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Asymptomatic vivax malaria is associated with an IFN-γ-program on adaptive immunity

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Abstract

The adaptive immunity against *Plasmodium vivax* is thought to be essential to limit parasite growth during asymptomatic malaria, preventing the occurrence of symptoms. However, the mechanisms governing clinical immunity during asymptomatic infections are not understood. Here, we investigated the adaptive cellular compartment in asymptomatic *P. vivax*-infected individuals (ASY) compared to symptomatic patients (SY) and healthy donors (CTL). Our integrative analysis revealed a T_H1-biased immune signature with expanded populations of T_H1 $CD4^+$ T cells associated with the asymptomatic infection. In addition, there is an expanded population of proliferating atypical memory B cells that correlate with IgG levels against *P. vivax* antigens and parasitemia. The absence of systemic inflammation based on a comprehensive panel of soluble markers and the lower expression of some regulatory markers suggests a controlled inflammatory response that can be derived from an effective control of parasite growth. Our findings suggest that ASY maintain a pool of IFN-γ-associated Th cell phenotypes that orchestrate the immune response, limiting parasitemia and preventing clinical malaria.

Keywords *Plasmodium vivax*, Asymptomatic malaria, Malaria, CD4 T cells, B cells

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Introduction

Malaria caused by *Plasmodium vivax* is a significant public health challenge worldwide, accounting for approximately 76% of the malaria burden in the Americas, estimated at 527,000 cases in 2021, most of them occurring in the Amazon [1]. Although *P. vivax* infection causes fever, anemia, and splenomegaly, it rarely causes life-threatening severe malaria, thus has been historically considered a benign infection. However, in the past decade, this concept was overthrown considering the vast negative effects on well-being and socioeconomic development [2].

The role of adaptive immunity to *P. vivax* infection is poorly understood. Adaptive immunity is critical to orchestrate effector mechanisms that control parasite replication during the blood-stage and solid evidence shows that protection is antibody-mediated [3–5]. Frequent exposure to *P. vivax* rarely induces sterile immunity, but recent infection provides short-term protection [3, 6]. The development of B cell antigen-specific clones after exposure, and therefore antibody secretion, is highly dependent on antigen-specific $CD4^+$ T cells, which are expanded after infection. The high levels of T cell-derived cytokines produced during acute malaria and the fact that T cells from *P. vivax*-exposed individuals respond to antigenic recall by producing IL-2, IFN-γ, and IL-10 support the importance of T cell responses towards protection. However, it is unclear whether and how certain $CD4^+$ T cell subsets (T_H1, T_H2, T_H17, T_{FH}, and T_{REG}) contribute to protection since several studies report different findings depending on the experimental design applied. Studies in mice and human suggest a pivotal role of IFN-γ, a T_H1-related cytokine, in controlling infection [7], although increased levels of this cytokine in human serum are correlated with severe disease [8, 9].

A significant proportion of *P. vivax*-infected individuals remain asymptomatic. Semi-immune individuals may present an asymptomatic infection that resolves spontaneously or persists for several days or weeks [3, 10]. Asymptomatic infections are usually not diagnosed due to a lack of active search for diagnosis or very low parasitemia, which are undetectable by conventional tests. Regardless of the low parasitemia, asymptomatic carriers can infect mosquitoes and remain undetectable as silent reservoirs contributing to *P. vivax* transmission in endemic areas [10, 11]. Recent findings estimate that more than 98% of total *P. vivax* biomass is retained in the spleen during asymptomatic *P. vivax* infection, suggesting that this organ is the main site of parasite interactions with the cells mediating innate and adaptive immunity [12]. In this sense, during ASY infections, the parasite biomass in the spleen might be enough to trigger the immune response, sustaining constant activation of T and B cell clones responsive to parasite antigens. However, the mechanisms underlying this process are mostly unknown. In this study, we explored several features of the adaptive immune response of asymptomatic individuals infected with *P. vivax.*

Methods

Patients and inclusion criteria

Screenings were conducted in Candeias do Jamari, Rondônia, a region endemic for malaria due to *Plasmodium vivax* in the Brazilian Amazon, as described previously [10]. The study enrolled adult asymptomatic individuals (ASY, $n = 41$, male/female ratio = 0.7) aged between 18 and 58 years old who had not experienced symptomatic malaria in the past two months. Exclusion criteria included self-reported pregnancy, breastfeeding, and current diagnoses of chronic inflammatory or infectious diseases. ASY individuals were identified using qPCR, in at least triplicate, from blood samples. Individuals with positive results for *P. vivax* in at least one replicate were included in the study. No ASY was positive by microscopy. Healthy controls (CTL, $n = 19$, m/f = 1.1) aged between 21 and 52 years and were from the same endemic area, did not present any symptoms of malaria or were taking medication for any chronic diseases. Also, they were negative by qPCR and microscopy and had not experienced any malaria episodes before the time of blood sample collection. Symptomatic patients (SY, $n = 28$, m/f = 3) aged between 21 and 59 years and were diagnosed by microscopy and reported symptoms (fever, fatigue, headache, and/or myalgia) at least 24 h before enrollment at the Infectious Diseases Outpatients at the Centro de Pesquisas em Medicina Tropical (CEPEM).

PBMC separation

Peripheral blood was collected in sodium heparin tubes, and plasma was removed by centrifugation at 1000 × g at room temperature (RT). Cells were diluted 1:1 (v/v) in sterile phosphate-buffered saline (PBS), and then peripheral blood mononuclear cells (PBMC) were separated by centrifugation at 410 × g for 40 min at 22 °C using Ficoll-Paque gradient (Cytiva). PBMC were washed twice in 45 mL cold PBS, and cell concentration was adjusted to 10^7 cells/mL in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco) and antibiotics (Penicillin 100 units/mL, streptomycin 0.1 mg/mL, Thermo Fisher Scientific).

Cell staining for flow cytometry

Flow cytometry was performed with fresh PBMC. Cells (1×10^6) were washed with PBS and then stained with antibodies against extracellular antigens in a staining buffer (PBS, 2% FCS) for 20 min. For panels with intracellular staining, cells were also fixed and permeabilized for 30 min at RT in the dark according to the manufacturer's

protocol (Foxp3 permeabilization buffer, Thermo Fisher Scientific) and then incubated for 30 min with antibodies against intracellular antigens. After two washing steps, cells were suspended in a staining buffer and acquired in a FACS-Celesta flow cytometer (BD biosciences, BD) at the Fiocruz-Rondônia Flow Cytometry facility. Details on the antibodies and gating strategy are available in Supplementary Table and Fig. 1.

Quantification of serum soluble mediators

Plasma chemokines (CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL10, and CCL11), cytokines (IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, TNF, and IFN-γ) and growth factors (FGF-basic, PDGF, VEGF, G-CSF, GM-CSF, IL-7, and IL-2) were quantified by a high-throughput microbeads array (Bio-Plex Pro™ Human Cytokine 27-plex Assay, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Data acquisition was carried out on a Luminex 200 System using the Bioplex Manager Software at the Fiocruz-Minas Flow Cytometry facility. Final concentrations of serum soluble mediators were estimated by 5-parameter logistic regression according to the standard curve. Results were expressed in pg/mL.

