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## **Vivax infection alters peripheral B cell profile and induces persistent serum IgM**

**Running Title: B cell perturbations in *P. vivax* malaria**

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## Abstract

B cell-mediated humoral responses are essential for controlling malarial infection. Studies have addressed the effects of *Plasmodium falciparum* infection on peripheral B cell subsets but not much is known for *P. vivax* infection. Furthermore, majority of the studies investigate changes during acute infection, but not after parasite clearance. In this prospective study we analyzed peripheral B cell profiles and antibody responses during acute *P. vivax* infection and upon recovery (30 days post-treatment) in a low-transmission area in India. Dengue patients were included as febrile-condition controls. Both dengue and malaria patients showed a transient increase in atypical memory B cells during acute infection. However, transient B cell-activating factor (BAFF)-independent increase in the percentage of total and activated immature B cells was observed in malaria patients. Naïve B cells from malaria patients also showed increased TLR4 expression. Total IgM levels

remained unchanged during acute infection but increased significantly at recovery. Serum antibody profiling showed a parasite-specific IgM response that persisted at recovery. A persistent IgM autoantibody response was also observed in malaria but not dengue patients. Our data suggests that in hypoendemic regions acute *P. vivax* infection skews peripheral B cell subsets and results in a persistent parasite-specific and autoreactive IgM response.

**Key words: vivax malaria, IgM repertoire, B cell development, immature B cells, dengue, PANAMA blot, atypical memory B cells, autoreactivity**

## 1. Introduction

Antibody-mediated immune response plays an important role in anti-malarial defence<sup>1</sup>. B cell development is crucial for proper functioning of humoral immunity. Two types of B cells are reported in murine peripheral blood—B1 cells, originating from the peritoneal cavity and B2 cells originating from the bone marrow. A third type of B cell subset—MZ (marginal zone) B cells—resides in the MZ of the spleen but also appears in peripheral circulation in humans<sup>2</sup>. B1 and MZ cells are considered innate-like, can respond to antigens independent of T cell help, and secrete broadly neutralizing IgM antibodies. Majority of the B cells in the peripheral blood are of the B2 type (hereafter referred to as B cells) and require T cell help to produce antigen-specific antibodies<sup>3</sup>. B cells emerging from the bone marrow are IgM and CD10 expressing transitional (immature) B cells that eventually lose their CD10 expression to become naïve B cells. Upon antigenic stimulation and cognate T cell interaction, naïve B cells undergo isotype switching and affinity maturation, and

differentiate to plasma cells and memory cells. In the absence of T cell help, B cells fail to switch the isotype but can differentiate to memory cells and plasma cells producing IgM of low affinity<sup>4</sup>. Plasma cells produced early in the immune response are short-lived, but later ones are long-lived, reside in the bone marrow, and continue secretion of antigen-specific antibodies for months or even years<sup>3,5</sup>.

Outside of Africa, malarial infections are predominantly caused by *Plasmodium vivax* and the species has wider distribution than *P. falciparum*. In 2016, *P. vivax* was estimated to have been responsible for 8.5 million malaria cases globally<sup>6</sup>. In 2014, particularly in urban India, 98% of all reported malaria cases were due to *P. vivax*<sup>7</sup>. Infections by *P. vivax* were generally considered to be benign and self-limiting. However recent reports indicate that *P. vivax* infections may lead to severe manifestations, including multi-organ dysfunction, cerebral malaria, and acute thrombocytopenia<sup>8</sup>. *P. vivax* and *P. falciparum* have distinct mechanisms of pathogenesis and also differ in host immunological responses they induce, e.g., cytokine/chemokine and antibody responses evoked<sup>9,10</sup>. In high-transmission areas chronic *P. falciparum* infections have been shown to cause alterations in peripheral B cell subsets. Atypical memory B cells<sup>11,12</sup> and elevated levels of immature B cells have been reported in peripheral blood of *P. falciparum*-infected adults and children respectively<sup>13</sup>. Malarial infection is also known to perturb generation of effective memory B cells, hampering the development of sterile immunity<sup>14</sup>. Scant data is available on the effect of *P. vivax* infection on peripheral B cell subsets, especially in hypoendemic regions of South Asia. This prospective study was undertaken to assess the kind of humoral response elicited in an Indian population by a first-time uncomplicated vivax infection, specifically, in terms of changes in peripheral B cell subsets observed and antibody profiles generated.

## 2. Materials and Methods

### 2.1 Study area

The study was undertaken in a tertiary medical centre (King Edward Memorial Hospital) situated at Mumbai in Western India. Malaria is seasonal in hypoendemic Mumbai, with higher number of clinical cases in the monsoon months (June to September)<sup>15</sup>.

### 2.2 Study population

All protocols used in this study were approved by Institutional Ethics Committee (IEC) of TIFR and IEC of Seth GS medical college and KEM hospital, Mumbai (EC/GOVT-3/2013). The study is registered with the Clinical Trials Registry of India (CTRI/2018/02/012087). Informed consent was obtained from all individuals before enrolling. Three groups of individuals who came to the out patient's department (OPD) of KEM hospital were invited to participate in the study. 1) Uncomplicated malaria patients (*P. vivax*), who reported to never having suffered from malaria before, and diagnosed by thick and thin blood smears and clinical features (<2000 parasites/ $\mu$ L and axillary temperature between 37–40°C), 2) Individuals who had accompanied the patients to the hospital and were willing to participate in the study were recruited as 'healthy controls' if they were malaria negative by the blood smear test, if they confirmed that they had never had malaria, if they did not present with any other symptoms and were not on medication for any chronic condition, 3) Non-malarial fever (dengue) patients, identified based on the clinical symptoms and confirmed as suffering from dengue infection by using Denguecheck Combo™ kit (Zephyr biomedical, Mumbai).

All enrolled individuals underwent a thorough physical examination by the attending physician. Their oral temperature, pulse rate, blood pressure, body weight, etc., were recorded. Data was also collected regarding previous episodes of malaria, episodes of pyrexia or other infections in the previous 6 months, and medications consumed in the prior 2 weeks. The day on which the patient came to the OPD was considered as day 1. 5–10 mL of blood was collected from all participants. Blood was also collected on day 30 (recovery sample) from the malaria patients, when the absence of parasites was confirmed by microscopy. We have previously shown that the host metabolic response reverts largely to baseline at this time point<sup>16</sup>. Hematological parameters were determined using Cell counter Mythic-18 (Spectrum, India) and were available for only 15 of 23 healthy donors enrolled. CD4/CD8 ratio was determined for all patients, and those with a CD4:CD8 ratio <1 were considered immunosuppressed and hence excluded from the study<sup>17</sup>. Also excluded were patients with falciparum infection as only 4 could be recruited during the research period, and patients reporting to have suffered from malaria before. The characteristics of the individuals included in the study are given in Table 1. Malaria patients were treated as per the WHO guidelines (chloroquine 25 mg/Kg body weight over 3 days followed by primaquine 0.25 mg/Kg body weight for 14 consecutive days). The patients self-reported treatment compliance.

### **2.3 Flow cytometry**

About 100 µL of whole blood was used for flow cytometric analysis. All antibodies were purchased from BD Biosciences, USA, and the catalogue numbers are indicated in table S1, unless otherwise mentioned. Lymphocytes, neutrophils and monocytes were

identified based on forward and side scatter. The B cell panel consisted of anti-CD19 BV-421, anti-CD20 PE, anti-CD21 FITC, anti-CD27 APC, anti-CD10 PE-Cy7, anti-CD69 PE-Cy5. A subset of samples was analyzed for changes in B1 cell profiles using anti-CD20 PE, anti-CD 43 BV421 and anti-CD27 APC. TLR4 expression was assessed using anti-TLR4-PE antibody. T cell panel consisted of anti-CD3 FITC (#555431), anti-CD4 PE (#555654) and anti-CD8 APC (#555438). Whole blood was incubated with the antibodies for 30 min on ice. Following incubation, RBCs were lysed using FACS lysis buffer (#349202, BD Biosciences) at 4°C for 10 min. The cells were washed with phosphate buffered saline (PBS) and the pellets were re-suspended in 500 µL of 2% Para-formaldehyde. The cells were acquired on BD LSR Fortessa and data analysed by FACS Diva software. Doublets were excluded on FSC-H vs FSC-A scatter plot. Various subsets and their phenotypic markers are given in Supplementary Table 1 and the gating strategy outlined in Figure 1.

