Interferon-γ signal drives differentiation of T-bet\textsuperscript{hi} atypical memory B cells into plasma cells following \textit{Plasmodium vivax} infection

Piyawan Kochayoo\textsuperscript{1}, Pongsakorn Thawornpan\textsuperscript{1}, Kittikorn Wangriatisak\textsuperscript{2}, Siriruk Changrob\textsuperscript{1}, Chaniya Leepiyasakulchai\textsuperscript{1}, Ladawan Khowawisetsut\textsuperscript{2}, John H. Adams\textsuperscript{3} & Patchanee Chootong\textsuperscript{1}*

For development of a long-lasting protective malaria vaccine, it is crucial to understand whether \textit{Plasmodium}-induced memory B cells (MBCs) or plasma cells develop and stably contribute to protective immunity, or on the contrary the parasite suppresses antibody responses by inducing MBC dysfunction. The expansion of T-bet\textsuperscript{hi} atypical MBCs is described in chronic \textit{Plasmodium falciparum}-exposed individuals. However, it remains unclear whether accumulation of T-bet\textsuperscript{hi} atypical MBCs is indicative of a protective role or rather an impaired function of the immune system in malaria. Here, the phenotypic and functional features of T-bet\textsuperscript{hi} atypical MBCs were studied in \textit{P. vivax} patients living in an area of low malaria transmission. During \textit{P. vivax} infection, the patients produced a twofold higher frequency of T-bet\textsuperscript{hi} atypical MBCs compared to malaria non-exposed individuals. This distinct atypical MBC subset had a switched IgG phenotype with overexpression of activation markers and FcRL5, and decreased Syk phosphorylation upon BCR stimulation. Post-infection, expansion of T-bet\textsuperscript{hi} IgG\textsuperscript{+} atypical MBCs was maintained for at least 3 months. Further studies of the contribution of T-bet\textsuperscript{hi} atypical MBC function to humoral immunity showed that synergizing IFN-γ with TLR7/8 and IL-21 signals was required for their differentiation into plasma cells and antibody secretion.

Antibodies play an essential role in protection against malaria. Anti-\textit{Plasmodium} antibodies prevent disease progression in several ways: (i) block sporozoites from invading hepatocytes\textsuperscript{1,2}; (ii) opsonize merozoites and activate cell-mediated death; (iii) prevent erythrocyte invasion and block Plasmodial proteins from binding to molecules on cell surfaces\textsuperscript{3}. However, the anti-\textit{Plasmodium} antibodies acquired following infection decline substantially within a few years in the absence of re-infection\textsuperscript{3,4}, and boost inconsistently upon antigen re-exposure\textsuperscript{5,6}. These impairments of long-lived MBC or plasma cell responses may contribute to an inefficient protective immunity against malaria.

Malaria infection triggers the response of \textit{Plasmodium}-specific MBCs as reported in several field studies\textsuperscript{7–11}. A controversy remains about the development and persistence of MBCs, whether their responses are short- or long-lived. Individuals residing in areas of high transmission appear to develop a low frequency of malaria-specific MBCs, even when parasite loads are sufficient and capable of inducing antibody responses after infection\textsuperscript{8,10}. However, long-lived responses of \textit{Plasmodium}-specific MBCs are reported in areas of low malaria endemicity, and stable \textit{Plasmodium}-specific MBCs occur without frequent boosting\textsuperscript{9,11}. These contrasting reports of MBC responses to malaria are probably influenced by differences in intensity of malaria transmission and nature of the antigens expressed, as well as by age and host genetics. More immuno-epidemiological studies of MBC responses in malaria endemic and epidemic populations may better inform the design of malaria vaccines.

