Proinflammatory Responses and Higher IL-10 Production by T Cells Correlate with Protection against Malaria during Pregnancy and Delivery Outcomes

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Pregnant women have a higher incidence of Plasmodium infection and clinical malaria than nonpregnant adults. Plasmodium falciparum and Plasmodium vivax infections during pregnancy cause adverse clinical outcomes such as maternal anemia and contribute to negative outcomes in the offspring, including low birth weight, prematurity, and infant deaths (1–3). Although the accumulation of parasites in the placenta through adherence to chondroitin sulfate A partially explains the higher susceptibility of pregnant women to infection, gestation-associated immunomodulation has been proposed to contribute to the burden of malaria in pregnancy, although this has yet to be proved (2, 4, 5). Placental monoinfection by Plasmodium falciparum infection, respectively. Higher intracellular IL-10 levels in T cells had a protective association with birth weight and future (delivery) Plasmodium falciparum infection, respectively. Higher intracellular IL-10 levels in T cells had a protective association with future Plasmodium falciparum infection and hemoglobin levels at delivery. The protective associations were found also with nonmalaria-specific T cell responses. Treg frequencies positively correlated with plasma eosinophil concentrations, but this subset did not express eosinophil receptor CCR3. Thus, activated immune system during pregnancy might contribute to protection against malaria during pregnancy and poor delivery outcomes. The Journal of Immunology, 2015, 194: 000–000.
expansion of CD4+CD25+ T cells in peripheral blood of pregnant women (7, 8), but subsequent studies with functional analyses and more detailed phenotyping (CTLA-4, CD25high, or FOXP3 markers) demonstrated that Tregs were indeed decreased in periphery during pregnancy as they migrate to the placenta (9, 10). Conversely, controlled proinflammatory responses during pregnancy play a critical role in embryo implantation, placentation, parturition, and defense against infections. However, excessive maternal inflammatory responses are associated with adverse pregnancy outcomes (11). Thus, immunoregulation during pregnancy seems essential not only to tolerate fetal Ags, but also to resolve the necessary inflammatory processes (12). Reduction of proinflammatory CD4+IL-10− IFN-γ+ T cells (Td41) responses is largely dependent on IL-10 (12). This modulation can be achieved through IL-10 produced extrinsically, for example, from Tregs, but also through intrinsic IL-10, in a process by which IFN-γ-only Td1 cells start coproducing IL-10 (which we term CD4+IL-10+ IFN-γ+ T cell [Tdpos]), acquiring self-regulatory properties (12). Although the role of Tregs in fetal tolerance has been documented, to our knowledge, there are no studies about the role of Tdpos or CD4+IL-10+IFN-γ− cells (Tr1) in pregnancy.

In the case of malaria, elevated Tregs, IL-10, and TGF-β levels have been described during P. vivax and P. falciparum infection in nonpregnant adults (13–17). In malaria, it is unknown whether Tregs contribute to the onset of the disease by driving a failure in the control of infection or they act to control immune-mediated pathology associated with malaria infection due to an excessive Td1 response (18). Whether the outcomes are favorable for the parasite or the host might depend on a variety of factors such as human genetic background, timing, and magnitude of proinflammatory responses to infection and/or level of previous exposure to the parasite (19). In addition, Tdpos have gained some attention lately in the field of malaria, as they have been suggested to play an important role in preventing immune-mediated pathology in this disease (20). Moreover, a recent study has identified this subset as the one dominating the P. falciparum–specific CD4+ T cell response in highly exposed children (21).

We speculated that a pregnancy-driven alteration of any regulatory T cell subset (Tregs, Tdpos, or Tr1) might affect susceptibility to malaria (and other infections) during pregnancy, resulting in poor delivery outcomes (1, 2, 22). Only three studies have described the Treg population in malaria during pregnancy, in which decreased peripheral levels in pregnant women with P. falciparum infection compared with uninfected women were observed, although this difference was not observed when women with submalaric parasitemia were included (23–25). Nevertheless, these studies did not include nonpregnant and nonmalaria-exposed individuals for comparisons, and did not address other regulatory T cell subsets. Thus, in this study, we set out to determine the effect of pregnancy in the T cell pool in a malaria-endemic area (Papua New Guinea [PNG]), compared with a malaria-free country (Spain) used as a control of “healthy” pregnancy. Upon that base, we describe the association of different subsets of T cells with present and future Plasmodium infection and delivery outcomes.

