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Submicroscopic *Plasmodium* infection during pregnancy is associated with reduced antibody levels to tetanus toxoid

C. Álvarez-Larrotta,* O.M. Agudelo,* Y. Duque,* K. Gavina,† S.K. Yanow,†‡ A. Maestre,* J. Carmona-Fonseca* and E. Arango*

**Grupo Salud y Comunidad, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia, †Department of Medical Microbiology and Immunology, Faculty of Medicine, University of Alberta, and ‡School of Public Health, University of Alberta, Edmonton, Alberta, Canada.*

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Correspondence: C. Álvarez-Larrotta, Grupo Salud y Comunidad, Facultad de Medicina, Universidad de Antioquia, Carrera 51D 62-29, oficina 336, Medellín, Colombia.
E-mail: catalinaalvarezl95@gmail.com

Summary

Submicroscopic *Plasmodium* infections in pregnancy are common in endemic areas, and it is important to understand the impact of these low-level infections. Asymptomatic, chronic infections are advantageous for parasite persistence, particularly in areas where the optimal eco-epidemiological conditions for parasite transmission fluctuate. In chronic infections, the persistence of the antigenic stimulus changes the expression of immune mediators and promotes constant immune regulation, including increases in regulatory T cell populations. These alterations of the immune system could compromise the response to routine vaccination. This study aimed to evaluate the effect of submicroscopic plasmodial infection with *P. falciparum* and *P. vivax* during pregnancy on the immune response to the tetanus toxoid vaccine in Colombian women. Expression of different cytokines and mediators of immune regulation and levels of anti-tetanus toxoid (TT) immunoglobulin (Ig)G were quantified in pregnant women with and without submicroscopic plasmodial infection. The anti-TT IgG levels were significantly lower in the infected group compared with the uninfected group. The expression of interferon (IFN)-γ, tumour necrosis factor (TNF) and forkhead box protein 3 (FoxP3) was significantly higher in the infected group, while the expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) and transforming growth factor (TGF)-β was lower in the group of infected. In conclusion, submicroscopic Plasmodium infection altered the development of the immune response to the TT vaccine in Colombian pregnant women. The impact of *Plasmodium* infections on the immune regulatory pathways warrants further exploration.

Keywords: cytokines, *Plasmodium*, regulatory pathways, submicroscopic infections, vaccines

Introduction

Infection with *Plasmodium falciparum* or *P. vivax* in pregnancy can cause adverse delivery outcomes, including maternal anaemia and low birth weight infants [1–4]. These outcomes have been well characterized in response to both microscopic and submicroscopic infections in pregnancy. Other adverse effects of malaria in pregnancy include immune tolerance [5], susceptibility to acquire malaria and other infections and alteration of the immune response to vaccination [6–9]. However, these immunological effects have only been studied in microscopic *P. falciparum* infections.

Submicroscopic *P. falciparum* infections are common in pregnant women [10]. These infections can be considered chronic, as they are not treated; therefore, there is persistent antigenic stimulation that causes changes to the cytokine environment and distribution of cell types in maternal peripheral blood and the placenta [11,12]. Chronic infections are defined by an altered transcriptional profile and for persistent inflammation [13,14]. A steady increase of proinflammatory cytokines such as interferon

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(IFN)-γ and tumour necrosis factor (TNF), as well as anti-inflammatory cytokines, such as transforming growth factor (TGF)-β and interleukin (IL)-10, has been associated with chronic submicroscopic plasmodial infections in pregnancy [11,12]. These changes in the cytokine environment may explain the increase in regulatory T (T_{reg}) forkhead box protein 3 ($FoxP3^+$) cells reported in placental and peripheral plasmodial infections [15,16].

An increase in T_{reg} cells and the role of the transcription factor, FoxP3, are associated with two important processes in the pathophysiology of malaria. First, T_{reg} cells and FoxP3 are associated with parasite growth *in vivo* and the development of severe malaria because of their role in negative regulation of inflammation [15]. Secondly, the increase in T_{reg} cells protects the host against inflammation [17,18]. The chronic infections are associated with exhausted T cells with less robust effector functions and with alteration in the differentiation of memory T cells [19]. The exhausted T cells manifest characteristic features, including sustained up-regulation and co-expression of multiple inhibitory receptors [programmed cell death 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG3) and T cell immunoglobulin and mucin-domain containing-3 (TIM3)] and failure to produce antigen-independent memory T cells [20]. Furthermore, T_{reg} cells can suppress unrelated immune responses in a non-antigen-specific manner by a mechanism known as bystander suppression [21]. Expression of the CTLA-4, also known as CD152 (cluster of differentiation 152), can indicate the suppressor capacity of the immune response because it is the key inhibitory receptor of T_{reg} cells [22,23]. Conversely, the programmed cell death ligand 1 (PD-L1), also known as cluster of differentiation 274 (CD274), is expressed in dendritic cells (DC) and is a ligand of PD-1 expressed in T_{reg} cells. A recent study indicates that PD-L1 supports T_{reg} induction and is an important receptor in the regulation of the immune response [24].

The increase of immune regulatory mediators and cells during chronic submicroscopic malaria infections could alter the immune response to vaccination. In particular, the effects of malaria on the effectiveness of immunization of pregnant women with tetanus toxoid (TT) need to be taken into consideration in public health programmes and need further study [25]. Tetanus is a life-threatening, vaccine-preventable infection that poses a significant risk to pregnant women and newborns. In 2015 it caused 34 000 neonatal deaths worldwide [26]. The majority of existing cases are found in sub-Saharan Africa and India, regions endemic for malaria. Several studies evaluated TT vaccine performance in relation to malaria infection. One aspect evaluated was the passive transfer of anti-TT immunoglobulin (Ig)G antibodies across the umbilical cord in placental *Plasmodium* infection; these were not affected by placental malaria, but infection affected the transfer of anti-IgG antibodies against measles [27]. Another study evaluated the effect of malaria chemoprophylaxis on the TT vaccine performance. The chemoprophylaxis with sulphadoxine–pyrimethamine administered to children did not affect serological responses to TT [28]. Similar results were observed in malaria chemoprophylaxis with amodiaquine hydrochloride prior to vaccination, and chemoprophylaxis did not change the immunogenicity of DTP and measles vaccines [29]. Additionally, altered cytokine responses to the TT and bacilli Calmette–Guérin (BCG) vaccines were observed in infants with antenatal exposure to *P. falciparum* [30].

