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<td><strong>Date</strong></td>
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Early Immune Regulatory Changes in a Primary Controlled Human *Plasmodium vivax* Infection: CD1c⁺ Myeloid Dendritic Cell Maturation Arrest, Induction of the Kynurenine Pathway, and Regulatory T Cell Activation

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**ABSTRACT** *Plasmodium vivax* malaria remains a major public health problem. The requirements for acquisition of protective immunity to the species are not clear. Dendritic cells (DC) are essential for immune cell priming but also perform immune regulatory functions, along with regulatory T cells (Treg). An important function of DC involves activation of the kynurenine pathway via indoleamine 2,3-dioxygenase (IDO). Using a controlled human experimental infection study with blood-stage *P. vivax*, we characterized plasmacytoid DC (pDC) and myeloid DC (mDC) subset maturation, CD4⁺CD25⁺CD127lo Treg activation, and IDO activity. Blood samples were collected from six healthy adults preinoculation, at peak parasitemia (day 14; ~31,400 parasites/ml), and 24 and 48 h after antimalarial treatment. CD1c⁺ and CD141⁺ mDC and pDC numbers markedly declined at peak parasitemia, while CD16⁺ mDC numbers appeared less affected. HLA-DR expression was selectively reduced on CD1c⁺ mDC, increased on CD16⁺ mDC, and was unaltered on pDC. Plasma IFN-γ increased significantly and was correlated with an increased kynurenine/tryptophan (KT) ratio, a measure of IDO activity. At peak parasitemia, Treg presented an activated CD4⁺CD25⁺CD127lo CD45RA⁻ phenotype and upregulated TNFR2 expression. In a mixed-effects model, the KT ratio was positively associated with an increase in activated Treg. Our data demonstrate that a primary *P. vivax* infection exerts immune modulatory effects by impairing HLA-DR expression on CD1c⁺ mDC, increased on CD16⁺ mDC, and was unaltered on pDC. Plasma IFN-γ increased significantly and was correlated with an increased kynurenine/tryptophan (KT) ratio, a measure of IDO activity. At peak parasitemia, Treg presented an activated CD4⁺CD25⁺CD127lo CD45RA⁻ phenotype and upregulated TNFR2 expression. In a mixed-effects model, the KT ratio was positively associated with an increase in activated Treg. Our data demonstrate that a primary *P. vivax* infection exerts immune modulatory effects by impairing HLA-DR expression on CD1c⁺ mDC while activating CD16⁺ mDC. Induction of the kynurenine pathway and increased Treg activation, together with skewed mDC maturation, suggest *P. vivax* promotes an immunosuppressive environment, likely impairing the development of a protective host immune response.

**KEYWORDS** *Plasmodium vivax*, Treg, dendritic cells, indoleamine 2,3-dioxygenase

*Plasmodium vivax* is a major cause of malaria morbidity, with an estimated 13.8 million cases annually (1). Outside of sub-Saharan Africa, *P. vivax* causes over 65% of malaria cases and is now recognized as a cause of severe and sometimes fatal disease (2, 3). As the incidence of falciparum malaria falls, the proportion of malaria cases due to *P. vivax* is predicted to increase, complicating efforts in malaria elimination (4). Immunity to malaria is...
slow to develop, incomplete, and short-lived (5). One possible explanation for impaired immunity is modulation of immune regulatory pathways by *Plasmodium*. Experimentally induced human *Plasmodium* infection, also referred to as controlled human malaria infection (CHMI), provides a unique opportunity to gain insights into the shaping of primary immune and regulatory responses in malaria-naive volunteers (6).

