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Analysis of Parasitological and Haematological Parameters and of $CD4^+$ and $CD8^+$ Cell Number in Patients with *Plasmodium vivax* Malaria

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ABSTRACT

The mechanisms of activation and regulation of T-lymphocytes and their cytokines in malaria is complex and poorly understood. This study evaluated the correlation of parasitaemia with the number of platelets and total leukocytes using Mann-Whitney U and Spearman's correlation coefficient test, identifies and quantifies subpopulations of Th1, Th2, Th17 and Treg cells during the infection by *Plasmodium vivax* by flow cytometry after lymphocytes isolated from the peripheral blood. There is a negative correlation between the parasitaemia and the number of platelets and depending on the amount of parasites, patients infected with *Plasmodium vivax* presents a higher level of thrombocytopenia. No correlation between the number of parasites and total leukocytes was observed in these patients and the number of total leukocytes did not differ when compared to healthy controls. The absolute number of Th1 and Treg cells did not differ between the groups. Patients infected with *Plasmodium vivax* showed increased number of Th2, Th17 and cytotoxic T-cells. The evaluation of proportion of $CD4^+$ and $CD8^+$ T-cells indicated no differences between the groups. The present indicate that infection induced by *P. vivax* active specific cells which will participate in immunoregulation against this parasite.

Key words: *Vivax* malaria, platelets, Th1 cells, cytotoxic T-cell

INTRODUCTION

Malaria is an infectious disease caused by a parasite called *Plasmodium*. Among the species causing this condition, the most prevalent in the world are *P. falciparum* and *P. vivax* (WHO., 2013). In the Amazon region, Brazil, the main transmitter is the *Anopheles darlingi* (Scarpassa and Conn, 2007). The pattern of malaria transmission in Brazil is characterized as hypoendemic to mesoendemic, its transmission is unstable and has annual seasonal fluctuations which is different from that observed in hyper-holoendemic areas (Camargo *et al*., 1996; Braga *et al*., 2002; Coura *et al*., 2006). The infection caused by *P. vivax* has long been considered a benign disease, especially when compared to infections caused by *P. falciparum*

(Anstey *et al*., 2009). However, the literature has shown that there was an increase in malignancy of this parasitosis and the most common consequences of these complications are severe anemia, respiratory distress and acute lung injury, coma, among other manifestations (Anstey *et al*., 2012). Constant prostration caused by infection is an important cause of morbidity and socioeconomic loss (Cui *et al*., 2003).

Evidence suggests that during the infection, malaria causes activation and dysfunction of T-cells and lymphopenia (Anstey *et al*., 2012). $CD8^+$ T-cells, the cytokines interferon-gamma (IFN-γ) and transforming growth factor alfa (TNF-α) confer protection against pre-erythrocytic *Plasmodium* parasites within hepatocytes (Kemp *et al*., 2002), whereas $CD4^+$ T-cells restrain the growth of erythrocytic *Plasmodium* parasites by secreting cytokines, activation of macrophages and the direction of humoral immunity (Schmidt *et al*., 2011). Recently, the involvement of regulatory T-cells (Tregs) in infection caused by *P. vivax* was demonstrated (Imai *et al*., 2010), suggesting that the balance between pro- and anti-inflammatory cytokines is needed to control changes related to malaria (Bueno *et al*., 2010). Humoral immunity appears to be reconstituted quicker than cellular immunity, after treatment (Jangpatarapongsa *et al*., 2006). The proportion of $CD4^+$ T-cells is high during acute infection by *P. vivax*. This is in contrast to what is normally seen during infections with *P. falciparum*, where the parasite-specific immune response is suppressed. It is likely that *P. vivax* induces a lower immune suppression than *P. falciparum* (Braga *et al*., 2002).

Another cell type that is related to suppression of cellular and humoral immunity during acute infection of *P. vivax* is the Treg cell which represents a subpopulation of T-cells characterized by expression of $CD25^+$ molecule and nuclear factor FOXP3 and the absence of CD127. It is involved in the control of homeostasis by preventing autoimmune diseases and regulation of chronic inflammation (Sakaguchi *et al*., 2008). Tregs cells induce suppression of effectors T-cells by blocking the activation and function of these lymphocytes, therefore, important in controlling the immune response to self antigens and non-self (Campbell and Ziegler, 2007; Sojka *et al*., 2008). The increased frequency of this cell type may benefit the survival of the parasite in the host. Evidences indicate that Treg cells can suppress T-cell responses through the production of IL-10 and TGF-β, while they can suppress B-cell maturation and differentiation directly or indirectly through regulation in the production of IL-2 or IL-4. This may explain the low level of parasite-specific antibodies seen in *P. vivax* patients (Rodrigues *et al*., 1991; Walther *et al*., 2009). Regarding the role of $CD4^+$ Th17 cells in protozoan infections, few studies have been conducted to determine the pathogenic or protector role triggered by the action of these cells. $CD4^+$ Th17 cells are distinguished by the production of IL-17 cytokine (IL17A and IL-17F) which induce recruitment of neutrophils to the site of inflammation and the production of chemokine and peptides microbicides by cellular tissue (Acosta-Rodriguez *et al*., 2007).