Materials and Methods

Study design and population

Malaria-naive nonpregnant (NNP) and malaria-naive pregnant (NP) donors were enrolled at the blood bank and at the antenatal care of the Hospital Clinic (Barcelona, Spain), respectively. In PNG, malaria-exposed pregnant (EP) women were recruited in the context of the PregVax project (FP7-HEALTH-201588, http://www.pregvax.net) at several Health Centres in the Madang Province between 2008 and 2010. At the time of starting the project, the region was characterized by high-level malaria transmission for both P. vivax and P. falciparum (26). P. vivax and P. falciparum par- asitemias (by microscopy and real-time PCR) and hemoglobin (Hb) levels were assessed at enrolment and delivery, and birth weight was recorded. For this study, infection was defined as a positive smear and/or PCR. Women with clinical symptoms who had a positive rapid diagnostic test and/or smear were treated according to the national guidelines. Malaria-exposed nonpregnant (ENP) women were recruited in one community in Madang province, PNG. A venous blood sample of 10 ml was collected in heparin Vacutainers from each donor and from pregnant volunteers at recruitment and delivery.

Ethical approval

Written informed consent was obtained from all study participants. The study was approved by the Medical Research Advisory Committee in PNG (MRAC 08.02) and by the Hospital Clinic of Barcelona Ethics Review Committee, Spain (Comité Ético d’Investigació Clínica).

Isolation of plasma and peripheral PBMCs

Plasma was separated from the cellular fraction within 16 h of collection by centrifuging at 600 × g for 10 min at room temperature, aliquoted, and stored at −80°C. Cells were further fractioned in a density gradient medium (Histopaque-1077; Sigma-Aldrich). PBMCs were frozen in FCS supplemented with 10% DMSO and stored in liquid nitrogen. PNG samples were shipped into liquid nitrogen to, and analyzed at, the Barcelona Centre for International Health Research.

Cellular stimulation and phenotyping

PBMCs were slowly thawed and their viability measured on a Guava Cytometer using Guava ViaCount Reagent (Millipore). All Abs and reagents came from BD Biosciences, unless otherwise indicated. For compensation controls, BD Comp Beads were used. Cells and beads were acquired on a BD LSR Fortessa cytometer. Acquired data were analyzed with FlowJo v9.7.5 (Tree Star, Ashland, OR). Raw data can be provided per request.

After thawing, one million PBMCs were rested overnight in RPMI 1640 medium supplemented with 10% FBS (Life Technologies) at 37°C, in duplicates when possible. The following day, GolgiPlug was added, and in one of the wells cells were cultured in the presence of anti-CD3 (precoated at 1 µg/ml in the well for 1 h; Abcam, Cambridge, U.K.) for 6 h. After incubation, PBMCs suspensions were stained with LIVE/DEAD Fixable Violet Dead Cell Stain kit (Invitrogen). After washing, cellular surface staining was performed with the following Abs: anti-CD14 Pacific Blue, anti-CD19 Horizon V450, anti-CD4 allophycocyanin-Cy7, anti-CD25 Alexa Fluor 700, and anti-CD127 PE. Cells were fixed and permeabilized using the Human FOXP3 Buffer Set, following manufacturer’s indications. Intracytoplasmic staining was performed with the following Abs: anti-CD3 PerCP, anti-FOXP3 Alexa Fluor 488, anti–IFN-γ PE/Cy7, and anti–IL-10 allophycocyanin. Tregs were analyzed in unstimulated samples and expressed as percentage of total CD3+CD4+ T cells. Stimu-

lated PBMCs were used to measure intracellular IL-10 and IFN-γ levels in the Treg population, analyzed as mean fluorescence intensity (MFI), and were used to measure other non-Treg (CD3+CD4+ “AND NOT” Tregs) subsets: Td1 (IL-10− IFN-γ−), Tr1 (IL-10+IFN-γ−), and Tdpos (IL-10+ IFN-γ−) cells. These three subsets were gated within and expressed as percentage of the non-Treg population. CCR3 (anti-CCR3 PE-CF594) expression was also analyzed in 12 samples before resting. Gating strategy is displayed in Fig. 1.

In a separate group of experiments, half a million PBMCs from 8 women with a P. falciparum infection at delivery (cases) and 16 P. falciparum–negative controls (all of whom were P. falciparum–negative at recruitment) were incubated for 16 h in the presence of cryopreserved P. falciparum (3D7)–infected RBCs (iRBC) or uninfected RBCs (uRBC) (ratio 1:3) and anti-CD3 (0.1 µg/ml) or medium. Then 30 µl medium was removed to analyze cytokine secretion in supernatant and was replaced by fresh medium containing GolgiPlug for an additional 6 h. Cells were analyzed with the same flow cytometry panel and gating strategy as described earlier.