ELISA for IgG and IgG subclasses

High-binding polystyrene plates (Nunc maxisorb, Merck) were sensitized overnight with 100 µL of recombinant *Pv*MSP-1_{19} [13] (0.2 µg/mL) and *Pv*AMA-1_{66} [14] (1 µg/mL) diluted in carbonate-bicarbonate buffer (0.2 mM Na_2CO_3, 46 mM $NaHCO_3$, pH 9.2). *Pv*DBPII$_{Brz-2}$ [15] (3 µg/mL) was diluted in PBS (pH 7.2). After they were washed twice, the plates were incubated for 2 h with a blocking solution (5% milk powder in PBS, pH 7.4). Plasma samples from individuals were diluted (1:101) in sample buffer (2.5% milk powder diluted in PBS, pH 7.4) and incubated for 30 min. Plates were washed three times with 250 µL of washing solution (PBS, 0.05% Tween 20) using an automated plate washer (Multiskan Go). Excess liquid from the plates was manually removed by inverting the plate onto absorbent filter paper. Goat anti-human IgG antibodies conjugated with horseradish peroxidase (HRP, Fapon Biotech) were added (1:100,000) and incubated for 30 (*Pv*MSP-1_{19}), 45 (*Pv*AMA-1_{66}), and 60 (*Pv*DBPII$_{Brz-2}$) minutes, respectively. For IgG subclasses, plasma was diluted (1:21) and incubated for 60 min. Mouse anti-human biotinylated antibodies anti-IgG1 (1:500, Sigma-Aldrich), anti-IgG2 (1:30,000, Sigma-Aldrich), anti-IgG3 (1:20,000, Sigma-Aldrich), and anti-IgG4 (1:30,000, Sigma-Aldrich) were added and incubated at 37 °C for 60 min, after which streptavidin-HRP (Sigma-Aldrich) was added for an additional 60-minute incubation at 37 °C. Reactions were developed with tetramethylbenzidine substrate solution (Moss, USA) for 30 min and stopped with 50 µL of 0.5 M sulfuric acid. The respective absorbances were measured using a VERSAmax microplate reader (Molecular Devices, USA) at 450 nm. Results were reported as a reactivity index (RI) according to the formula: RI = Sample optical density/mean + 3 standard deviation of the optical density of healthy individuals.

Statistical analysis

Cytometry data was analyzed in FlowJo (version 10). The Shapiro-Wilk test was used to assess whether the variables in each clinical group had a parametric distribution. For comparisons between clinical groups, the Kruskal-Wallis test was used followed by the Dunn's test for multiple comparisons with the family error rate corrected by the Benjamini-Hochberg method. Data were represented in dot plots over boxplots, considering the median and the interquartile ranges. Significant differences ($p < 0.05$) were represented with asterisks. For statistical analysis, we used R (version 4.3.0) and rstatix package (v0.7.2). Sparse partial least squares discriminant analysis was performed using functions from the mixOmics package (v 6.24.0). Missing values were imputed using a Non-linear Iterative Partial Least Squares algorithm (NIPALS). The dataset was 5-fold segregated into training and testing samples.

Results

An overview of the differences between ASY and SY compared to CTL

To explore the immunity of ASY individuals, 348 immunological features were analyzed, including T and B cell phenotypes and soluble markers (antibodies, cytokines, chemokines, and growth factors). Out of 120 features that were different between SY and CTL (Fig. 1), 89 features were higher in SY, including the levels of antibodies against *Pv*MSP1_{19}, *Pv*AMA1_{66}, and *Pv*DBPII$_{Brz2}$, cytokines (IFN-γ, IL-10, IL-1RA, and IL-6), growth factors (G-CSF, GM-CSF), and chemokines (CXCL8, CXCL10, CCL2, CCL3, and CCL4). In contrast, 31 features were lower in SY compared to CTL, including soluble factors (IL-4, IL-13, IL-7, IL-9, CCL11 and PDGF-bb), and $CD4^+$ T cells co-expressing $CXCR3^+CCR6^+$, hereafter named atypical T_H1 (a-T_H1). The canonical T_H1, $CXCR3^+CCR6^-$ T cells, were increased among follicular helper T cells (T_{FH}).

Differences between ASY and CTL were lesser in contrast to SY and CTL. Out of 74 features, 31 were increased, and 43 were decreased in ASY compared to CTL. Antibodies against *P. vivax* antigens were the most increased features in ASY compared to CTL. Cell frequencies related to the expression of CXCR3 were also increased in ASY, while some soluble markers and cell populations were decreased (Fig. 1). Ages of ASY and

Fig. 1 Integrative data analysis of immune features indicates a T_H1-biased T cell response in asymptomatic malaria. Data from T and B cell frequencies of diverse phenotypes, plasma cytokines (cross), chemokines (square) and growth factors (crossed-square), antibodies (circles), and from symptomatic (SY, turquoise, $n=28$), asymptomatic (ASY, salmon, $n=41$) and healthy controls (CTL, $n=21$) were compiled and analyzed. Volcano plot of SY and ASY *P. vivax*-infected individuals. Each dot represents a feature. Data from Dunn's tests, using CTL as a reference group, show in the *y*-axis the *p*-value (log) and in the *x*-axis as the fold change of the mean of each group over CTL. The red horizontal line represents a confidence level of 0.95 for *p*-values. Gray dots are features with no statistical significance over CTL

CTL, and ASY and SY were similar ($p>0.05$), although CTL were younger than SY ($p=0.03$). Age and sex were correlated with some variables, with no biological relevance in the context of our analysis. We next explored the features differentially expressed in ASY compared to CTL and SY.

ASY infection induces minimal interference in plasma cytokines, chemokines, and growth factors while levels of IgG are increased

We evaluated the levels of 27 soluble factors, including cytokines, chemokines and growth factors, and the levels of IgG and IgG subclasses against *P. vivax*-antigens in infected individuals and CTL. As expected, SY infection was marked by increased concentration of several serum cytokines (IL-10, IL-6, IL-1RA, and IFN-γ), chemokines (CCL3, CCL4, CXCL8 and CXCL10), and the growth factor G-CSF. On the contrary, the levels of IL-4, IL-9, IL-13, IL-17, PDGF-bb and CCL11 were lower in SY compared to CTL (Fig. 2A, left panel, supplementary Fig. 2). No cytokine, chemokine, or growth factor were increased in ASY, but the levels of IL-4, IL-5, IL-6, IL-9, IL-12, IL-13, and TNF were decreased in ASY compared to CTL (Fig. 2A). Compared to SY, the levels of the soluble markers IL-1RA, IL-6, IL-10, IL-12, IFN-γ, TNF, CCL2, CCL3, CCL4, CXCL8, CXCL10, and G-CSF were lower in ASY while IL-17, CCL11, PDGF-bb, and VEGF were higher in ASY (Supplementary Fig. 2).

As for antibodies, both SY and ASY displayed increased levels of IgG against the three *P. vivax*