Based on the literature, the aim of this study was to compare the total number of leukocytes and platelets between a group of patients infected with *P. vivax* and a group of healthy controls, besides investigating possible correlation between parasitemia with these hematological parameters of patients with *P. vivax* malaria, identify and quantify cell subpopulations: Th1($CD3^+$ $CD4^+$ IFN-γ^+), Th2 ($CD3^+$ $CD4^+$ $IL4^+$), Th17 ($CD3^+$ $CD4^+$ IL-17^+), Treg ($CD4^+$ $CD25^+$ $CD127^-$) and cytotoxic T ($CD3^+$ $CD8^+$) by membrane markers and the presence of their specific cytokines and evaluate the proportion of $CD4^+$/$CD8^+$ T-cells in *P. vivax* malaria patients and healthy controls.

MATERIALS AND METHODS

Study population: Patients (n = 148) with a *P. vivax* malaria diagnostic were selected to participate in this study. They were recruited from the Julio Muller University Hospital (HUJM)

of the Federal University of Mato Grosso State (UFMT), located in Cuiabá, MT, Brazil. This service acts as a state reference for diagnosis and treatment of malaria cases receiving from all over the state of Mato Grosso and possibly other states in the North of Brazil. The predominant parasite species in the service is the *P. vivax*. For the group of healthy controls, 25 subjects clinically healthy and non-residents of endemic malaria area were selected from the Center of Hematology and Hemotherapy of Mato Grosso. All the study subjects were both gender.

Malaria patients: A group of 148 patients diagnosed with *P. vivax* malaria, randomly selected from the database of the HUJM, is represented by adults (age>18 years), who did not have any other acute or chronic infection or pregnancy and were not in treatment with immunosuppressive drugs. The diagnosis of *P. vivax* infection was based on examination of thick blood smears stained with 5% Giemsa solution. The age, number of previous episodes of malaria and other infectious diseases history of each participant were recorded using a standard questionnaire. Individuals with a positive diagnosis accepted to participate in present study after reading and signing a consent form that formalizes the participation of these individuals as volunteers. Patients who have had some type of antimalarial treatment and patient with mixed infection or infection with *P. malariae* detected by Polymerase Chain Reaction (PCR) were excluded from this study. This study was developed under the standards of the Ethics Committee of the Julio Muller University Hospital (Protocol 633/CEP-HUJM/09) and was developed under the terms of Resolution 196/96 of the National Health Council.

Healthy controls: In this study 25 clinically healthy individuals, selected for blood donation at the Center of Hematology and Hemotherapy of Mato Grosso, with no history of malaria and residents of non-endemic areas for the disease were evaluated. The consent of all subjects was obtained for the study. Hematologic data, such as platelets and leukocytes numbers were obtained for comparison analyzes. Of this group, 10 individuals were randomly selected for analysis of immunophenotyping, 07 males and 03 females, aged 35-55 years and negative test for malaria (thick smear test).

Blood collection: Two blood samples were taken from each patient, obtained during clinical procedures performed in HUJM. The same procedure was performed in the group of healthy controls. A finger-tip smear was used for the parasitological diagnosis and then 15 mL of venous blood was collected for analysis of cell phenotype. The Blood was drawn aseptically into Vacutainer ® tubes (Becton Dickson and Company, Franklin Lakes, NJ, USA) with EDTA. Haemoglobin (Hb), hematocrit (HCT), leukocytes and platelet levels were measured using a blood cell counter (ABX Pentra 90, Horiba Diagnostics, Kyoto, Japan). The data related to the number of total leukocytes and platelets was compared between patients with *P. vivax* malaria and healthy controls.

Parasitological diagnosis: Thick blood smears were stained with 5% Giemsa solution and examined to determine the species of *Plasmodium* by two well-trained microscopists. The final parasitaemia of patients was expressed as parasites per μL of blood from each individual. The estimated parasitaemia was classified by the methods described as follows.

Traditional semi quantitative method of crosses (+): The criterion is the number of parasites found in each of the 100 fields examined according to the following description: The 40-60 parasites

per 100 fields = (+/2 cross); 1 parasite per 1 field = (+); 2-20 parasites per 1 field = (++); 21-200 parasites per 1 field = (+++) and more than 200 parasites per 1 field = (++++) (Ministerio da Saude Brasil, 2005).

Method of quantitative evaluation per 100 microscopic fields: The 100 microscopic fields used for semi quantitative evaluation are equivalent to 0.2 μL of blood, thus parasitaemia per μL of blood is estimated as follows: The 40 to 60 parasites per 100 fields = (+/2 cross) = 200-300 parasites per mm^3; 1 parasite per 1 field = (+) = 301-500 parasites per mm^3; 2-20 parasites per 1 field = (++) = 501-10.000 parasites per mm^3; 21-200/1 field = (+++) = 10.001-100.000 parasites per mm^3 and more than 200 parasites per 1 field = (++++) = 100.000 or more parasites per mm^3 (Ministerio da Saude Brasil, 2005).

Correlation analysis of parasitological and haematological parameters: All patients with *P. vivax* malaria (n = 148) were included in the study to examine possible correlation between their levels of parasitaemia (mm^3) and haematological evaluation. Platelets counts (mm^3) and total leukocytes (%) were used for evaluation of the inflammatory response in these patients at the time of positive *P. vivax* malaria diagnosis. The data was subjected to linear regression analysis made by GraphPad PRISM 5.4 program.