Quantification of Abs and cytokines

Ab responses were analyzed using Luminex technology with a panel developed in-house, as described before (27). Because of Ag-coupled beads limitation, the measurement of IgG Ab levels was done in the first 78 samples analyzed and did not include the ENP women from PNG.
Plasma cytokines were quantified using the Cytokine Magnetic 30-Plex Panel (Life Technologies) multiplex suspension detection system, according to the manufacturer’s instructions. Pro-TGF-β was measured using an ELISA kit (R&D Systems, Abingdon, U.K.).

**Plasmodium spp. detection by PCR**

Plasmodium species molecular detection by PCR methods was performed at the Istituto Superiore di Sanità (Rome, Italy) or at the Institute of Medical Research (Madang, PNG). The protocols followed have been previously described (28, 29). In brief, in Italy, DNA was extracted from dried blood spots following manufacturer’s instructions (PureLink Genomic DNA Kit; Life Technologies) and eluted in 150 μl elution buffer. *P. vivax* and *P. falciparum* parasites were detected using species-specific primers and probes and LightCycler 480 Instrument. The PCR was composed of 1 cycle at 95˚C 10 min; 50 cycles at 95˚C 10 s, 50˚C 20 s, 72˚C 5 s; and 1 cycle 40˚C 1 min. The threshold for positivity for each species was established as a cycle threshold < 45, according to negative controls.

For each species, 45 cycles of 15 s at 95˚C and 1 min at 58˚C. The threshold for positivity for each species was established as cycle threshold < 40, according to negative controls.

**Statistical analysis**

Quantitative variables for cell population frequencies, cellular marker MFI values, Ab levels (MFI), cytokine concentrations (pg/ml), and age (y) were summarized using the arithmetic or geometric means and their SD or 95% confidence interval (CI), or with the medians and interquartile ranges, as appropriate. T cell variables could not be log-transformed because of the presence of “0” and negative numbers; thus, median regressions were performed.

**Results**

**Study population**

Participants in this study were grouped as: 1) NNP, 2) NP, 3) ENP, and 4) EP. NNP donors (n = 20, 6 women and 14 men) and NP donors (n = 24) were residents in Spain and had never traveled to malaria-endemic areas. ENP (n = 38, all women) and EP (n = 113) donors were enrolled in PNG. In the EP group, 101 women were randomly selected at their first antenatal visit (recruitment) or delivery, and 12 additional women were selected for having being enrolled in the first trimester of pregnancy. One objective of the study was to compare T cell frequencies in pregnant versus nonpregnant individuals; therefore, samples in the pregnant cohort were not paired (recruitment and delivery), to increase the sample size in the pregnant cohort in relation to the nonpregnant cohort. Only PBMC samples with a viability >70% were used for the assays. After discarding samples below cutoff (NP 6 samples, ENP 1 sample, EP 12 samples), mean viability after thawing PBMCs was 90% and after overnight resting 86%. When cell counts were low, we prioritized the staining of unstimulated T cells to analyze Tregs (see Materials and Methods), resulting in lower sample size of anti-CD3–stimulated T cells in the NP (n = 8) and EP groups (n = 95).

**FIGURE 1.** Gating strategy. After discarding debris, viable cells were shown according to FSC and a dump channel containing a viability marker, CD14 and CD19. Viable cells were displayed according to CD3 and CD4 expression, and the double-positive population was gated for CD25+ cells. Then Tregs were gated according to the positive expression of FOXP3 and low levels of CD127. Percentage and production levels of IL-10 and IFN-γ were assessed within the Treg subset. In samples stimulated with anti-CD3, a boolean gate containing CD3+CD4+ “AND NOT” Tregs was built to identify non-Treg cells, and this population was further divided according to the production of IL-10 and/or IFN-γ in Tr1 (IL-10+IFN-γ−), Tdpos (IL-10+IFN-γ+), and TH1 (IL-10−IFN-γ+) cells. CCR3 expression levels were also analyzed in some samples. FSC, forward side scatter.
pared with the ENP group (0%; \( p = 0.033 \)). Fig. 1 displays the gating strategy for each T cell population.

**Plasmodium exposure and pregnancy effects on T cell subpopulations**

One of the objectives of this study was to analyze the effect of pregnancy on the T cell pool in a malaria-endemic area to discard differences with a “healthy” pregnancy, by comparing the four study groups: NNP, NP, ENP, and EP. We found fewer CD4\(^+\) CD25\(^{hi}\)CD127\(^{-}\)FOXP3\(^+\) cells (Tregs) in NP and EP women than in the nonpregnant counterpart groups (Fig. 2A), although differences did not reach statistical significance in the malaria-naive group (NNP versus NP), probably because of a lower number of samples and high variability in the data. No differences were

**FIGURE 2.** Effect of malaria exposure and pregnancy in the levels of regulatory T cells and intracytoplasmic cytokine levels. Peripheral blood T cells were characterized by flow cytometry in the different groups: NNP (\( n = 20 \)), NP (Tregs: \( n = 18 \); Tr1, Tdpos, and TH1: \( n = 8 \)), ENP (\( n = 37 \)), and EP (Tregs: \( n = 101 \); Tr1, Tdpos, and TH1: \( n = 95 \)).

