Human Host-Derived Cytokines Associated with *Plasmodium vivax* Transmission from Acute Malaria Patients to *Anopheles darlingi* Mosquitoes in the Peruvian Amazon

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Abstract. Infection of mosquitoes by humans is not always successful in the setting of patent gametocytemia. This study tested the hypothesis that pro- or anti-inflammatory cytokines are associated with transmission of *Plasmodium vivax* to *Anopheles darlingi* mosquitoes in experimental infection. Blood from adults with acute, non-severe *P. vivax* malaria was fed to laboratory-reared F1 *An. darlingi* mosquitoes. A panel of cytokines at the time of mosquito infection was assessed in patient sera and levels compared among subjects who did and did not infect mosquitoes. Overall, blood from 43 of 99 (43%) subjects led to mosquito infection as shown by oocyst counts. Levels of IL-10, IL-6, TNF- α , and IFN- γ were significantly elevated in vivax infection and normalized 3 weeks later. The anti-inflammatory cytokine IL-10 was significantly higher in nontransmitters compared with top transmitters but was not in TNF- α and IFN- γ . The IL-10 elevation during acute malaria was associated with *P. vivax* transmission blocking.

INTRODUCTION

Malaria patients are not equally infectious to mosquitoes.^{1–5} A variety of determinants modulate transmission, including gametocyte quality, vector competence, and human innate and acquired immunity. Although host antibodies^{6–8} have been shown to affect parasite infectivity for mosquitoes, transmission inefficiency is still observed in malaria hypoendemic regions where presence of antibody is thought to be low.^{2,9} Previous work in Sri Lanka has suggested that humoral factors associated with the malarial paroxysm reduces parasite infectivity for mosquitoes.^{5,10} In this study, we hypothesized that cell-mediated immunity as assessed by serum cytokines as a biomarker would influence transmission.

Cell-mediated immunity, with surrogate markers including pro-inflammatory cytokines, has been shown to play a role in controlling malaria parasitemia^{11,12} and symptom severity,^{13–16} and to likely impact transmission.^{17–19} Interferon-gamma (IFN- γ), produced by Th1 and natural killer cells, is involved in activation of macrophage and iNOS and is an important signal in fighting intracellular infections. Tumor necrosis factor α (TNF- α), also part of the Th1 response, induces inflammation and cell death. The TNF- α receptor blockade has been suggested to increase *Plasmodium chabaudi chabaudi* transmission to mosquitoes,^{17,20} and TNF- α itself has been shown to inactivate gametocytes in the presence of leukocytes.²¹ The transforming growth factor β (TGF- β) has been studied in its actions within the mosquito, and is thought to regulate mosquito midgut immunity via anopheline nitric oxide synthase, thus impacting transmission.^{18,19}

We hypothesized that immunomodulatory cytokines might affect the ability of *Plasmodium vivax* gametocytes to infect mosquitoes and that the identification of such factors might be a useful biomarker of transmission. To test this hypothesis, we used a multiplex cytokine panel to determine the relationship between IFN- γ , TNF- α , interleukin-2 (IL-2), IL-4, IL-5, granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-1 β , and IL-8 as related to oocyst development in experi-

mental infection using *ex vivo*-obtained *Plasmodium vivax* during acute symptomatic infection where gametocytes are clearly patent. Because there are no reference ranges for cytokine values, a ratio of cytokine levels of acute infection and at 3 weeks after drug treatment was used as a benchmark of cytokine response during acute malaria.

MATERIALS AND METHODS

Study sites. Subjects were recruited from the Ministry of Health health posts in the region of Iquitos, Peru, the capital of the Loreto Department in the Peruvian Amazon. This city is located at 3°45' South, 73°15' West and is surrounded by the Amazon rainforest, the Itaya River to the east, and the Nanay River to the west, both of which feed the Amazon River to the north.