Flow cytometry analysis: To perform the cell phenotyping of subpopulations of Th1, Th2, Th17 and Treg cells, 10 patients with *P. vivax* malaria and 10 healthy controls were selected from the initial population of study. For inclusion, those patients with *P. vivax* malaria were randomly selected and presented the following characteristics: Two or less crosses in the parasitaemia classification (Ministerio da Saude Brasil, 2005) and weren't primoinfected.

Isolation of peripheral blood mononuclear cells: Peripheral Blood Mononuclear Cells (PBMCs) were obtained when 15 mL of peripheral blood from each individual were collected in EDTA tubes and the same volume of Ficoll-Hypaque (Histopaque® 1.077 Sigma, USA) was added. All content was divided into five 50 mL tubes of polypropylene (Falcon 2074, BD Biosciences, USA) and centrifuged at 400 g for 30 min at room temperature. The ring containing the mononuclear cells formed at the interface between plasma and erythrocytes were collected and washed three times at 400 g for 10 min with PBS. Finally, the cells were resuspended in 1 mL of PBS and the count was carried out by Neubauer chamber with the cells diluted (1:10) in Turk dye. The final volume was adjusted to 1×10^6 cells per mL.

Cell phenotyping by flow cytometry and intracellular staining: Extracellular staining was preceded by incubation of 100 μL of FACS solution containing specific combinations and concentrations, according to the manufacturer's instructions, of monoclonal antibodies labeled with different fluorochromes: Phycoerithrin (PE)-conjugated mouse anti-human CD3 and CD25; Alexa Fluor® 488-conjugated mouse anti-human CD4; fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD8 (all from Invitrogen, USA) and Alexa Fluor® 647-conjugated mouse anti-human CD127 (BD Pharmingen™) were incubated in the dark, on ice for 30 min. After incubation, PBMCs were washed twice for 5 min at 350 g with 200 μL of FACS solution. Intracellular staining for cytokines was performed using the BD-Becton Dickinson fixation/permeabilization buffer kit following manufacturer's instructions. After that, PBMCs were incubated with specific volume of allophycocyanin (APC)-conjugated mouse anti-human IFN-γ and

APC-conjugated mouse anti-human IL-4 and Alexa Fluor® 647- conjugated mouse anti-human IL-17 (BD Pharmingen™) and incubated in the dark, on ice for 30 min. After incubation, the cells were centrifuged for 5 min at 350 g, resuspended in 200 μL of FACS solution and then the phenotypic analyses were performed using BD Accuri™ C6 flow cytometer (Becton Dickinson, USA). Fifty thousand events were acquired and analyzed by the BD Accuri™ C6 software (BD Biosciences, EUA). Isotype controls FITC, PE and APC were used in all experiments. The boundaries of the quadrants were always based on the negative population.

Statistical analysis: The statistical analysis was determined by GraphPad Prism 5.4 software (La Jolla, CA, USA). Data was expressed as Mean±Standard error of the mean (SEM). To compare the haematological parameters, proportion of T-cells and immunophenotyping of infected patients and healthy controls, the Mann-Whitney U test was used. For the correlation analysis between the parasitaemia and platelets and between parasitaemia and total leukocytes, the data were subjected to linear regression analysis by the Spearman's correlation coefficient test. The p-value <0.05 was considered statistically significant.

RESULTS

Comparative analysis of the platelets and total leukocytes blood counts between groups: All 148 individuals diagnosed with *P. vivax* malaria and all 25 healthy controls were used in the analysis. The results indicated that there was a statistically significant difference, demonstrating that patients with *P. vivax* malaria have thrombocytopenia during the *P. vivax* malaria when compared to healthy controls ($p<0.0001$) (Fig. 1a). However, when comparing the number of total leukocytes from patients infected with *P. vivax* and healthy controls, no significant difference was observed between groups ($p<0.645$) (Fig. 1b).

Correlation between the level of parasitaemia and platelets blood counts and between the level of parasitaemia and total leukocytes in infected patients with *P. vivax* malaria: A multiple linear regression was created to verify the correlation between parasitaemia in the *P. vivax* malaria patients (n = 148) and the platelets blood counts at the time of diagnosis and the correlation between the parasitaemia (mm^3) and the total number of leukocytes. A significant negative correlation between the level of parasitaemia (mm^3) and the platelet counts was detected