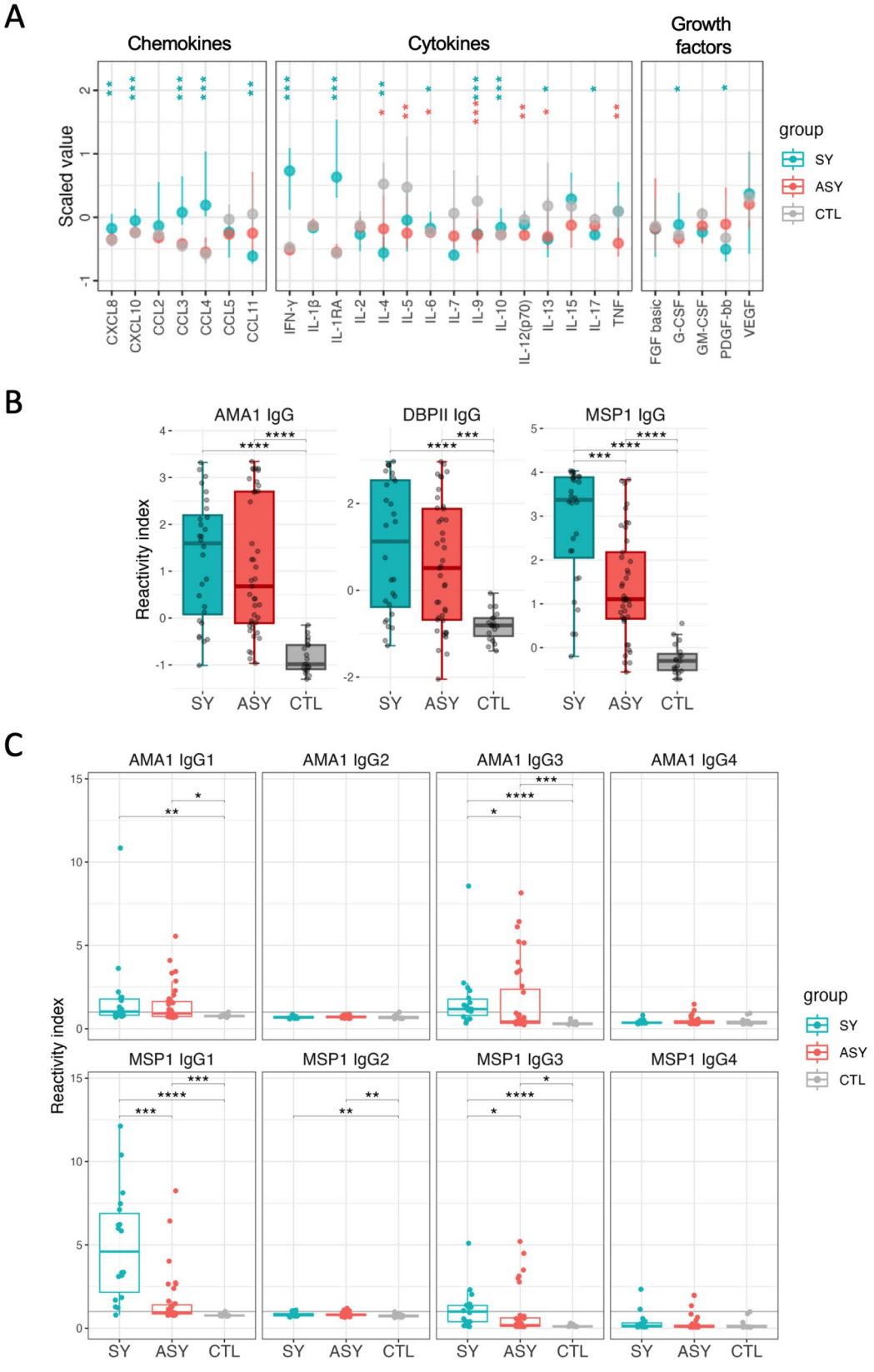

Fig. 2 (See legend on next page.)

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Fig. 2 High IgG levels and absence of systemic inflammation during ASY malaria. Plasma samples were used in a bead fluorometric assay measurement of 27 serum cytokines, chemokines, and growth factors. (**A**) Statistical summary of all soluble factors relative to each group. Data was scaled using z-scores. Dot represents the median, and vertical lines represent the interquartile range for each group represented by colors: symptomatic (SY, turquoise, $n = 28$), asymptomatic (ASY, salmon, $n = 41$), and healthy controls (CTL, gray, $n = 21$). Asterisks represent differences compared to CTL. (**B**) Levels of IgG against *Pv*MSP1$_{19}$, *Pv*AMA1$_{66}$, and *Pv*DBPII$_{brz-2}$ (B) and IgG subclasses against *Pv*MSP1$_{19}$ and *Pv*AMA1$_{66}$ (**C**) by group. Gray lines represent the reactivity index cut-off. Dunns' test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

erythrocytic-stage antigens compared to CTL (Fig. 2B). Levels of IgG1 and IgG3 against *Pv*AMA-1$_{66}$ and IgG1, IgG2, and IgG3 against *Pv*MSP-1$_{19}$ were increased in SY compared to CTL (Fig. 2C). Among ASY, levels of IgG3 against *Pv*AMA-1$_{66}$ and levels of IgG1 and IgG2 against *Pv*MSP-1$_{19}$ were increased compared to CTL. Only IgG1 levels against *Pv*MSP-1$_{19}$ were lower in ASY compared to SY (Fig. 2C). We found mild to moderate correlations between age and IL-13 among ASY (*rho* = 0.32, $p = 0.041$), CCL11 and G-CSF among SY (*rho* = 0.51, -0.42, $p = 0.009$, 0.035, respectively) and CXCL10 among CTL (*rho* = 0.48, $p = 0.041$). Although these differences were significant, we believe they have limited biological relevance in the context of our study.

Regulatory markers are less expressed in ASY

The ASY infection led to significantly lower levels of many cytokines compared to CTL, some of which could be attributed to helper T cells' reduced production. By evaluating the non-exclusive expression of these regulatory markers, we found higher frequencies of cells expressing PD-1 or ICOS in SY compared to both ASY and CTL. In contrast, cells expressing TIM-3 were significantly decreased in ASY compared to both SY and CTL (Fig. 3A). Cells expressing CD69 were increased in SY compared to ASY (Fig. 3A).

To explore the activation status of CD4$^+$ T cells and to associate the interrelationship between them and the decreased cytokine production, we investigated the expression of specific regulatory molecules by using a Boolean strategy.

By evaluating the exclusive expression or co-expression of these regulatory markers, we found that, on average, 90.2% of CD4$^+$ T cells in ASY did not express any of the investigated regulatory markers, while 88.4% and 85.4% did from CTL and SY, respectively (Supplementary Fig. 3). Cells expressing only PD-1, CD69, or ICOS were predominant in all groups, with less than 25% of cells co-expressing 2, 3, or 4 markers (Fig. 3B). Notably, SY malaria induced an expansion of cells co-expressing 2, 3, and 4 markers, mainly characterized by higher frequencies of CD69$^-$ICOS$^+$PD-1$^+$TIM-3$^-$CD4$^+$ T cells and CD69$^+$ICOS$^+$PD-1$^+$TIM-3$^-$CD4$^+$ T cells compared to CTL. This trend was not observed in ASY (Fig. 3B and C, Supplementary Fig. 3). Conversely, the frequencies of CD69$^+$ICOS$^-$PD-1$^-$TIM-3$^+$CD4$^+$ and CD69$^-$ICOS$^+$PD-1$^-$TIM-3$^-$CD4$^+$ T cells were lower in ASY compared to CTL (Fig. 3B and C). Finally, regulatory T cells were lower in ASY compared to CTL ($p = 0.01$), but no other relevant difference was observed, including in the correlation analysis (Fig. 1).

In summary, ASY infection is associated with lower cytokine levels and a lower proportion of CD4$^+$ T cells expressing regulatory markers than CTL. In contrast, SY malaria exhibits an expansion of cells co-expressing multiple regulatory markers.

Expansion of T_H1 cells is associated with ASY infection

Since most of the cytokines observed at lower levels during ASY infection compared to CTL were related to a T_H2 response, we assessed the frequency of CD4$^+$ T cell subsets, here defined by the expression of CXCR3 and CCR6 (Fig. 4A). No differences were observed in the frequencies of atypical-T_H1 (CXCR3$^+$CCR6$^+$), T_H2 (CXCR3$^-$CCR6$^-$), and T_H17 (CXCR3$^-$CCR6$^+$) between ASY and CTL (Fig. 4B). However, T_H1 cells were increased in ASY compared to both SY and CTL. In addition, ASY displayed lower frequencies of T_H2 and higher frequencies of a-T_H1 cells compared to SY. The frequencies of a-T_H1 subset were lower in SY compared to ASY and CTL (Fig. 4B).

We next evaluated the combined expression of regulatory markers among memory T helper cells, as defined by the expression of CD45RO. Among memory T_H1 cells, we found increased frequencies of cells co-expressing 3 or 4 markers in SY compared to CTL, except for the CD69$^+$ICOS$^-$PD-1$^+$TIM-3$^+$ phenotype. Among cells co-expressing 2 markers, only the CD69$^-$ICOS$^+$PD-1$^+$TIM-3$^-$ phenotype was increased in SY compared to CTL (Fig. 4C). Frequencies of cells expressing a single marker were similar between SY and CTL. Regarding ASY, cells expressing solely PD-1 (CD69$^-$ICOS$^-$PD-1$^=$TIM-3$^-$) or not expressing any of these markers were most frequent compared to CTL. In contrast, cells expressing solely ICOS (CD69$^-$ICOS$^+$PD-1$^-$TIM-3$^-$) were less frequent among ASY compared to CTL (Fig. 4C).