Subject recruitment. Subjects with fever and diagnosed microscopically with *P. vivax* malaria were invited to participate in the study. Adults 18 years of age and older able to provide informed consent and adolescents 15 to 17 years of age able to assent with parents' informed consent were enrolled. Pregnant women and patients with severe malaria symptoms requiring immediate medical attention were excluded as were patients with mixed *Plasmodium* infection, or patients who had already initiated antimalarial therapy. All participation was voluntary and the decision to participate had no effect on treatment, which is provided free of charge by the Peruvian government according to the Peruvian Ministry of Health guidelines.

Ethics. This study was approved by the Institutional Review Boards/Ethical Committees of the University of California San Diego, La Jolla, CA, Universidad Peruana Cayetano Heredia, Lima, Peru, and Asociación Benéfica Prisma, Lima, Peru. Approval to carry out the study was also obtained from the Loreto Directorate of Health, Iquitos, Peru.

Questionnaire. Patients answered a questionnaire about their symptoms (length of time with fever, nausea, malaise, etc.), malaria and health history (episodes of vivax, falciparum, mixed infections in the past and time of last infection, and other health issues), medications, and exposures (use of bed net, travel history, and household members with fever/malaria).

Membrane feeding assays. A sample 13.5 mL of venous blood was drawn into 1.3 mL of anticoagulant citrate-phosphate

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dextrose. Of this, 6 mL of this blood was used (within 5–10 minutes) in a standard membrane feeding assay (MFA) with F1 generation *Anopheles darlingi* as previously described.² Mosquitoes were allowed to feed until engorgement for ~15–30 minutes. Non-engorged mosquitoes were removed before analysis.

Mosquitoes were dissected on Day 7. Mosquito midguts were dissected in 0.004% merthiolate in phosphate buffered saline. Oocysts were stained a faint pink and then enumerated and recorded per midgut.

Sample preservation. At the time of blood draw, plasma was separated from blood cells by centrifugation at $400 \times g$ for 6 minutes and then preserved at -70°C . Plasma remained frozen until processed at the University of California San Diego, La Jolla, CA.

Follow-up. Three weeks after enrollment, subjects were visited for symptom evaluation and a second venipuncture. A sample of 5 mL of blood was collected, centrifuged at $400 \times g$ for 6 minutes, and plasma was removed and stored at -70°C continuously until processing.

Cytokine analysis. Plasma samples were thawed for the first and only time at the time of processing. One hundred fifty microliters (150 μL) of plasma was centrifuged at $10,000 \times g$ for 10 minutes. Fifty microliters (50 μL) of the centrifuged sample was used in duplicate per patient for analysis using a multiplex bead assay (Human Cytokine 10-Plex Panel, Invitrogen Catalog no. LHC0001, Carlsbad, CA), according to manufacturer's instructions.

Statistical analysis. A ratio of cytokine levels at the time of infection to 3 weeks after treatment was compared using paired *t* tests. Clinical characteristics of transmitters and nontransmitters were compared using independent *t* tests.

The top 13 transmitting subjects were selected based on their high infectivity for mosquitoes (> 8 oocysts identified per surviving mosquito dissected). Each of these 13 transmitters was matched with a nontransmitter based on similar parasitemia as judged by light microscopy (Table 1). These 13 matched pairs ($N = 26$) were then compared for differences in clinical characteristics using *t* tests. The cytokine levels in each of the two groups were evaluated for normal distribution and then compared between groups using the Wilcoxon rank-

sum test. Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL) statistical package.

RESULTS

Subjects. Ninety-nine subjects (57 men and 42 women) with acute, symptomatic *P. vivax* malaria were enrolled in the study. Subjects' mean age was 32 ± 12 (SD) years. All had headache as a symptom and all but one had fever. Most patients (96%) were seen within 7 days of onset of illness. Seventy-six percent of patients gave a history of prior infection with malaria. At the time of membrane feeding, 56% of subjects had fever $\geq 38^{\circ}\text{C}$.

MFA. A total of 7,419 *An. darlingi* mosquitoes were engorged on infected blood in MFAs, of which 5,610 survived (mean 25 mosquitoes) until dissection on Day 7. Forty-four percent of patient specimens infected mosquitoes.