(A) Dot plots show the median percentage of regulatory T cells and TH1 cells in the four groups. (B) Bars show mean \pm SEM IL-10 or IFN-\( \gamma \) MFI of the Treg and non-Treg populations. (A and B) Age-adjusted median regression models were estimated and effects were assessed comparing the four study groups (*\( p < 0.05 \)). (C) Scatter plot shows distribution of values for the frequencies of Tregs, Tdpos, and TH1 cells in the EP group, \( n = 95 \). The \( p \) value corresponds to Spearman’s correlation test. rho, Spearman’s coefficient.
observed between malaria-exposed and naive groups (NNP versus ENP and NP versus EP), suggesting that malaria exposure had no effect on Treg frequencies.

A pregnancy-associated expansion of Tr1 (FOX3−IL-10−IFN-γ−) cells was found in the unexposed cohort, whereas a pregnancy-associated expansion of Tdpos (FOX3−IL-10−IFN-γ+) and Th1 (FOX3−IL-10−IFN-γ+) cells was observed in the malaria-exposed cohort (Fig. 2A). When the effect of malaria exposure was studied, we found lower proportions of Tr1 and Tdpos cells in malaria-exposed donors compared with malaria-naive individuals, both in the pregnant and the nonpregnant cohorts, and a reduction of Th1 cells only in the nonpregnant cohort (Fig. 2A).

Next, we investigated whether the production of IL-10 and IFN-γ by different T cell subsets was also affected by pregnancy and malaria exposure. Compared with NNP, NP women had lower intracytoplasmic levels of IL-10 (MFI) in both Treg and non-Treg subsets; however, there were no differences in IL-10 expression between ENP and EP groups (Fig. 2B). With regard to IFN-γ, EP women produced more Treg–IFN-γ than the ENP group (Fig. 2B). Malaria exposure was associated with decreased levels of IL-10 (Fig. 2B) and IFN-γ (Fig. 2B) in the nonpregnant cohort, and increased levels of IL-10 in the pregnant cohort.

To ascertain whether the changes observed in the malaria-exposed groups were indeed associated to malaria exposure, we analyzed the correlation of T cell proportions and intracytoplasmic cytokine levels with plasma IgG Ab responses to a total of 19 *Plasmodium* Ags, which are well-known markers of malaria exposure. No significant correlation was found between IgG responses against malaria Ags and Tr1s, Th1, Tr1, or Tdpos cells (Supplemental Table I) or cytokine expression levels (data not shown). We also assessed the correlations between the frequencies of the four different T cell subsets. Our data showed that Th1 cells positively correlated with Tdpos and negatively with Tr1s (Fig. 2C), whereas no correlation was observed with Tr1 cells (data not shown).

All together, these findings confirm that pregnant women in PNG have fewer peripheral Tregs than nonpregnant individuals, as shown in other malaria-free countries, but point to differences in other immune mediators: whereas in “control” pregnancies in Spain there was a pregnancy-associated reduction of IL-10 expression by T cells and no changes in IFN-γ, pregnancy in PNG was associated with an expansion of IFN-γ–producing cells and IFN-γ levels. Nevertheless, changes associated to country of origin did not seem to be due to malaria exposure, as measured by Ab immunity.

**Association of T cells at recruitment with delivery outcomes:**

To understand which variables during pregnancy might affect the proportions of T cells and intracellular cytokine levels in the EP group (Table I displays the characteristics of this cohort), median regressions were estimated with the following variables: parity, age, gestational age, time point of bleeding (enrolment versus delivery), and *P. vivax* and *P. falciparum* infection at time of bleeding. Univariate analysis showed that proportion of Treg decreased with gravidity, and Th1 increased with gravidity, and Th1 increased with age (Table II). Women with a *P. falciparum* infection had more Tdpos cells (Fig. 3A, 3B, and Table II) but unaltered Treg frequencies. *P. vivax* infection and time point did not have any impact on the frequencies of the different T cell subsets. Adjusted analyses showed the same results.

Others have reported decreased Treg frequencies in *P. falciparum*–infected pregnant women compared with uninfected women in the first antenatal visit, but not at delivery (24). Thus, we compared Treg frequencies in three groups: uninfected nonpregnant malaria-exposed women, and uninfected and infected (*P. falciparum* and/or *P. vivax*) pregnant women from the same area. No differences were observed between infected or uninfected pregnant women either at recruitment or delivery, but both groups have fewer Tregs than nonpregnant women at both time points (Fig. 3C).