Remarkably, in SY, an expansion of cells expressing all regulatory markers was mostly significant among T_H17 and a-T_H1 cells, with the former composing more than 50% of the cells expressing regulatory markers (Supplementary Figs. 3 and 4).

Expansion of T_H1-committed follicular helper T cells

Follicular helper T cells (T_{FH}) are defined as memory $CD4^+$ T cells co-expressing $CXCR5^+ICOS^+PD\text{-}1^+$. They provide help to B cells in the germinal centers to produce memory B cells and plasma cells, and their function overlaps with cells committed to T_H1, T_H2, and T_H17 phenotypes during physiological conditions and infections [16, 17]. While T_{FH} cells are expanded during SY malaria, there are no alterations in ASY compared to CTL (Fig. 5A and B). However, we found increased frequencies of T_H1-like cells among T_{FH} in both ASY and SY compared to CTL. In addition, decreased frequencies of a-T_H1-like and T_H17-likeT_{FH} were also observed between ASY, SY and CTL (Fig. 5C).

Proliferating atypical B cells are increased during ASY malaria

We next investigated the B cell compartment, and the memory populations of B cells defined by the expression of CD21 and CD27. A dimensionality reduction overview of the B cell populations by group is shown in Fig. 6A.

Plasma cells have been defined by the expression of CD38 in $CD19^+CD20^-CD21^-$, but since >90% of $CD20^-CD21^-$ B cells are also $CD38^+$, almost all cells identified using this strategy are plasma cells [16–18]. Plasma cells ($CD20^-CD21^-CD10^-CD19^+$) were increased in SY compared to ASY and CTL. SY also displayed an increased frequency of atypical memory B cells (AtM, $CD21^-CD27^-CD10^-CD20^+CD19^+$) compared to CTL (Fig. 6A and B). Conversely, SY had a decreased frequency of classical memory B cells (CM, $CD21^+CD27^+CD10^-CD20^+CD19^+$) compared to both ASY and CTL. A higher frequency of proliferating cells, evidenced by the expression of Ki67, was observed between SY and both CTL and ASY among the four populations of B cells (Fig. 6C). Among AtM, ASY displayed a higher frequency of $Ki67^+$ B cells than CTL (Fig. 6C).

We next evaluated the association of $Ki67^+$ AtM B cells with levels of antibodies and parasitemia. There was a positive correlation between the frequency of $Ki67^+$ AtM B cells and the parasitemia measured by qPCR (Fig. 6D). IgG levels against MSP-1 and AMA-1, but not for DBP-II, were positively correlated with $Ki67^+$ AtM B cells (Fig. 6E). Altogether, these data suggest an active and constant activation of atypical B cells dependent on antigenic stimulation.

Integrative data analysis of immune features indicates a T_H1-biased T cell response in asymptomatic P. vivax infection

To assess the immune signature of ASY infection, we employed a sparse partial least squares discriminant analysis (sPLS-DA). This method enables the identification of the most predictive features for sample classification [19]. While there is a significant discrimination of SY in latent component 1 (LC 1), ASY and CTL could be discriminated in latent component 2 (LC 2) (Fig. 7A). A receiver-operating characteristic (ROC) curve analysis indicates a high precision of the model's capability to distinguish the groups in the testing sample. The area under the curve (AUC) analysis indicates a good efficiency of the model in segregating ASY from CTL or SY using the two components (AUC = 0.91, $p < 0.001$, Fig. 7B). Among the important variables that contribute to the segregation of LC 2, T_H1 in CD4, $CXCR3^+CCR6^-$ in $CD69^+CD4^+$, and $CXCR3^+CCR6^-$ in $TIM\text{-}3^+CD45RO^+CD4^+$ phenotypes were the most frequently associated with ASY infection (Fig. 7C).

Altogether, ASY infection displays an immune signature that correlates with an increased T_H1 response and a lower expression of regulatory molecules and key cytokines that may contribute to the asymptomatic status or a higher threshold for symptoms development.

Discussion

Herein, we found that asymptomatic malaria is associated with a higher frequency of cells that are associated with IFN-γ-signaling, along with a decreased frequency of cells expressing regulatory markers and with lower levels of plasma cytokines related to a T_H2 response. Combined as an immune signature, these features can effectively segregate ASY from CTL and SY, providing a better understanding of the immunity during asymptomatic malaria. We define an immune signature as an emerged product of regulatory processes involving activation and inhibition of effector functions in both cellular and humoral compartments. Our findings corroborate with the study done by Ioannidis et al. (2021), which found an IFN-γ-driven response in asymptomatic individuals infected with *P. vivax* from Indonesia [20].

The release of cytokines and chemokines is frequently associated with the onset of the malaria paroxysm. During symptomatic *P. vivax* malaria, IFN-γ, IL-10, IL-1β and IL-6 are released contemporaneously with the onset of symptoms such as fever, headaches, and chills [21, 22]. Less explored, the levels of the chemokines CCL3, CCL4, CXCL8, and CXCL10 are also increased during acute disease [23]. Interestingly, we found no increase in TNF levels in SY, as corroborated by others [22]. Although TNF correlates with the paroxysms in *P. falciparum* malaria [24], its relation with paroxysms in *P. vivax* malaria seems to be less clear [25], and apparently important during severe *P. vivax* malaria [8].

While most of the immune mediators involved in the innate immune response are not altered in ASY, levels of the pro-inflammatory cytokines TNF, IL-6, and IL-12 are decreased, suggesting a suppressed inflammatory signaling during sub-patent parasitemia. The T_H2-related

