

RESEARCH ARTICLE

Soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) and other inflammatory mediators in malaria by *Plasmodium vivax* during enteroparasites coinfection

Myrela Conceição Santos de Jesus¹, José Hugo Romão Barbosa², Rubens Alex de Oliveira Menezes³, Margarete do Socorro Mendonça Gomes⁴, Lays Gisele Santos Bomfim⁵, Tamirys Simão Pimenta⁶, Andrea Regina de Souza Baptista¹, Ricardo Luiz Dantas Machado^{1,2}, Tatiana Rodrigues de Moura^{2,5,7*}, Luciane Moreno Storti-Melo^{2,8}



1 Programa de Pós-Graduação em Microbiologia e Parasitologia Aplicadas, Instituto Biomédico, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brasil, **2** Programa de Pós-Graduação em Biologia Parasitária, Universidade Federal de Sergipe, São Cristóvão, Sergipe, Brasil, **3** Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá, Macapá, Amapá, Brasil, **4** Superintendência de Vigilância em Saúde do Estado do Amapá, Macapá, Amapá, Brasil, **5** Health Sciences Graduate Program, Federal University of Sergipe, São Cristóvão, Brazil, **6** Instituto Evandro Chagas / Secretaria de Vigilância em Saúde / Ministério da Saúde, Ananindeua, Pará, Brasil, **7** Departamento de Morfologia, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Sergipe, São Cristóvão, Sergipe, Brasil, **8** Departamento de Biologia, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Sergipe, São Cristóvão, Sergipe, Brasil

✉ These authors contributed equally to this work.
* tmoura.ufs@gmail.com

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Abstract

Malaria is a major health issue with more than 200 million cases occurring annually. Moreover, in Malaria endemic area are frequently observed Malaria-enteroparasite co-infections associated with the modulation of inflammatory response. In this aspect, biomarkers play an important role in the disease prognosis. This study aimed to evaluate inflammatory mediators in malaria during coinfection with enteroparasites. A subset of serum samples already collected was analyzed and divided into four groups: Malaria (n = 34), Co-infected (n = 116), Enteroparasite (n = 120) and Control (n = 95). The serum levels of sTREM-1 and IL-6 were measured by ELISA. TNF- α , and IL-10 levels were previously carried out by flow cytometry. Higher serum levels of sTREM-1 and IL-6 were showed in malaria patients compared to healthy controls. In co-infected malarial patients sTREM-1 serum levels were similar to control group. Interestingly, co-infected malaria patients showed IL-6 serum levels decreased compared to individuals only infected with *P. vivax*. However, in Malaria patients and co-infected there was a positive correlation between the IL-6 and IL-10 levels (P < 0.0001). This is the first report of sTREM-1 levels in *P. vivax* infected. Moreover, the results revealing a divergent effect of co-infection with the increased balance between pro-and anti-inflammatory cytokines and reduced IL-6 levels but increases the anemia occurrence. The

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results also highlight the potential use of IL-6 as a biomarker for *P. vivax* and enteroparasites coinfection.

Introduction

Malaria is a major parasitic disease with high morbidity and mortality worldwide, with 228 million cases occurring only in the year 2018 [1]. In humans this disease is caused by five different species of *Plasmodium* [2] of which *P. vivax* is the species with greatest epidemiological interest as it is the most widely distributed species in regions outside of the African continent. *P. vivax* is responsible for high morbidity caused by the infection, and brings social and economic losses to the countries where this disease is present [3, 4]. Cases of severe malaria due to infection by this species have been described in the literature more frequently [5–8].

In 2018 more than 194,000 cases of the disease occurred in Brazil, of which more than 99% are from the Amazon region [9]. Another important health issue in the same area is the high circulation of enteroparasites [10–12] which, during a co-infection with *Plasmodium vivax*, may alter the immunopathological course. A previous study conducted by our group [13] analyzed the impact of co-infection *Plasmodium*-enteroparasite in the municipality of Oiapoque, located in a border area between Brazil and French Guiana. The results showed an increase in the levels of the cytokines TNF- α , and IL-10 in individuals co-infected with *P. vivax* and enteroparasitoses. However, this data raises new questions about the impact of other inflammatory immunological markers since it was not possible to verify the influence of co-infection on the cytokines profile that characterizes the Th1 and Th2 pattern.