*Plasmodium* infection at the time of bleeding was associated with changes in IFN-γ production in the Treg population. Interestingly, *P. vivax* infection was associated with a decrease whereas *P. falciparum* infection was associated with an increase in the levels of this cytokine (Fig. 3D).

In summary, we show a lack of effect of malaria infection on Treg frequencies but increased Tdpos proportions and Treg–IFN-γ production associated to *P. falciparum* infection and decreased Treg–IFN-γ production in *P. vivax* infection.

**Association of T cells at recruitment with delivery outcomes:**

**Hb, birth weight, and *P. falciparum* infection**

We estimated regression models to study the association of T cell frequencies and intracellular production of IL-10 and IFN-γ measured at recruitment with Hb levels at delivery and birth weight. Th1 at recruitment had a positive association with birth weight (unadjusted effect estimate on birth weight [g] per 1% increase in Th1: 51.2 U [g], CI: 6.6; 95.9, *p* = 0.026, *n* = 33). However, the effect was not observed when we adjusted the model by age, gestational age, number of previous gestations, *P. falciparum* infection and decreased *P. falciparum* infection. Interestingly, IL-10 MFI levels in both Tregs and non-Tregs at recruitment had a positive association with Hb levels at delivery, and this was maintained after adjusting for the other variables (Table III).

Because the prevalence of infection was relatively low in our cohort, we did not have power to assess longitudinal associations of T cell responses with *P. falciparum* infection at delivery. Therefore, we performed a case–control study with additional samples from the Pregvax immunological cohort to investigate the associations of T cell and intracytoplasmic cytokine expression at recruitment with *P. falciparum* infection at delivery. We assessed nonspecific responses as described earlier and *P. falciparum*–specific T cell responses. All women were *P. falciparum*–negative at recruitment.

iRBC triggered recall responses in all T cell subsets (Fig. 4A); however, no differences in malaria-specific T cell frequencies were observed between women suffering or not a future *P. falciparum* infection (Fig. 4B). Nevertheless, fewer nonspecific (anti-CD3–stimulated) Tdpos cells were observed at recruitment in pregnancy.
women infected with *P. falciparum* at delivery, although the difference was not significant when adjusted for multiple comparisons (Fig. 4B). Lower *P. falciparum*–specific Treg–IL-10 levels and lower nonspecific Treg–IL-10 and non–Treg–IL-10 levels were observed in women with a future *P. falciparum* infection (Fig. 4C). Only nonspecific Treg–IL-10 differences remained significant after multiple comparison adjustment (*p = 0.060*; Fig. 4C).

Cytokine concentration in supernatant was also analyzed in the case–control study. PBMCs from women suffering a future *P. falciparum* infection secreted less IL-2, TNF, IFN-γ, MCP-1, and monokine induced by γ IFN (MIG), independently of the stimulus (iRBC or anti-CD3, Table IV). Moreover, iRBC-stimulated PBMCs from infected women secreted less IFN-γ–induced protein 10 (IP-10) and epidermal growth factor (EGF; Table IV). After multiple comparison correction, only IL-2 secretion after iRBC stimulation was different between infected and uninfected women (Table IV).

All together, these data show that production of proinflammatory cytokines by PBMCs and Th1 frequencies, together with increased intracytoplasmic IL-10 levels in T cells, may have a protective role against *P. falciparum* malaria infection during pregnancy and poor delivery outcomes. Of note, these protective responses might not necessarily be malaria specific. Also, they confirm that classical FOXP3+ Tregs do not have an apparent role in susceptibility to peripheral malaria infection during pregnancy or poor delivery outcomes.

**Correlation between T cells and plasma cytokine and chemokine concentrations**

To provide some insights into the immunological pathways involved in the altered distribution of regulatory T cells observed in pregnancy and their association with clinical parameters, the correlation between the T cell subsets and cytokines, chemokines, and growth factors was assessed. In Table V, a heat map shows the Spearman’s *p* coefficients for each cellular subset and plasma cytokine/chemokine concentrations, with a scale of colors ranging from dark gray (Spearman’s *p = 0.4*) to white (*p = −0.4*). The *p* values were adjusted for multiple comparisons (q value). Tdpos negatively correlated with IL-4 (q = 0.027), and Th11 cells negatively correlated with IL-12 (q = 0.003) and with IL-4 (q = 0.012). Tregs showed a positive correlation with IL-2 (q = 0.002) and eotaxin (Fig. 5A), and a negative correlation with TGF-β (q = 0.009). Eotaxin levels are decreased during pregnancy and in individuals exposed to malaria (27, 30). We have previously demonstrated that memory B cells express eotaxin receptor CCR3 (27) and activated murine Tregs express also this receptor (31). Thus, although eotaxin is not a classical T cell chemokine, we decided to investigate whether human Tregs express CCR3 and might respond to eotaxin. Low amounts of CCR3 were found in peripheral Treg (CD3+CD4+FOXP3+CD127−) frequencies were not associated with lower CD4−CD8−CD19− frequencies, which are present in the healthy women.