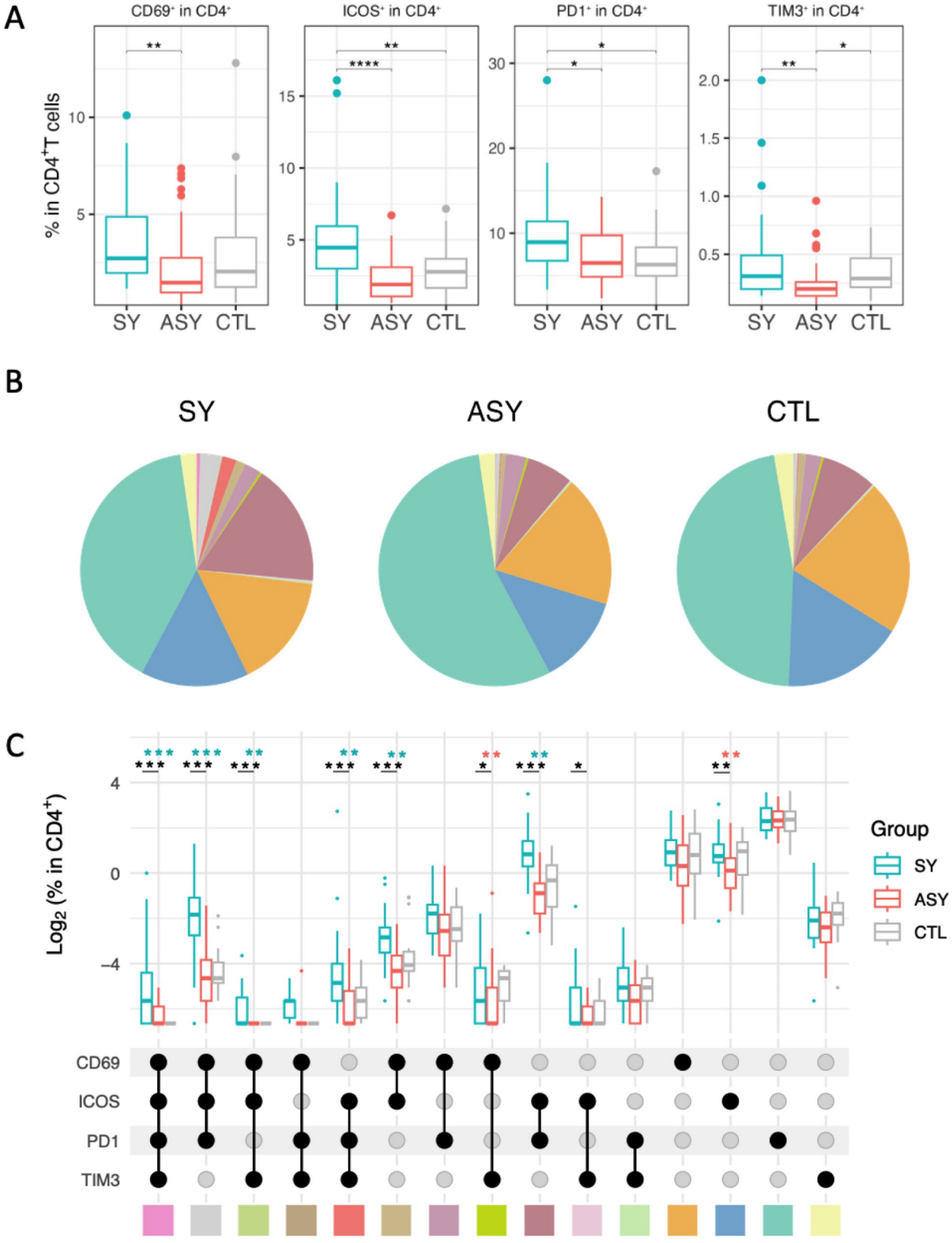

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Fig. 3 Lower frequencies of CD4$^+$T cells expressing regulatory markers are correlated with asymptomatic malaria. PBMC from SY, ASY, and CTL were analyzed by flow cytometry. (**A**) Boxplots with median and interquartile ranges represent frequencies of CD4$^+$ T expressing ICOS, PD-1, TIM-3, or CD69 in each group. Asterisks over connecting lines represent significant differences between groups by Dunn's test. Dots over boxplots represents outliers over the 3rd quartile. (**B**) Pie charts depicting the frequencies of Boolean-defined phenotypes by group. Colors represent the phenotypes shown in C. (**C**) Boxplots with median and interquartile range represent frequencies of CD4$^+$ T cells co-expressing ICOS, PD-1, TIM-3, and CD69 determined by a Boolean analysis. Asterisks over connecting lines represent significant differences between groups by Dunn's test. Colored asterisks represent differences between the group with the same color and CTL. Combinations of markers are shown underneath the plot. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

cytokines IL-4, IL-5, IL-9, and IL-13 are also decreased in ASY compared to CTL. It has been shown that IL-10 levels were lower in severe cases of *P. vivax* malaria than in asymptomatic cases, suggesting that IL-10 could mediate clinical immunity by controlling pro-inflammatory response [8]. These findings were not confirmed by us and others [25, 26]. Our results suggest a downregulated/silenced immunity that could be maintained by a low but continuous *P. vivax*-antigen stimulation during ASY infection. The absence of proinflammatory mediators in ASY could be an outcome of low parasitemia, which is insufficient to maintain innate immunity activation. Additionally, the parasitemia threshold to trigger symptoms is possibly higher in ASY individuals. According to this hypothesis, adaptive immunity would be responsible for orchestrating parasite control, although the quality or intensity of the responses would not be able to eliminate the infection completely. However, the fact that some ASY individuals display very low levels of specific antibodies (anti-MSP1, -AMA1, or -DBPII) suggests that other mechanisms might be involved in the control of parasitemia in ASY. An alternative hypothesis is that low persistent parasitemia desensitizes cells and, therefore, basal cytokine production is compromised. If this hypothesis is true, T cell stimulation or stimulation of innate cells with pathogen-associated molecular patterns should not induce high levels of cytokines or induce a strong response.

We previously showed that cytokine release upon restimulation of PBMC from SY *P. vivax*-infected patients is impaired due to increased expression of inhibitory receptors such as TIM-3, CTLA-4, and PD-1 on CD4$^+$ T cells [27]. During ASY malaria, we found similar frequencies of cells expressing these regulatory molecules compared to CTL, except for TIM-3$^+$CD4$^+$ T cells, which are less frequent in ASY than CTL and SY. In humans, TIM-3 deficiency is associated with autoimmunity and malignancy, while high TIM-3 expression correlates with inhibited effector T cell responses and T cell dysfunction during viral infections and cancer [28]. Also, CD4$^+$ T cells expressing exclusively ICOS (ICOS$^+$CD69$^-$PD-1$^-$TIM-3$^-$CD4$^+$ T cells) were lower in ASY than CTL. ICOS is expressed in CD4$^+$ T cells upon TCR engagement, and it is essential for germinal center development and T cell-dependent B cell maturation [29]. Lack of ICOS leads to a T_H1 expansion in murine model and is associated with stronger immunity against some intracellular pathogens [29]. Also, the absence of ICOS in the *Plasmodium chabaudi* mouse model resulted in an increased T_H1 population with higher levels of IFN-γ early during infection [30]. Conversely, human deficiency of ICOS leads to low antibody production and the inability to mount a specific antibody response [31], while ICOS co-activation in naïve T_H cells is associated with higher production of IFN-γ depending on the cytokine milieu [32]. Lower frequencies of ICOS and TIM-3 expressing T_H cells in ASY could be a consequence of sub-patent parasitemia, with lower TCR engagement and inflammatory response.

Atypical Th1 cells have been implicated in both physiological and pathological conditions, as they contribute to immune defense against chronic infections by promoting a sustained immune response. These cells are highly responsive to antigens from *Candida albicans* and *Mycobacterium tuberculosis*, secreting IFN-γ, IL-17, and IL-22, simultaneously [33]. The frequency of a-T_H1 cells is lower in patients chronically infected with HIV, despite antiviral therapy. In these patients a-T_H1 is the major source of TNF and CCL20 and are highly permissive to HIV replication, with potential to infiltrate and recruit CCR6$^+$ T cells into sites of viral replication [34]. In our study, their frequency was similar between ASY and CTL, suggesting a limited importance during asymptomatic infection. However, these cells are diminished in circulating PBMC of SY patients, in both memory and T_{FH} compartment, which suggest depletion or sequestration of these cells during the symptomatic infection.

IFN-γ is suggested to play controversial role during malaria by either promoting symptoms or being crucial for protection [7]. However, several soluble mediators are released during clinical malaria, resembling a cytokine storm as described for other diseases [6]. Thus, although IFN-γ is produced during *P. vivax* infection, its beneficial effects might be overshadowed by the release of concurrent cytokines (IL-1RA), chemokines (CXCL8, CCL2, CCL3, CCL4), and growth factors (G-CSF) during the symptomatic disease. We propose that the inflammatory environment induced during symptomatic infection might negatively interfere in the development of an effective immunity, which has been shown to be highly dependent on IFN-γ signaling [6].