The effects of malaria infection could be reflected in a decrease in serum IgG levels against TT vaccine administered to pregnant women during antenatal care. The immune response against TT is characterized by strong differentiation of T cells and high production of IFN-γ after vaccination [31]. It was determined *in vitro* that the TT-specific IFN-γ secretion was mediated exclusively by $CD4^+$ T cells [T helper type 1 (Th1) response] [32]. An adequate amplification of the immune response of T cells and a potent IFN-γ production are fundamental to B cell differentiation and suitable production of anti-TT IgG [33].

There are no reports of maternal tetanus in South America or of levels of anti-TT IgG. In the northwestern region of Colombia *P. falciparum* and *P. vivax* are endemic, and a high frequency of pregnancy-associated submicroscopic plasmodial infections have been reported in peripheral blood during the course of pregnancy and in placental blood (23 and 4.9%, respectively) [34]. Little is known about the effects of those infections on immunity in mothers and babies. This study aimed to evaluate the effect of chronic submicroscopic *Plasmodium* infection during pregnancy on the immune response to the TT vaccine in Colombian women. Expression of different immune mediators and levels of anti-TT IgG were quantified in pregnant women with and without submicroscopic plasmodial infection during pregnancy.

Methods

Study site

Women were enrolled between September 2013 and May 2016 as part of a larger study in the municipality of Puerto Libertador, Department of Córdoba, in Northwestern Colombia (07°53′35′ N, 75°40″16″ W). This region has an estimated area of 43 506 km^2, and a population of 2·5 million at risk of malaria [35,36]. The region has stable malaria transmission intensity and is

homogeneous in terms of eco-epidemiology and malaria transmission. The region has a high malarial incidence, with a mean annual parasite index of 35·8 cases/1000 inhabitants. *P. vivax* and *P. falciparum* co-exist, but *P. vivax* prevails (60–70% of the total) [36].

Study design and sample selection

A total of 401 pregnant women with antenatal care were recruited sequentially in the main study. Colombian pregnant women received the tetanus, diphtheria and acellular pertussis vaccine (Tdap) as a single dose after the 26th week, between weeks 27 and 30 of gestation. Based on the availability and quality of the material collected from peripheral and placental blood, a subset of women was selected for this study in order to explore the effect of submicroscopic plasmodial infection during pregnancy on the immune response to the TT vaccine. A sample of 62 women was selected in this study: 18 with confirmed history of submicroscopic plasmodial infection at different times in pregnancy (submicroscopic plasmodial infection in pregnancy (SPIP) group) and 44 women without microscopic or submicroscopic plasmodial infection during pregnancy (no-SPIP group). The pregnant women selected were enrolled, on average, during the 20th week of gestation. The status of infection was determined from peripheral blood samples collected at monthly antenatal visits and from both peripheral and placental blood at delivery. Plasmodial infection was diagnosed by thick blood smear (TBS) and quantitative real-time PCR (qPCR). Submicroscopic infection was defined as a positive result by qPCR and a negative result by microscopy. Women in the no-SPIP group had negative results with both tests (qPCR and TBS) in peripheral blood during all antenatal visits, including at delivery, and in placental blood. Blood smears were stained with Field's stain and read by an experienced microscopist; samples were considered negative if no parasites were detected in 200 fields (1000 × total magnifications).

Inclusion and exclusion criteria

Inclusion criteria of the main study were voluntary acceptance and informed consent, permanent residency (> 1 year) in the malaria-endemic region, no history of pre-eclampsia and negative HIV and TORCH tests. The exclusion criterion was withdrawal of consent.

Data and specimen collection

After inclusion, a survey with maternal information was completed, which recorded data including age, number of pregnancies, number of malaria episodes during the ongoing pregnancy (based on antenatal records) and antimalarial treatment administered, weight, height and haemoglobin level. Newborn information included Apgar score, birth weight, length and head circumference.

During pregnancy, peripheral blood samples were collected during monthly antenatal visits. At delivery, placental blood was taken immediately after birth and maternal peripheral blood was obtained within 24 h. All peripheral and placental blood samples were collected in both ethylenediamine tetraacetic acid (EDTA) and dry tubes to obtain serum, plasma, packed red blood cells (RBC) and buffy coat. After sample collection in the field, the serum and plasma were separated immediately and stored in liquid nitrogen to be transported to the laboratory. RBC and buffy coat were homogenized with Trizol (Invitrogen, Carlsbad, CA, USA) (1 : 4) and stored in liquid nitrogen to be transported to the laboratory. In the laboratory, all samples were stored at –80 °C until processing.

Thin blood smears and blood spots on filter paper Whatman #3 were prepared from each sample (peripheral and placental blood).

Detection of Plasmodium infection by qPCR

Total nucleic acid was extracted from packed RBCs using a MagMAX 96 DNA Multi-Sample Kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed as described elsewhere [37]. Samples were first tested for *Plasmodium* DNA using genus-specific primers and a hydrolysis probe (Plasprobe). PCR was run on the ABI 7500 FAST platform. Samples with a cycle threshold (Ct) < 45 were tested in two single species-specific reactions for *P. falciparum* and *P. vivax* using RNA. DNA copy number was quantified from the genus-specific reaction against a standard curve using a plasmid containing a fragment of the 18S gene from *P. falciparum*.