Infectivity of *P. vivax* patients for *An. darlingi* mosquitoes. Forty-four of 99 (44%) subjects led to infection of mosquitoes (transmitters). Transmitters were compared with the nontransmitters. Groups were similar for age, sex, days of symptoms, and parasitemia, heart rate, respiratory rate, and hematocrit, but were significantly different for temperature, which was on average 0.6°C higher among the nontransmitters (Table 1), as seen previously.²

Transmission association with parasitemia. Infection per parasitemia range was analyzed (Figure 1). Of the 10 subjects with $< 1,000$ parasites/mL of blood, only one sample infected 1 of 63 mosquitoes. Thus, parasitemia overall was important to transmission, with parasitemia $< 1,000$ parasites/mL limiting the likelihood of transmission among subjects with symptomatic parasitemia.

Top transmitters. Given the non-normal distribution of oocyst number and infection prevalence in experimental mosquito

TABLE 1
Comparison of clinical aspects of patients who transmitted (transmitters) versus those who did not transmit (nontransmitters) *Plasmodium vivax* infection to mosquitoes

	% Transmitters	% Nontransmitters	P value
Total no. subjects	44	55	
Age (years)			
Mean (range)	33.2 (15–60)	31.8 (15–64)	0.573†
Sex			
% Male	55	60	
Temp at blood raw			
Mean $\pm$ SD	$37.4^{\circ}\text{C} \pm 0.8^{\circ}\text{C}$	$38.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$	0.001†
Heart rate			
Mean $\pm$ SD	83 ± 13.5	89 ± 15	0.069†
Hematocrit			
Mean $\pm$ SD	40.2 ± 4.0	39.8 ± 3.6	0.562†
Days of symptoms			
Mean (range)	3.9 (1–7)	4.9 (2–20)	0.057†
History of malaria (%)	73	78	
Parasitemia			
Mean (range)	4485 (60–14,477)	4016 (358–26,190)	0.497*

*The groups differed significantly for temperature at blood draw.

†By independent *t* test.

FIGURE 1. Infection by parasitemia grouping. When parasitemia is grouped, it is evident that for parasitemia $< 1,000$ in this group of symptomatic subjects, the likelihood of transmission is low.

TABLE 2

Top transmitters (> 8 oocysts/mosquito) and nontransmitters matched for parasitemia*

	Top 13 transmitters	Matched 13 nontransmitters	P value
Age in years			
Mean (range)	39 (15–60)	34 (15–59)	0.347†
Sex			
% Male	54	54	
Temp at blood draw			
Mean $\pm$ SD	37.3 $\pm$ 0.6°C	38.3 $\pm$ 0.8°C	0.003†
Heart rate			
Mean $\pm$ SD	78 $\pm$ 10.5	92 $\pm$ 10.8	0.003†
Mean resp rate	19	19	0.767†
Hematocrit			
Mean	41.8	40.4	0.509†
Days of symptoms			
Mean (range)	3.6 (1.1)	5.0 (2.9)	0.133†
Parasitemia			
Mean (range)	5383 (2048–11,231)	5268 (2058–9,000)	0.893†

*The groups differed significantly for temperature at blood draw and average heart rate.

†By independent *t* test.

infections, phenotypic extremes were focused on for analysis of transmission. Thirteen of the 44 subjects that infected mosquitoes were classified as top transmitters as their blood samples infected at a mean of ≥ 8 oocysts per mosquito. These 13 subjects were matched by parasitemia with 13 nontransmitters. Comparison between these groups shows that in addition to a significant difference in temperature at blood draw ($P = 0.003$), the groups also had significantly different heart rates ($P = 0.003$) with nontransmitters with an average 14 beats/minute greater than transmitters (Table 2).