Impairment of the numbers and functions of dendritic cells (DC) is one possible immune regulatory strategy that *Plasmodium* parasites may implement to subvert host immune responses (7). DC are a heterogeneous population of professional antigen-presenting cells that can uniquely prime naive T cells (8). In *Plasmodium falciparum* CHMI, we previously reported loss of total peripheral blood myeloid DC (mDC) and plasmacytoid DC (pDC) (9), as well as CD1c+ mDC dysfunction, characterized by impaired expression of major histocompatibility complex (MHC) class II and skewed proinflammatory cytokine production (10). In areas where malaria is endemic, there is no consensus as to whether *P. vivax* infection leads to stable pDC and mDC numbers (11), decreases pDC and mDC numbers (12, 13), or differentially affects pDC and mDC numbers (14). In adults with patent or subpatent asymptomatic *P. vivax* infection, pDC and mDC numbers are retained (13, 15). Although DC numbers have been evaluated during *P. vivax* malaria, it remains to be determined if *P. vivax* impairs DC subset maturation in a primary infection.

A key immune regulatory enzyme in DC activation is indoleamine 2,3-dioxygenase (IDO) (16, 17), an intracellular, nonsecreted enzyme that mediates the first and rate-limiting step in the extrahepatic tryptophan-kynurenine pathway. It metabolizes the essential amino acid tryptophan (which is central to immune regulation [18]) to the metabolite kynurenine. The resulting metabolic changes in the local milieu can contribute to immunogenic tolerance via activation of regulatory T cells (Treg) and suppression of effector T cell responses (reviewed in reference 19). DC and other cells can express IDO in response to interferon gamma (IFN-γ). IDO enzymatic activity is reflected in the ratio of kynurenine (K) to tryptophan (T) concentrations (the KT ratio) (20). IDO activity correlates with disease severity in chronic HIV disease (20) and cancer malignancy (21) and is associated with dysregulated immune responses and impaired microvascular reactivity in sepsis (22). Induction of the kynurenine pathway has been shown in murine (23) and falciparum (24–26) malaria, but its role in vivax malaria has yet to be investigated.

To examine perturbations of immune regulation in *P. vivax* infection, we assessed longitudinal changes in DC subset phenotypes and numbers, tryptophan metabolism via plasma amino acid concentrations, and Treg activation in six healthy adults during the course of experimental *P. vivax* infection. We report that primary *P. vivax* infection reduced DC subset numbers and selectively altered their HLA-DR expression, induced kynurenine production, and activated Treg, all of which likely impair the development of a protective host immune response.

RESULTS

Infection and parasite detection. Following infection, all the subjects experienced some symptoms of malaria (with a mean onset on day 12), including fever of ≥39°C (n = 5) (27). Parasites were detectable by PCR in all the subjects from day 8 after inoculation of the *P. vivax*-infected erythrocytes (pRBC), as previously reported (27, 28). On day 14, at a median peak parasitemia of 31,395 parasites/ml, individuals were treated with artemether-lumefantrine (Table 1). All the subjects cleared parasitemia 48 h after commencement of antimalarial therapy (27). Automated whole-blood white cell counts revealed a significant decline in the number of circulating lymphocytes and platelets at peak parasitemia (Table 1) (P = 0.03). No changes in circulating granulocyte or monocyte counts were observed.

Primary *P. vivax* infection reduces DC numbers and CD1c+ mDC maturation. The effect of a first *P. vivax* blood-stage infection on circulating DC subset numbers and phenotype was examined. DC were identified as lineage marker negative and HLA-DR positive. Subsets were divided by expression of CD123 (pDC) and CD11c (mDC), and mDC were further subdivided by unique expression of CD16 (FcyIII receptor), CD1c
Plasma at any time point examined. There was no detection of IL-2, IL-4, or tumor necrosis factor (TNF) in (median, 123 pg/ml; IQR, 67 to 654 pg/ml; 17 to 981 pg/ml; IQR, 0.6 (0.4–1.4) 0.3 (0.2–0.4) 0.2 (0.1–0.4)b 1.7 (1.6–1.9) 0.8 (0.3–1.1) 0.6 (0.6–0.7)b 2.4 (2.0–2.9) 1.1 (0.6–1.4) 0.8 (0.7–1.0)b 55 (40–72) 22 (6–37) 9 (3–17)b 183 (137–338) 96 (30–206) 65 (33–110)b NK cells (cells/μl) 86 (79–111) 10 (5–15) 16 (12–41)b 15 (7–30) 14 (7–23) 19 (12–37) b 46 (33–59) 32 (12–36) a 18 (14–21)a