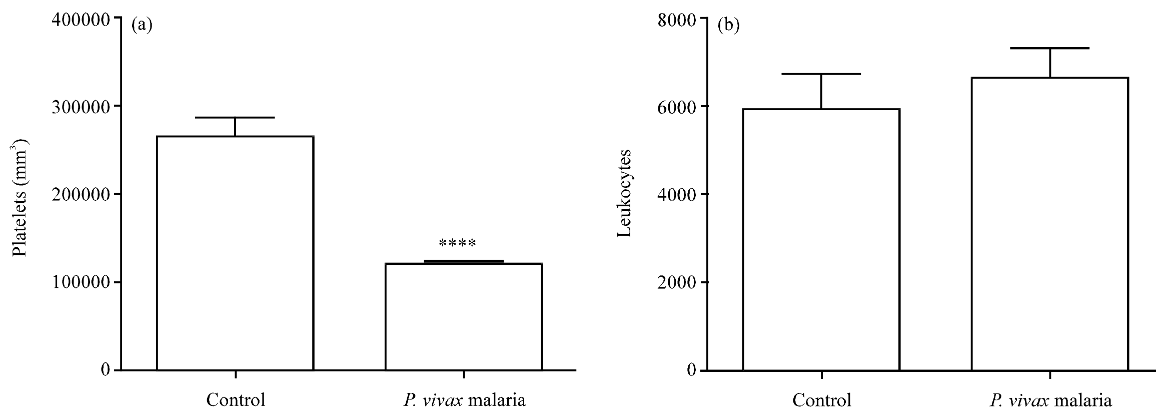

Fig. 1(a-b): Comparison analysis of haematological parameters (a) Platelet blood count (mm^3) and (b) Total number of leukocytes in peripheral blood of patients infected with *P. vivax* (n = 148) and healthy controls (n = 25), ****$p<0.0001$ (Mann Whitney U test)

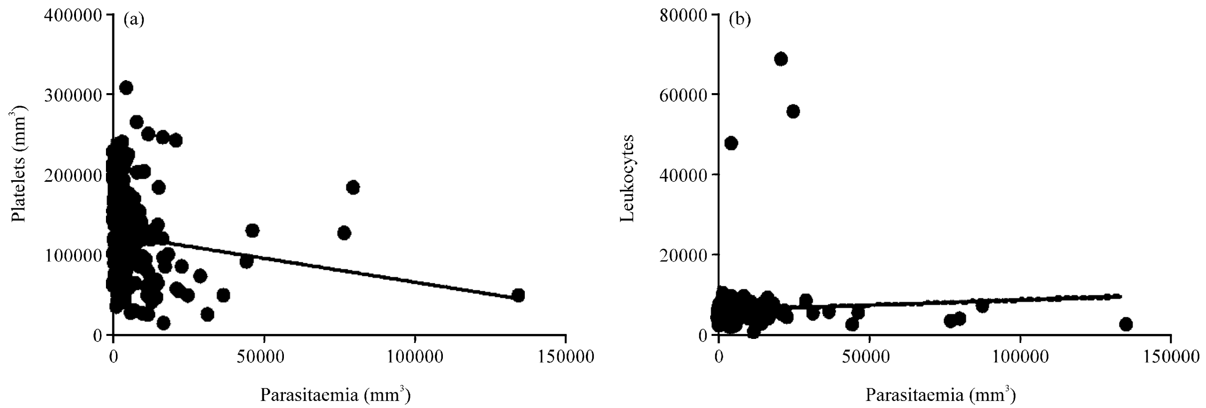

Fig. 2(a-b): Correlation analysis between the parasitaemia and haematological parameters (a) Presence of the negative correlation between parasitaemia (mm^3) and platelet (mm^3) count ($R = -0.29$; $p<0.0005$) and (b) Absence of correlation between the number of parasitaemia (mm^3) and total leukocytes of patients infected with *P. vivax* (n = 148), respectively (Spearman's correlation coefficient test)

Table 1: Clinical and haematological parameters from patients with *P. vivax* malaria analyzed by flow cytometry

Gender	PAR. (mm^3)	Crosses	Hb ($g\ dL^{-1}$)	HCT (%)	PLAT (mm^3)	LEUC. (%)	Fever (°C)	Time/days	No. of prev.mal.
M	1175	++	12.07	40.0	63.000	4700	38.5	4	20
M	3133	++	12.07	36.7	95.000	6500	39.0	7	15
M	400	+	13.80	33.2	77.000	4200	38.5	3	30
F	1900	++	13.10	41.0	45.000	4500	39.1	4	5
F	525	++	12.70	31.2	183.000	4410	38.8	2	3
M	3000	++	12.90	33.0	73000	4980	39.0	2	15
M	1300	++	13.10	41.2	36000	3850	40.2	3	7
F	4140	++	13.10	39.0	65000	4200	38.5	1	3
M	650	++	12.00	33.8	76000	2680	39.5	3	30
F	2550	++	12.40	37.5	86000	4000	39.0	2	10

Reference values: PAR.: Parasitaemia (mm^3), Crosses: + (301-500 parasites per mm^3), ++ (501-10.000 parasites per mm^3) (Ministerio da Saúde Brasil, 2005), Hb: Haemoglobin, HCT: Hematocrit, PLAT: Platelets, LEUC: Leukocytes, prev.mal: Previous malaria

($R = -0.29$ and $p<0.0005$) (Fig. 2a) but there was no significant association between the level of parasitaemia and the total number of leukocytes of this same patients ($p<0.509$) (Fig. 2b).

Comparison of T-cells subpopulations between groups: PBMCs from patients infected with *P. vivax* (n = 10) and healthy controls (n = 10) were identified by flow cytometry as described in the methodology. The clinical and haematological parameters of patients diagnosed with *P. vivax* malaria selected for immunophenotyping analyzes and used in our study are identified in Table 1.