\textsuperscript{1}Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand. \textsuperscript{2}Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. \textsuperscript{3}Department of Global Health, University of South Florida, Tampa, FL 33612, USA. \textsuperscript{*}email: pchooton@gmail.com
Transcription factor T-bet plays critical roles as a transcriptional regulator in several immune lineages, including interferon γ (IFN-γ)-secreting CD4+ Th1 cells and B cells. T-bet+ B cells are found in both mice and humans. They promote antibody class switching to IgG2a/c in murine and IgG1/IgG3 in human B cells, and control viral infections and control malaria transmission areas produce a high number of T-bet+ atypical MBCs. In chronic immune activation, this cell population diminishes B cell receptor (BCR) signaling and limits antibody production. Although T-bet+ atypical MBCs are shown to be exhausted cells in chronic antigenic stimulation, they appear to play a role in autoantibody secretion, functional responses to yellow fever, vaccination, and influenza virus vaccination, as well as primary HIV infection. P. falciparum-exposed individuals from moderate to high malaria transmission areas produce a high number of T-bet+ atypical MBCs.

The booster response of this cell population was shown in a human immunization study. At present, atypical MBCs are proposed to potentially function as: (i) pre-antibody secreting cells (pre-ASCs), (ii) activated B cells in normal immune responses to vaccination and some infections, (iii) immune regulators which upregulate FcRL5 inhibitory receptor in the presence of high IgG levels, or (iv) anergic MBCs that contribute to inefficient humoral immunity. Despite extensive data showing an expansion of atypical MBCs with malaria exposure, the exact role of these cells under transcription T-bet control remains incompletely understood. Most of reports are documented in P. falciparum malaria but lacking in P. vivax, which has unique biological characteristics such as dormant liver-stages and relapsing fever. Given interspecies variation, their functional roles in triggering of immune responses might be different. Future studies of the phenotypic characteristics, dynamic responses, and functions of atypical MBCs in geographical settings with varying malaria transmission intensities, as well as the drivers of their stimulation and differentiation, are required to clarify their role in humoral immunity.

Here, the frequency, phenotypic characteristics and kinetics of T-bet+ atypical MBCs were analysed in P. vivax-infected patients in a low transmission intensity setting. The function of T-bet+ B cells (atypical MBCs), and the signals that induce their differentiation into plasma cells, were evaluated in these patients. Our findings may benefit the further development of efficacious malaria vaccines.

Results

Increased proportion of T-bet+ atypical MBCs during acute P. vivax infection. To observe the acquisition of T-bet+ atypical MBC responses in natural P. vivax infection in the setting of low-level transmission, the frequency of T-bet+ B cells was determined during acute malaria. Most (85%) subjects possessed a significantly higher frequency of these cells than did healthy controls (HCs) (Fig. 1a, b and Supplementary Table S1). As the T-bet+ MBC population is heterogeneous, to understand their function as contributors to antibody responses, T-bet profiling (based on low, intermediate, and high expression on CD19+ B cells and among MBC subsets) was determined (Fig. 1c). Of T-bet+ B cells, 69.31%, 15.64% and 3.55% exhibited atypical, activated and classical MBC phenotypes, respectively. T-bet+ B cells made up 38.33% of atypical, 15.36% of activated, 12.50% of classical MBCs. In addition, among T-bet- B cell subsets, 13.52% were atypical, 3.95% were activated, and 10.85% were classical MBCs (Fig. 1d).

T-bet+ atypical MBCs were skewed toward IgG class switching. We investigated whether the expanded T-bet+ atypical MBCs during acute P. vivax infection mainly expressed switched or unswitched MBC phenotypes. Of the 26 samples from acutely infected patients, 16 were initially detected of IgM and IgD expression in the T-bet+ B cells (Supplementary Fig. 1 and Supplementary Table S1). There was a higher percentage (62%) of T-bet+ switched (IgM+ IgD-) than unswitched (IgM+, IgD+) MBCs (Fig. 2a). We next addressed whether the T-bet+ switched MBCs were to IgM or IgG. Significantly more IgG than IgM was detected in this population (Fig. 2b). Further analysis of T-bet+ switched (IgM or IgG) MBCs (from CD21, CD27 expression) showed that the phenotypic markers were mainly on atypical MBCs (T-bet+ IgM; average 59.63%, SEM 4.58% and T-bet+ IgG 27.10%, SEM 4.17%) (Fig. 2c, d).