Receptors present on the surface of innate immune cells can recognize components of microorganisms and initiate the inflammatory response. Among these, the Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1) is a receptor expressed in macrophages, monocytes, and neutrophils that is known to increase inflammatory responses of myeloid cells in innate and adaptive responses that were initiated by the Toll-like receptors [14, 15]. The increase in serum levels of the soluble form of TREM-1 (sTREM-1) has been associated with more severe profiles in other parasitic diseases, such as visceral leishmaniasis [16], and has also been reported in malaria by *P. falciparum* [17–19].

In addition to sTREM-1, several cytokines have been described that play a role in controlling infection and symptom development [20]. Between them, TNF- α , interleukin 10 (IL-10) and IL-6 are molecules that can be a powerful inflammatory marker of severity during malaria. IL-6 has often been associated with increased severity during malaria caused by *P. vivax* in Brazil's extra-amazon [21] and Amazon regions [22] however this cytokine has not yet been evaluated in this scenario of malaria coinfection with enteric pathogens.

This study therefore aims to evaluate the levels of inflammatory mediators in malarial patients and to compare with individuals co-infected with enteroparasites residents of the municipality of Oiapoque, Amapá state, North Brazil.

Material and methods

Subjects and sample collection

A subset of samples already collected was analyzed out of individuals previously evaluated by Menezes et al. [13]. The samples were collected between November 2014 and November 2015 in the municipality of Oiapoque, Amapá State, in northern Brazil. Only participants who were native to Oiapoque were included and signed a consent form to participate in the research

study. Participants were over seven years of age and agreed to provide blood and stool samples.

Participants were divided into four groups: (1) Malaria: individuals with malaria by *P. vivax* who were negative for intestinal parasites (n = 34); (2) Co-infected: individuals with malaria and intestinal parasite co-infection (n = 116); (3) Enteroparasite: individuals without malaria and positive for an intestinal parasite (n = 120); (4) Control: individuals who were negative for malaria and intestinal parasite (n = 95). Blood collection was performed during diagnosis for malaria so none of the participants were using anti-malarial drugs to the time of blood collection. Ten millilitres of venous blood was collected from each patient by venipuncture. Four millilitres was dispensed into a tube with EDTA to perform haematological analysis and to prepare thick blood smear slides, and six millilitres was dispensed into a tube with no anticoagulant for immunological analysis. The serum samples were stored at -20°C in the Center for Microorganisms' Investigation of the Fluminense Federal University until use. The diagnosis and pro and anti-inflammatory cytokine profiles was conducted according to the methodology described below [13].

Estimation of the hematological and parasitological parameters against *P. vivax* and enteroparasite infection.

The diagnosis for malaria, the quantification of parasitemia, the quantification of gametocytes, and hemoglobin dosage have been previously performed, as well as the enteroparasites diagnosis [13]. The diagnosis for malaria was made by microscopic analysis and confirmed by molecular diagnosis, following a previously described protocol [23]. The fecal diagnosis was performed using the technique and/or methods of Hoffman-Pons-Janer and Faust. For each sample, two slides were examined for detection of parasites by two investigators with identification experience, using optical microscopy (Nikon, Japan) with magnifications of 100X and 400X. For the negative cases, three fecal samples were requested on alternate days to increase the detection sensitivity.

In addition, hemoglobin dosage was performed according to the protocol recommended by the World Health Organization through hematocrit index. The hematological parameters evaluated were the total number of erythrocytes (RBC; reference range: male 4.5–6.5 x 10⁶/μL, female 3.9–5.6 x 10⁶/μL and children aged 7–11 years 4.5–4.7 x 10⁶/μL) and hemoglobin levels (Hb; males 13 g/dL, females 12 g/dL and children 11 g/dL). Individuals were considered anemic when their haemoglobin blood levels were 13 g/dL for males, 12 g/dL for females, and 11 g/dL for the children.