#### **2.4 Enzyme linked immunosorbant assay (ELISA)**

Antigen-specific IgM and IgG antibody profiles were determined by ELISA as reported previously<sup>18</sup>. Briefly, microtitre polystyrene plates (#9018, Corning, USA) were coated with 100 ng of antigen in 100 µL of PBS per well and blocked with 1% gelatin in PBS. The peptides were all obtained from Mimotopes, Australia and consisted of *P. vivax* merozoite surface protein-1 (MSP-1; aa 20-ETESYKQLVANVDKLEALVV-39), apical membrane antigen-1 (AMA-1; aa 21-CGRNQKPSRLTRSANNVLEK-42), gametocyte surface antigen-1 (GAM-1; aa 1262-CPLPTWEVLHDGC-1272)<sup>19</sup>, and the highly conserved phosphoriboprotein P2 found on the surface of infected RBCs<sup>20</sup> (P2; aa 27-NVLGAVNADVEDEVLNNFIDSLK-49). Plasma samples were diluted 1:100 in PBS for use. The plates were developed by using

horse-radish peroxidase labeled anti-human IgG (#054220) /IgM (#054920) (Invitrogen Biosciences, India) and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (#10102946001, Roche, Germany). The absorbance was read at 405 nm. The baseline was considered as mean OD of sera from all the healthy controls (n=23) + 2 SD (standard deviations). The results were expressed as Reactivity Index (RI) calculated as the ratio of the serum sample OD to the baseline OD. Samples were considered negative if RI < 1. Total IgG (#ab100547, abcam, USA), IgM (#ab137982, abcam, USA) and BAFF (#ab119579, abcam, USA) concentrations were determined using commercially available kits as per the manufacturer's instructions.

## **2.5 Autoantibody repertoire analysis**

PANAMA blot method was used to analyze autoantibody repertoire of sera samples from malaria and dengue patients and healthy participants (eight sera from each group), as described earlier<sup>21</sup>. In brief, brain lysate proteins (200 µg/gel; Medley, Ozyme, France) were resolved on 10% SDS-PAGE and transferred on nitrocellulose membrane. The membranes were blocked with PBS-T (PBS containing 0.2% Tween 20) for 1 h before adding sera using an immunoblot cassette. After overnight incubation at 4°C, the membranes were washed and incubated with alkaline phosphatase labeled anti-IgM or anti-IgG secondary antibody (#A3275, Sigma, USA) for 2 h. The membranes were washed again and developed using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) substrate (#S3771, Promega, France). The bands developed in the anti-IgG probed membranes were too faint for meaningful analysis and were not processed further. Membranes probed with anti-IgM antibodies were dried and scanned by a high resolution scanner (600 DPI) using an



8-bit linear grayscale as described<sup>21</sup>. Subsequently, the proteins transferred to membranes were stained with colloidal gold (Protogold, British-BioCell, GB), and the stained membranes scanned again. Using colloidal gold staining, immunoreactivity profiles were adjusted for migration inequalities using IGOR software as described<sup>21</sup>. Intensities between membranes were adjusted by a standard consisting of a pool of sera from the vivax patients. Profiles of reactivity of the samples with brain proteins were separated into 17 sections. Each section corresponded to the IgM antibodies recognizing a band with a defined molecular weight.

## 2.6 Statistical Analysis

Normality test was performed on all data before statistical analysis. Paired T test was used for comparisons between day 1 and day 30 results. One-way ANOVA and Kruskal-Wallis test and post hoc Dunn multiple comparison test was used to compare greater than two groups. Spearman's correlation was used to assess correlations between B cell subsets and BAFF levels. All analysis was done using Prism 6 software (Graphpad Software, Inc, USA). Immunoblot data were analyzed by multivariate statistical methods, using IGOR software (Wavemetrics, Lake Oswego, USA), including specially written software packages. The standard migration scale was divided into sections around individual peaks of immunoreactivity. Section-wise absorbance values were subjected to principal component analysis (PCA) based on covariance calculation. PCA factor 1 is the linear combination of single reactivity measurements that represents the maximum of information about a multivariate data set in terms of total variance. In this study, factor 1 represented 65% of total variance and had exclusively positive factor loads, so that it can be seen as a univariate approximation of IgM repertoire reactivity. p values <0.05 were considered statistically significant in all cases.

### 3. Results

#### 3.1 Study participants and clinical parameters

Data from 71 patients who approached the hospital with acute *P. vivax* infection and reported suffering from malaria for the first time (56 males, 15 females) was included in the study. In agreement with previous reports we noted an adult male bias in malaria patients<sup>15</sup>. The median parasite density was 1560 (IQR: 300–1960)/ $\mu$ L blood which is in concordance with earlier reports<sup>9</sup>. Recovery (day 30) samples could be obtained only from 15 of the 71 patients. Data from twenty-three healthy participants who did not have a history of malaria were included as controls. To ensure that results were malaria-specific and not due to pyrexia, we included data from 21 dengue fever patients. The characteristics of all individuals enrolled in the study are presented in Table 1.

Analysis of hematological parameters revealed that none of the malaria patients suffered from anemia—Hb levels of malaria patients were similar to that of controls (Table 1). However, both malaria and dengue patients exhibited significant leukopenia and thrombocytopenia in the acute phase of the infection (Table 1) as has been reported earlier<sup>22</sup>. Unlike what was observed for first-time malaria sufferers in Brazil<sup>23</sup> there was no change in the number of monocytes in our malaria cohort (Table 1) neither did we observe lymphopenia in our cohort as reported<sup>24</sup>. All these changes were transient and reverted to base line at day 30 (Table 1).

### 3.2 Acute *P. vivax* infection causes specific changes in peripheral B cell subsets

B cell subsets were defined as naïve ( $CD19^+CD20^+CD21^+CD10^-CD27^-$ ), immature ( $CD19^+CD20^+CD21^+CD10^+CD27^-$ ), classical memory cells (MBCs;  $CD19^+CD20^+CD21^+CD10^+CD27^+$ ), atypical MBCs ( $CD19^+CD21^-CD10^-CD27^-$ ), and plasma cells ( $CD19^+CD20^-CD21^-CD10^-CD27^-$ ) based on the markers expressed. Atypical memory B cells were identified using a modified gating strategy published earlier<sup>12</sup>. A subset of samples (n=10) was also analyzed for B1 B cell profile. Figure 1 shows the gating strategies used.

The percentage of B cells in the peripheral lymphocyte pool of malaria patients was significantly decreased on day 1 (Figure 2A) and remained at that level at recovery (Figure 3A). In concordance with published reports, naïve B cells were the most frequent and plasma cells the least frequent in the blood of healthy controls (Figure 2 B,E)<sup>12</sup>. Naïve B cells decreased significantly during acute malaria infection (Figure 2B). Although there was an apparent, albeit moderate, increase in the naïve B cell frequency in 10 out of 15 patients, the average frequency remained at the levels of day 1 (day 1  $34.7 \pm 12.4\%$  vs day 30  $39.8 \pm 16.6\%$ ; Figure 3B ) and was significantly lower than the frequency observed in the healthy population ( $52.5 \pm 18.6$ ;  $p < 0.01$  healthy vs day 30). The percentage of immature B cells and plasma cells, on the other hand, increased significantly during acute malaria (Figure 2 C,E) but reverted to baseline by day 30 (Figure 3 C,E). There was no change in the percentage of classical memory B cells on day 1 (Figure 2D), but their percentage increased significantly at recovery (Figure 3D). Interestingly, there was a significant increase in the percentage of atypical memory B cells during acute infection but these reverted to baseline at recovery (Figure 2F). It was not possible to test for B1 B cells in all the samples, hence we investigated the percent of B1 cells in a subset of malaria patients (n=10). There was no

change in % B1 cells in these patients (controls  $22.10 \pm 20.77$ ) vs patients ( $26.03 \pm 15.23$ ;  $p > 0.05$ ).