Expression analysis of cytokines and other immune molecules

A reverse-transcription real-time PCR assay with relative quantification (qRT–PCR) was used to evaluate the expression of the cytokines IL-10, TGF-β, IFN-γ and TNF and the molecules FoxP3, CTLA-4 and PD-L1 in maternal peripheral blood. Each analyte per sample was tested in triplicate. For each molecule a standard curve was made to determine the efficiency of the reaction. The efficiencies obtained were between 94 and 106%. For each molecule analysed by qPCR, a Ct less than 40 was obtained. Buffy coat homogenized with Trizol (Invitrogen) was used for extraction of total RNA, following the manufacturer's instructions. The EXPRESS OneStep Superscript® qRT–PCR kit (Invitrogen) was used for reverse transcription and amplification of each molecule, using a StepOnePlus real-time PCR system (Applied Biosystems) and the primers and probes described in Table 1. The OneStep software version 2.3 was used for data analysis.

Table 1. Primers and probes used in this study

Gene	Forward primer (5–3)	Reverse primer (5–3)	Probe (5–3)	Product Size (bp)
IL-10	CCTGGAGGAGGTGATGCCCCA	CAGCGCCGTAGCCTCAGCC	CAAGGCGCATGTGAACTCCCTG	131
TGF-β	TCAGAGCTCCGAGAAGCGGTA	GTTGCTGTATTTCTGGTACAT	CCGGGCAGAGCTGCGTCTGCTGA	92
IFN-γ	GAAGAATTGGAAAGAGGAGAGTGA	TGGACATTCAAGTCAGTTACCG	TTCCTTGATGGTCTCCACACTCTTTTGG	218
TNF	GCCCAGGCAGTCAGATCA	GCTTGAGGGTTTGCTACAACA	CCCGAGTGACAAGCCTGTAGCCC	74
FOXP3	GAGAAGCTGAGTGCCATGCA	GGAGCCCTTGTCGGATGAT	CCACCTGGCTGGGAAAATGGCAC	87
CTLA4	GCTCAGCTGAACCTGGCTAC	CGTGCATTGCTTTGCAGAAGAC	CCTGCACTCTCCTGTTTTTTC	88
PD-L1	CTGTGAAAGTCAATGCCCCATAC	CAGTTCATGTTCAGAGGTGACTG	CCAAAGAATTTTGGTTGTGGAT	80
β-actin	CGAGCGCGGCTACAGCTT	CCTTAATGTCACGCACGATT	ACCACCACGGCCGAGCGG	58

The PCR reaction efficiencies were determined based on mRNA extracted from stimulated peripheral mononuclear cells from malaria-naive donors. Relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method [38] and normalized with β-actin as the reference gene. A pool of peripheral mononuclear cells from healthy pregnant women was used as a calibrator. All experiments included a no-template control.

Quantification of cytokines and IgG against TT

Levels of anti-TT IgG and the cytokines IL-10, TGF-β, TNF and IFN-γ were quantified by enzyme-linked immunosorbent assay (ELISA) in the serum samples obtained from maternal peripheral blood at the time of delivery. The antibodies were quantified with the tetanus IgG ELISA kit (RE56901; IBL International, Hamburg, Germany) and the cytokines were quantified using the ELISA kit from Human OptEIA™ (BD Biosciences, Inc., San Jose, CA, USA) following the manufacturers' instructions. Eight-point standard curves with serial twofold dilutions were used for each cytokine, with the following concentration ranges, including a point with a concentration of 0·0: IL-10 from 7·8 to 500 pg/ml; TGF-β from 125 to 8000 pg/ml; TNF from 7·8 to 500 pg/ml; IFN-γ from 4·7 to 300 pg/ml; and IgG anti-TT from 0·0 to 5·0 UI/ml. Three replicates of each point were used. The samples were tested in triplicate and diluted 1 : 2 with the test diluent. Plates were read in a Labsystems Multiskan microplate reader (Thermo Scientific, Waltham, MA, USA) at 450 and 620 nm. The calculations and regression analyses were performed manually in Microsoft Excel 2013. In the process it was necessary to dilute (1 : 4) several samples because they exceeded the highest point of the curve. For IL-10 only one sample from the SPIP group was diluted and for TGF-β four samples were diluted, two from the SPIP group and two from the non-SPIP group.

Statistical analysis

Most of our data were not normally distributed, based on the Kolmogorov–Smirnov test; thus, the non-parametric Mann–Whitney *U*-test and Kruskal–Wallis test were performed to evaluate differences between the groups. The IBM SPSS Statistics (version 24) was used. Comparisons were made between the non-infected group (no-SPIP) *versus* the infected group (SPIP), and additional comparisons were made between the groups of women infected by *P. vivax* (SPIP by *P. vivax*) with the no-SPIP group. Significance was accepted for all analyses at $P < 0.05$. Spearman's rho was used to measure the correlation between the variables. Following a significant Kruskal–Wallis test, Dunn's test of multiple comparisons was performed for adjustment.

Ethics

The study protocol was reviewed and approved by the Ethics Committee of the Instituto de Investigaciones Médicas, Universidad de Antioquia. Each participant gave full informed consent according to the Helsinki convention and the Colombian regulations for this type of research. Each subject voluntarily agreed to participate in the study.

Results

The general characteristics of the study women and their newborns are shown in Table 2. Some data were not available from the clinical history. Most women (58%; 33 of 57 women with data) were multiparous. Haemoglobin levels were measured at delivery in 23 women and the frequency of anaemia was 39%, with no difference between the SPIP and no-SPIP groups ($P = 0·5$). The birth weight was similar in all study groups, and all the newborns weighed more than 2500 g. Women with SPIP had babies with smaller head circumference compared with babies of healthy women, and this finding was also observed in newborns of women with infection by *P. vivax*. In addition, newborn length was significantly lower in the infected group compared with the non-infected group (Table 2).