Assessment of TNF- α , and IL-10 response against *P. vivax*

Cytokine levels were detected by flow cytometry according to previously published guidelines in the plasma samples [13]. Quantification of cytokines was conducted using a cytometric bead array kit (BD). A standard curve was performed for each cytokine and analyzed using FACSDiva software (Becton Dickinson, San Jose, CA, USA). The bead populations were displayed according to their respective fluorescence intensities, from dimmer to brighter. In the CBA, the cytokine capture beads were mixed with detection antibody conjugated with PE fluorochrome and then incubated with the test samples to form a "sandwich" test. The acquisition tubes were prepared with 50 μL of the sample, 50 μL of bead mix, and 50 μL of Th1/Th2 PE detection reagent (Human Th1/Th2 PE Detection Reagent/1 vial, 4 mL). The results were presented on graphs and in tables using FCAP Array 3 software (Becton Dickinson, San Jose, CA, USA). Raw MFI (median fluorescence intensity) values were quantified for each cytokine. The values were expressed in pg/mL for each cytokine in comparison to the standard curve. Three hundred events were considered for each cytokine.

Quantification of sTREM-1 and IL-6 by ELISA

In addition to the existing cytokine panel, IL-6 and sTREM-1 levels were also dosed. The levels of sTREM-1 in the serum were measured by using Human TREM-1 DuoSet ELISA kit (R&D Systems, Minneapolis, MN, EUA) and IL-6 levels were measured by IL-6 Human Uncoated ELISA Kit (Thermo Fisher Scientific, Waltham, MA, EUA). The absorbance was measured using a microplate reader Epoch (BioTek, Winooski, VT, EUA) at 450 nm and a wavelength correction at 570 nm to subtract the background. A standard curve was generated for each plate using the manufacturer's recommended protocol.

Statistical analysis

Differences between the two groups were calculated using the Mann–Whitney U-test, or the Kruskal-Wallis test with the Dunn showing mean and standard error. Differences with $p < 0.05$ were considered statistically significant. Analyses were performed using Prism 8.4.3 software (GraphPad).

Ethical considerations

The samples included in this study were collected with the approval of the Research Ethics Committee of the Federal University of Amapá. The participants who agreed to participate signed a written informed consent form. For minor participants, their parents or guardians signed the consent forms.

This study was also approved by the Ethics Committee of the Fluminense Federal University (CAAE number 40790120.7.0000.0003) to reuse the samples and waiving the consent of the participants and parents or guardians of the minors for this new analysis.

Results

Serum levels of sTREM-1 and IL-6 were measured in patients with malaria and controls. Considering the high prevalence of enteroparasitosis circulating in the region and that this infection can alter the immune course of malaria this was also measured the levels in two other comparison groups: co-infected and enteroparasites. Results showed a higher level of sTREM-1 and IL-6 in individuals with malaria compared with individuals without the infection ($P < 0.05$). The level of sTREM-1 was similar between the groups, although a decreasing trend was seen for the co-infected group (Fig 1A). Interestingly for IL-6, in the malaria group the level was 6.9 higher than control ($P < 0.0001$) and the results showed higher levels of this cytokine compared to all other groups ($P < 0.005$). In the co-infected group, the IL-6 levels were higher than in the control group ($P < 0.002$) (Fig 1B).

Given the importance of the pro-and anti-inflammatory balance during malaria, a comparison was performed between the ratio of the main pro-inflammatory (IL-6 and TNF- α) and anti-inflammatory (IL-10) cytokines identified in the malaria and coinfecting groups. In this analysis, the coinfecting group had a higher level of IL-10 than the inflammatory cytokines, compared to the profile seen in the malaria group (Fig 1C and 1D).

Considering previous findings that these samples showed an increase in anemia in the coinfecting group [13], an evaluation of the three main inflammatory mediators identified in the coinfecting group with and without anemia was carried out to identify if there is a difference in the levels of these cytokines in these groups. It was observed from this analysis that the group of individuals with anemia had higher levels of IL-6, TNF- α , and IL-10 cytokines than the individuals without anemia ($P < 0.05$) (Fig 2A–2C).