In contrast to acute malaria patients, the percentage of B cells in the peripheral lymphocyte pool remained unchanged in dengue patients (Figure 2A). Similar to what we observed in malaria patients, naïve B cells decreased (Figure 2C) and plasma cells and atypical memory B cells increased significantly during acute dengue infection (Figure 2E,F), while classical memory B cells remained unchanged (Figure 2D). Unlike malaria patients, however, the percentage of immature B cells was significantly lower in dengue patients (Figure 2B).

We quantified the serum BAFF content in a subset of acute vivax and dengue patients. Both febrile conditions showed a comparable increase in BAFF levels (Figure 4). Spearman's correlation analysis revealed that although a positive correlation was found between % of immature B cells and BAFF levels in healthy controls (Figure S1A;  $p = 0.017$ ,  $r = 0.636$ ), there was no correlation between BAFF levels and B cell subsets in patients of either malaria or dengue (Figure S1B,C).

### **3.3 *P. vivax* infection elicits a persistent antigen-specific IgM response**

We measured total and parasite-specific IgM and IgG antibodies in the sera of malarial patients using ELISAs. Although the total IgM levels on day 1 in malaria patients were indistinguishable from those of healthy individuals, these levels were significantly higher at recovery (Figure 5A). By contrast, total IgG levels whether on day 1 or on day 30 were similar to the control IgG levels (Figure 5B). We used immunodominant peptides derived from four surface proteins expressed at different erythrocytic stages *viz.* AMA-1,

GAM-1, MSP-1, and P2 to determine anti-parasite IgM and IgG response. Of these, the first three are commonly used to determine anti-vivax responses in vaccination studies<sup>19</sup> whereas anti-P2 peptide antibodies have been found to be present in Indian falciparum patients (unpublished data). About 12% of the malaria patients had IgM antibodies recognising all the 4 peptides; the corresponding figure for IgG was a meagre 1%. The IgM positivity rate was  $\approx$ 50% for individual peptides except for GAM-1, which was recognized by 73% of the patients (Table S2). Chi-square analysis of IgM and IgG positivity rates showed a significant increase in IgM and IgG anti-MSP-1 positivity rates (Table S2). By contrast, IgM and IgG positivity rates remained unchanged for AMA-1, P2, and GAM-1 (Table S2).

Although positivity rates for different peptides were around 50% or more (table S2), the levels of antigen-specific antibodies in the serum of individual patients were low; the median RI for both IgM and IgG responses against MSP-1, AMA-1, and P2 was at or below the cut off (Figure 6A,B). GAM-1 was the only exception (Figure 6A,B). The anti-GAM-1 response in the healthy controls was 10 times lower than that of the other peptides, which could be partially responsible for the high RI observed for this antigen. Paired T test analysis of day 1 and day 30 antibody responses revealed that anti-MSP-1 IgM and IgG responses increased at recovery (Figure 6C). By contrast, anti-AMA-1 IgM response decreased at recovery with no concomitant increase in the IgG response (Figure 6D). Anti-P2 and anti-GAM-1 IgM and IgG antibody responses, on the other hand, remained at the same level at peak and recovery (Figure 6E,F). IgM:IgG ratios remained statistically indistinguishable whether on day 1 or day 30 (Figure 6 G–J), suggesting that the IgM response was sustained throughout the observation period. Thus patients suffering from

malaria for the first time exhibited an antigen-specific IgM response and this response persisted at the same level after 30 days.

### **3.4 *P. vivax* infection generates an autoantibody response**

Malaria is characterized by polyclonal B-cell activation and hyper gammaglobulinemia<sup>25,26</sup> with the production of a diverse repertoire of autoantibodies<sup>26,27</sup>. *P. vivax* infections have also been shown to result in the generation of anti-cardiolipin, anti-erythrocytic, and anti-phospholipid antibodies<sup>28,29</sup>. However, no studies investigate the generation of autoantibodies using an unbiased quantitative approach that allows the assessment of the total repertoire of self-reactive antibodies generated during *P. vivax* infections and the complex interactions between these components. We investigated brain protein reactivity as a surrogate for autoreactivity in a subset of sera samples (n=8 each for malaria patients, dengue patients, and controls) using a combination of quantitative immunoblotting (PANAMA blot) and multivariate statistics to compare the reactivity of circulating IgM to human brain proteins<sup>21</sup>. The number of bands recognized were significantly greater in malarial patients as compared to dengue patients and healthy controls (Figure 7A,B). We compared profiles of antibody reactivity from each group by Principal Component Analysis (PCA). Malaria patients had higher PCA factor 1 compared to both healthy controls and dengue patients, indicating that vivax infection induced a significant IgM autoantibody response both in terms of diversity and intensity (Figure 7C). Spearman's rank correlation analysis revealed that there was also no correlation between the infection-induced autoantibody response and antigen-specific IgM response ( $p>0.05$ ). We also analyzed day 1 and day 30 samples to understand if the autoreactivity persisted 30

days post-treatment. The number of bands and PCA 1 factor for sera samples on day 30 was similar to that observed on day 1 (Figure 7D–F), indicating that not only did the infection induce autoreactivity, but that this autoreactivity persisted in the recovery phase. Except for reactivity to one band, the profile of bands recognized by day 1 and day 30 sera were statistically indistinguishable (Figure 7G).

### 3.5 B cell-surface TLR4 expression is elevated in *P. vivax* patients

Our data showed a persistent IgM positivity rate as well as antigen-specific IgM response in *P. vivax*-infected patients whether in the acute phase or 30 days later. A predominant IgM response that does not switch to IgG is a characteristic of T-independent immune responses<sup>30</sup>. Toll like receptors (TLRs) and specifically TLR4 is known to promote T-independent responses<sup>31</sup>. Hence we investigated the expression of TLR4 on the leukocytes of malaria and dengue patients using flow cytometry. Monocytes and neutrophils from dengue patients showed elevated levels of TLR4 expression—mean fluorescence intensity (MFI) of  $1263 \pm 967$  for patient monocytes vs  $412 \pm 169$  those of healthy controls; MFI of  $477 \pm 271$  for patient neutrophils vs  $129 \pm 63$  for healthy controls. Malaria patients also exhibited similar increase in TLR4 expression on monocytes (MFI:  $676 \pm 361$  vs  $412 \pm 169$  for healthy control) and on neutrophils (MFI:  $324 \pm 315$  vs  $412 \pm 169$  in healthy controls). However, only *P. vivax*-infected patients showed increased TLR4 expression on B cells (Figure 8A). Next we investigated if this elevated TLR4 expression was restricted to a particular type of B cell using a subset of patient samples. Only naïve B cells exhibited increased expression of TLR4 (Figure 8B). The increased TLR4 expression on naïve B cells led us to ask the question if these cells were activated by assessing their CD69 expression

(Figure 8C,D). Both immature and naïve B cells showed significant CD69 expression (Figure 8D).