Atypical memory B cells were found to be increased during symptomatic malaria in several studies [17, 35, 36]. It is hypothesized that IFN-γ signaling, possibly induced by subpatent parasitemia, contributes to AtM

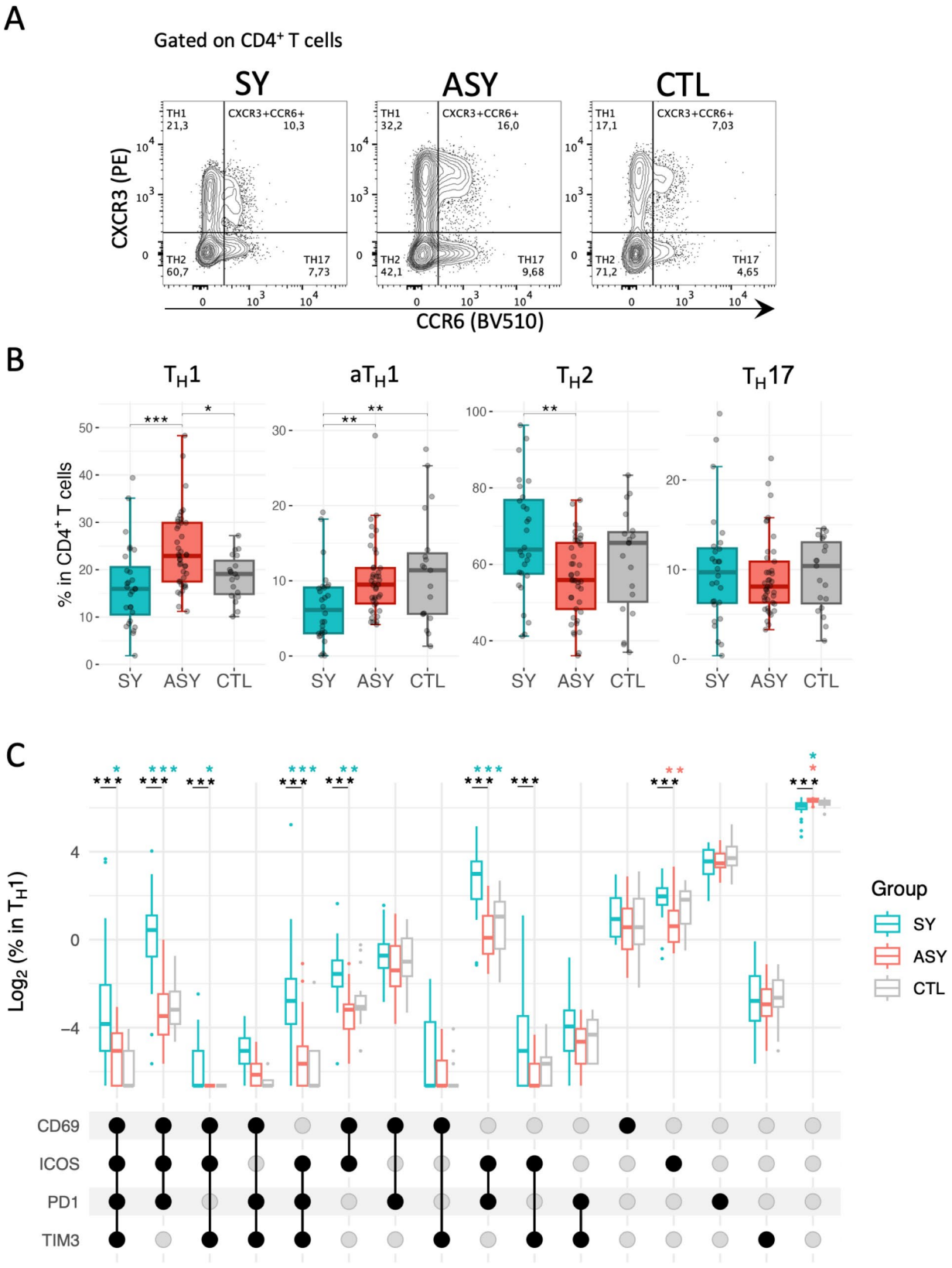

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Fig. 4 ASY infection is marked by increased T_H1 $CD4^+$T cell frequencies. PBMC from SY, ASY, and CTL were analyzed by flow cytometry analysis. (**A**) Representative contour plots show the gating strategy to assess the T_H1 ($CXCR3^+CCR6^-$), atypical-T_H1 ($CXCR3^+CCR6^+$), T_H2 ($CXCR3^-CCR6^-$) and T_H17 ($CXCR3^-CCR6^+$) populations among $CD4^+$ T cells from SY, ASY and CTL. (**B**) Frequencies of $CD4^+$ T cells subpopulations by group. Data is represented as scattered dot-plots over boxplots with median and interquartile range. Each dot represents a single individual. Asterisks over connecting lines represent significant differences between groups by Dunn's test. (**C**) Boolean analysis of the regulatory markers among T_H1 cells. Data is represented as boxplots with median and interquartile ranges, and a dot represents outliers. Asterisks over connecting lines represent significant differences between groups. Colored asterisks represent differences between the group with the same color and CTL. Combinations of markers are shown underneath. Dunn's test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

B cell expansion. Additionally, the interaction between T_H1-like T_{FH} cells and B cells may play a role in promoting AtM differentiation. These cells are also increased during autoimmune diseases such as erythematosus systemic lupus and rheumatoid arthritis [37] and infection with hepatitis C virus and human immunodeficiency virus [38]. AtM usually express Tbet, a transcription factor initiated by IFN-γ-mediated signals and can be derived from both memory and naïve B cells. In naïve B cells IFN-γ signaling, BCR crosslinking, and activation of TLR-7 or TLR-9 pathways all contributed to inducing elevated T-bet expression levels, and it has been suggested that these cells are generated in the extrafollicular environment [39–42]. These cells have controversial roles during infection, either by not responding to parasite antigens [36, 37] or by producing IgG in certain conditions [43, 44]. Emerging evidence suggests that T_{FH} cells play a crucial role in shaping B cell responses, including AtM formation, even outside the classic germinal center environment [45, 46]. Finally, peripheral T_{FH} cells may influence the generation of extrafollicular AtM, contributing to sustained immune activation without excessive inflammation, which might be required for malaria resistance. All in all, while these controversial findings suggest an unexplored heterogeneity among AtM, their role during *P. vivax* malaria is unknown.

The increased frequencies of both $CXCR3^+$ T_{FH} cells and AtM B cells suggest an interplay of these cells in germinal centers, mediated by IFN-γ. Interestingly, while higher levels of IFN-γ are found during SY malaria, no alterations were observed in ASY infections. These findings suggest that previous symptomatic infections can trigger $CD4^+$ T cells to produce IFN-γ, providing a signal in germinal centers that induces the production of effector T and B cells and antibodies that can persist after immune contraction, providing clinical protection to new infections. Another possibility is that, in the absence of systemic inflammation, even low levels of IFN-γ produced by *P. vivax*-specific $CD4^+$ T cells provide sufficient signaling to control infection. This response would thus be enough to prevent an increase in parasitemia and avoid symptoms, allowing the ASY state to be sustained or prolonged by low, oscillating parasitemia.

Our study has limitations that must be considered. First, the similar age ranges observed across the ASY, SY, and CTL groups indicate minimal confounding from age. However, the male/female ratio varied slightly among groups (ASY: 0.7, SY: 3, CTL: 1.1). The unbalanced ratio observed among SY is due to epidemiological characteristics of the region and sampling bias (males are more exposed due their working activities) that have no influence on the results. Each variable was carefully evaluated to ensure that the differences reported and discussed are consistent regardless of sex differences. We found no statistically significant correlations between sex and the key immunological markers in our study, suggesting limited influence of this factor. The sample sizes may have limited the statistical power, particularly for subgroup analyses. However, this is an exploratory study aiming to identify potential markers that correlate with ASY infection. Despite the limitation of a small sample size, the significant findings provide valuable insights. Functional assays with *P. vivax*, which are essential for validating our findings and providing deeper insights into the role of T_H1 cells, are challenging due to the parasite's inability to be cultured and the difficulty of producing antigen extracts. Therefore, we emphasize the need for future studies validate our findings and further investigate the immune mechanisms underlying the asymptomatic infection.