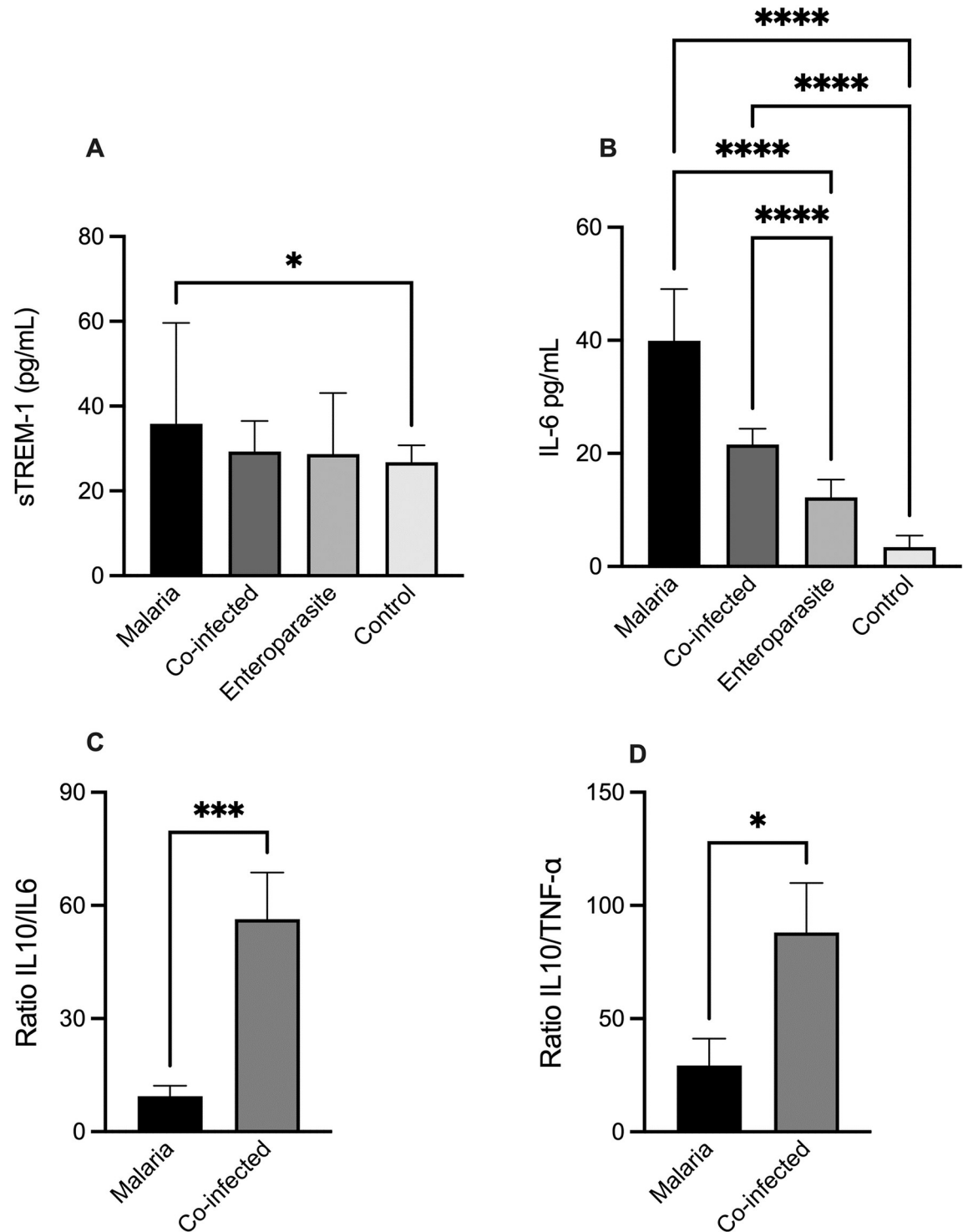


Fig 1. Differential levels of inflammatory mediators during malaria with sTREM-1 and IL-6 serum levels were associated with malaria infection and IL-6 serum levels were also associated during enteroparasites co-infection. (A) sTREM-1 measured in serum samples from malaria-infected individuals (n = 34), co-infected patients (n = 116), enteroparasite patients (n = 120) and healthy controls (n = 95). Bars represent the mean \pm standard error (* $P < 0.05$, by Mann Whitney test). The asterisk represents a statistically significant difference between the malaria and the control and group. (B) IL-6 measure in serum samples from malaria-infected individuals (black; n = 34), co-infected patients (blue; n = 116), enteroparasite patients (purple, n = 120) and healthy controls (pink, n = 95). Bars represent the mean \pm standard error (**** $P < 0.0001$, by Mann Whitney test). The asterisk represents a statistically significant difference between the malaria and the control and group. (C) The ratio between the levels of IL-10 and IL-6 in serum