TABLE 1 Peripheral blood cell populations

<table>
<thead>
<tr>
<th>Population</th>
<th>Preinfection</th>
<th>Peak parasitemia</th>
<th>24 h posttreatment</th>
<th>P valuea</th>
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</thead>
<tbody>
<tr>
<td>Pre c/-peak parasitemia</td>
<td>Pre c/-posttreatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasite genomes (genomes/ml whole blood)</td>
<td>0</td>
<td>31,395 (22,119–89,145)</td>
<td>94 (6–112)</td>
<td>NA</td>
</tr>
<tr>
<td>Platelets (10⁹/liter)</td>
<td>209 (1,188–231)</td>
<td>159 (136–179)</td>
<td>113 (78–139)a</td>
<td>0.03</td>
</tr>
<tr>
<td>Granulocytes (10⁹/liter)</td>
<td>2.5 (2.1–5.3)</td>
<td>2.8 (1.8–4.3)</td>
<td>1.5 (1.4–5.3)b</td>
<td>NS</td>
</tr>
<tr>
<td>Monocytes (10⁹/liter)</td>
<td>0.6 (0.4–1.4)</td>
<td>0.3 (0.2–0.4)</td>
<td>0.2 (0.1–0.4)b</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes (10⁹/liter)</td>
<td>1.7 (1.6–1.9)</td>
<td>0.8 (0.3–1.1)</td>
<td>0.6 (0.6–0.7)b</td>
<td>0.03</td>
</tr>
<tr>
<td>PBMC (10⁹/liter)</td>
<td>2.4 (2.0–2.9)</td>
<td>1.1 (0.6–1.4)</td>
<td>0.8 (0.7–1.0)b</td>
<td>0.03</td>
</tr>
<tr>
<td>CD14+ cells (cells/μl)</td>
<td>55 (40–72)</td>
<td>22 (6–37)</td>
<td>9 (3–17)b</td>
<td>0.06</td>
</tr>
<tr>
<td>B cells (cells/μl)</td>
<td>183 (137–338)</td>
<td>96 (30–206)</td>
<td>65 (33–110)b</td>
<td>0.03</td>
</tr>
<tr>
<td>CD4 T cells (cells/μl)</td>
<td>722 (604–938)</td>
<td>410 (194–527)</td>
<td>318 (279–381)b</td>
<td>0.03</td>
</tr>
<tr>
<td>CD8 T cells (cells/μl)</td>
<td>137 (106–184)</td>
<td>81 (26–104)</td>
<td>19 (13–87)b</td>
<td>0.06</td>
</tr>
<tr>
<td>Treg (cells/μl)</td>
<td>46 (33–59)</td>
<td>32 (12–36)a</td>
<td>18 (14–21)a</td>
<td>NS</td>
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aH = 4, 
bH = 5.

CD1c, increased HLA-DR expression at peak parasitemia and 24 h after drug treatment (Fig. 2A). CD8 and CD141 (BDCA-1), or CD141 (BDCA-3) (Fig. 1A). At peak infection, circulating pDC, CD1c+ mDC, and CD141+ mDC declined and did not recover 24 h after drug administration (Fig. 1B). CD16+ mDC numbers dropped in four of six participants at peak infection (Fig. 1B). To examine whether these cells were undergoing programmed cell death, we examined intracellular active caspase 3 expression (29). Cleaved active caspase 3 significantly increased in pDC, CD16+ mDC, and CD1c+ mDC at peak infection (Fig. 1C), suggesting the cells were undergoing apoptosis. The low frequency of CD141+ mDC precluded an accurate assessment of caspase 3 staining in this population. There was no statistically significant evidence of caspase 3 staining at peak infection in B cells, CD4+ T cells, or CD8+ T cells (see Fig. S1 in the supplemental material).