During “healthy” pregnancies, Tregs migrate to the placenta, resulting in reduced peripheral levels (9, 10). In this study, peripheral Treg (CD3+CD4+FOXP3+CD127−) frequencies were also found to be lower in pregnant women compared with nonpregnant women in PNG, a high-malaria transmission area. However, lower frequencies were not associated with *Plasmodium* infection at any time point (recruitment or delivery) or with delivery outcomes (Hb levels and birth weight). Our results contrast with previous find-
ings showing lower Treg frequencies in P. falciparum–infected pregnant women (23, 24), compared with uninfected pregnant women. Our discrepant results might arise from the inclusion of very low parasitemias (submicroscopic) in the definition of infection; as in previous studies, Treg frequency negatively correlated with parasitemia in pregnant women (24), and no differences in Treg frequencies were observed between uninfected and infected pregnant women when submicroscopic infections were accounted for (25).

Interestingly, although Treg frequencies in pregnancy followed a similar pattern in both malaria-free and malaria-endemic countries, indicating their importance in fetal tolerance, we did observe differences in other T cell populations. In control pregnancies, we observed an expansion of Tr1 cells, whereas pregnancy in PNG was associated with an expansion of Tdpos and TH1 cells. It could be speculated that the expansion of TH1 cells, although counterproductive for development of the fetus if excessive (11), might be necessary in tropical areas to protect the mother and fetus from common infectious diseases, which are largely associated with poor delivery outcomes (1, 22, 32, 33). Indeed, in our study, proinflammatory cytokines and TH1 frequencies at recruitment had a protective association with P. falciparum infection at delivery and birth weight, respectively. However, harmful excessive inflammation might be prevented by coexpression of IL-10, as part of a successful

Table III. Association between cellular variables at recruitment and Hb levels at delivery

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\*Effect estimate (Eff) per 1% increase in T cell frequency.
\*Effect estimate per 100-U increase in MFI. Simple and adjusted median regression models were performed. Bold represents \( p < 0.05 \). 

FIGURE 3. Present Plasmodium infection is associated with changes in Tdpos proportions and expression of cytokines in Tregs. (A) Representative dot plots of the CD4+FOXP3+CD127+ non-Treg subset distribution in pregnant women with or without present P. falciparum infection are displayed. Numbers indicate the percentage of cells within each gate. (B) Dot plots represent the median percentage of Tregs, Tr1, and Tdpos in pregnant women with or without present P. falciparum infection at any time during pregnancy. (C) Dot plots represent the median percentage of Tregs, Tr1, and Tdpos in pregnant women with or without present P. falciparum infection at any time during pregnancy. (D) Bars show the mean + SEM of IFN-γ MFI in Tregs in individuals with or without P. falciparum and P. vivax infections at any time during pregnancy. (B and D) The \( p \) value corresponds to a simple median regression model adjusted by age, gestational age, number of previous gestations, P. vivax infection, and P. falciparum infection. P. falciparum: U Tregs \( n = 93 \), Tr1 and Tdpos \( n = 88 \); I Tregs \( n = 8 \), Tr1 and Tdpos \( n = 7 \). P. vivax: U Tregs \( n = 86 \), I Tregs \( n = 8 \). *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \). I, infected; U, uninfected.
effector response (12). Certainly, Tdpos cells were also expanded in pregnancy in PNG and presented a significant correlation with TH1 cell frequencies. Moreover, Tdpos were also associated with protection from future *P. falciparum* infection, pointing again to the close relation between TH1 and Tdpos during pregnancy in PNG. Of note, nonspecific and *P. falciparum*–specific T cell responses were associated with protective responses, and T cell frequencies did not correlate with malaria-specific Ab levels analyzed in plasmas from the same women. This strongly suggests that the proinflammatory profile observed in pregnancy in PNG is pregnancy regulated or at least not malaria specific, and therefore it is tempting to speculate that it might protect against other harmful infectious diseases during pregnancy such as influenza or tuberculosis. Nevertheless, this has to be proved with an appropriate study. It could also be that the differences observed in pregnancies between Spain and PNG are due to different ethnic or genetic backgrounds.