samples from malaria-infected individuals (n = 34) and co-infected patients (n = 116). Bars represent the mean \pm standard error (** $P < 0.001$, by Mann Whitney test). The asterisk represents a statistically significant difference between the malaria and the control and co-infected group. (D) Ratio between the levels of IL-10 and TNF- α in serum samples from malaria infected (n = 34) and co-infected individuals (n = 116). Bars represent the mean \pm standard error (* $P < 0.05$, by Mann Whitney test). The asterisk represents a statistically significant difference between the malaria and the control and co-infected group.

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Discussion

The inflammatory response generated during malaria is essential for parasite clearance however enhanced inflammation can trigger immunopathological damage to the host [24, 25]. This is the first study that describes the levels of sTREM-1 in individuals infected with *P. vivax* and has identified significantly increased levels in infected individuals ($P < 0.05$).

The sTREM-1, the soluble portion of TREM-1, has been extensively investigated as a potential biomarker of severity [16, 20, 26–28]. In parasitic infections, high levels of sTREM-1 were observed in patients with Visceral Leishmaniasis who died, and the levels of sTREM-1 were negatively correlated with leukocyte count and hemoglobin concentration and were associated with increased parasitemia [16]. Similarly, a study conducted in Uganda with patients infected with malaria by *P. falciparum* identified sTREM-1 as a new biomarker of severe and fatal malaria in children with this malaria profile [26].

Our study is thus the first to include only non-severe patients infected by *P. vivax* which is the most widely distributed species of *Plasmodium* worldwide [1, 3] and observe a difference in sTREM-1 levels when compared to healthy controls. However, when comparing with the coinfecting and enteroparasite groups no significant difference was seen, although a decreasing trend in the coinfecting group was observed. This may be due to the fact that all the individuals included here had mild symptoms and the main findings already described with sTREM-1 and parasitic diseases correlate this protein with a severity prognosis [16, 20, 26].