Cell surface expression of the MHC class II molecule HLA-DR was measured to assess DC maturation in response to infection. Varying effects were observed among different DC subsets. HLA-DR expression was significantly reduced on CD1c+ mDC at peak parasitemia and after drug treatment compared to baseline, while CD16+ mDC had significantly increased HLA-DR expression at peak parasitemia and 24 h after drug treatment (Fig. 2A). In contrast, HLA-DR expression on pDC remained stable (Fig. 2A). Surface expression of CD1c, a glycolipid-presenting molecule, was significantly reduced on CD1c+ mDC, markedly so in two of six participants (Fig. 2B), although this did not compromise CD1c+ mDC identification. Surface expression of CD16 (FcγII receptor) on mDC did not change significantly following infection, increasing in four of six participants and falling in two of six participants (Fig. 2B). Interleukin 3α (IL-3α) receptor (CD123) surface expression on pDC decreased at peak parasitemia (Fig. 2B). Together, these results indicate significant and varied changes to DC subsets during primary P. vivax infection.

Increase in plasma IFN-γ. Infection with P. vivax led to significantly elevated plasma IFN-γ concentrations at peak parasitemia (median, 176 pg/ml; interquartile range [IQR], 17 to 981 pg/ml; P = 0.03), which remained significantly elevated 24 h after treatment (median, 123 pg/ml; IQR, 67 to 654 pg/ml; P = 0.03) (Fig. 3A). Plasma levels of IL-10 and IL-6 were increased 24 h after drug treatment, although concentrations were low (medians, 8 [IQR, 5 to 11] pg/ml [P = 0.03] and 13 [IQR, 7 to 21] pg/ml [P = 0.06], respectively). There was no detection of IL-2, IL-4, or tumor necrosis factor (TNF) in plasma at any time point examined.

Increase in IDO activity. IFN-γ is an inducer of IDO, an enzyme that mediates the first step in the kynurenine pathway of tryptophan metabolism. As kynurenine is the primary metabolite of tryptophan metabolism, the KT ratio in plasma was used as a surrogate marker of IDO activity (22, 30). Kynurenine was elevated at peak parasitemia compared to baseline (mean, 3.6 ± 1.6 μmol/liter versus 1.5 ± 0.3 μmol/liter; P = 0.02) and remained significantly elevated at 24 (5.7 ± 1.1 μmol/liter; P = 0.0004) and 48
Tryptophan concentrations decreased at peak infection (mean, 28.5 ± 10.0 μmol/liter versus 47.3 ± 5.0 μmol/liter at baseline; P = 0.003) and further declined at 24 (21.2 ± 6.0 μmol/liter; P = 0.0006) and 48 (22.8 ± 8.6 μmol/liter; P = 0.002) h after treatment (Fig. 3D). The KT ratio was also elevated at peak parasitemia (mean, 161 ± 147 versus 32 ± 5 at baseline; P = 0.08) and remained elevated at 24 (mean, 305 ± 156; P = 0.02) and 48 (mean, 272 ± 168; P = 0.03) h post-antimalarial treatment (Fig. 3E). Despite a relatively small sample size, there was a very strong association between the plasma KT and the plasma IFN-γ concentration (Spearman r = 0.94; P = 0.02) (Fig. 3B), with the kynurenine concentration also correlating with plasma IFN-γ (Spearman r = 0.94; P = 0.02) at the peak of infection. A mixed-effects model confirmed that the increase in the KT ratio is associated with an increase in IFN-γ (P < 0.001). There was no relationship between the plasma KT and parasitemia in these six individuals. The high-pressure liquid chromatography (HPLC) assay used to determine plasma kynurenine and tryptophan levels measured 27 other amino acids (see Table S1 in the supplemental material). Notably, only kynurenine, and consequently the KT ratio, was significantly elevated, while concentrations of 12 amino acids, including the immunoactive amino acids tryptophan and L-arginine, were all significantly reduced at peak parasitemia.
Activation of regulatory T cells. We next examined whether IDO activation coincided with Treg activation. Treg were identified as CD4^+ CD25^+ CD127^lo T cells, and activation was measured by loss of CD45RA expression. Resting Treg (rTreg) expressed CD45RA, and activated Treg (aTreg) were defined as CD45RA negative with high expression of CD25 (Fig. 4A) (31, 32). We observed a significant increase in aTreg (day 0 median, 13% [IQR, 10 to 16%]; day 14 median, 18% [IQR, 15 to 22%]; day 15 median, 23% [IQR, 18 to 30%]) and an apparent decline in rTreg (day 0 median, 17% [IQR, 15 to 33%]; day 14 median, 12% [IQR, 6 to 15%]; day 15 median, 7% [IQR, 4 to 12%]) (Fig. 4B) at peak parasitemia and 24 h after drug treatment. The increase in aTreg was matched by a significant increase in overall Treg expressing tumor necrosis factor receptor 2 (TNFR2) (day 0 median, 23% [IQR, 11 to 33%]; day 14 median, 17% [IQR, 15 to 33%]; day 15 median, 12% [IQR, 6 to 15%]; day 15 median, 7% [IQR, 4 to 12%]) (Fig. 4B), a molecule crucial for Treg suppressive function (33). CD45RA-negative Treg predominantly expressed TNFR2 (less than 5% of rTreg expressed TNFR2). Notably, in a mixed-effects model, there was a strong positive longitudinal association between the increases in aTreg and in IDO activity (the plasma KT ratio) (P < 0.001), while there was no relationship between aTreg and parasitemia (P = 0.67). Importantly, at peak parasitemia, the plasma kynurenine concentration was positively correlated with the proportion of circulating aTreg (Spearman r = 0.88; P = 0.03).