Cytokines in *P. vivax* infection. Ten cytokines were simultaneously analyzed for each of the 99 subject samples at both the time of infection and at ~ 3 weeks after starting antimalarial therapy. The 3-week samples were collected from 91 of 99 subjects and cytokine levels from these samples were used as an internal baseline for each subject as there is no existing reference range for these molecules. For those subjects lost to follow-up, the mean 3-week cytokine value from collected samples was used as an estimate for those patients' 3-week control.

The IL-10 and IL-6 showed dramatic elevation in *P. vivax* infection compared with the 3-week convalescent controls

TABLE 3

Levels of IL-10 and IL-6 during *Plasmodium vivax* infection

Cytokine	Mean (standard error of the mean) (pg/mL)	P value by paired <i>t</i> test	P value by Wilcoxon signed-rank test for related samples
IL-10			
@Time 0	2637.3 (568.0)	< 0.001	< 0.001
@3 weeks	24.5 (1.5)		
IL-6			
@Time 0	508.6 (109.7)	< 0.001	< 0.001
@3 weeks	21.36 (1.9)		

(Figure 2, Table 3). These elevations are similar to what has been shown previously in severe *P. falciparum* infection.^{22,23}

The TNF- α and IFN- γ were also elevated in subjects during infection compared with post-treatment, though not uniformly (Figure 3), in contrast to what has recently been described in reports of severe vivax malaria.²⁴ The IL-8 levels seemed to be increased substantially in several subjects in the convalescent sample compared with the levels during infection, though this trend did not reach statistical significance using the nonparametric Wilcoxon signed-rank test (Table 4). Except for IL-1 β and IL-8, these cytokines tended to be elevated during symptomatic *P. vivax* infection compared with convalescent state.

Cytokine response and parasitemia, fever. Regression analysis was used to compare parasitemia quantified by light microscopy with cytokine levels. Cytokine levels did not consistently predict parasitemia. Enumeration of parasites by light microscopy is known to be variable because of variations in thick smear samples, the fields counted on each slide, and calculations used to determine parasitemia that may have affected these outcomes.^{25–27} Regression analysis was also used to evaluate whether cytokine levels (log-transformed) predicted elevated temperature at time of blood draw. Previously described associations between IFN- γ and TNF- α with fever were not observed.¹⁵ The IL-10 levels (ratio of acute: post-treatment, log-transformed), however, did predict elevation in temperature, though the correlation was small ($R = 0.108$, $P = 0.001$). The IL-6 (ratio of acute: post-treatment, log-transformed) was similarly associated with temperature ($R = 0.135$, $P < 0.001$). The IL-10 and IL-6 ratios were closely correlated ($R = 0.65$, $P < 0.001$).

Cytokines in top transmitters versus matched nontransmitters. The ratios of cytokine levels at infection to convalescence

FIGURE 2. IL-10 and IL-6 levels at enrollment and at 3 weeks after treatment (A) IL-10 and (B) IL-6.

FIGURE 3. IFN- γ and TNF- α at time 0 (infection with *Plasmodium vivax*) and at 3 weeks after treatment initiation. (A) IFN- γ and (B) TNF- α .

(fold-change) from top transmitters and matched nontransmitters were compared using the nonparametric Mann-Whitney *U* test. Nontransmitters tended toward higher elevations from baseline levels of IL-10 and IL-6 than top transmitters, but this reached statistical significance only for IL-10 ($P = 0.036$). There was no difference in top transmitters and matched nontransmitters in the ratios of IFN- γ , TNF- α , or IL-8 (Figure 4). Analyses for all transmitters versus nontransmitters showed a trend toward increased IL-10 in nontransmitters, but did not reach statistical significance ($P = 0.089$), likely a result of sample size.