## Discussion

Peripheral B cell subset skewing has been reported in chronic HIV, HCV, and *P. falciparum* infections<sup>12,32,33</sup> and may be the result of compartmental redistribution, enhanced production due to specific or nonspecific activation stimuli, or alteration in cell death. An early and effective B cell response is essential for clearing pathogens and alterations in the homeostasis of peripheral B cell compartment are likely to result in a suboptimal humoral response. This study investigates the effects of acute vivax infection on peripheral B cell subsets and its consequences on parasite-specific antibody responses in the hypoendemic region of Mumbai, India, with annual frequency of parasite detection between 0.07 to 4.6%<sup>15</sup>. We also assessed if the perturbations caused by the infection persisted post parasite clearance. We observed that acute vivax infection resulted in a decline in the percentage of B cells in the peripheral lymphocyte pool and this decline persisted 30 days post parasite clearance. However, the percent immature B cells and atypical memory B cells increased in the acute phase but reverted to baseline at day 30. Interestingly, both naïve B cells and immature B cells showed an activated phenotype. In concordance with earlier reports the number of classical memory cells was significantly increased at recovery<sup>13</sup>. Analysis of serum antibody profiles revealed that the malarial infection resulted in a persistent IgM response characterized by the induction of both antigen-specific and autoantibodies.



There are contrasting observations regarding the effect of malarial infection on peripheral B cell pool. Figueiredo et al.<sup>34</sup> did not observe any change in the frequency of either total B cells or immature B cells in the peripheral pool. By contrast several other studies report that malarial infection affects B cell lymphopoiesis with an attendant decrease in the total number of B cells in peripheral blood of mice and humans similar to what we have observed<sup>11,23,35,36</sup>. Although, the studies by Figueiredo et al.<sup>34</sup> and Chaves et al.<sup>23</sup> are situated in Brazil and investigate the effect of primary vivax infection in adults, the median parasitemia is an order of magnitude higher in the Chaves et al. study and our study, suggesting that apart from parasite and host genetics, other factors such as parasitemia levels may also affect B cell lymphopoiesis. Decrease in total B cells in the peripheral pool has been linked to an increase in pro-inflammatory cytokines and increased serum BAFF<sup>37,38</sup> levels. Children in malaria hyperendemic regions have been reported to have increased immature B cells<sup>13</sup> and survival of these cells in the periphery is also linked to increased serum BAFF levels<sup>39</sup>. Although we did observe a decreased frequency of total B cells and an increased frequency of immature B cells in acute malarial patients, non-malarial fever (dengue) patients did not exhibit either of these features. Indeed, there was a decrease rather than increase in the percentage of immature B cells in dengue patients. However, serum BAFF levels were elevated in both dengue and malaria patients during acute infection. Furthermore, there was no significant correlation between BAFF levels and total, immature, or naïve B cells in the patients. It is therefore likely that decline in peripheral B cells and increase in immature B cells may not be a direct consequence of an inflammatory response, but caused by malarial parasite-specific factors.

Increased atypical MBCs have been reported in chronically infected HIV individuals<sup>32</sup> and *P. falciparum*-infected adult patients in hyperendemic regions<sup>11</sup> as well as in *P. vivax* infections in hyperendemic areas of Papua New Guinea<sup>40</sup> and hypoendemic areas of Brazil<sup>34</sup>. Increase in BAFF levels and proliferation of atypical MBCs have been correlated in controlled human malaria infections with *P. falciparum*<sup>41</sup>. However, we found no correlation between increased BAFF levels and increase in atypical MBCs in our cohort. Recent observations raise the possibility of a link between inflammation and atypical MBC expansion<sup>42</sup>. IFN- $\gamma$  in conjunction with antigen-specific cross-linking by IgM has been shown to drive the expansion of T-bet<sup>hi</sup> atypical memory B cells in children with repeated malarial episodes<sup>42</sup>. Our own observation of increase in atypical MBCs in two different infections suggests that formation of these cells is likely a feature of multiple febrile conditions that result in augmented systemic IFN- $\gamma$  production. Further studies are warranted to understand the role, if any, of atypical MBCs in resistance to infection.

Investigations in the antibody profiles of malaria patients revealed that in concordance with an earlier study<sup>34</sup>, the concentration of total IgM antibodies at day 1 was similar to those found in healthy controls. However, total IgM levels were significantly higher than healthy controls at recovery. Such variations were not observed in total IgG levels. Increase in total IgM levels during acute phase has been reported in *P. falciparum* infections central India<sup>21</sup>, however the study did not investigate IgM levels in the recovery phase.

In agreement with previous reports, we observed considerable heterogeneity in the antigen-specific humoral response profiles<sup>43</sup>. The seropositivity rate for GAM-1 was similar to that reported in an earlier study conducted in another hypoendemic region of India<sup>19</sup>.

The response to the highly conserved phosphoriboprotein peptide P2 was similar to that observed for MSP-1 and better than that of AMA-1, suggesting P2's potential as a diagnostic candidate. Both anti-MSP-1 and anti-AMA-1 responses are generally found to correlate well with vivax infection<sup>43,44</sup>. We found a 50% positivity rate for these antigens in our cohort. Interestingly, we found the anti-MSP-1 response (both IgM and IgG) increased significantly at recovery ( $p < 0.05$ ). This is in contrast to data reported from Brazil where anti-MSP-1 IgM and IgG responses were found to decline 30–45 days after treatment<sup>34</sup>. This clearly underscores the need to identify country-specific indicators of exposure and immunity, as they may vary depending on the epidemiology of malaria in different parts of the world and as previously noted, the predominance of data from Brazil in the field may have implications for the generalizability of findings to other *P. vivax* endemic regions in South America and the Asia-Pacific<sup>44</sup>.

Our study was conducted in a tertiary clinic, where the patients reported malaria-like symptoms that had persisted for at least a few days before their visit. Seroconversion from IgM to IgG has been reported to occur within 3 days of the appearance of malarial symptoms<sup>45</sup>, yet we found that the patients continued to exhibit a sustained antigen-specific IgM antibody response even after 30 days at recovery. Furthermore, Spearman's rank correlation analysis revealed that there was no significant correlation between IgM and IgG response except for P2 which showed a positive correlation (data not shown) clearly indicating that patients with high anti-P2 IgG response were also exhibiting a high IgM response. Our data thus makes a case for analysing for both IgM and IgG antibody responses in low-transmission areas. The reason for the persistence of the IgM response in our cohort

merits further investigations as host genetics have been suggested to influence the overall antigen specificity of IgM and IgG responses to malaria<sup>46</sup>.

*P. falciparum* infection is known to induce elevated titers of autoantibodies<sup>21</sup>.

Presence of anti-cardiolipin antibodies have also been reported in *P. vivax* infections and have been linked to anemia<sup>47</sup> and cerebral malaria<sup>48</sup>. Generation of autoantibodies in *P. falciparum* has been attributed to polyclonal B cell activation<sup>49</sup> with the involvement of TLRs via PfEMP-1<sup>50</sup>. Although a similar polyclonal B cell activation has not been observed for *P. vivax* infections<sup>47</sup>, our observation of increased TLR4 expression on naïve B cells, suggested the possibility of their polyclonal activation. We used an unbiased quantitative approach to detect autoantibody generation in vivax malaria using brain lysate as a surrogate for autoantigens. Several lines of evidence indicate that irrespective of the targeted organ (e.g., thyroid, pancreas, joints, brain, or skin), a significant proportion of autoantigens are highly expressed in the synaptic compartment of the central nervous system<sup>51</sup>. We found increased diversity in the repertoire of autoantibody produced in vivax patients compared to dengue patients. The level of these self-reactive antibodies being maintained at recovery suggests that they may contribute to protective immune mechanisms that trigger parasite containment<sup>52</sup>. The increased reactivity to section 7 at recovery suggests that it may be a signature of protection in vivax malaria. The IgM autoantibodies observed at recovery might be derived from expanded classical B cells. Alternatively, some of these autoantibodies could be 'natural antibodies' that are characterized by low affinity for the antigen and polyreactivity. To our knowledge this is the first study reporting generation of IgM autoantibodies to brain lysate proteins in non-anemic, mild, vivax malaria patients reportedly suffering from malaria for the first time. This generation of autoantibodies was

clearly malaria-specific and persisted largely unchanged—both, in terms of levels and repertoire—even after parasite clearance. Clearly, the existence of autoantibodies does not necessarily imply development of autoimmune disease. The debris-clearing activity of IgM autoantibodies has been well established, and mice experimentally deprived of these autoantibodies demonstrate lupus-like symptoms<sup>53</sup>. Certain levels of antibodies to different proteins of the nervous tissue are present in the blood of healthy persons<sup>51,53</sup> and the generation of autoantibodies to brain lysate proteins may reflect a change or shift in the autoantibody repertoire of malaria patients. The mechanism(s) responsible for the increased TLR4 expression on B cells as well as autoantibody generation and their ramifications need to be examined.