Expansion of T-bet+ atypical MBCs was stably maintained post-infection. We observed kinetic responses of T-bet+ B cells and their subsets during and after P. vivax infection. Of the 26 patients, 9 with higher frequencies of T-bet+ B cells than did HCs (2.34%; average + 2SD of HCs) were recruited as a cohort for tracking changes in the cell frequencies during acute malaria infection (AC) and after infection (Supplementary Table S1). During AC, all patients in this cohort presented higher total T-bet+ B cells (average, 4.42%; SEM 0.73%) compared to the percentage in HCs (average 1.27%; SEM 0.18%). Post-infection, the population of T-bet+ B cells expanded further as found at the 1-month sampling (average 2.59%; SEM 0.65%). However, the frequency of these cells was significantly decreased at the 3-month post-infection time-point compared to AC (Fig. 3a). Four and 2 patients maintained elevated T-bet+ levels 3 and 6 months after infection, respectively (Fig. 3a).

The tracking of unswitched (IgM+ IgD+), switched IgM and switched IgG MBCs among T-bet+ B cells showed that the switched IgG MBCs found during AC (average 25.51%; SEM 6.02%) were further increased 3-month post-infection (average 42.39%; SEM 4.26%) and then declined to baseline levels 6 months after parasite clearance (average 25.19%; SEM 5.53%). In contrast, much lower frequencies of T-bet+ switched IgM (average 9.92%; SEM 2.47%) and unswitched MBCs (average 13.46%; SEM 2.72%) were observed during AC. These tended to decrease at 1-month post-infection (T-bet+ switched IgM; average 2.93%; SEM 0.77% and unswitched MBCs; average 12.34%; SEM 3.49%) and continued to decline over subsequent timepoints, indicating that T-bet+ unswitched MBCs were likely switched into T-bet+ IgG MBCs after infection (Fig. 3b and Supplementary Fig. 2). Further kinetic analysis of T-bet+ IgG atypical MBCs showed that cell frequency during AC (average 71.61%; SEM 6.26%) remained stably detectable at all follow-up times with no significant difference of those frequencies through...
Figure 1. Increased proportion of T-bet\textsuperscript{hi} atypical MBCs during acute *P. vivax* infection. (a) Representative flow cytometry plot indicating expression of T-bet in CD19\textsuperscript{+} B cells. (b) The frequency of T-bet\textsuperscript{-} B cells is shown from acute *P. vivax* patients (AC; *n* = 26) compared to healthy controls (HCs; *n* = 22). (c) Representative flow cytometry plot indicating profiling expression of T-bet, basing on low, intermediate or high expression in total CD19\textsuperscript{+} B cells. Among T-bet\textsuperscript{neg}, T-bet\textsuperscript{int} and T-bet\textsuperscript{hi} B cells, activated, classical and atypical MBCs were defined. (d) The frequency of each subpopulation was determined. The horizontal lines represent mean values ± SEM. Statistical analyses were performed using Mann–Whitney U tests (for unpaired variables) and Wilcoxon signed rank tests (for paired variables). ***P < 0.001; ****P < 0.0001.
the recovery phase. However, the frequency of T-bet\textsuperscript{hi} IgG activated MBCs (average 22.76%; SEM 6.41%) was relatively low and stayed so to the 6-month blood collection (Fig. 3c and Supplementary Fig. 3).

**T-bet\textsuperscript{hi} atypical MBCs from acute *P. vivax* patients had upregulated activation markers and FcRL5.** To understand whether and how T-bet\textsuperscript{hi} atypical MBCs participate in immune responses to acute
Plasma cells.