**FIGURE 4.** Association of malaria-specific and nonspecific T cell responses with future *P. falciparum* infection. (**A**) Graph depicts the percentage of each T cell subset after stimulating PBMCs with uRBC or iRBC. The p value corresponds to the Wilcoxon matched-pairs signed-rank test. (**B**) Dot plot shows the differences in T cell frequencies at recruitment between *P. falciparum*–infected (I) and *P. falciparum*–uninfected (U) pregnant women at delivery after stimulating PBMCs with iRBC or anti-CD3. (**C**) Bars represent IL-10 and IFN-γ expression by Tregs and non-Tregs after stimulation with anti-CD3 or iRBC. (**B** and **C**) The p value corresponds to the Mann–Whitney U test, and q represents the adjusted p value after multiple comparison correction using the Benjamini–Hochberg method.
cytoplasmic IL-10 levels were associated with protection against low Hb levels and future (delivery) *P. falciparum* infection. These are remarkable results because anemia is one of the most common complications of pregnancy and malaria in pregnancy (2, 34). However, the mechanism might not be so evident. A recent study has demonstrated that IL-10 triggers hepcidin release by macrophages in vitro (35). Hepcidin blocks iron release by macrophages and intestinal absorption, lowering plasma levels. Thus, IL-10 has been proposed as a mechanism contributing to anemia during *P. falciparum* infection (35). Indeed, others have reported higher IL-10 plasma levels in pregnant women with *P. falciparum* infection and anemia at delivery (25). In contrast, hepcidin has been shown to block *P. falciparum* liver stages (36); thus, T cell–produced IL-10 might protect against future malaria infection (and consequent anemia) through hepcidin release. Finally, it is also possible that T cell IL-10 is not directly involved in protection against *P. falciparum* infection and poor delivery outcomes, but our results are just confounding associations related to TH1-protective answers, due to the apparent tight regulation between proinflammatory cytokine responses and future *P. falciparum* infection, for which only IL-2 remained significant after multiple comparison correction. However, taken together, all these results point in the same direction.

Tregs might exert their immunomodulation through cell–cell contact or by production of cytokines. In this study, we examined cytokine production as a measure of Treg activity. Lower intracytoplasmic IL-10 levels were found in Tregs and FOXP3+ regulatory T cells in control (Spanish) pregnant women compared with nonpregnant individuals. Downregulation of IL-10 expression by T cells during pregnancy is not surprising, because currently it is widely accepted that inflammation is necessary for critical steps in early pregnancy (11). Regardless, the lower expression of IL-10 was not observed when we compared pregnant and nonpregnant individuals from PNG. Interestingly, this seems again a process specifically regulated in tropical areas (or at least PNG) during pregnancy, to prevent deleterious delivery outcomes associated with infectious diseases. In this regard, higher intra-

Table IV. Differences in cytokine concentration in PBMC-stimulated supernatant between women with and without future *P. falciparum* infection

<table>
<thead>
<tr>
<th></th>
<th>Anti-CD3</th>
<th>iRBC</th>
</tr>
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<tr>
<td></td>
<td>z^a</td>
<td>p^b</td>
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<tr>
<td>EGF</td>
<td>0.707</td>
<td>0.480</td>
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<tr>
<td>EOTAXIN</td>
<td>1.838</td>
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<tr>
<td>FGF</td>
<td>0.707</td>
<td>0.480</td>
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<td>G-CSF</td>
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<td>GM-CSF</td>
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<td>HGF</td>
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<tr>
<td>IFN-α</td>
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<tr>
<td>IFN-γ</td>
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<td>0.270</td>
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<tr>
<td>IL-10</td>
<td>0.122</td>
<td>0.903</td>
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<td>IL-12</td>
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<td>IL-13</td>
<td>2.145</td>
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<td>IL-15</td>
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<td>IL-17</td>
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</tr>
<tr>
<td>IL-1RA</td>
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<td>1.000</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.980</td>
<td>0.327</td>
</tr>
<tr>
<td>IL-2</td>
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</tr>
<tr>
<td>IL-2R</td>
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<tr>
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<td>IL-5</td>
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<td>IL-7</td>
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<td>IL-8</td>
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<td>IP-10</td>
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<td>MCP-1</td>
<td>2.390</td>
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<tr>
<td>MIG</td>
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<td>MIP-1α</td>
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<td>MIP-1β</td>
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<td>RANTES</td>
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<tr>
<td>TNF</td>
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</tr>
<tr>
<td>VEGF</td>
<td>1.011</td>
<td>0.312</td>
</tr>
</tbody>
</table>

Uninfected women: n = 16; *P. falciparum*-infected women: n = 8.

^a-z: coefficient representing differences between infected and uninfected women. z > 0 signifies higher cytokine/chemokine concentration in uninfected women.

^b: p corresponds to Mann-Whitney U test.

^c: q adjusted p value (Benjamini-Hochberg method).