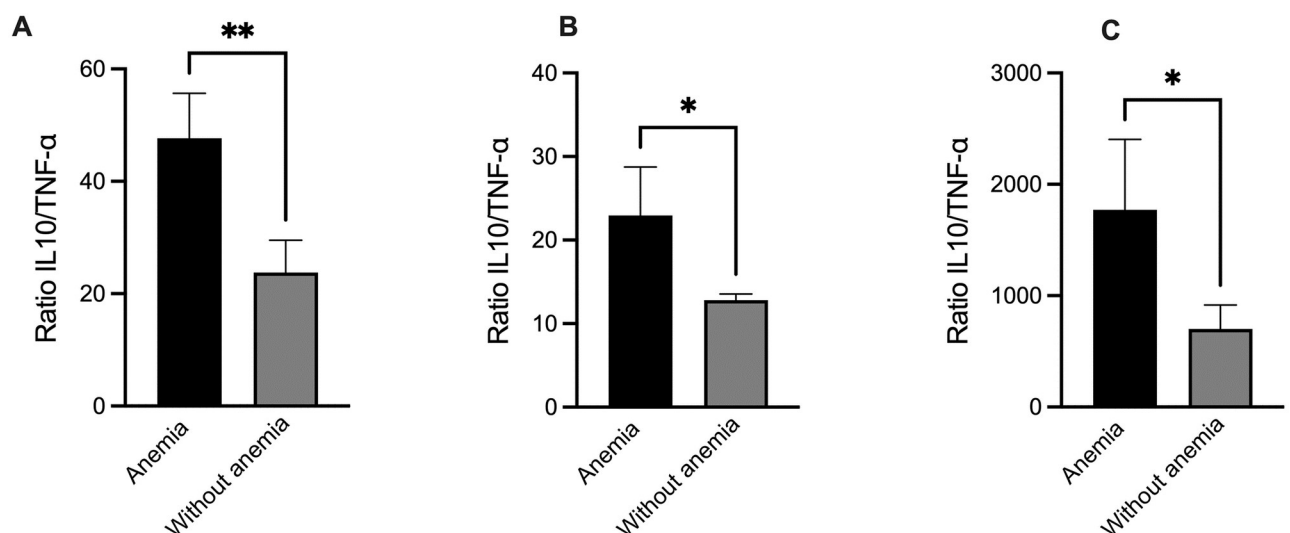


Fig 2. Higher levels of inflammatory mediators were correlated with anemia in patients coinfecting with *P. vivax* and enteroparasites. (A) IL-6 levels in serum samples from co-infected patients with anemia (n = 15) and without anemia (n = 48). Bars represent the mean \pm standard error (* $P < 0.05$, by Mann Whitney test). The asterisk represents a statistically significant difference between the malaria and the control and co-infected group. (B) TNF- α levels in serum samples from co-infected patients with anemia (n = 15) and without anemia (n = 48). Bars represent the mean \pm standard error (* $P < 0.05$, by Mann Whitney test). The asterisk represents a statistically significant difference between the malaria and the control and co-infected group. (C) IL-10 levels in serum samples from co-infected patients with anemia (n = 15) and without anemia (n = 48). Bars represent the mean \pm standard error (* $P < 0.05$, by Mann Whitney test). The asterisk represents a statistically significant difference between the malaria and the control and co-infected group.

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Furthermore, experimental studies of sTREM-1 induction with LPS in animal models demonstrated that when low-dose LPS (2 ng/kg) was used, the increase in sTREM-1 was transient and decreased progressively within the first 24 hours [29]. If the same concept applies for malaria, the low parasitemia should influence the data found here considering that many samples have not detected sTREM-1 levels. It must be considered that the serum used in this analysis was collected and refrigerated between 2014 and 2015 years and the sTREM-1 in some samples may have degraded over time. Therefore, the results described here could differ if they are made with fresh samples, we acknowledge.

Our results also demonstrated that IL-6 serum levels increased 6.9 times in malaria patients compared to healthy individuals. In addition, IL-6 was associated positively with IL-10, which is compatible with the mild forms in the patients included in this study. Although, higher IL-6 production during *P. falciparum* infection was observed to be associated with increased clinical episodes [30], the IL-6 levels do not change the memory response development in *P. vivax*, since no variations were observed in the production of anti-PvMSP-1₁₉ IgG antibodies in our previous study with these same samples [13].

In contrast to what was found for sTREM, IL-6 levels were significantly lower in the coinfecting group compared to the malaria group. The malaria group showed higher values of this cytokine compared with all other groups, demonstrating that IL-6 levels are highly stimulated during *P. vivax* infection. Interestingly, the difference observed between malaria and the coinfecting group seems to indicate a modulation of inflammation in individuals coinfecting with enteroparasites. As expected, the IL-10/IL-6 ratio was lower in malaria group when compared to the coinfecting, showing that in the coinfecting the response was leaning more towards a suppressive profile. This may indicate a potential protective effect of co-infection on malaria by *P. vivax*. In malaria by *P. falciparum*, this protective effect has already been seen in acute renal failure and jaundice [31] and co-infection with *Ascaris lumbricoides* was protective against cerebral malaria [32]. A study conducted in Africa with people coinfecting with *P. falciparum* and soil borne helminth, coinfection led to increased IL-10 and IL-6 levels and a down-regulation of TGF- β [33]. In our study with *P. vivax*, the coinfecting had increased IL-10/IL-6 and IL-10/TNF- α ratios and decreased IL-6 levels when compared to patients who were infected with malaria infection only.

However, another possible scenario must be considered. In the acute phase of infection, a predominantly pro-inflammatory response is important for parasitemia control and parasite clearance [34]. Given that all the individuals were included in the study at the time of diagnosis it is likely that most of them were in the acute phase and therefore this immunoregulation may be favorable to the parasite, ensuring maintenance of the infection.

Additionally, increased anemia was seen in the coinfecting compared to the malaria group [13] which may support the hypothesis that the suppressive profile may not be beneficial for the patients. Anemia during malaria is a multifactorial etiology. The incidence and severity increase in specific groups such as children under 5 years old, pregnant women, and can also be caused due to resistance during anti-malarial treatment, hemolysis, malnutrition, and other associated infections such as HIV, parvovirus B19, Intestinal helminths [35]. However, among these characteristics, only enteroparasite coinfection was present in the individuals included in our study.