In T-bet hi activated nor classical MBCs (data not shown). Further investigation of functional capacity of these

found to express CD11c+, CXCR3+, FcRL5+ phenotypes and exhibited reduced BCR signaling17, 21, 24. Moreover,

in children and adults living in moderate and high transmission settings. These expanded cells were commonly

MBCs into plasma cells, stimulation in vitro with TLR7/8 (R848) and B-cell activating factors (IL-2 and BAFF)21. We initially stimulated T-bethi atypical MBCs (atypical MBCs) under conditions which readily drove classical

P. vivax plasma cells compared to the same condition without IFN-γ stimulation (Fig. 6a, b), indicating that atypical
differentiation. In the presence of IFN-γ, atypical MBCs from P. vivax patients and HCs generated twofold more

differentiation of these cells in vitro. The combination of IL-21 with TLR7/8, IL-2 and BAFF resulted in approxi-
mately 2.5-fold higher induction of atypical MBCs into plasma cells (average 8.82%; SEM 0.84%) compared to the

same condition without IL-21. Compared with HCs, atypical MBCs from patients had a slightly lower ability
to generate plasma cells (HC; average 12.90%; SEM 1.36%) (Fig. 6a, b), suggesting that the IL-21 signal could

help in their development. Since IFN-γ signal was reported to drive double negative 2 (DN2) (CD27−, IgD−,
CXCR5 and CD11c+) B cell differentiation into plasma cells in autoimmune diseases following stimulation with

TLR7/8 and IL-2122, 27, we next included IFN-γ, Th1-derived signals, in atypical MBC culture to enhance their
differentiation. In the presence of IFN-γ, atypical MBCs from P. vivax patients and HCs generated twofold more

plasma cells compared to the same condition without IFN-γ stimulation (Fig. 6a, b), indicating that atypical

MBCs required signal from Th1 cells to promote differentiation into plasma cells.

In addition, the functional ability of atypical MBCs to secrete antibody was observed upon stimulation with various signals. Upon TLR7/8, IL-2 and BAFF stimulation, total IgG was greatly detected in classical MBC culture supernatant, whereas these signals did not induce antibody secretion from atypical MBCs (Fig. 6c). Addition of T-cell-derived cytokines into atypical MBC cultures (TLR7/8, IL-2 and BAFF) showed that IL-21 increased total IgG antibodies and IFN-γ significantly increased IgG levels in supernatant (Fig. 6c). Importantly, P. vivax antigen-specific antibody was specifically detected in classical MBC cultures, from 2 of 3 patients. The undetectable level of anti-P. vivax antibody upon TLR7/8, IL-21 and IFN-γ stimulation of atypical MBCs was observed due to the low frequency of antigen specific atypical MBCs in blood circulation (Fig. 6d).

Discussion

Expansion of T-bet+ B cells has been described in the context of various chronic antigenic stimulations, including aging16, 31, autoimmunity22, 32, vaccination16, 23, and infectious diseases such as HIV, HCV, and P. falciparum malaria16, 17, 29. This cell population expressed the phenotype of as atypical MBCs (CD21−CD27−), and also expressed CD11c, CXCR3 and FcRL517, 25, 26. However, the function of high expression of T-bet in atypical MBCs in these models, as well as the triggers of their differentiation into plasma cells, is unclear. Our results revealed an increase of T-bet hi B cells during acute P. vivax infection. Most of the responses were T-bet hi atypical MBCs with IgG class switching, and were stably maintained the responses in the convalescence phase for at least 3 months. These T-bet hi atypical MBCs overexpressed CD11c, CD69, CD86 and IL-21R, as well as FcRIL5 and CD95. Functional analysis of these MBCs exhibited diminished BCR signaling following cross-linking. To drive the functional role of T-bet hi atypical BCR differentiation into plasma cells and antibody secretion, stimulation with a combination of TLR-7/8 and T cell-derived cytokines (IL-21 and IFN-γ) was required. Together, our data suggest that P. vivax infection triggered an expansion of T-bet hi atypical MBCs and that these expanded cells played a role in anti-malarial humoral immunity under TLR7/8 ligands, and Th1 and Th1-derived signals.