In the SPIP group ($n = 18$), the qPCR results were as follows: 10 (55%) had *P. vivax infection (SPIP by P. vivax),* three *(*17%) had *P. falciparum infection* and five (28%) had mixed infection (positive for both species). In 10 (56%) women, the infection was detected at delivery: six

Table 2. General characteristics of pregnant women and newborns according to infection status by quantitative polymerase chain reaction (qPCR)

	No-SPIP group			SPIP group			SPIP by *P. vivax* group			
Variable	*n*	Median	IQR 25–75%	*n*	Median	IQR 25–75%	*n*	Median	IQR 25–75%	P^a
					Maternal data					
Age (years)	41	20	19–26	17	21	20–31	9	20	16–34	0·881
Weight (kg)	39	58	51·5–63·0	17	58	51–61	9	57	50–60	0·901
Height (cm)	39	158	151–162	17	158	152–159	9	158	150–159	0·705
Haemoglobin (g/dl)	15	11·1	10·6–11·9	8	11·8	9·85–12·4	5	11·5	10·1–12·1	0·856
Number of pregnancies	40	2	1–3·75	17	2	1–3	9	2	1–3	0·782
					Newborn data					
Apgar test 1	41	9	8–9	17	9	8–9	9	8	8–9	0·423
Apgar test 2	11	9	9–10	11	10	9–10	6	10	9·25–10	0·412
Birth weight (g)	41	3300	3100–3700	17	3300	2800–3600	9	3300	2800–3600	0·569
Length (cm)	41	51	50–52	17	49	48–52	9	49	48–51	**0·041**
Head circumference (cm)	41	34	33–35	17	33	32–34	9	33	31·5–34	**0·019**

P^a-value based on the Kruskal–Wallis statistical test, comparison of no-microscopic or submicroscopic plasmodial infection during pregnancy (no-SPIP) group, SPIP group and SPIP by *Plasmodium vivax*. IQR = interquartile range.

had a placental infection (PI); of these, the date of infection for four women are not known and four gestational infections at delivery (GID); only one of these had a history of infection at week 27 of gestation. Regarding gestational infection during pregnancy (GIP), the eight included women attended three to four antenatal visits. These visits were between weeks 12 and 36 of pregnancy. In most women the infection was detected at more than one antenatal visit. At the first antenatal visit, two women were infected; at the second antenatal visit, eight of the women were infected; at the third antenatal visit, four women were still infected; and in the quarter antenatal visit, five women were still infected. All women included in this group were negative at delivery (Supporting information, Table S1; Fig. 1a). In general, the level of infection in these women was very low and all samples had < 2 DNA copies/μl of blood.

Anti-tetanus toxoid IgG levels

The anti-TT IgG levels in the SPIP group were 2·99 ± 0·79 IU/ml, compared with 4·02 ± 0·54 IU/ml ($P < 0·001$) in the no-SPIP group (Fig. 1b). Moreover, women infected with *P. vivax* also had significantly lower levels of anti-TT IgG (3·32 ± 0·75 IU/ml) than non-infected women ($P = 0·007$) (Fig. 1c). The adjustment for multiple comparisons with Dunn's test showed a significant difference between the no-SPIP and SPIP groups ($P < 0·001$), and between the no-SPIP and SPIP by *P. vivax* groups ($P < 0·05$). These findings suggest that submicroscopic plasmodial infection in general, and specifically submicroscopic *P. vivax* infection, decreased the level of antibodies generated after vaccination with TT in pregnant women.

Expression of molecules associated with immune responses in infected and non-infected pregnant women

To determine transcriptional changes resulting from chronic plasmodial infection, the expression of different mediators of the immune response in the study groups was measured (Figs. 2 and 3). The expression of IFN-γ and TNF (Figs. 2a and 2b) and the regulatory molecule FoxP3 (Fig. 3a) was higher in the SPIP group, compared with the no-SPIP group, while the expression of TGF-β and CTLA-4 (Figs. 2d and 3b) was lower in the SPIP group. In addition, the expression of PD-L1 was similar in both groups (SPIP *versus* no-SPIP) (Fig. 3c). The expression of CTLA-4 was significantly lower in women infected by *P. vivax* compared with the no-SPIP group ($P = 0·009$) (Fig. 1d). After adjustment for multiple comparisons with Dunn's test, the difference in the expression of TGF-β was maintained between the no-SPIP and SPIP groups, and between the no-SPIP and SPIP by *P. vivax* ($P < 0·01$). Similarly, after adjustment, differences in expression of FoxP3 were sustained between the no-SPIP and SPIP groups ($P < 0·05$), and differences in expression of CTLA-4 was maintained between the no-SPIP and SPIP by *P. vivax* groups (< 0·05).

Cytokine levels in peripheral blood of infected and non-infected pregnant women

Cytokine protein levels were quantified to determine if the transcription profile is concordant with changes in