DISCUSSION

Using a controlled human malaria infection, we showed that a first blood-stage \textit{P. vivax} infection selectively suppressed CD1c^+ mDC maturation, increased the plasma KT ratio, and activated circulating Treg. Furthermore, a strong positive association between plasma IFN-\gamma, the plasma KT ratio, and the magnitude of Treg activation was observed. It is notable that these marked changes were induced at a lower blood parasitemia than is usually seen in clinical disease caused by \textit{P. vivax} (3).
The effects of *P. vivax* on DC HLA-DR expression differed substantially among blood DC subsets. In accordance with *P. falciparum* sporozoite CHMI trials (34), we report retained CD16^+^ mDC in the periphery, alongside increased HLA-DR expression at peak infection. In contrast, CD1c^+^ mDC, the subset that routinely express high HLA-DR and are considered potent professional antigen-presenting DC (8), were uniquely altered, with diminished HLA-DR expression at peak parasitemia. Reduced HLA-DR expression on mDC has been associated with impaired T cell activation and proliferation (35), suggesting that in an early primary *P. vivax* infection the T cell response may be shaped by CD16^+^ mDC rather than CD1c^+^ mDC. In *P. falciparum* blood-stage CHMI, we recently reported similarly impaired HLA-DR expression on CD1c^+^ mDC, as well as a skewed proinflammatory TNF cytokine response (10). Together, these reports suggest that, at least in a first infection, *P. vivax* and *P. falciparum* exert comparable effects on mDC subsets. In clinical vivax malaria, mDC subsets and pDC decline (12, 14), with one study reporting stable pDC numbers (11). However, no studies have reported mDC subset activation, and few studies have assessed total DC activation in clinical vivax malaria (11, 12). In studies examining total DC, CD86 (11, 12) and HLA-DR (12) expression were both significantly reduced. Complexities in MHC class II antigen-processing pathways provide multiple targets for pathogen interference, from modulation of de novo synthesis (36) to enhanced ubiquitination of existing MHC class II molecules (37). Our previous report of stable HLA-DR on total mDC (9) highlighted the importance of assessing DC subsets individually. Other potential effects of the altered DC subset phenotype on immune responses during early *P. vivax* infection may include the reduced ability of CD1c^+^ mDC to present glycolipids to αβ- or γδT cells via CD1c (38), as well as the reduced ability of pDC to bind IL-3, a T cell-derived glycoprotein that supports the viability and differentiation of hematopoietic cells (39). Additional research is required to understand the mechanisms and/or consequences of the altered DC phenotype we describe for malaria immunity. It remains to be determined if DC subsets are similarly affected in clinical *P. vivax* malaria.