A more detailed understanding of the generation and persistence of the atypical MBCs associated with malaria infection is useful for vaccine development. In P. falciparum malaria, an increase of T-bet hi atypical MBCs was observed during P. falciparum infection is useful for vaccine development. In P. falciparum malaria, an increase of T-bet hi atypical MBCs was observed during...
that exposure to parasite drives expansion of T-bet\textsuperscript{hi} atypical MBCs and that the chronic presence of parasites induces accumulation of this MBC subset with reduced B cell effector function. Here, we observed the functional characteristics of T-bet\textsuperscript{hi} atypical MBCs in \textit{P. vivax} subjects who lived in an area of Thailand with seasonal malaria

**Figure 4.** T-bet\textsuperscript{hi} atypical MBCs from acute \textit{P. vivax} patients had upregulated activation markers and FcRL5. Ex vivo expression of cell surface protein on CD19\textsuperscript{+} B cells stratified by level of T-bet expression in acute patients (\(n = 10\)). The horizontal lines represent mean values ± SEM. Statistical analyses were performed using Wilcoxon signed rank tests; **\(P < 0.01\); ***\(P < 0.001\); ****\(P < 0.0001\).
transmission (a single rainy season peak). During acute malaria, T-bethi atypical MBCs were expanded, mainly switched to IgG subclass, and had elevated expression of activation markers (CD11c, CD69, CD86, IL-21R), FcRL5 and CD95. Moreover, CXCR5 and CCR7 were downregulated among the T-bethi atypical MBCs. After parasite clearance, the switched IgG+ T-bethi atypical MBCs persisted for at least 3 months. Our data proved that non-chronic infection to P. vivax infection drives an expansion of T-bet hi atypical MBCs which exhibit T-dependent responses such as upregulation of activation markers and IL-21R. A possible explanation of the T-bethi atypical MBC response with IL-21R+ phenotype in vivax malaria is that B cells encounter parasite antigen via BCRs and receive signals from CD4+ T cells which subsequently drive migration of B cells into germinal centers (GCs)33, 34. Within these GCs, T-B interactions may induce upregulation of IL-21R on B cells, which in turn supports the activation of IL-21 signaling in GCs. Tfh cells, with polarizing IFN-γ and IL-21, contribute to the upregulation of T-bet and development of an atypical phenotype (CD21−CD27−) on these cells. In addition, they potentially participate in isotype switching to IgG and persist after parasite clearance17, 33, 35. The lower expression of the GC-positioning receptors CXCR5 and CCR7 on these cells might explain their migration outside GCs to play a crucial role in humoral immunity. The overexpression of FcRL5 on IgG+ T-bethi atypical MBCs in vivax malaria might be part of a feedback mechanism which downregulates the hyperactivated state of atypical MBCs, or they may be markers of durable and robust responses to re-infection as previously proposed in P. falciparum studies36. In-depth investigations regarding these pivotal signals of the immune mechanisms that drive T-bet hi atypical MBCs to play effector function are still needed for improved malaria vaccine design.

The role of T-bethi atypical MBCs in the immune system remains elusive. To date, the signals inducing high expression of T-bet in atypical MBCs, as well as the function of this cell population in the immunity, require more understanding. In malaria, it has been reported that P. falciparum-infected erythrocytes drive expansion of T-bet in B cells37, and that IFN-γ is the key driver for T-bethi atypical MBCs differentiation17, 20. With these findings, a functional role of T-bet hi atypical MBCs might be immunosuppression, as suggested by the upregulation of multiple inhibitory receptors with reduced BCR-mediated signaling and limited antibody production17, 20, 21. Up to now, there is no report in P. vivax malaria which differs from P. falciparum since it has a dormant stage in the human liver (hypnozoites) and a relapsing fever. Moreover, different malaria transmission settings may influence the functional roles of atypical MBCs. Here, we found that T-bethi atypical MBCs from P. vivax-infected patients had significantly reduced Syk BCR signaling upon cross-linking. This indicates that these cells might have less activation in response to BCR signaling or require additional signals for transition from resting to a highly active state. Our functional analysis focused on antibody responses demonstrated that, unlike classical MBCs, atypical MBCs did not differentiate into plasma cells and secrete IgG antibodies upon TLR7/8, IL-2 and BAFF stimulation. These results suggested that atypical MBCs require different or more signals for plasma cell generation. Previous reports documented that T cell-derived cytokines (IL-21 and IFN-γ) were significantly increased in sera from patients with autoimmune disease38, 39 and P. falciparum malaria infection40, 41. Of note, we found upregulation of IL-21R on these cells. The addition of IL-21 to atypical MBCs increased the capacity to induce plasma cell generation, indicating that IL-21 is one of the most potent cytokines derived from Th1 cells in regulating atypical MBCs. Furthermore, we observed their functional capacity by adding Th1-derived cytokines.