It is worth of noting that despite the decrease in IL-6, this coinfecting group showed higher values of TNF- α than malaria [13]. TNF- α is an important pro-inflammatory cytokine and during malaria has been correlated with the development of anemia [36]. The anemia in these patients may thus be developed as a result of the inflammatory response of these cytokines and the generation of a damage-associated molecular pattern (DAMPs) [37, 38] and/or due to a

strong TH2 response that favors the persistence of the parasite and is generated by the natural cycle of *Plasmodium* replication that leads to red blood cells lysis.

As expected, in malaria patients, parasitemia was positively correlated with the anti and pro-inflammatory cytokines IL-10 and TNF- α . In a study conducted in a municipality in Amazonas, Brazil increased IL-10 and IL-6 were associated with parasite load [22]. In our study, although there was a tendency to increase, no significant association was found. For sTREM-1 no association with the cytokine panel or with the clinical variables parasitemia, gametocytes, and hemoglobin levels has been identified.

We also demonstrated a potential modulation of IL-6 during coinfection with intestinal parasites. It is important to mention that most malaria-endemic regions have a high prevalence of enteroparasites [31, 32, 39]. Nevertheless, few studies have focused on assessing the impact of co-infection on malaria prognosis. One limitation that we acknowledge is the inability to separate enteroparasites into the helminth, protozoan, and both groups in our analysis due to the low number of individuals in the groups.

Therefore, studies are needed to determine the impact of these immune molecules on each of these groups, especially sTREM-1 which has been scarcely studied in these populations. Moreover, studies have demonstrated the impact of polymorphisms in the *TREM-1* gene on the susceptibility, inflammatory role and prognosis of malaria and other diseases [17, 40–44]. It is also necessary therefore to assess the genetic variability of *TREM-1* in populations of endemic areas and their impact on the course of malaria by *P. vivax*. Finally, is the coinfection with enteroparasites beneficial during *P. vivax* infection in non-severe cases, and is the anemia developed during coinfection caused by immune damage or due to parasite persistence? are two key questions that need further clarification.

Supporting information

S1 Data.

(XLSX)

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Author Contributions

Conceptualization: Rubens Alex de Oliveira Menezes, Margarete do Socorro Mendonça Gomes, Andrea Regina de Souza Baptista, Ricardo Luiz Dantas Machado, Tatiana Rodrigues de Moura, Luciane Moreno Storti-Melo.

Data curation: Tamirys Simão Pimenta, Ricardo Luiz Dantas Machado.

Formal analysis: Margarete do Socorro Mendonça Gomes.

Funding acquisition: Andrea Regina de Souza Baptista, Ricardo Luiz Dantas Machado.

Investigation: Myrela Conceição Santos de Jesus, José Hugo Romão Barbosa, Tamirys Simão Pimenta, Andrea Regina de Souza Baptista, Ricardo Luiz Dantas Machado, Tatiana Rodrigues de Moura, Luciane Moreno Storti-Melo.

Methodology: Myrela Conceição Santos de Jesus, José Hugo Romão Barbosa, Rubens Alex de Oliveira Menezes, Lays Gisele Santos Bomfim, Ricardo Luiz Dantas Machado, Tatiana Rodrigues de Moura, Luciane Moreno Storti-Melo.

Project administration: Ricardo Luiz Dantas Machado.

Resources: Rubens Alex de Oliveira Menezes.

Supervision: Andrea Regina de Souza Baptista, Ricardo Luiz Dantas Machado, Luciane Moreno Storti-Melo.

Visualization: Tamirys Simão Pimenta.

Writing – original draft: Myrela Conceição Santos de Jesus, Margarete do Socorro Mendonça Gomes, Andrea Regina de Souza Baptista, Ricardo Luiz Dantas Machado, Tatiana Rodrigues de Moura, Luciane Moreno Storti-Melo.

Writing – review & editing: Myrela Conceição Santos de Jesus, Margarete do Socorro Mendonça Gomes, Tamirys Simão Pimenta, Andrea Regina de Souza Baptista, Ricardo Luiz Dantas Machado, Tatiana Rodrigues de Moura, Luciane Moreno Storti-Melo.

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