DISCUSSION

In this study using experimental *P. vivax* infections of *An. darlingi* in the Peruvian Amazon, we found that several

key cytokines in acute symptomatic vivax malaria infection were elevated, in particular IL-10 and IL-6, but also IFN- γ and TNF- α among others, consistent with prior studies on *P. falciparum* and *P. vivax* infections.^{24,28,29} Of the highest significance from the data presented here, IL-10 was found to be associated with *P. vivax* infectivity for mosquitoes. The finding of an inverse association of IL-10 with the ability of patients with *P. vivax* malaria to transmit to mosquitoes found in this study is the first time a connection between IL-10 and malaria transmission has been made, and has important implications for understanding the complex dynamics of host-pathogen interactions vis à vis mosquito infections. These results are surprising and counterintuitive because IL-10 is considered an anti-inflammatory cytokine. In accordance with previous work, we had similarly hypothesized that pro-inflammatory cytokines such as IFN- γ and TNF- α would be associated among subjects in whom transmission is reduced or prevented because of damage to gametocytes in the setting of inflammation associated with IFN- γ and TNF- α . Nonetheless, unlike previous studies, we did not detect a relationship between elevated TNF- α and IFN- γ with decreased transmission.^{30,31}

We hypothesized that pro-inflammatory cytokines such as IFN- γ and TNF- α would be elevated among subjects in whom transmission is reduced or prevented because of damage to gametocytes in the setting of inflammation associated with IFN- γ and TNF- α . Unlike previous studies, we did not detect a relationship between elevated TNF- α and IFN- γ with decreased transmission.^{30,31} Surprisingly, instead, elevation of IL-10, an anti-inflammatory cytokine, was associated with transmission blocking of vivax malaria to *An. darlingi* in acute infection in this study.

Potential mechanistic role of IL-10. The IL-10, first described as cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine mostly produced by macrophages, but its source includes all leukocytes. Monocytes and macrophages are its primary target cells, resulting in suppression of pro-inflammatory cytokine production and inhibition of antigen presentation. The IL-10 also targets CD4 cells in which it causes suppression of cytokine synthesis and decreased production of CD4 cells themselves (reviewed in Niikura³²).

The IL-10 has been shown to reduce the inflammatory immune reaction mediated by pro-inflammatory cytokines such as IFN- γ and TNF- α , but at the expense of host ability

TABLE 4

IFN- γ , TNF- α , IL-2, IL-4, IL-5, GM-CSF, IL-1 β , and IL-8, were measured at 0 and 3 weeks*

Cytokine	Mean (standard error of the mean)	<i>P</i> value by paired <i>t</i> test	<i>P</i> value by Wilcoxon signed-rank test for related samples
IFN- γ			
@Time 0	166.4 (13.8)	< 0.001	< 0.001
@3 weeks	87.3 (5.8)		
TNF- α			
@Time 0	119.1 (6.18)	< 0.001	< 0.001
@3 weeks	71.0 (4.4)		
IL-2			
@Time 0	23.4 (2.1)	< 0.001	< 0.001
@3 weeks	13.8 (1.1)		
IL-4			
@Time 0	305.6 (14.6)	< 0.001	< 0.001
@3 weeks	190.1 (12.6)		
IL-5			
@Time 0	9.1 (0.6)	< 0.001	< 0.001
@3 weeks	5.7 (0.4)		
GM-CSF			
@Time 0	56.0 (3.9)	< 0.001	< 0.001
@3 weeks	30.4 (2.7)		
IL-8			
@Time 0	215.5 (39.7)	< 0.001	0.280
@3 weeks	960.1 (198.8)		
IL-1 β			
@Time 0	27.4 (3.3)	0.386	0.453
@3 weeks	24.6 (2.4)		

*GM-CSF = granulocyte/macrophage-colony stimulating factor.