FIG 3 Increased KT ratio in response to *P. vivax* infection. (A) Cytometric bead arrays were used to measure the longitudinal plasma IFN-γ concentration. (B) Spearman’s correlation of IFN-γ and KT ratio at peak infection (day 14). Using HPLC, the plasma concentrations of kynurenine (C) and tryptophan (D) were determined. (E) The longitudinal plasma KT ratio was calculated by dividing the kynurenine concentration (in micromoles per liter) by the tryptophan concentration (in micromoles per liter) and multiplying the quotient by 1,000. The values at preinfection (day 0), peak parasitemia (day 14), 24 h after drug treatment (day 15), and 48 h after drug treatment (day 16) are shown (n = 6).
The decline in circulating CD1c<sup>+</sup> mDC and pDC in early *P. vivax* infection was in accord with data from previous studies of uncomplicated vivax malaria (12). Active caspase 3 staining indicated that apoptosis at least partially contributed to the decline in circulating DC subsets, especially in CD1c<sup>+</sup> mDC. The reduction of circulating DC subsets could also be attributed to DC migration (40), as well as impaired repopulation of DC precursors from the bone marrow (41). In early *P. vivax* infection, we did not observe T cell or B cell apoptosis, suggesting the reduction in lymphocyte subsets may be attributable to lymphocyte migration, as reported in *P. falciparum* CHMI (42–44).

We report a rise in the plasma KT ratio shortly after the onset of malaria symptoms, indicating an increase in systemic IDO activity. In accordance with the original reports (16), plasma IFN-γ was positively associated with IDO activity. Modulation of microenvironment tryptophan availability through increased IDO activity is a well-characterized mechanism of immune regulation by tolerogenic DC (19). While IDO activity has been demonstrated for human pDC (45–47) and CD1c<sup>+</sup> mDC (48), we did not measure DC-specific IDO expression in this study, and the observed drastic increase in the plasma KT ratio is unlikely to be attributable to DC alone. Mouse models of malaria have shown that IDO is not essential for the clinical resolution of malaria infection (49).
identified the vascular endothelium as a primary site of IDO expression (23, 30), particularly sites of vascular obstruction (23). Here, we show for the first time induction of systemic IDO activity in vivax malaria, with activation of the kynurenine pathway occurring early in primary infection and remaining significantly elevated for at least 48 h post-antimalarial treatment. At baseline, the KT ratio was comparable to that reported in healthy individuals (20, 22, 49). At peak infection, however, the KT ratio (median, 105) was similar to that reported in nonsevere sepsis (median KT ratio, 82) and increased after drug treatment (median KT ratio, 229) to levels higher than those identified in severe sepsis patients (median KT ratio, 162) (22). The physiological or pathophysiological relevance of this striking increase remains to be determined. A reduction in tryptophan and an increase in kynurenine concentrations may drive T cell differentiation (via nutrient sensing or kynurenine binding to the aryl hydrocarbon receptor [50]), as well as potentially expanding and activating Treg (51, 52). In murine malaria models, systemic IDO activity is associated with increased immunopathology and impaired T cell priming (53). Imbalances in downstream kynurenine metabolites have also been associated with pathology in falciparum malaria (24–26). An improved understanding of how and where Plasmodium activates IDO may permit the development of novel adjunctive interventions. In fact, an antimalarial compound with IDO-inhibitory properties was recently described (54).