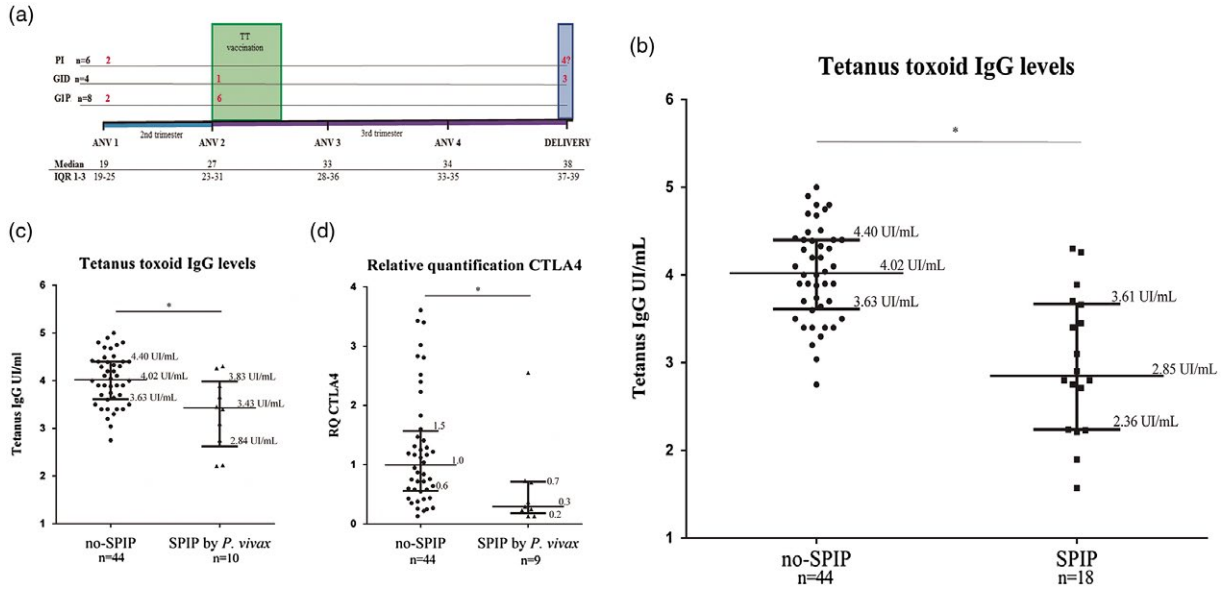

Fig. 1. (a) Timeline of infection and vaccination against TT in the SPIP group. Placental infection (PI). Gestational infection at delivery (GID). Gestational infection during pregnancy (GIP). The green box indicates the period of TT vaccination in pregnant women (between weeks 27 and 30). The blue box shows the timing of quantification of IgG against TT in the groups evaluated. The red numbers indicate the number of pregnant women with a history of malaria during pregnancy. Each number is located in the week of gestation in which the infection was detected. In cases of placental infection, all were detected at the time of delivery and 4 pregnant women had no history of malaria during pregnancy and the time of infection is unknown (4?). (b) IgG against tetanus toxoid in pregnant women according to infection status by qPCR. Median and interquartile ranges of IgG against tetanic toxoid. * $P = 0{\cdot}000$ Kruskal Wallis test. IgG against tetanus toxoid (c) and CTLA4 relative quantification (d) in no-SPIP and SPIP by P. vivax groups. Median and interquartile ranges of IgG against tetanic toxoid. * $P = 0{\cdot}007$ Mann-Whitney U test. Median and interquartile ranges of RQ CTLA4 * $P = 0{\cdot}009$ Mann-Whitney U test.

the levels of proteins in peripheral maternal blood. Table 3 shows the levels of each cytokine in serum of infected and non-infected women. All cytokines had similar levels in women without infection and those with plasmodial infection.

Significant correlations among the relative expression or serum levels of the different immune mediators

We hypothesized that submicroscopic plasmodial infection during pregnancy alters the immune response of the host. We found that anti-TT IgG levels did not correlate with any of the immune mediators evaluated in the two study groups. However, significant correlations ($P < 0{\cdot}05$) or highly significant correlations ($P < 0{\cdot}01$) were observed between the different cellular immune mediators (Fig. 4) (Supporting information figures show scatterplots with rho and *P*-values). In the no-SPIP group (Fig. 4a), most of the significant correlations between the relative expressions of the different immune mediators were positive (i.e. when a variable increased or decreased, the other variable did the same). However, most of the correlations were moderate or low (rho < 0·5), and a high correlation (rho > 0·5) was only observed between TNF and IFN-γ. Expression of the anti-inflammatory cytokine TGF-β was correlated significantly with the proinflammatory cytokines IFN-γ and TNF, and with the regulatory mediators CTLA-4, FoxP3 and PD-L1. Expression of IFN-γ was also correlated with TNF and the regulatory mediators CTLA-4, FoxP3 and PD-L1. Interestingly, expression of PD-L1 was correlated with both proinflammatory (IFN-γ and TNF) and anti-inflammatory (TGF-β) cytokines, as well as with FoxP3 associated with T_{reg} cells. Conversely, the serum levels of TGF-β also correlated positively with the levels of IFN -γ and TNF, and serum levels of these two proinflammatory cytokines (IFN-γ and TNF) showed a strong positive correlation, which confirms their co-dependence during inflammatory processes. Negative correlations were observed between the protein level of IFN-γ and expression of TGF-β and CTLA-4 and the protein level of TNF and IFN-γ with expression of CTLA-4.

In the SPIP group (Fig. 4b), correlations were weaker compared with the no-SPIP group. Expression of TNF correlated strongly with expression of IFN-γ and PD-L1, while expression of CTLA-4 correlated with expression of the anti-inflammatory cytokines TGF-β and IL-10. Furthermore, the protein levels of TNF correlated positively with levels of TGF-β and negatively with expression of CTLA-4. It is important to note that all correlations observed in the SPIP group were also observed in the no-SPIP group.