Figure 5. T-bet hi atypical MBCs of P. vivax-infected patients reduced BCR signaling. (a) Representative histograms indicating expression of pSyk, pBLNK, and pPLCγ2 in MBC subsets cross-linked for 5 min with anti-IgM/IgG Abs (black line tracing) and uncross-linked (shaded gray area). (b) Mean fluorescence intensity (MFI) of pSyk, pBLNK, and pPLCγ2 in T-bethi activated and atypical MBCs after BCR cross-linking (n = 5). The horizontal lines represent mean values ± SEM. Statistical analyses were performed using Wilcoxon signed rank tests; *P < 0.05.
Our results showed that plasma cell differentiation was enhanced by IFN-γ, as it showed a higher frequency of plasma cells in atypical MBC culture compared to the condition without IFN-γ. Collectively, the findings indicated that atypical MBCs required multiple stimuli including TLR7/8, IL-21 and IFN-γ signals, to induce plasma cell generation. Of note, anti-\textit{P. vivax} specific antibodies were found in classical MBC cultures of some patients, but not in atypical MBC cultures, likely reflecting the low level of \textit{P. vivax} specific atypical MBCs in circulating blood. Thus, there is a need to develop high sensitivity techniques for detection of the rare population of malarial specific MBCs in producing anti-malarial specific antibodies as well as their protective roles in further studies.

For the studies of MBC responses in malaria, the complex life cycle, antigenic variation/polymorphism, and its ability to establish chronic infection could well influence development and longevity of MBC responses. \textit{P. vivax} parasites have unique biological features compared to \textit{P. falciparum}. The main differences of \textit{P. vivax} parasite include: (i) dormant hypnozoite in liver, which often escapes immune response and leads to latent state\textsuperscript{42, 43}, (ii) recurrent parasitemia implicating in relapsing symptoms\textsuperscript{44}, (iii) preference for invading reticulocytes and increasing their deformability and fragility leading to evasion of immune system\textsuperscript{45, 46}. Given these different characteristics, the detailed knowledge of atypical MBC function in \textit{P. falciparum} infection might not explain in \textit{P. vivax}-exposed individuals. In comparison of T-bet\textsuperscript{hi} atypical MBC responses in malaria, these cells were increased in frequency and activated by showing upregulation of activating markers (CD11c, CD69) and co-stimulatory molecules (CD86 and IL-21R) in both \textit{P. falciparum} and \textit{P. vivax} infection\textsuperscript{17, 20, 47}. For the contribution
of atypical MBCs in humoral immunity, the BCR signaling (PLCy2, pBLNK and Syk) of *P. falciparum*-associated atypical MBCs was impaired\(^1\), thereby requiring *Staphylococcal enterotoxin B* and Tfh cell interaction for plasma cell differentiation\(^2\). In *P. vivax*, only greatly reduced Syk BCR signaling was detected in T-bet\(^{hi}\) atypical MBCs from infected patients, resulting in requirement of additional signals from TLR7/8 and T cell-derived cytokines (IL-21 and IFN-γ) for driving into plasma cell differentiation and antibody secretion. Future exploration is required to determine the role of Tfh cells in contribution to activation and differentiation of T-bet\(^{hi}\) atypical MBCs into plasma cells.