In this study, the plasma KT ratio and the plasma kynurenine concentration were strongly correlated with Treg activation, indirectly supporting an immune regulatory function of systemic IDO activity in vivax malaria, an infection in which Treg are known to be induced (11, 14, 55, 56). However, it is not clear whether Treg contribute to the onset of disease by dampening or preventing the initiation of effector immune responses or whether they act to control immune-mediated pathology associated with malaria. In this study, of primary P. vivax infection, we identified increased activation of Treg (percentages of aTreg and Treg expressing TNFR2) at peak parasitemia but no association between Treg activation and parasitemia in this cohort of six individuals. Our data are consistent with previous findings of increased Treg activation in adults with clinical illness from vivax malaria (56). In falciparum malaria, Treg were also activated, and this was associated with increased TNFR2 expression in severe, but not uncomplicated, malaria (57). Our observation of increased ex vivo Treg TNFR2 expression in primary P. vivax infection contrasts with a lack of increase in Treg TNFR2 expression reported in acute vivax malaria in regions where vivax malaria is endemic (55), with the latter likely to be recurrent and not primary infections. Frequent reexposure to Plasmodium spp. is thought to decrease TNFR2+ Treg (58), with recurrent infections thereby potentially reshaping the host regulatory immune response.

Overall our data support the notion that both Plasmodium species activate Treg and that in P. vivax this occurs early in blood-stage infection, with a strong association between measures of IDO activity and the proportion of activated Treg. While these associations do not demonstrate a causal effect, we speculate that asexual blood-stage Plasmodium infection triggers IDO activation via IFN-γ, which may contribute to activation of Treg. Altogether, we show early perturbation of immune regulatory pathways following first blood-stage P. vivax infection. Reduced DC subset numbers, selective modulation of CD1c+ mDC HLA-DR expression, increase in IDO activity, and Treg activation are all likely to impair host immune responsiveness in primary P. vivax infection. Finally, our data support the modulation of tryptophan metabolism in response to a primary P. vivax infection as a key mechanism contributing to immune suppression and regulatory T cell expansion.

MATERIALS AND METHODS

Study cohort. This study was nested in a clinical trial conducted to confirm the safety and reproducibility of the human P. vivax-induced blood-stage malaria model. The conduct of the clinical study (trial registration ACTRN12613001008718) and the PCR method used to quantify parasitemia were described in detail elsewhere (27, 28). Six healthy adults (3 males and 3 females), 22 to 32 years (median, 26 years [IQR, 24 to 31 years]) of age, received ~100 pRBC via intravenous injection in saline (27). Antimalarial drug treatment was administered on day 14 using artemether-lumefantrine (27). The study was approved by the Human Research Ethics Committees of QIMR Berghofer Medical Research Institute (clinical and laboratory study) and the
modulation of IDO Treg and DC

Human Research Ethics Committees of the NT Department of Health and Menzies School of Health Research (Laboratory study).

**Blood parasitemia.** Parasitemia was monitored daily 8 days after infection using quantitative PCR (with a limit of detection of 64 parasites/ml), as previously described (27).

**Blood collection.** Blood samples anticoagulated with acid citrate dextrose (ACD) were collected prior to inoculation and at the same time each morning on days 14 and 15. On these days, plus day 16, plasma samples anticoagulated with CTAD (a mixture of citrate, theophylline, adenosine, and dipyridamole) were collected. Blood collection time points were determined by the primary clinical study. All flow cytometric assays were undertaken using ACD whole blood and processed within 2 h of collection. The plasma was cryopreserved within 30 min of collection.