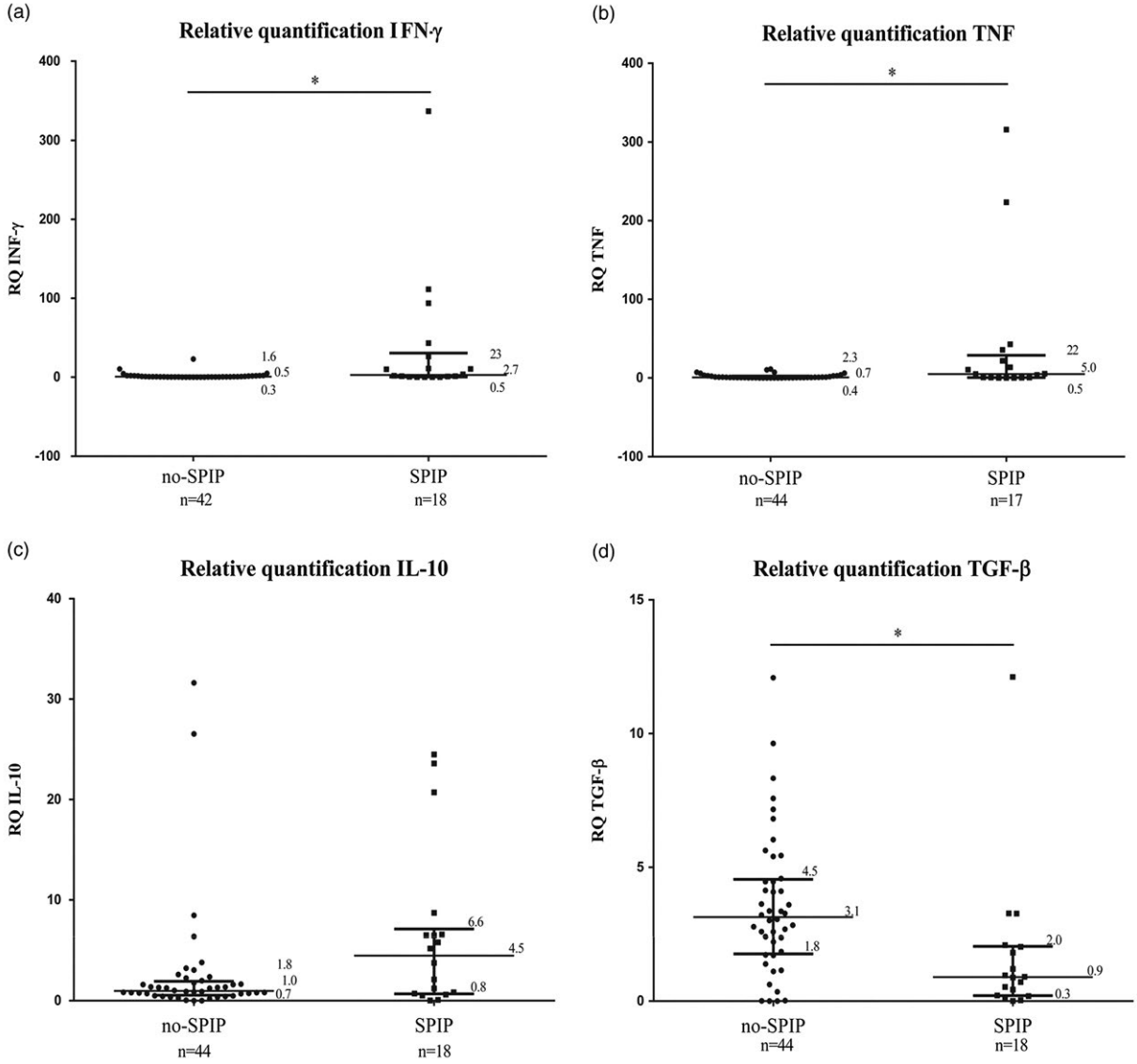

Fig. 2. Relative quantification of cytokine expression in pregnant women according to infection status by qPCR. Median and interquartile ranges of relative quantification (RQ) of IFN-γ (a), TNF (b), IL-10 (c), TGF-β (d) and in the two study groups. *P* values * < 0·05 Kruskal Wallis test.

In the group of pregnant women infected with *P. vivax*, the same correlations were observed as in the SPIP group. Additionally, it was observed that the protein levels of IFN-γ correlated positively with levels of IL-10.

Discussion

In this pilot study, despite the small number of samples, a marked decrease in the levels of anti-TT IgG was found in Colombian pregnant women with submicroscopic plasmodial infection. In Kenya, tetanus antibody levels were significantly lower in women with active chronic or past placental malaria detected by histopathology, with the adjusted reductions between 36 and 41% [7]. Although the women studied here had submicroscopic infection and those studied by Cumberland *et al.* had microscopic infection (haemozoin and/or parasites in placental tissue detected by histopathology), in both studies women had chronic infections with unknown timing and/or duration but which were associated with an impaired immune response to vaccination. Furthermore, the frequency of memory B cells with specificity for TT were not modified by *P. falciparum* infection status in a cohort of primigravid women at the time of delivery [39]. This finding does not support the results obtained in our study. However, it is important to note that the frequency of specific memory B cells does not necessarily reflect the levels of circulating specific IgG antibodies.