Cytotoxic T-cell $CD3^+$ $CD8^+$ and analysis of the proportion of $CD4^+/CD8^+$ T-cells: Comparing the group of patients infected with *P. vivax* and the group of healthy controls, a statistically significant difference was detected in the absolute number of $CD8^+$ T-cells, indicating an increase of the $CD8^+$ T-cells in patients infected with *P. vivax* when compared to the healthy control group ($p<0.05$) (Fig. 3a). However, the $CD4^+/CD8^+$ ratio showed no statistical difference between the groups ($p<0.217$) (Fig. 3b).

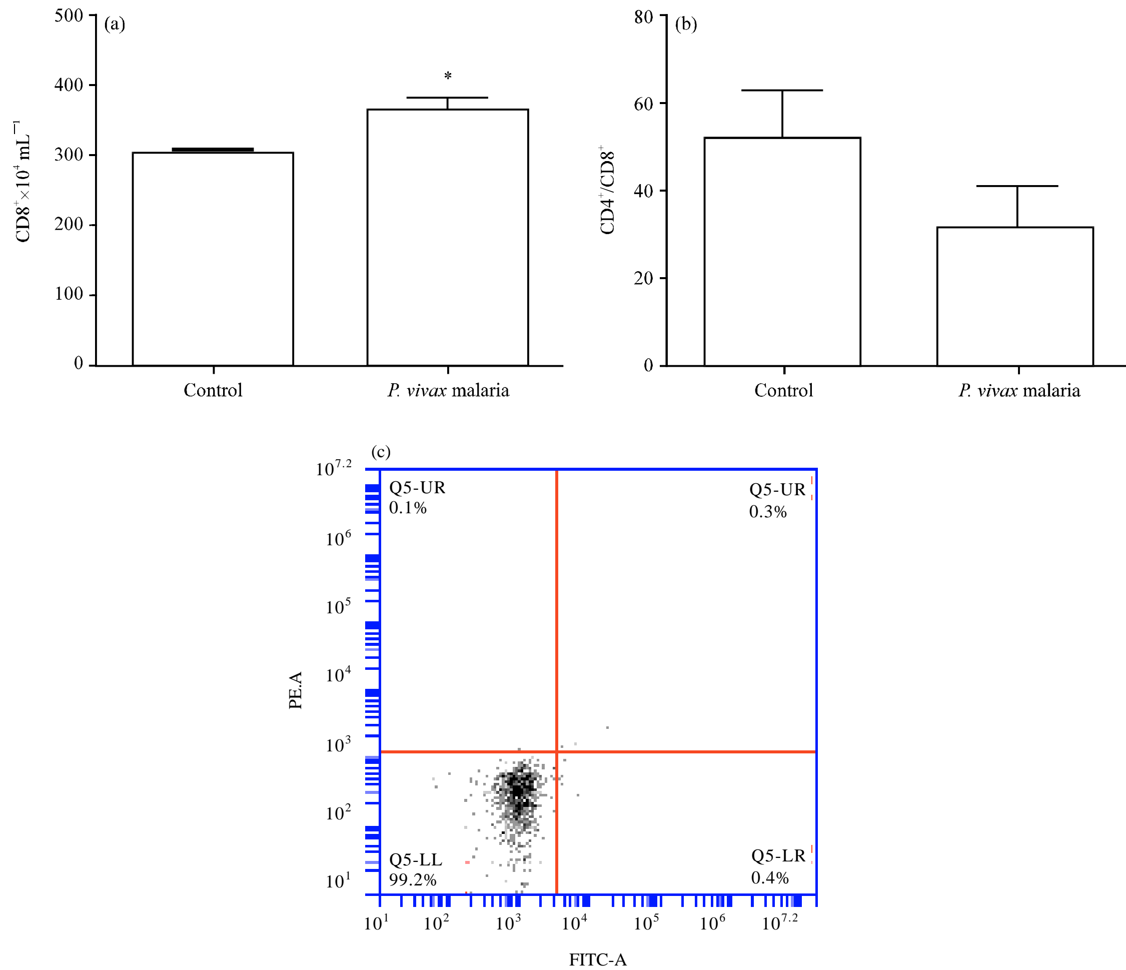

Fig. 3(a-c): Absolute number of $CD8^+$ and proportion of $CD4^+/CD8^+$ T-cells (a) Increase of the absolute number of $CD8^+$ T-cells of patients infected with *P. vivax* (n = 10) compared to healthy controls (n = 10) *$p<0.05$, (b) No significance difference when comparing the $CD4^+/CD8^+$ ratio of patients infected with *P. vivax* (Mann Whitney U test) and (c) Dot plots illustrating isotype control used to make the quadrants

Frequency of Th1 ($CD3^+$ $CD4^+$ IFN-γ^+), Th2 ($CD3^+$ $CD4^+$ IL-4^+) and Th17 ($CD3^+$ $CD4^+$ IL-17^+) cells: The Th1 cells, were determined from the absolute number of IFN-γ^+ in the T-cells subpopulation ($CD3^+$ $CD4^+$ IFN-γ^+) and demonstrated no statistically significant difference when comparing healthy control group and the patients infected with *P. vivax* ($p<0.070$) (Fig. 4a). Statistically significant difference was demonstrated when we compared the absolute number of Th2 ($CD3^+$ $CD4^+$ IL-4^+) and Th17 ($CD3^+$ $CD4^+$ IL-17^+) cells, by means of the quantification of IL-4^+ and IL-17^+ cytokines, showing an increased in the absolute number of this cytokines in patients infected with *P. vivax* when compared with healthy controls group ($p<0.01$ Fig. 4b and $p<0.05$ Fig. 4c, respectively).