B1 B cells are a population of B cells that were originally identified in mice and shown to produce high levels of low-affinity natural IgM antibodies. Recently Quách et al.<sup>54</sup>, have suggested that human B1 B cells might be preferentially selected for autoreactivity. Given that we found a persistent IgM anti-malarial response and that the infection also induced autoreactive antibodies, we investigated a subset of the patient samples for B1 B cells. Although malarial patients had reduced frequency of total B cells, we found no significant changes in the frequency of peripheral B1 B cells. Apart from B1 B cells, MZ B cells, found in human peripheral blood are also known to produce IgM in response to antigenic stimulation<sup>2</sup>. We did not have enough samples to investigate if the frequency of MZ cells increased in response to vivax infection. However, apoptosis of MZ B cells has also been observed in infection with *P. chabaudi* in the murine model<sup>36</sup>. MZ B cells have also been shown to undergo apoptosis with a consequent decrease in numbers in the blood of

patients suffering from repeated falciparum infections<sup>55</sup>. The role of these cells in acute vivax infection, especially in hypoendemic areas, needs to be investigated.

Increased frequencies of immature or transitional B cells have been found in a number of immune deficiencies and are characterized by a dysregulated or impaired humoral immune response<sup>56,57</sup>. Immature (transitional) B cells have been shown to give rise to memory B cells independent of germinal centre reaction and may serve as precursors for plasma cells involved in innate antibody production<sup>58</sup>. There seems to be a correlation between increased frequency of immature B cells and increased autoantibody generation<sup>59,60</sup>. A recent report shows that BAFF promotes humoral autoimmunity *via* direct, TACI-dependent activation of transitional B cells<sup>61</sup>. We found that *P. vivax* infection induced an IgM response that had both an antigen-specific and an autoreactive component. Coupled to the increased BAFF levels and increased activated immature B cells in the periphery our data suggests that the antibody profile generated by the infection could, at least in part, be a consequence of the increased frequency of activated immature B cells.

After immunization or infection, activated naïve B cells switch from producing low-affinity IgM antibodies to high-affinity IgG antibodies in a T-dependent manner. By contrast, T-independent responses are typically of the IgM isotype. LPS stimulation of TLR4 expressed on immature B cells has been shown to increase the production of IgM significantly in murine models<sup>62</sup>. The persistent IgM response prompted us to investigate expression of TLR4 on B cells. We found increased expression of TLR4 on naïve B cells. Although we did not find increased expression of TLR4 on immature B cells, the possibility of increased expression of other TLRs (e.g., TLR9, a known ligand for heme produced during malarial infection<sup>63</sup>) cannot be ruled out. Both these B cell subsets also exhibited an activated

phenotype. The role of T cells in generating an effective humoral response in malaria remains unresolved. Long-lived *Plasmodium*-specific somatically hypermutated IgM<sup>+</sup> memory B cells capable of giving rise to plasma cells in both T-independent and T-dependent manner have been demonstrated in the murine model of malaria<sup>64</sup>. Generation of long-lived IgM-secreting plasma cells to T-independent antigens and in *P. chabaudi* *chabaudi* infection have been reported previously<sup>65,66</sup>. The half-life of human IgM is known to be ~5 days, the continued high IgM levels suggests that vivax infection induced long-lived IgM-secreting plasma cells in our cohort. Together with the increased TLR4 expression this suggests that at least part of the IgM response was T-independent.

In conclusion, we report distinct perturbations in the B cell profile of vivax malaria patients in the Indian population with a specific increased influx of immature B cells in the periphery. We find that acute *P. vivax* infection induces the generation of antigen-specific IgM antibodies during acute as well as recovery phase. Part of the induced IgM response was self-reactive similar to what has been reported for falciparum malaria in high-transmission regions. Given that vivax infections have become virulent over the years, a better understanding of the immune responses to vivax malaria will assist in tackling the disease and will aid in the development of more targeted interventions such as a vaccine for vivax malaria.

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## Figure legends:

**Figure 1. Gating strategy for flow cytometric analysis of B cell subsets.** A) B2 cells (conventional B cells) were identified on the basis of CD19 expression and subsets were identified based on the expression of CD20, CD21, CD27, and CD10 markers. B) Atypical MBCs were identified as CD10<sup>-</sup> population that did not express CD21 and CD27. C) B1 cells were identified as CD20<sup>+</sup> cells expressing CD43 and CD27. Shown are representative dot plots from one *P. vivax* infected patient.

**Figure 2. B cell subsets in peripheral blood of participants on day 1.** A) B cells (expressed as a percent of the total lymphocyte population) in healthy controls, *P. vivax* patients and dengue patients on day 1. (B–F) B cell subsets (expressed as a percent of total CD19<sup>+</sup> B cells) as defined in Figure 1; naïve (B), immature (C), classical memory (D), plasma cells (E) and atypical memory B cells (F). Each dot represents individual participant and horizontal lines indicated mean of values of respective group (■: healthy controls, n= 23; ●: *P. vivax* naïve patients, n=71; ▼: Dengue patients, n=21 ). Statistical significance between the healthy controls and patient groups was determined using Kruskal-Wallis test and post hoc Dunn multiple comparison test. \*p< 0.05, \*\*p < 0.01, \*\*\*p<0.001and \*\*\*\*p < 0.0001

**Figure 3. B cell subsets at acute infection with *P. vivax* on (day 1) and at recovery (day 30).** Percentages of B cell subsets (of total B cells) were determined by phenotypic analysis on day1 and after recovery on day 30 (n=15) as outlined in Figure 1. (A) B cells expressed as percentage of total lymphocytes. (B–F) B cell subsets (expressed as a percent of total CD19<sup>+</sup> B cells): naïve (B), immature (C), classical memory (D), plasma cells (E) and atypical memory B cells (F). Statistical significance between the groups was determined using paired t test.\*p< 0.05, \*\*p < 0.001 and \*\*\*\*p < 0.0001

**Figure 4. Serum BAFF concentrations.** Concentration of BAFF in pg/mL in healthy controls (○, n=8), *P. vivax* patients (●, n= 16), and dengue patients (▲, n=8). Each dot represents one individual participant. Horizontal lines indicate mean values±SD. One way ANOVA was used for comparisons between the groups, \*p < 0.05 and \*\*p < 0.001.

**Figure 5. Concentration of total IgM and IgG antibodies in sera of participants.**

Concentration of total IgM (A) and IgG (B) in healthy controls (○, n=23) and naïve *P. vivax*-infected individuals on day 1 (▼,n=15) and at recovery (day 30, ▲, n=15). Kruskal-Wallis test and post hoc Dunn multiple comparison test was used for comparisons between the three groups and paired t test for comparison between day 1 and day 30 data \*\*p < 0.001.