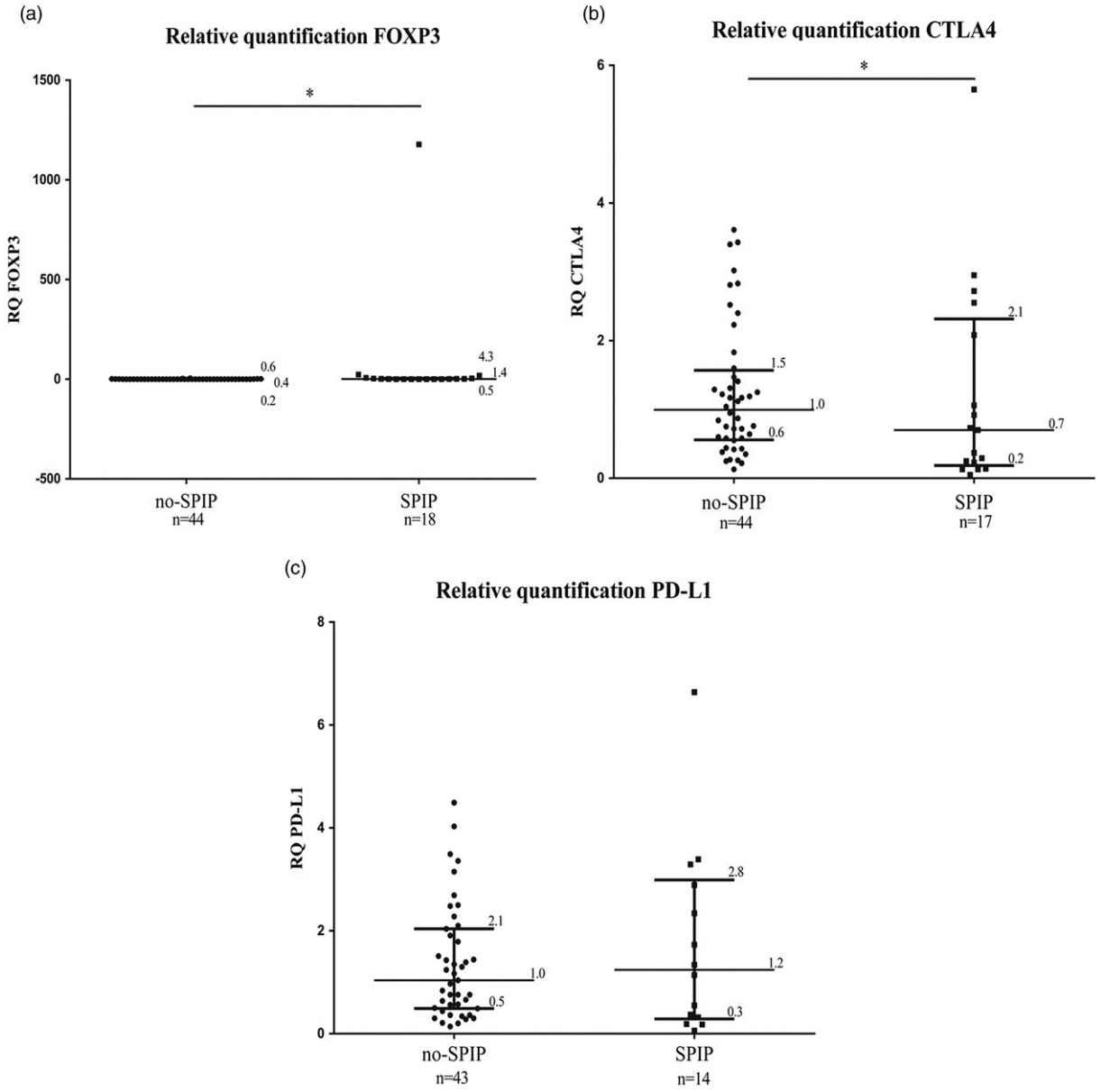

Fig. 3. Relative quantification of regulator molecules in pregnant women according to infection status by qPCR. Median and interquartile ranges of relative quantification (RQ) of FOXP3 (a), CTLA4 (b), and PDL1 (c) in the two study groups. *P* values *< 0·05 Kruskal Wallis test.

There are no antibody values that determine protection from tetanus; the amount of antibody that ensures immunity to tetanus is assay-specific [40]. We used ELISA to determine anti-TT IgG levels and with this assay, concentrations of at least 0·1–0·2 IU/ml are defined as protective. However, cases of tetanus have been reported in individuals within this range of antibody concentrations [41]. The levels of anti-TT IgG observed in our study far exceed these thresholds. It is important to highlight that the World Health Organization recommends two doses of vaccine as a minimum to ensure protection against maternal and neonatal tetanus [42]. In Colombia, the expanded immunization plan includes only a single dose during pregnancy, without taking into account a woman's vaccination history. At the same time, there is no notification of maternal tetanus and the neonatal tetanus notification rate is so low that it is not known whether the vaccination coverage is sufficient or if there is underreporting of cases.

Although our study includes a limited number of samples, these preliminary findings suggest that submicroscopic plasmodial infection in general, and specifically by *P. vivax*, modulates the immune response to the TT vaccine in pregnant women. This is consistent with studies that

Table 3. Levels of cytokines in maternal peripheral blood according to infection status by quantitative polymerase chain reaction (qPCR)

Cytokine (pg/ml)	No-SPIP *n* = 44		SPIP *n* = 18		SPIP by *P. vivax* n =10		
	Median	IQR 25–75%	Median	IQR 25–75%	Median	IQR 25–75%	P^a
IL-10	5·8	4·9–7·0	7·3	5·7–8·6	6·9	4·9–9·6	0·566
TGF-β	2657	1514–5307	3502	1924–5407	4030	2191–5294	0·431
TNF	4·0	2·8–5·6	3·2	2·1–6·6	3·9	2·2–6·6	0·824
IFN-γ	3·4	2·5–10·3	3·2	2·9–14·1	3·0	2·8–6·0	0·799

P^a-value based on the Kruskal–Wallis statistical test, comparison of no-microscopic or submicroscopic plasmodial infection during pregnancy (no-SPIP) group, SPIP group and SPIP by *Plasmodium vivax*. IQR = interquartile range; IL = interleukin; TGF = transforming growth factor; TNF = tumour necrosis factor.

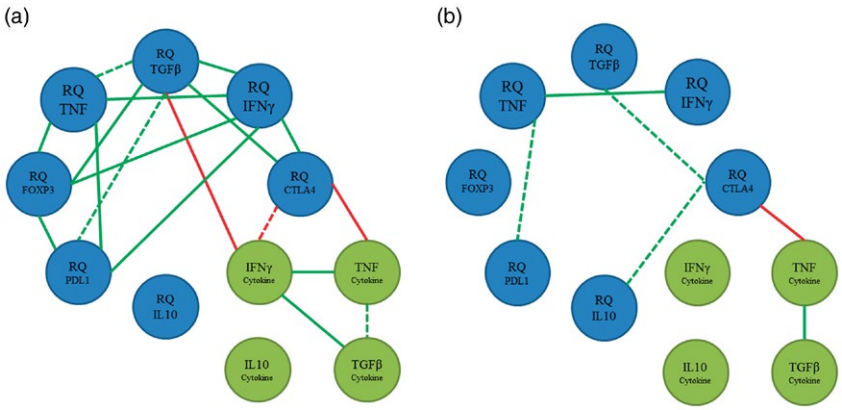

Fig. 4. Significant correlations between protein levels and expression of cytokines. Correlation analysis (rho Spearman) of the protein levels and the expression of immune mediators in peripheral blood of no-SPIP group (a) and SPIP group (b). Blue circles: relative quantification of the expression of each cytokine. Green circles: levels of cytokines (level of protein). Green lines: positive correlation. Red lines: negative correlation. Continuous lines: statistical significance $P < 0{\cdot}01$. Dotted lines: statistical significance $P < 0{\cdot}05$.