**Flow cytometric analysis.** Cytometric bead arrays (BD Biosciences) were used to measure levels of TNF, IFN-γ, IL-2, IL-4, IL-6, and IL-10 in plasma according to the manufacturer’s instructions. DC were enumerated from 200 μl of whole blood using lineage markers: anti-CD3 (HI134), CD14 (MSE2), CD19 (HIB19), CD20 (2H7), CD56 (HCD56), and CD34 (56E), each conjugated to phycoerythrin (PE), and anti-CD1c (L161) fluorescein isothiocyanate (FITC), anti-CD123 (6H6) PE-Cy7, anti-CD11c (Bly6) V450, anti-CD141 (M80) allophycocyanin (APC), anti-CD16 (3G8) APC-Cy7, and anti-HLA-DR (L243) peridinin chlorophyll protein (PerCP) (PerCP) (Fig. 1). Regulatory T cells were characterized in 200 μl of whole blood with anti-CD4 (M46), anti-CD25 (M-A251) PE, anti-CD127 (R34.34) PC7, anti-CD3 (UCHT1) APC-eFluor780, and anti-CD45RA (HI100) V450 (Fig. 4). The antibodies were sourced from BioLegend, except CD3 APC-eFluor780 (eBioscience). Following surface staining, RBC were lysed with fluorescein isothiocyanate (FACS) lysis buffer (BD Biosciences) and washed in phosphate-buffered saline (PBS). The cells were resuspended in PBS-1% (wt/vol) paraformaldehyde (surface only) and acquired on a FACSCanto II (BD Biosciences).

The absolute number of DC was determined by adding the automated-cell-counter-derived lymphocyte and monocyte counts (Table 1) (109 cells/liter), dividing by 100, and then multiplying by the percentage of dendritic cells, with multiplication by 1,000 to give the cell count per microliter. Lymphocyte subset counts were determined using the lymphocyte gate.

**Apoptosis.** Intracellular active caspase 3 staining was assessed as previously described (59). DC subset expression of active caspase 3 was evaluated in 1,000 μl of whole blood using the panel from Fig. 1A, with the following substitutions: anti-active caspase 3 (C92-605 BD Biosciences) FITC and CD1c (L161) APC. Active caspase 3 expression in non-DC was evaluated in 100 μl of whole blood using anti-active caspase 3 (C92-605) FITC, anti-CD56 (HCD56) PE, anti-CD4 (RPA-T4) PerCP, anti-CD25 (B1.49.9), anti-CD14 (MSE2) APC and anti-CD20 (2H7) APC-Cy7, and anti-CD3 (UCHT1) V450 and anti-CD8 (RPA-T8), each purchased from BioLegend. Briefly, whole blood was stained with surface antibodies, RBC were lysed with FACS lysing solution (BD Biosciences), and the cells were permeabilized using 1× Perm/Wash (BD Biosciences) and stained with active caspase 3 antibody (C92-605). The cells were resuspended in PBS-2% fetal calf serum (FCS) and acquired on a FACSCanto II (BD Biosciences).

**Amino acid measurement.** Plasma amino acids (Fig. 3 and Table S1 in the supplemental material) were measured in CTAD plasma, using HPLC (Shimadzu, Kyoto, Japan) with UV (250 nm) and fluorescence (excitation, 250 nm; emission, 395 nm) detection, as previously described (60). The plasma KT ratio, a measure of systemic IDO activity (22), was calculated by dividing the kynurenine concentration (in micromoles per liter) by the tryptophan concentration (in micromoles per liter) and multiplying the quotient by 1,000.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc.) and STATA 14 (STATA Corp. LP). Depending on the data distribution, the Wilcoxon signed-rank test was used for analysis of longitudinal data. The Spearman rank test was used for correlation analyses. Mixed-effects models were used to examine longitudinal associations. A P value of <0.05 was considered significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00986-16.

**SUPPLEMENTAL FILE 1,** PDF file, 0.4 MB.

**ACKNOWLEDGMENTS**

We thank the Q-Pharm staff, who conducted the human infection studies, in particular Suzanne Elliott and Alice Lau, for supporting the research. We thank Paul Griffin for his advice and clinical assistance. We thank the volunteers who participated in the clinical trials.


This study was funded by the Australian National Health and Medical Research Council.
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