In summary, this study comprehensively characterizes the immunological signature of asymptomatic individuals infected with *P. vivax*. Our findings underscore the intricate interplay between regulatory molecules and T and B cell subsets, contributing to a deeper understanding of the immune dynamics in asymptomatic *versus* symptomatic *P. vivax* infections.

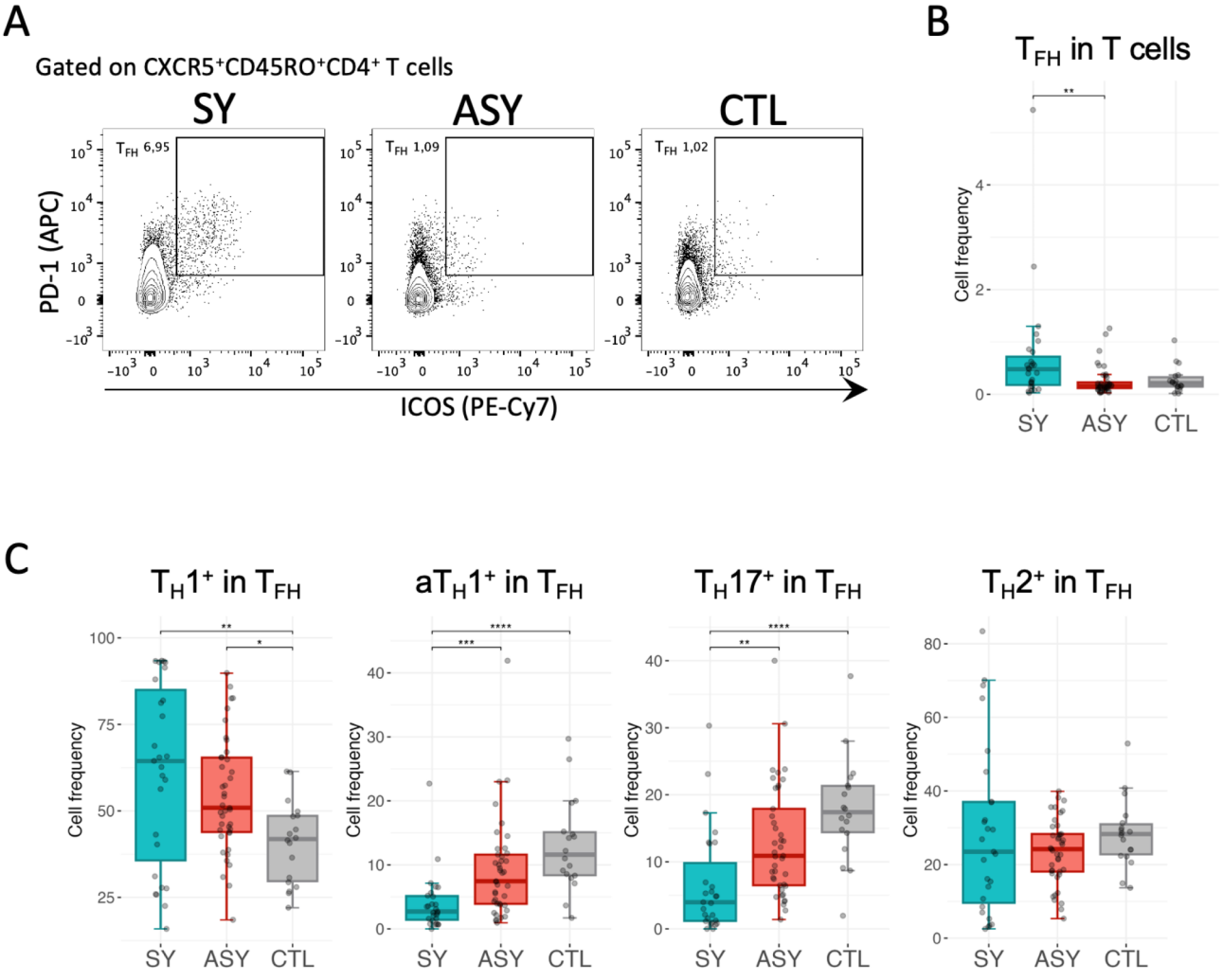

Fig. 5 *P. vivax*-infected individuals exhibit increased frequencies of T_H1-committed T_{FH}. (**A**) Representative contour plots show the gating strategy to assess T_{FH} population ($ICOS^+PD\text{-}1^+CXCR5^+CD45RO^+CD4^+$ T cells). (**B**) Boxplots with median and interquartile ranges represented the frequencies of T_{FH} in $CD4^+$ T cells and (E) of $CD4^+$ T cell subpopulations among T_{FH}. Asterisks over connecting lines represent significant differences between groups. Dunn's test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

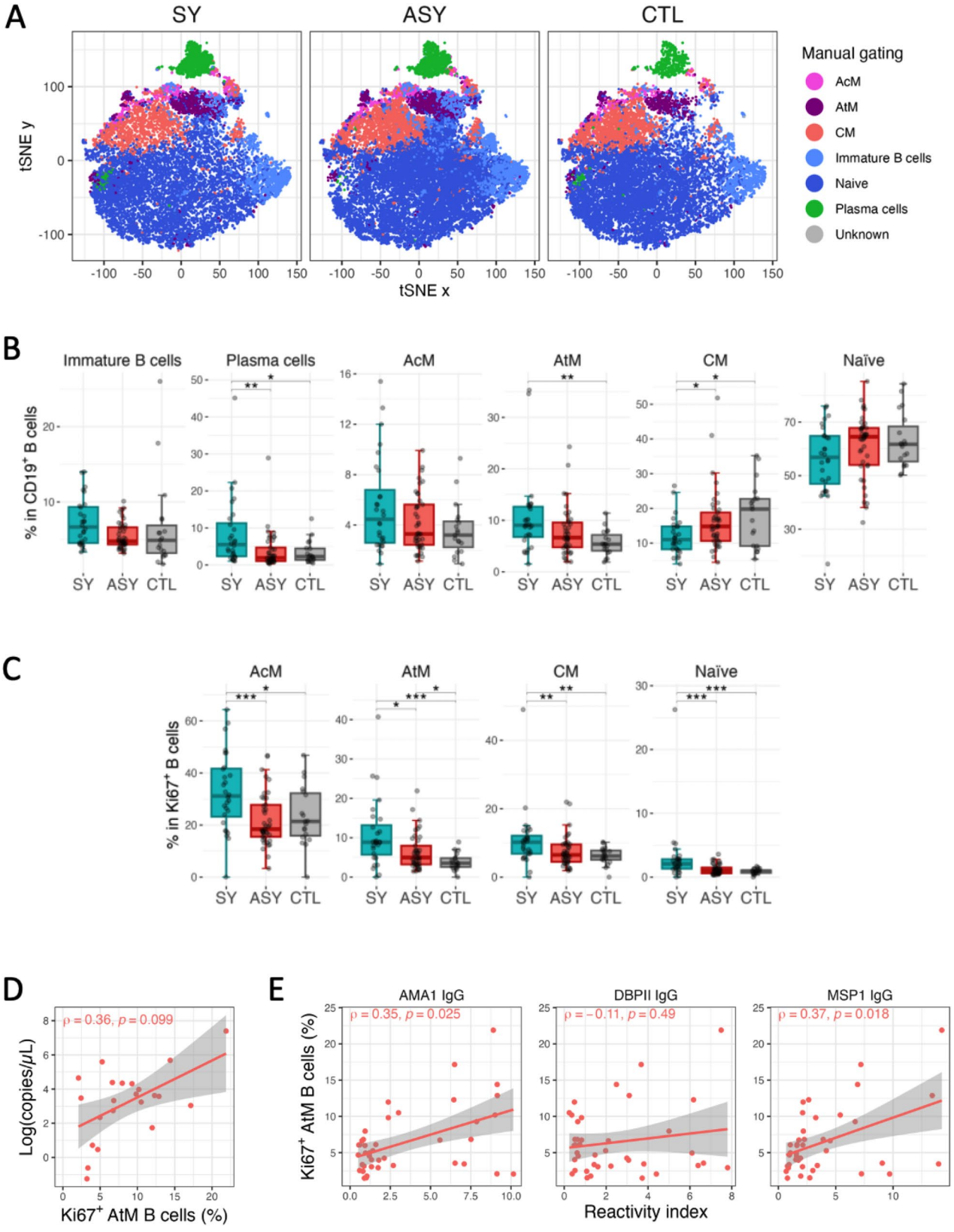

Fig. 6 (See legend on next page.)