Limitation of this study is a small sample size for tracking kinetic responses of expanded T-bet\(^{hi}\) atypical MBCs in cohort analysis. Nine of total 26 who had higher T-bet\(^{hi}\) atypical MBC frequencies than HCs (average + 2 SDs of HCs) were recruited in a 6-month cohort study. Thus, this observation should be considered as preliminary result to demonstrate the maintenance of T-bet\(^{hi}\) IgG atypical MBCs in response to *P. vivax* infection. More screening of expanded T-bet\(^{hi}\) atypical MBCs samples at acute disease and cohort study design would help to ensure their kinetic responses after parasite clearance as well as to clarify their functional roles in malaria immunity.

In summary, *P. vivax* infection was found to be associated with T-bet\(^{+}\) B cell activation and expansion. The predominant response was of T-bet\(^{hi}\) atypical MBCs with an IgG phenotype. These cells showed a profile of upregulated activation markers and FcRL5, and exhibited reduced responsiveness to pSyk following cross-linking. Notably, the signals from TLR7/8 and T cell-derived cytokines (IL-21 and IFN-γ) appeared to drive their function in anti-malarial humoral immunity. A better understanding of the regulators and functions of these expanded cells, in settings of chronic stimulation by various antigens, will provide further insight into the mechanism of their differentiation, and may improve vaccine strategies and therapeutic interventions.

**Methods**

**Ethical statement.** Ethical approval was obtained from the Committee on Human Rights Related to Human Experimentation, Mahidol University, Thailand (MUIRB 2012/079.2408). The study participants gave written informed consent to enroll in this study before collecting blood samples. All experiments involving human subject were conducted in accordance with relevant guidelines and regulations.

**Study subjects and sampling.** Twenty-six patients with acute *P. vivax* malaria living in an area of low intensity malaria transmission (Kraburi, Ranong Province, a village near the Myanmar border in Southern Thailand), were enrolled in the study for determination of frequency and phenotype of T-bet\(^{hi}\) atypical MBCs. Of the 26, the 9 who had T-bet\(^{hi}\) atypical MBC frequencies more than the average + 2 standard deviations (SDs) of that in HCs were enrolled in a 6-month cohort sub-study to track the kinetic changes of these cells with follow-up visits at 1, 3 and 6 months after infection. To further explore the profile of T-bet\(^{hi}\) atypical MBCs during *P. vivax* infection, 10 subjects were recruited for analysis of activation and inhibition marker expression. The function of T-bet\(^{hi}\) atypical MBCs was analysed for upregulation of BCR signaling molecules, differentiation into total IgG, and anti-*P. vivax* antigen-specific antibodies after in vitro stimulation (n = 5).

*Plasmodium vivax* infection was confirmed by microscopic examination and nested PCR of peripheral blood. Patients were evaluated for sub-patent parasitemia every 2 weeks. Data on past malaria infections were obtained from the records of the Vector-borne Disease Unit. Twenty-six malaria non-infected Thai residents who lived in non-endemic areas (Bangkok, Thailand) were recruited as HCs.

**Flow cytometry.** Flow cytometry of peripheral blood mononuclear cells (PBMCs) was performed as previously described\(^1\). Briefly, PBMCs were incubated with Live/Dead fixable stain (Invitrogen, USA) and monoclonal antibodies (mAbs) (Supplementary Table S2). Cells were then fixed and permeabilized according to the manufacturer’s instructions (Transcription Factor Buffer Set, Biolegend, USA). Intracellular targets were stained with a transcription factor T-bet. Analyses were done with a BD FACS Canto II flow cytometer (BD Biosciences, USA).