**Figure 6 : Antigen-specific antibodies in sera of malaria patients.** Reactivity index (RI) of antigen specific IgM (A) and IgG (B) antibodies in healthy participants (H; n=23, grey) *P. vivax* patients (I; n=71, black). RI was calculated as the ratio of the serum sample OD to the baseline OD where the baseline was considered as mean OD of sera from all the healthy controls + 2 SD (standard deviations). Sera with RI<1 were considered negative (below dashed line). The line within a box represents median RI value of individual group, and whiskers define minimum and maximum values. (C–F) Reactivity index of antigen specific IgM and IgG antibodies against C) MSP-1, D) AMA-1, E) P2, and F) GAM-1 in patients on day 1 and day 30 (n=15). Sera with RI<1 were considered negative (below dashed line). (G–J) Ratio of IgM RI to IgG RI for G) MSP-1, H) AMA-1, I) P2, and J) GAM-1. Statistical significance for c to f was calculated using paired t test. \*p < 0.05, \*\*p < 0.001

**Figure 7. Analysis of IgM autoantibody profiles using PANAMA blots.** A) Representative immunoblot of select individuals showing IgM autoreactivity against brain extract from sera of healthy controls (n=6), *P. vivax* (n=8), and dengue (n=8) patients. Each lane represents a different individual's serum. Std is the standard. B) Median number of bands recognized by healthy controls, and malaria and dengue patients. C) PCA factor 1 scores from IgM reactivity profiles showing group wise distribution. D) Representative immunoblot of select



individuals showing IgM autoreactivity against brain extract from sera of *P. vivax* patients on day 1 and day 30 (n=3 each). E) Median number of bands recognized by malaria patients on day 1 and day 30 (n=10 each). F) PCA factor one scores from IgM reactivity profiles from day 1 (n=10) and day 30 (n=10). G) Mann-Whitney test was used for statistical evaluation of band intensities of 17 sections of day 1 and day 30 groups. Inset, comparison of band intensities of section 7 of day 1 and day 30. Horizontal lines indicate mean. Error bars represent SD. Statistical significance was determined using Mann Whitney test for two groups and Kruskal Wallis test for greater than 2 groups. \*  $p < 0.05$  \*\*  $p < 0.001$ .

**Figure 8. Surface TLR4 and CD69 expression on B cells and their subsets by flow cytometry.**

A) TLR4 expression measured as Mean fluorescence intensity (MFI) on total B cell populations in healthy individuals (○, n=23), *P. vivax* infected patients (■, n=71); and dengue patients (▲, n=21). B) Expression of TLR4 on immature B cells (○,●), naïve B cells (□,■), classical MBCs (◇,◆), plasma cells (△,▲), and atypical MBCs (▽,▼) in healthy (H; open symbols, n=10) and *P. vivax*-infected (I, closed symbols, n=15) individuals. C) CD69 expression on B cells in healthy individuals (○, n=10) and *P. vivax*-infected patients (■, n=15). D) Expression of CD69, on immature B cells (○,●), naïve B cells (□,■), classical MBCs (◇,◆), plasma cells (△,▲), and atypical MBCs (▽,▼) in healthy (H; open symbols, n=10) and *P. vivax*-infected (I, closed symbols, n=15) individuals. Statistical significance between two groups was determined using Mann-Whitney test; for more than two groups Kruskal-Wallis test and post hoc Dunn multiple comparison test was used. \*  $p < 0.05$  \*\*  $p < 0.001$ .

**Table 1. Characteristics and haematological parameters of participants**

Parameters	<i>P. vivax</i>		Healthy* (day 1)	Dengue (day 1)
	(day 1)	(day 30)		
Participants: Male (Female)	56 (15)	12 (3)	18 (5)	19 (2)
Mean age $\pm$ SD	34.25 $\pm$ 13.0	--	32.3 $\pm$ 10.8	32.8 $\pm$ 11.6
Parasite count/ $\mu$ L blood (IQR)	1560 (300–1960)	0	NA	NA
% Parasitemia ( $\pm$ SD)	0.031 ( $\pm$ 0.02)	NA	NA	NA
Hemoglobin (g/dL)	12.7 (11.6 – 13.8)	12.1 (11.7 – 14.8)	13.7 (12 – 13.4)	13.5 (12.5 – 14)
RBCs ( $\times 10^6/\mu$ L)	4.6 (4.2 – 5.2)	4.14 (3.9 – 4.7)	4.4 (4.1 – 5.6)	4.7 (4.3 – 5.7)
WBCs ( $\times 10^6/\mu$ L)	5.7 (4.6 – 7.3) <sup>a</sup>	6.7 (5.3 – 10.4)	7.0 (6.3 – 8.1)	4.4 (3.4 – 7.1) <sup>a</sup>
Neutrophils (%)	70 (63 – 77)	67 (58.5 – 73)	64 (58.8 – 69)	63 (55 – 82)
Lymphocytes (%)	25 (20 – 32)	30 (24.5 – 38)	34 (28 – 38.2)	31 (9.4 – 37)
Monocytes (%)	2 (2 – 3)	2 (2 – 3)	2 (2 – 3)	4 (2 – 6) <sup>c</sup>
Platelets ( $\times 10^3/\mu$ L)	128 (98 – 166) <sup>a</sup>	302 (248 – 308) <sup>b</sup>	289 (253 – 375)	129 (72 – 163) <sup>a</sup>

**Table 1:** Characteristics and hematological parameters of enrolled patients and healthy participants

Legend: a =  $p < 0.001$  *P. vivax*/dengue compared to healthy, b =  $p < 0.001$  *P. vivax* day 1 compared to day 30, c =  $p < 0.01$  dengue compared to healthy/*P. vivax*. IQR values mentioned in brackets, \*based on 65% of the samples. Hematological parameters are reported as mean (IQR) NA: Not Applicable