Identification of Treg cell ($CD4^+$ $CD25^+$ $CD127^-$): The absolute number of Treg cell represented by the subpopulation ($CD4^+$ $CD25^+$ $CD127^-$) from patients infected with *P. vivax* and the healthy control, showed no statistically significant difference between the groups ($p<0.797$) (Fig. 5a).

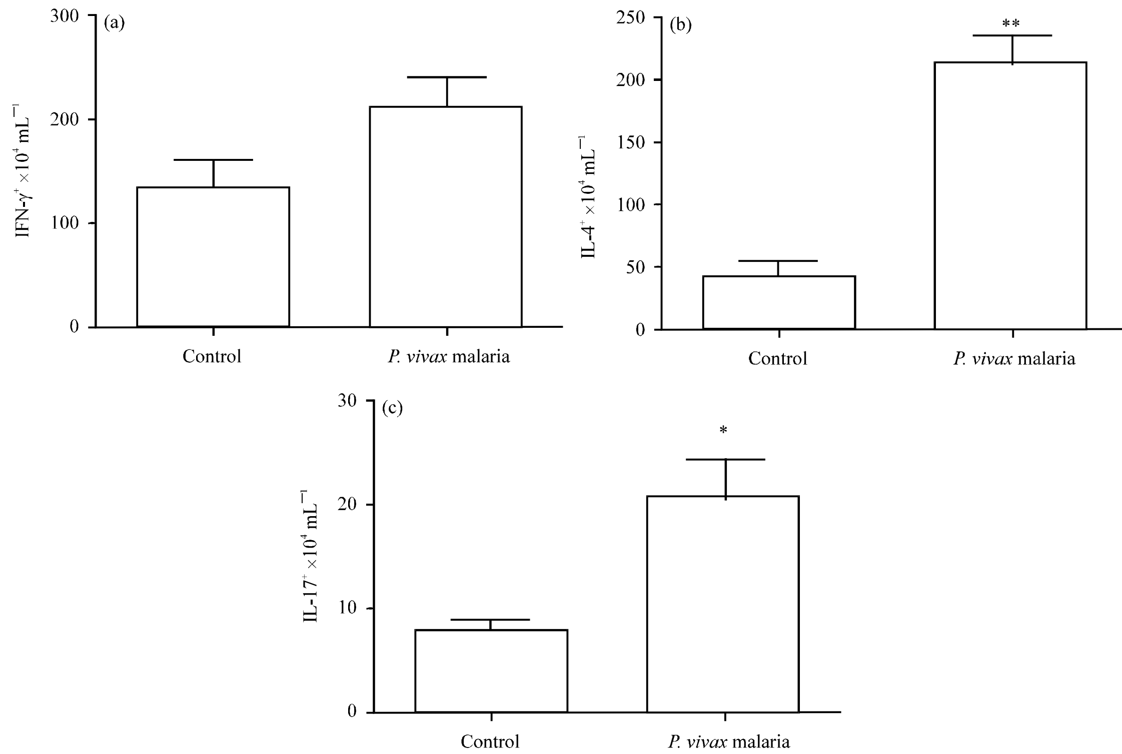

Fig. 4(a-c): Analysis of Th1, Th2 and Th17 profile (a) No significance difference when comparing the absolute number of IFN-γ^+ in subpopulations of T-cells $CD3^+$ $CD4^+$ IFN-γ^+ of patients infected with *P. vivax* (n = 10), Healthy controls (n = 10). Increase in the absolute number of IL-4^+ (b) **$p<0.01$ and IL-17^+ (c) *$p<0.05$, respectively in subpopulations of T-cells $CD3^+$ $CD4^+$ IL-4^+ and $CD3^+$ $CD4^+$ IL-17^+ of patients infected with *P. vivax* (n = 10) when comparing to healthy controls (n = 10) (Mann Whitney U test)

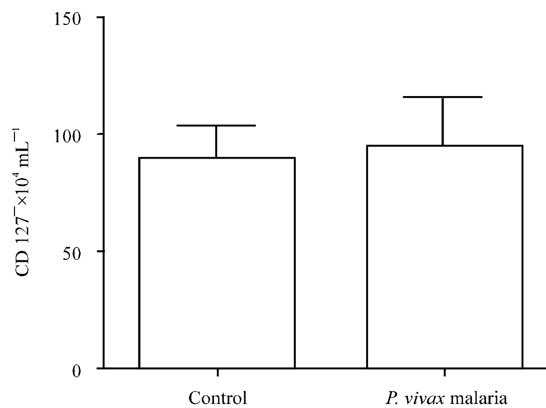

Fig. 5: Evaluation of the absolute number of Treg cells. Absolute number of Treg cells in the subpopulation of Treg cells ($CD4^+$ $CD25^+$ $CD127^-$) from patients infected with *P. vivax* (n = 10) and healthy controls (n = 10), showing no significance difference in the absolute number of these cells in the control group compared to the group of patients infected with *P. vivax*