FGF: basic fibroblast growth factor; HGF: hepatocyte growth factor; VEGF: vascular endothelial growth factor.

Notably, some of our results lost significance when adjusted for other confounding variables or for multiple comparisons. Although these statistical corrections are important to distinguish true associations from confounding and random associations, they also increase the chances of false-negative results. For that reason, we focused our interpretation of data on consistency of results and biological plausibility. That is the case for the protective association found between TH1 frequencies and birth weight, which was lost in the adjusted regression, or the association between proinflammatory cytokine responses and future *P. falciparum* infection, for which only IL-2 remained significant after multiple comparison correction. However, taken together, all these results point in the same direction.

Tregs might exert their immunomodulation through cell–cell contact or by production of cytokines. In this study, we examined cytokine production as a measure of Treg activity. Lower intracytoplasmic IL-10 levels were found in Tregs and FOXP3+ regulatory T cells in control (Spanish) pregnant women compared with nonpregnant individuals. Downregulation of IL-10 expression by T cells during pregnancy is not surprising, because currently it is widely accepted that inflammation is necessary for critical steps in early pregnancy (11). Regardless, the lower expression of IL-10 was not observed when we compared pregnant and nonpregnant individuals from PNG. Interestingly, this seems again a process specifically regulated in tropical areas (or at least PNG) during pregnancy, to prevent deleterious delivery outcomes associated with infectious diseases. In this regard, higher intra-

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a previous study showing that this T cell phenotype is the
P. falciparum--specific CD4$^+$ T cell dominant response in children
with high exposure (more than two episodes), in contrast with
a dominant TNF response in children with low exposure (21).
P. falciparum--infected pregnant women had higher IFN-$\gamma$
levels (MFI) in the Treg subset than noninfected pregnant women. This
is in consonance with a previous study in which Tregs induced in
the presence of P. falciparum showed a population of FOXP3$^+$
cells producing proinflammatory cytokines, mainly IFN-$\gamma$ (37).
We also observed that P. vivax infection was associated with a
decrease in Treg--IFN-$\gamma$ production levels (MFI), in contrast with
the results reported in nonpregnant individuals (14). A different
population delineation marker (ours included CD127) or the
particularity of pregnancy could be the cause of this discrepancy.
We attempted to suggest immunological mediators of the altered
frequencies of T cells during pregnancy. Eotaxin concentrations
were decreased in pregnancy (27, 30) and correlated very well with
Treg frequencies, but the latter did not express the main eotaxin
receptor CCR3, which suggests that this chemokine is not in-
volved in the redistribution of Tregs during pregnancy. We were
surprised to find a relatively strong negative correlation between
IL--12 concentration and T$_H^1$ cell frequency and between TGF--$eta$
concentration and Treg frequency, because these cytokines are
known to trigger differentiation of those cellular subsets. Thus, we
interpret the negative correlation as a redistribution of the T cell
subsets to other tissues.

Our study presents some limitations. First, we could not provide
absolute counts but frequencies (proportions) of T cells; thus, some
cautions must be taken when interpreting the data. Second, pe-
ripheral blood represents a limited view of the immunological
compartment, and expansion/reduction of certain T cell pop-
ulations might be a consequence of redistribution to other tissues
like placenta, as already described for Tregs (9). However, our
study has the advantage of studying human samples throughout
the whole pregnancy (first trimester to delivery); therefore, col-
clection of placentas in early pregnancy was not possible. Also,
placental blood was not available for this analysis. Finally, Tr1
and Tdpos frequencies were somewhat low; however, they were in
similar ranges as other publications detecting IL-10 in vitro by
intracellular cytokine staining in malaria patients (21).

In conclusion, we demonstrated that nonspecific proinflam-
matory responses at recruitment, which have been widely be-
lieved to be counterproductive in pregnancy, are indeed associ-
ated with protection against P. falciparum malaria at delivery and
poor delivery outcomes. This proinflammatory response is ac-
companied by a tight increase of intracytoplasmic IL-10 levels in
T cells, which could prevent excessive inflammation, but that
might represent a protective mechanism itself. This has impor-
tant consequences in the design of malaria (and other) vaccines
for pregnancy. Moreover, if larger studies confirm these results,
some of these markers might be considered as early predictors of
poor delivery outcomes associated to malaria in pregnancy.

**FIGURE 5.** Plasma eotaxin correlates with Tregs, but these do not ex-
press CCR3. (A) Scatter plots show the
distribution of values for Tregs (%) and plasma eotaxin concentration in
the four groups, n = 118. The p value
for multiple comparison corre-
tion using the Benjamini–Hochberg
method. (B) Histograms represent CCR3
expression in different populations.
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Disclosures

The authors have no financial conflicts of interest.

References