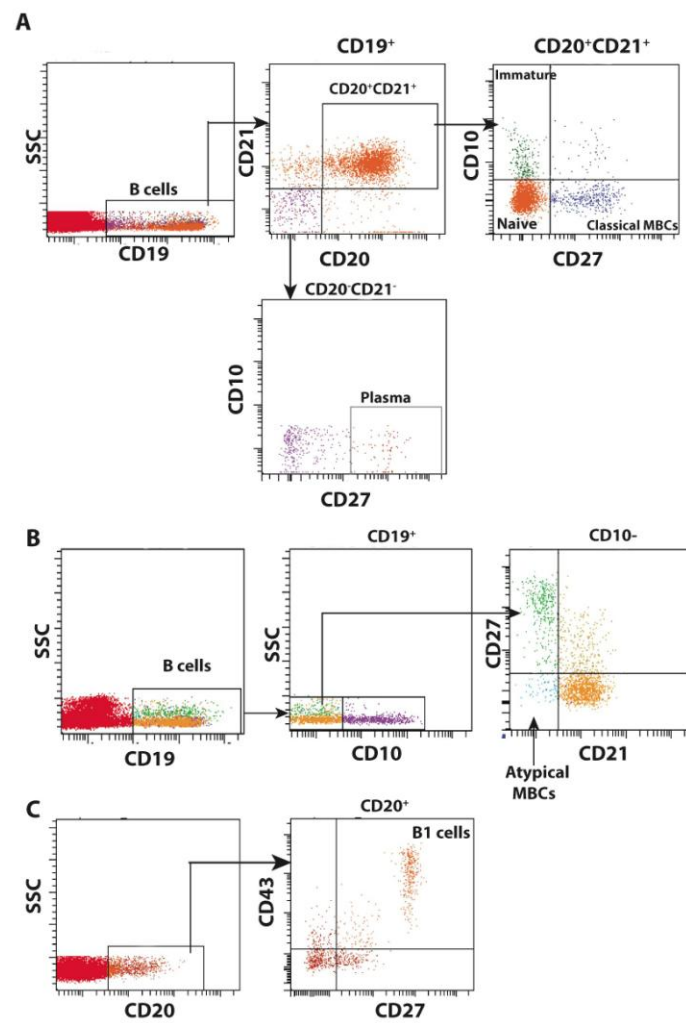


Figure 1

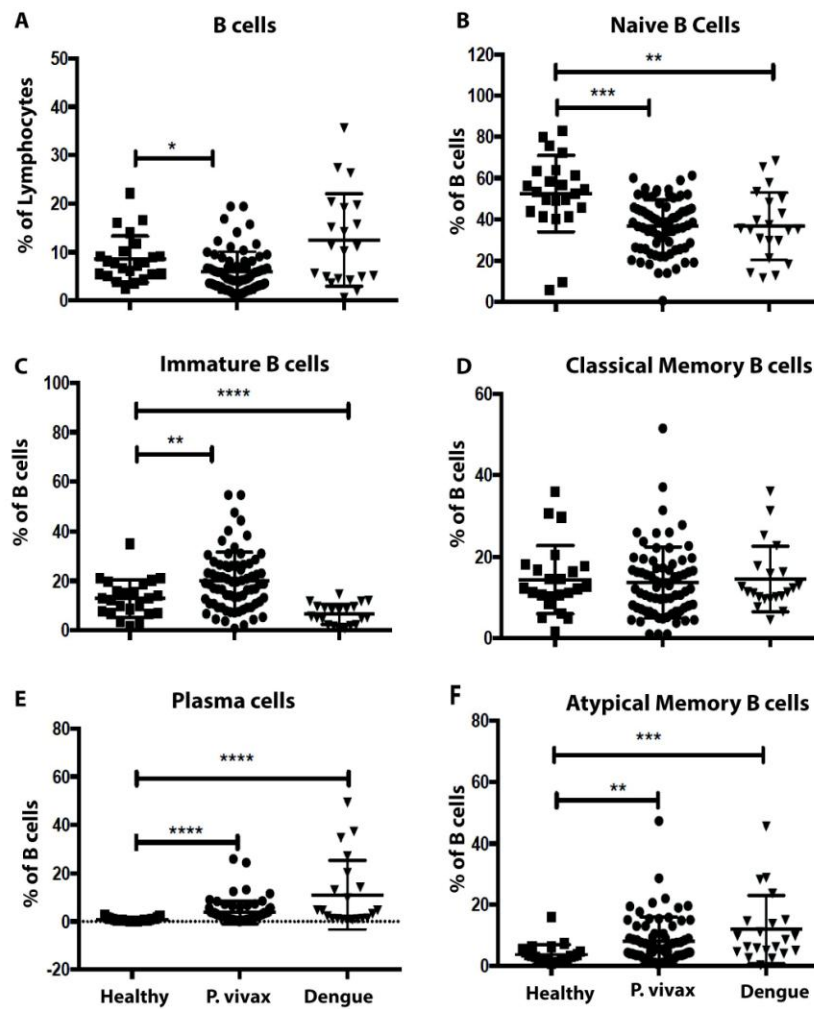


Figure 2

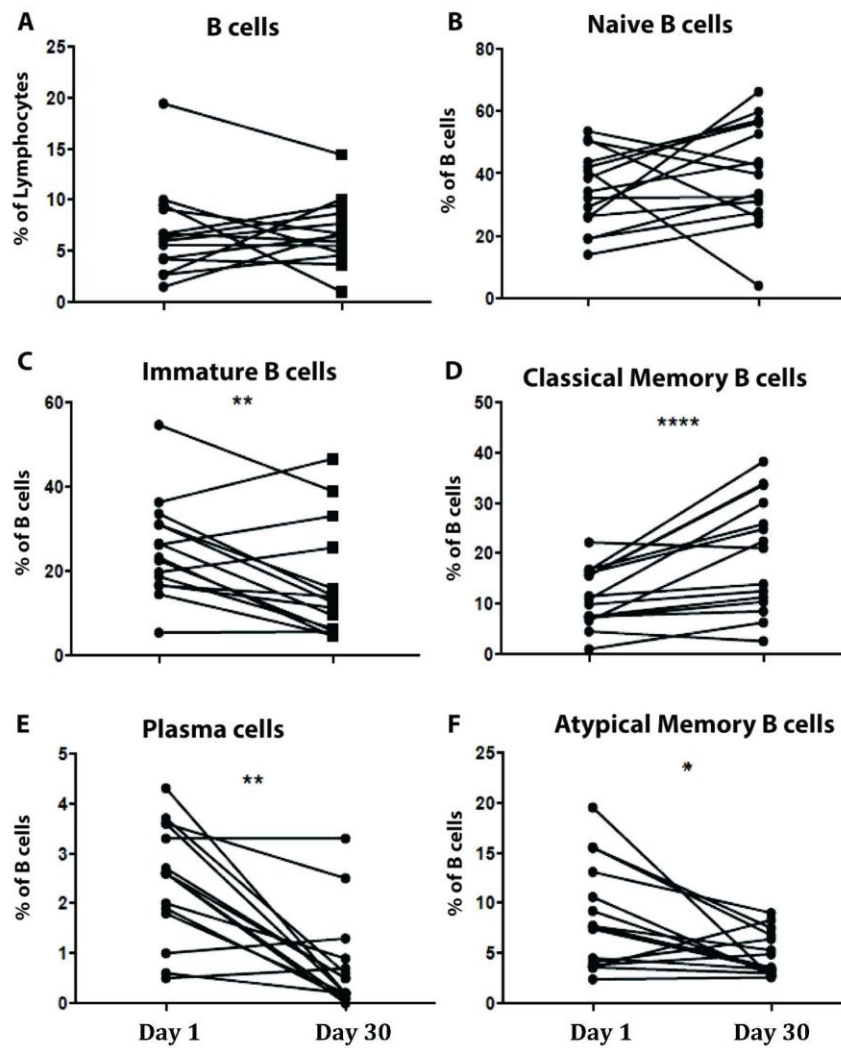


Figure 3

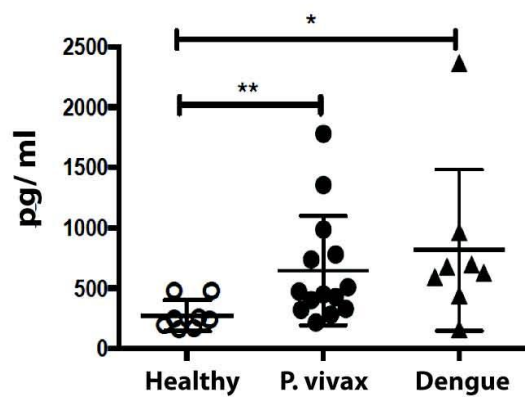


Figure 4

