** The proportion of T-helper 2 cells in peripheral blood decreases after antimalarial treatment. Isolated peripheral blood mononuclear cells were stained and measured by flow cytometry, and a representative example of the gating on CD4+ T cells is shown in panel A. Percentages of Tbet* (B and D) and GATA3* (C and E) CD4+ T cells are shown for the cross-sectional subset (B and C; 22 infected individuals and 47 uninfected individuals) and the longitudinal study subjects (D and E; 11 individuals). Closed symbols represent *Plasmodium*-infected individuals, open symbols indicate uninfected subjects, and squares represent individuals infected with 2 species of *Plasmodium*. For the cross-sectional comparison, geometric means are depicted. Lines connect data from the same individual. *P<.05.
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 Tregs in peripheral blood in infected subjects in our cross-sectional study, yet after treatment, Treg numbers decreased, albeit nonsignificantly. Discrepancies found in studies of Tregs likely reflect differences in study population, presence of other coinfections, and the Treg phenotypes studied. For example, in our study, malaria intensity was different in the groups participating in the cross-sectional and the longitudinal studies. In the cross-sectional study, infection was detected by PCR, which has the sensitivity to detect lower-intensity infections, while in the longitudinal study, infection was determined using microscopy, which will only detect higher-intensity infections. It is possible that assessment of CD25 and FOXP3 expression involves 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 Tregs, as a more specific marker of suppressive function of Tregs [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 in individuals 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 after treatment. So far, few immunological studies of malaria have considered Th2 cell responses in any detail. In a study in Papua, interleukin 4 (IL-4) responses to *P. falciparum*–infected RBCs did not differ between infected and uninfected children [21]. IL-4 production in response to *P. falciparum* schizont lysates was also unaffected by administration of intermittent preventive treatment with sulfadoxine-pyrimethamine to infants in Mozambique [22]. However, in the same study, the level of IL-13, the Th2 cytokine examined in our study, was elevated in the plasma of children treated with sulfadoxine-pyrimethamine [22]. Interestingly, *P. falciparum* lysate–specific immunoglobulin E antibodies, which are 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

Figure 3. Suppression of tumor necrosis factor (TNF) and interleukin 13 (IL-13) but not interferon γ (IFN-γ) production in response to *Plasmodium falciparum*–infected red blood cells (PRBCs). Peripheral blood mononuclear cells from Indonesian children were cultured with PRBCs for 4 days. Culture supernatants were analyzed for levels of TNF (*A* and *D*), IFN-γ (*B* and *E*), and IL-13 (*C* and *F*). Geometric means of cytokine production were compared between 19 *Plasmodium*-infected and 39 *Plasmodium*-uninfected children (*A*–*C*). Squares represent individuals infected with 2 species of *Plasmodium*. Treatment effects on cytokine production were tested by paired analysis (*D*–*F*, 15 individuals); lines connect data for the same individual. Cytokine levels are not comparable between the 2 study subsets, as multiplex assays were performed on different days. *P*<.05.
clinical malaria episodes and plasmodial parasitemia, expression of GATA3 and IL-4 genes is 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 with the response in 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 with responses in 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 class switching.

Along with IL-13, TNF production induced by *P. falciparum*-infected RBCs in vitro was suppressed during asymptomatic infection. Although we hypothesized that TNFRII+ Tregs would suppress cytokine production in response to *P. falciparum*-infected RBCs, we found a positive association between TNFRII expression levels on Tregs, with both TNF and IL-13 levels stimulated by *P. falciparum*-infected RBCs. 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, 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 Tregs and TNF, as well as our observations that TNFRII expression decreased after parasite elimination and that the correlation of TNFRII-expression on Tregs with *P. falciparum*-infected RBCs-induced cytokines waned, support the notion that there is a dynamic interaction between parasites, TNF, and TNFRII-expressing Tregs.

The positive association of TNFRII-expressing Tregs 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, there may be an indirect causal relationship, owing to a possible correlation between IL-13 and TNF production and the fact that TNF can induce TNFRII expression. The numbers in the separate studies were too small for analysis, but when data from all malaria-infected individuals in the 2 study groups were combined, indeed TNF and IL-13 were significantly correlated (data not shown). It is also known that Th2 cells can have a more proinflammatory character when coexpressing cytokines such as TNF [27],

Figure 4. Tumor necrosis factor receptor type II (TNFRII) expression on regulatory T cells (Tregs) is associated with cytokine production in response to *Plasmodium falciparum*-infected red blood cells (PRBCs) during plasmodial infection and stabilizes after treatment. Peripheral blood mononuclear cells were assessed for TNFRII expression on CD25+FOXP3+ Tregs and stimulated with PRBCs to detect cytokine production in culture supernatants. Mean fluorescence intensity (MFI) of TNFRII expression on Tregs is depicted on the x-axis, and tumor necrosis factor (TNF; A and C) or interleukin 13 (IL-13; B and D) production in response to PRBCs is depicted on the y-axis for time points before treatment (A and B; 19 individuals) and after treatment (C and D; 14 individuals). Squares represent individuals infected with 2 species of *Plasmodium*. Spearman correlation coefficients (rho) and P values are indicated.
whereas modified Th2 cells coexpressing antiinflammatory cytokines [28] could play a role in controlling proinflammatory responses. In addition, a recent article has shown a tight coregulation and cooperation of FOXP3 and GATA3 transcription [29]. Therefore, our data could suggest that the proinflammatory Th2 cells are suppressed during infection while the anti-inflammatory Th2 cells are correlated with TNFRII expression on Tregs. Studies are needed to better characterize cytokine coexpression by single cells to determine the contribution of different cell subsets to malarial immunology.

In conclusion, since a considerable proportion of individuals in malaria-endemic areas may be asymptomatic parasite carriers, this group needs to be studied more intensively. On the basis of our data, we propose that in vivo malaria–induced TNF upregulates TNFRII on Tregs, 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.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copylefted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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