reported low production of IFN-γ, low response of T cells to some mitogens and increased T_{reg} cells in cord blood of neonates born to women with placental malaria [43–47]. These alterations in the host immune response, associated with microscopic *P. falciparum* malaria, result in increased susceptibility to bacteraemia in children [48,49] and alteration of the vaccine response [7,9,50]. Therefore, it is essential in future to evaluate the effect of submicroscopic maternal infections in our setting on the immunity of neonates and children post-vaccination.

Chronic placental infection by *Plasmodium* induces an expansion of FoxP3 [16], a transcription factor that regulates the expression of inhibitory molecules and anti-inflammatory cytokines. Consistent with this, we found that infected women had higher expression of FoxP3, a measure of T_{reg} cells [51–53], than uninfected women. Additionally, we evaluated the role of the receptors PD-L1 and CTLA-4 and their association with anti-TT IgG levels and infection status. We observed no difference in the relative expression of PD-L1 between the groups. Interestingly, there was a significant decrease in the expression of CTLA-4 in the infected women (Fig. 3b) and in the SPIP by *P. vivax* (Fig. 1d) group compared with the no-SPIP group. In previous studies, decreased numbers of activated T lymphocytes were observed in *P. vivax* infection compared with healthy subjects living in the same endemic zone [54]. It is important to highlight that 83% of the pregnant women in this study were infected with *P. vivax*; probably there were not enough activated T lymphocytes compared to our control group. It would be interesting to investigate if this was due to a low amount of activated T lymphocytes. Other studies of the immune response to *P. falciparum* acute infections in humans [55] and to *P. berghei* ANKA infections in mice [56] reported increases in CTLA-4 expression in activated T lymphocytes. In mononuclear cells isolated from cord blood of women with *P. falciparum* placental malaria, an increase in antigen-specific $CD4^+$ regulatory cells $CD4^+CD25^+CTLA\text{-}4^+$ with suppressive characteristics was observed [57]. This demonstrates the active role of CTLA-4

to maintain an immunological balance against *Plasmodium* as a negative regulator of T cell activation. Conversely, our results show a negative correlation of the relative expression of CTLA-4 with the levels of proinflammatory cytokines in the two groups evaluated. In cytotoxic T lymphocytes, CTLA-4 controls the translational inhibitor programmed cell death protein 4 (PDCD4) associated with the inhibition of TNF and INF-γ [58]. Interestingly, this could explain the correlation found, and it would be important to evaluate the role of CTLA-4 in translational inhibition in $CD4^+$ T cells.

As for IL-10, we did not observe any significant differences between the no-SPIP and SPIP groups; however, an increase in the expression of IL-10 was evident in the SPIP group. In different studies, IL-10 is increased in cases of acute and asymptomatic malaria [59] and is associated with protection against symptoms and immunopathology of the disease. In the SPIP study group with chronic submicroscopic infections, a positive correlation was found between the expression of IL-10 and CTLA-4 (i.e. when IL-10 increases, CTLA-4 increases); this correlation could indicate a role for IL-10 in the limitation of the effector immune response. No correlation was found in the no-SPIP group.

Due to the exploratory nature of this study, all the significant *P*-values, even without adjustment for multiple comparisons, must be considered relevant and suggestive of further research. However, it is important to remark that after adjustment the differences in the expression of the cytokine TGF-β and the regulatory molecules FoxP3 and CTLA-4 were maintained. The increase in the expression of FoxP3 and TGF-β in the group of infected women suggests an inclination towards a regulatory profile of the immune response which supports our hypothesis.

The largest number of correlations was observed in the no-SPIP group compared to the SPIP group. Additionally, we did not observe correlations between the expression and the levels of the cytokines in the groups evaluated. It is important to note that the correlations are very susceptible to the number of samples and it is probably necessary to obtain more samples in order to observe concordance between the expression and cytokine levels. An increase in both expression and cytokines was observed even in the absence of correlations in the group of infected women.

We recognize that the small number of samples analysed is an important limitation of our study that can affect the correlations reported here. Additionally, quantification of cell populations and their phenotyping would be important for future analyses. It is important to highlight that the women were vaccinated between weeks 26 and 30 of pregnancy, but we do not know the exact date of vaccination in relation to the delivery, which could influence the levels of IgG against TT found at the time of delivery.

In conclusion, our pilot study demonstrated that submicroscopic plasmodial infections were associated with a decrease in the production of IgG against the tetanus toxoid vaccine administered in a single dose in Colombian pregnant women. Despite this, it is not known if lower anti-TT levels compromise protection against maternal and neonatal tetanus. It is possible that the impact of lower anti-tetanus toxoid IgG is greater in microscopic gestational and placental infections compared with submicroscopic infections [2·05 UI/ml [6] *versus* 2·99 UI/ml in this study (P = 0·009)]. Furthermore, in Colombia, the existing social conditions in malaria-endemic areas facilitate parasitic co-infections which could potentiate the alterations in the effector immune response.

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Disclosures

The authors declare that they have no conflicts of interest.

Author contributions

All authors have contributed significantly to the design, execution, analysis and writing of this report, and have seen and approved the final submitted version.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Significant correlations between expressions of cytokines and levels of cytokines in no-SPIP group. Correlation analysis (rho Spearman). **P* value < 0·05; ***P* value < 0·01.

Fig. S2. Significant correlations between expressions of cytokines and levels of cytokines in SPIP group. Correlation analysis (rho Spearman). **P* value < 0·05; ***P* value < 0·01.