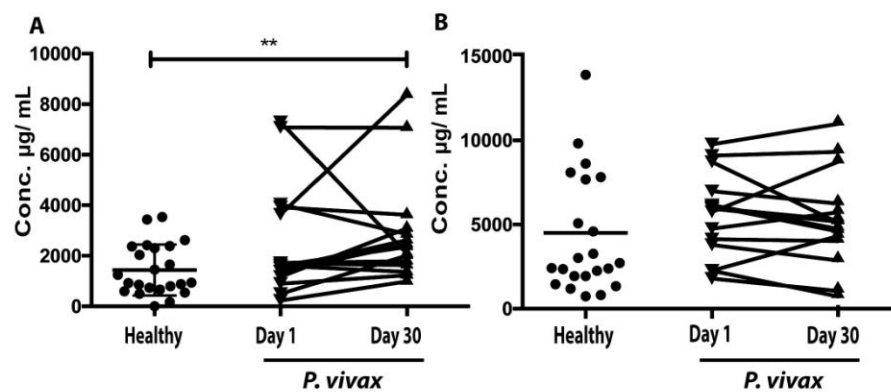


Figure 5

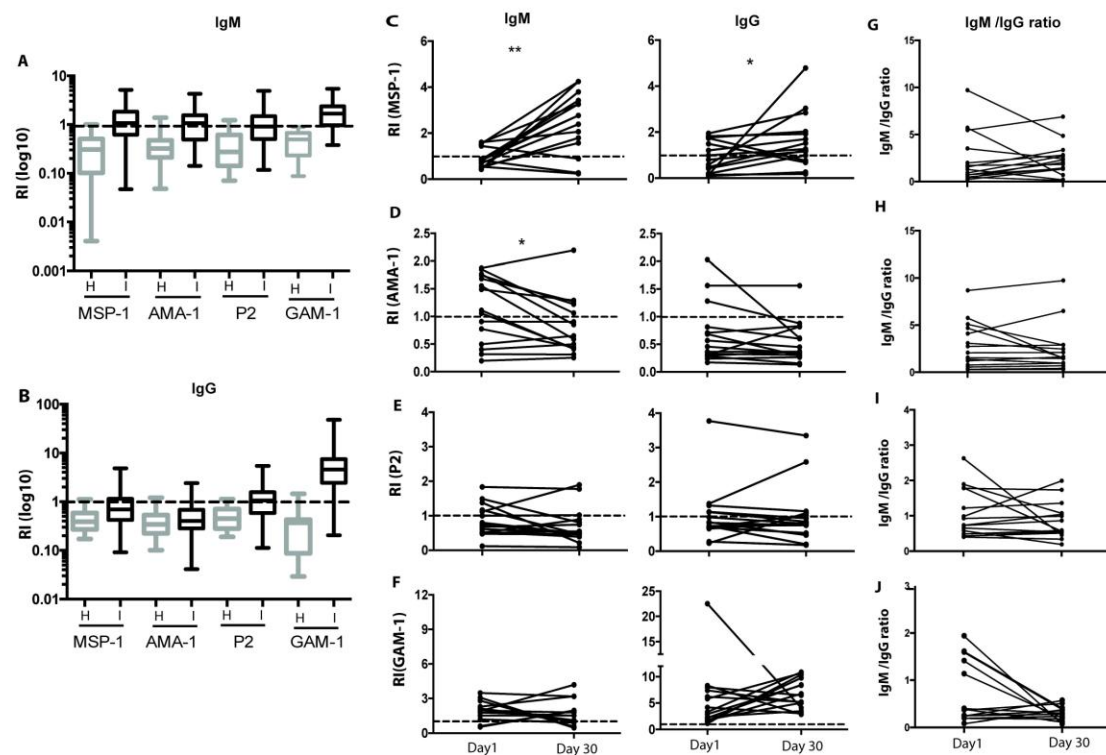


Figure 6



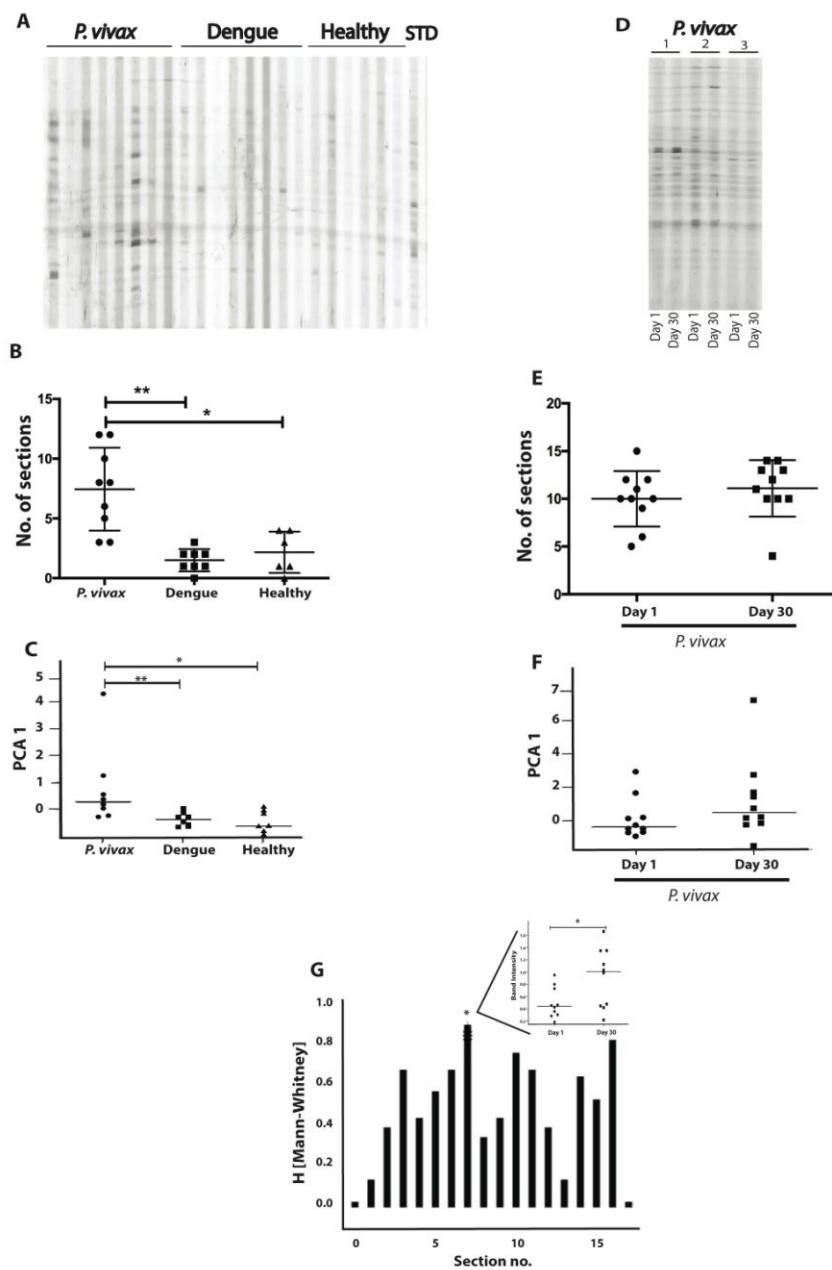


Figure 7

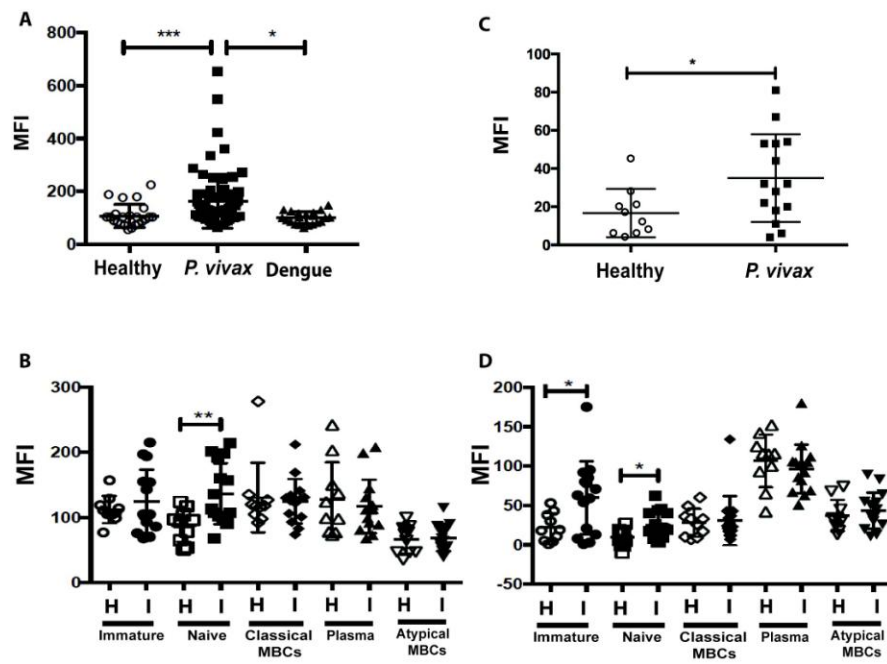


Figure 8