FIGURE 4. Cytokine elevations in acute vivax infection compared between nontransmitters and top transmitters. (A) IL-10 is elevated significantly more in nontransmitters than transmitters ($P = 0.036$). (B) IL-6 is more elevated in nontransmitters than transmitters, but does not reach statistical significance ($P = 0.064$). (C, D, and E) IFN- γ , TNF- α , and IL-8 elevations in vivax infection are similar between transmitters and nontransmitters ($P = 0.205$, $P = 0.762$, and $P = 0.277$, respectively).

to control the infecting parasite.^{33–36} Mouse studies have consistently shown IL-10 to be an important cytokine in decreasing inflammation and protecting the host in murine malaria models.^{37–39} In human malaria, several studies have demonstrated association of low IL-10 levels with more severe disease such as cerebral malaria and severe anemia.^{37,40,41} Elevated levels of IL-10 have been associated with clinical protection from these severe manifestations of malaria³⁷ at the expense of persistent and/or elevated parasitemia.^{12,42,43}

The unexpected association of transmission blocking with higher IL-10 levels, however, is consistent with the current understanding of gametocytogenesis and malaria transmission. Malaria transmission (infection of mosquitoes from human infection) occurs by sexual reproduction by sexually dimorphic gametocytes taken up in a blood meal by a feeding female *Anopheles* mosquito. These gametocytes make up only < 10% of the overall parasite population in the circulating blood.⁴⁴ Gametocytogenesis is variable within and among malaria infections (reviewed by Alano⁴⁵) and what triggers schizonts to be sexually differentiated remains obscure. Despite gaps in knowledge regarding the mechanisms of *P. vivax* gametocytogenesis, it is likely that increased gametocytes seen in circulation correlates with increased transmission.^{46,47} Both gametocytogenesis and transmission seem to occur more in settings of stress to the parasite, reflected by an association with IL-10.

It is important to note that IL-10 did have a small but significant association with fever in this study. This may be a small confounder because previous reports suggest that fever is associated with decreased transmission.^{48,49} Higher temperature itself may adversely affect gametocyte quality in some way, as a previous study surmised that contents of the serum during fever actually somehow adversely impact gametocyte viability.⁵⁰ The correlation of IL-10 and degree of fever in this study was small ($R = 0.1$), hence it is unlikely to confound the association of IL-10 with transmission blockade, though more precise studies might shed light on this phenomenon. A causal relationship between IL-10 and fever is unlikely, as the opposite has been shown that IL-10 has been associated with asymptomatic pregnant patients without fever⁵¹; interestingly, higher fever did not correlate with higher TNF- α in our subjects, in contrast to earlier studies.⁵⁰

We also showed that in symptomatic subjects, the level of parasitemia contributed to the likelihood of successful transmission because among subjects with low parasitemia (< 1,000 parasites/ μ L), there was negligible transmission. A confounder not addressed in this study is naturally occurring transmission-blocking antibodies, an issue that we have addressed in other studies (McClellan C and Vinetz J, manuscript in preparation). The low likelihood of low parasitemia to transmit may be caused by a lack of gametocytes in the blood meals fed to mosquitoes or may have been from host-related immunity, as parasitemia levels have been shown to be associated with gametocytemia levels.⁴⁴ This trend in infectivity is relevant to enrollment procedures in future transmission studies, however may not hold true in asymptomatic subjects. This distinction is of epidemiological significance because asymptomatic hosts are thought to be an important part of the *Plasmodium* reservoir that allows persistence of the disease in human populations.^{52,53} Dynamics of asymptomatic malaria infection and its immune correlates are poorly understood. The IL-10 and associated cytokines

will be important to study in asymptomatic versus symptomatic hosts in the setting of transmission.

In this study, the cytokine-mediated factor only partially accounted for transmission success to *An. darlingi* mosquitoes, and clearly is variable among *P. vivax*-infected subjects. Our data suggest that IL-10 is associated with suppression of transmission. In addition to cytokines, there are several other important variables at work. Some of these factors include non-human factors such as the mosquito immune system and the impact of the mosquito midgut microbiome on transmission.⁵⁴ Currently, the development of transmission-blocking vaccines focuses on potential antibodies that interfere with transmission and several candidate antigens are being tested.^{55–58} We also hypothesize that parasite reproductive behavior is guided by the parasite's nutritional milieu, i.e., the nutritional status of the host.⁵⁹ These many factors all play a role in the complex, dynamic process of transmission, and warrant further study in the pursuit of developing better tools with which to combat the burden of malaria on infected people and societies.

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