DISCUSSION

Haematological alterations are the most common complications in malaria and have an important role in the severity and fatality of the disease. In malarial infection, studies evaluating the immunological mechanisms involved in infection indicate that the host immune system carries a potent response against the parasite, causing changes in almost all components of the immune system (Riley *et al*., 2006). Regarding haematological data of the evaluated patients, despite the levels of hemoglobin and leukocytes counts were within the reference standards for the majority of individuals; in this study the patients infected with *P. vivax* presented thrombocytopenia. However, this had a negative correlation with the level of parasitaemia. Present is in agreement with Grynberg *et al*. (2007), who observed similar data and have associated this clinical condition to the presence of a polymorphism at the *P. vivax* ama-1 domain I gene. Although, the etiology of this thrombocytopenia is not yet confirmed, it is also a characteristic of patients with *P. vivax* malaria, thus corroborating with other studies that had demonstrated this characteristic in patients infected with *P. vivax* (Dale and Wolff, 1973; Lee *et al*., 2001). Several hypotheses have been postulated as causes of thrombocytopenia associated with malaria, among them, disseminated intravascular coagulation, immune mechanisms, increased splenic sequestration, or the possible presence of parasites in the red lineage cells in the bone marrow, leading to reduction in the population of platelets in the circulation (Lacerda *et al*., 2008).

In *P. vivax* infection, the total leukocytes blood count was not affected, regardless of the level of parasites. However, when the T-cells subpopulations were evaluated, present study showed a significant increase in the absolute number of subpopulations of T-cells $CD3^+$ $CD8^+$ in the group of patients infected, suggesting that they have a role in malaria infection, since $CD8^+$ T-cells act through mechanisms of cytotoxicity against liver stages of the parasite (Torre *et al*., 2002). In humans, the response of $CD8^+$ T-cell to epitopes of the liver stage of *P. falciparum* and more recently to *P. vivax*, have been reported in individuals exposed to malaria. These studies have shown the participation of MHC class I molecules in the antigen presentation in the liver stage of *P. vivax* malaria to $CD8^+$ T-lymphocytes (Arevalo-Herrera *et al*., 2002). Subsequently, the $CD8^+$ T-cells secrete IFN-γ which eliminates infected hepatocytes or inactive intracellular parasite (Lee *et al*., 2001; Klotz *et al*., 1995). The proportion of $CD4^+/CD8^+$ T-cells of patients infected with *P. vivax* showed no statistically significant difference when compared to the healthy control group. Similar data were observed by other authors in the literature (Borges *et al*., 2013; Riccio *et al*., 2003). However, some studies indicate that the proportion of $CD4^+$ T-cells is increased during acute infection by *P. vivax* malaria which is not normally seen during infections with *P. falciparum*, in which the parasite-specific immune response is suppressed. It is probable that *P. vivax* induce immune suppression less than *P. falciparum* (Braga *et al*., 2002).

Treg cells ($CD4^+$ $CD25^+$ $CD127^-$) have a key role in controlling the immune response, both self and non-self antigens, besides pathogens and commensals (Melo and Carvalho, 2009). Consistent evidence, based on experimental models and in *P. falciparum* infections, suggest that Treg cells may have an important role during malarial infection (Walther *et al*., 2009). The evidence of the role of these cells during infection was demonstrated by *P. vivax* functional assay in which there was a reduction in PBMCs proliferation in naturally infected individuals in the presence of T-cell ($CD4^+$ $CD25^+$). Indeed, it is important to emphasize that regulatory cells may limit the pathology caused by the parasite during infection, controlling the inflammatory response but may also compromise the mechanism of destruction of the parasite (Riley *et al*., 2006). Nevertheless, the association between Treg cells and *P. vivax* infection is poorly understood. Evidence in murine

models and in humans, suggests that Treg cells expand and become activated during malaria infection. However, it is not completely clear whether this expansion occurs directly from proliferation, the type of cytokines present in the environment or because of recruitment of secondary lymphoid organ (Bueno *et al*., 2010). The expansion of Treg cells in human and experimental malaria infection appears to be associated directly with the parasitaemia. In adults and children infected with *P. falciparum* the proportion of T-cells $CD4^+$ $CD25^+$ $CD127^-$ $FOXP3^+$ was correlated with the number of circulating parasites (Walther *et al*., 2009) and with the presence of Histidine 2-rich protein of *P. falciparum*, a marker for the total biomass of the pathogen (Minigo *et al*., 2009). In this study, the presence of a subpopulation of T-cells ($CD4^+$ $CD25^+$ $CD127^-$) showed no statistically significant difference between groups. We evaluated only patients with low parasitaemia is evaluated and this could explain why present data differ from most studies with these cells described in the literature. Another possibility for this result would be that low expression of CD127 ($CD127^-$) is not an intrinsic characteristic of Treg and that the differential CD127 expression on Treg depends on its location and its activation status, because the differential regulation of CD127 expression between conventional T $CD4^+$ and regulatory cells after activation is dependent on the availability of IL-7 and the place where this activation occurs (Simonetta *et al*., 2010). In individuals infected with *P. vivax* in Thailand, an expansion of Treg cells in peripheral blood was observed during the acute phase and a greater expansion of these cells in cultures of PBMCs stimulated by parasite antigen (Jangpatarapongsa *et al*., 2008).