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Fig. 6 B cell signature suggests a T_H1-biased commitment of B cell lineages due to increased AtM compartment. B cells from SY, ASY, and CTL were analyzed by flow cytometry analysis. (**A**) Representative dot-plot showing a t-SNE projection of B cell populations analyzed by group: Activated memory B cells (AcM, $CD27^+CD21^-CD20^+CD10^-CD19^+$), Atypical memory B cells (AtM, $CD27^-CD21^-CD20^+CD10^-CD19^+$), Immature B cells ($CD10^+CD19^+$), Naïve B cells ($CD27^-CD21^+CD20^+CD10^-CD19^+$), Plasma cells ($CD21^-CD20^-CD10^-CD19^+$), Classical memory B cells (CM, $CD27^+CD21^+CD20^+CD10^-CD19^+$). (**B**) Frequencies of Immature B cells, Plasma Cells, CM, AcM, AtM, and Naïve B cells in $CD19^+$ B cells by group. Data is represented as scattered dot-plots over boxplots with medians and interquartile range. Each dot represents a single individual. Asterisks over connecting lines represent significant differences between groups by Dunn's test. (**C**) Frequencies of Ki67-expressing cells among CM, AcM, AtM, and Naïve over total $CD19^+$ B cells. (**D**) Spearman correlation between relative parasitemia (copies/µL) and the frequency of $ki67^+$ atypical memory B cells from ASY. (**E**) Spearman correlation between antibody levels against $PvAMA1_{66}$, $PvMSP1_{19}$, and $PvDBPII_{brz-2}$ and the frequency of $Ki67^+$ atypical memory B cells from ASY. Spearman's R and *p*-values are displayed on graphs. The line represents the trend line by a linear regression model

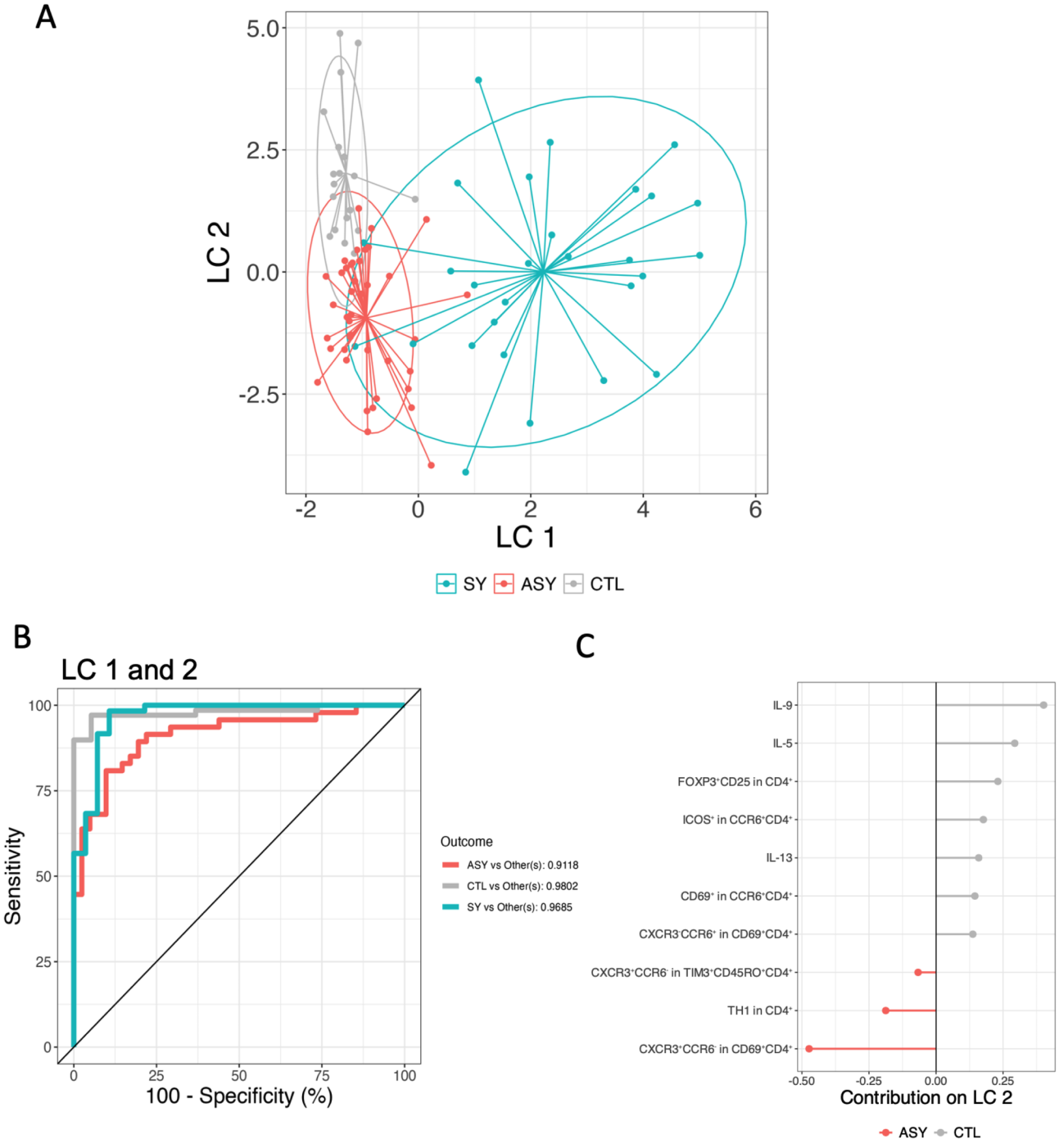

Fig. 7 Sparse partial least squares discriminant analysis (sPLS-DA) indicates a T_H1 biased signature in ASY. (**A**) sPLS-DA model score plot with confidence ellipses (95%) shows the group's segregation after feature selection. Each dot represents a participant. Lines connect dots to a centroid. (**B**) ROC curves of the model with performances of each group vs. the others. Legends display AUC scores for each group. (**C**) The loading plot of the features on the latent component 2 depicts the top variables to segregate ASY and CTL. Features are ranked according to their loading weight

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06353-1.

Supplementary Material 1

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Author contributions

LRVA and RTG conceived and designed the study. GGA, LRVA, PACC, CMC, KT, BVSV, JRS, AM, and BCC conducted the field sample collection, qPCR diagnosis, and PBMC separation. GGA, LRVA, PACC, GRG, MMF, CMC, and AFC performed the experiments. DBP, AM, and MST conducted clinical examination, sociodemographic data, and sample collection. MCC and JP supervised the data storage. ISS and DTG provided reagents. GGA and LRVA performed the analysis. GGA, LRVA, and RTG wrote the manuscript. LRVA supervised the study.

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Data availability

The data that support the findings of this study are available within the article and its Supplemental Tables and Figures. Raw data and scripts are available in https://dataverse.harvard.edu/.

Declarations

Ethics approval and consent to participate

This study was performed under protocols reviewed and approved by the Ethical Committees on Human Experimentation from Instituto René Rachou, Fiocruz, and National Ethical Council (CAAE: 59902816.7.0000.5091). All participants were informed about the objectives and procedures of the study, with voluntary participation through written informed consent.

Conflict of interest

The authors have declared that no conflict of interest exists.

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