Recently, Scholzen *et al*. (2010) suggested the existence of different subpopulations of the Treg cells during malaria infection, as evidenced by intracellular IFN-γ, IL-4 and IL-17 production and intermediate expression of FOXP3 molecule. Regarding the understanding of the regulation of the effector immune response in *P. vivax* infections little is yet known. Therefore, further studies are needed to determine the role of Treg cells in populations exposed to this infection.

In the current study, the involvement of cytokines is analyzed which are related to the Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17) immune response. Present data show that although a few cell populations are increased, significant changes in cytokine expression profile was observed. In present study, patients with *P. vivax* malaria showed no difference in the absolute number of Th1 cells represented by quantification of IFN-γ. The balance between Th1 and Th2 cytokines seem to be crucial in the control of clinical symptoms of malaria. A protective immune response against malaria is initiated by antigen-presenting cells which ultimately activate $CD4^+$ and $CD8^+$ T-cells.

The protection against infection resulting from Th1 immune response is largely mediated by pro-inflammatory cytokines IFN-γ and TNF-α (Artavanis-Tsakonas and Riley, 2002). The IFN-γ is a pro-inflammatory cytokine produced by $CD8^+$ and Th1 cells and in *P. vivax* malaria it has a direct anti-parasitic action. The action of IFN-γ, as well as other pro-inflammatory cytokines, should be controlled so that the production level is appropriate and, therefore, elimination of infection with less damage to the host (Artavanis-Tsakonas and Riley, 2002). Again, it is clear that the immune response differs according to the species of *Plasmodium* infecting and it is important to emphasize that this study was conducted where *P. vivax* is the most prevalent specie and due to the response against *Plasmodium* be influenced by a variety of factors, not all information obtained in this study can be extrapolated to other areas of the world. However, the lack of correlation of the cellular response profile observed in our results and variables such as parasitaemia, number of previous malaria episodes and clinical status, prevents us from making any associations that suggest protection cell markers or susceptibility to malaria. Therefore, further studies are needed to determine the possible mechanisms involved in the pathogenesis of this important infection.

The Th2 immune response, characterized by the absolute number of IL-4 in the subpopulation of $CD3^+$ $CD4^+$ IL-4^+ T-cells was detected elevated in present study during the infection by *P. vivax*. Alam *et al*. (2008) detected the average concentrations of IL-4 in the amount of 151.04 pg mL^{-1} in individuals infected with *P. vivax* malaria. Zeyrek *et al*. (2006) also detected elevated serum levels of IL-4 during infection by *P. vivax*. IL-4 appears to play a regulatory role in malarial infection by inhibiting the Th1 pathway and inducing specific antibodies which can be protective (Paludan, 1998). Although, well described the participation of the humoral immune response in malaria, it can be maintained independent of IL-4 or serum levels of this cytokine may increase or decrease significantly over the course of infection (Medina *et al*., 2011). Studies indicate that IL-4 acts to prevent the development of cerebral malaria and are associated with severe anemia (Cabantous *et al*., 2009).

Another cell population recently associated with parasitic infections are the Th17 cells which are mediated by IL-17A and IL-17F. They induce the expression of pro-inflammatory cytokines as a result of the recruitment and activation of multiple lineages of leukocytes (Bueno *et al*., 2012). Controversial studies have demonstrated that Th17 cells mediate protection against *Trypanosoma cruzi* (Miyazaki *et al*., 2010) and *Toxoplasma gondii* (Iwakura *et al*., 2008). Moreover, in the human cutaneous leishmaniasis, there was a relationship promoting pathogenesis (Bacellar *et al*., 2009), suggesting that the role of Th17 in parasitic infections remains to be elucidated. Ishida *et al*. (2010) demonstrated that the absence of IL-17 in knockout mice infected with *P. berghei* ANKA developed experimental cerebral malaria. In the current study, an increase in Th17 cells, represented by the high number of IL-17 was verified in patients infected with *P. vivax* compared to healthy controls. This data do not allow any clear conclusion about the role of these cells in protection or pathogenesis of this disease. However, Bueno *et al*. (2012) demonstrated that elevated levels of Th17 cells were detected in patients with *P. vivax* malaria from the state of Amazonas, Brazil and these finds are associated with high production of IFN-γ, IL-10 and TGF-β.

The triggering of effector immune response during malaria infection is a complex process that has been characterized over the past decades. Further immunological and molecular studies should be conducted with a focus on understanding the immune response against *P. vivax* infection, in order to elucidate the pattern of response and protective factors associated with immunopathology of *P. vivax*.

CONCLUSION

Thrombocytopenia is a haematological parameter present during the *P. vivax* malaria and this has a negative correlation with the level of parasitaemia. However, the level of parasitaemia does not influence the number of total leukocytes in patients with *P. vivax* malaria. In addition, there is no variation in the number of total leukocytes nor in the $CD4^+/CD8^+$ ratio during the acute infection by *P. vivax* but there is an increase in the absolute number of cytotoxic T-cells, Th2 and Th17 cells during the infection with *P. vivax*, indicating that infection active specific cells which will participate in immunoregulation against this parasite. The Th1 and Treg cells number were not altered by infection with *P. vivax*.

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