**BCR signaling assay.** Two million PBMCs were stained with anti-CD19, -CD21 and -CD27 mAbs. Cells were washed and incubated at 37 °C for 30 min before adding F(ab’)2 anti-IgM and anti-IgG (Southern Biotech) at a final concentration of 20 μg/ml and incubated at 37 °C for 5 min. For detection of phosphoproteins, cells were fixed, permeabilized and stained with antibodies specific for phospho-Syk (Y352) (pSyk), pBLNK (Y84) and pPLCγ2 (Y759) (BD Biosciences) and T-bet (Supplementary Table S2).

**B cell sorting and stimulation.** B cell subsets were sorted using FACS Aria III (BD Biosciences). The gating strategy of cell sorting was shown in Supplementary Fig. 4. Sorted cells (5 × 10⁴) were cultured in RPMI media supplemented with 3 conditions of stimuli; Stimuli 1 (S1): 1 μg/ml R848 (Invivogen, USA), 10 ng/ml IL-2 (Peprotech, USA), 10 ng/ml BAFF (Peprotech); S2: 1 μg/ml R848, 10 ng/ml IL-2, 10 ng/ml BAFF and 100 ng/ml IL-21 (Peprotech); S3: 1 μg/ml R848, 10 ng/ml IL-2, 10 ng/ml BAFF, 100 ng/ml IL-21 and 20 ng/ml IFN-γ (Invivogen), at 37 °C, 5% CO₂ for 11 days. Culture supernatants were tested by IgG ELISA and the cells were harvested and stained with CD19, CD27 and CD38 antibodies before acquisition on a flow cytometry.

**IgG ELISA.** Culture supernatants were assessed for total IgG and anti-PvDBL-TH2 antibodies by indirect ELISA as previously reported\(^1\). Briefly, 1 μg/ml of anti-human IgG (clones MT91/145; Mabtech, Sweden) or 2 μg/ml PvDBL-TH2 were coated on 96-well plates followed by blocking with 5% BSA-PBS. Undiluted supernatant was added to wells followed by detection with goat anti-human IgG conjugated to horseradish peroxidase (HRP).
Statistical analysis. Statistical analyses and graphing were carried out using GraphPad Prism 8.2.1 software, GraphPad Software, USA, www.graphpad.com. Mann–Whitney U tests (for unpaired variables) and Wilcoxon signed rank tests (for paired variables) were performed. P-values < 0.05 (2-tailed) were considered statistically significant.

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References
1. Michon, P., Fraser, T. & Adams, J. H. Naturally acquired and vaccine-elicited antibodies block erythrocyte cytoadherence of the Plasmodium vivax Duffy binding protein. Infect. Immun. 68, 3164–3171. https://doi.org/10.1128/IAI.68.6.3164-3171.2000 (2000).
2. Teo, A., Feng, G., Brown, G. V., Beeson, J. G. & Rogerson, S. J. Functional antibodies and protection against blood-stage malaria. Trends Parasitol. 32, 887–898. https://doi.org/10.1016/j.pt.2016.07.003 (2016).
3. Ceravolo, I. P. et al. Naturally acquired inhibitory antibodies to Plasmodium vivax Duffy binding protein are short-lived and allele-specific following a single malaria infection. Clin. Exp. Immunol. 156, 502–510. https://doi.org/10.1111/j.1365-2249.2009.09391.x (2009).
4. Muggenyi, C. K. et al. Declining malaria transmission differentially impacts the maintenance of humoral immunity to Plasmodium falciparum in Children. J. Infect. Dis. 216, 887–898. https://doi.org/10.1093/infdis/jix370 (2017).
5. Baird, J. K. et al. Age-dependent acquired protection against Plasmodium falciparum in people having two years exposure to hyperendemic malaria. Am. J. Trop. Med. Hyg. 45, 65–76. https://pii:10.4269/ajtmh.1991.45.65 (1991).
6. Akpogheneta, O. J., Dunyo, S., Pinder, M. & Conway, D. J. Boosting antibody responses to Plasmodium falciparum merozoite antigens in children with highly seasonal exposure to infection. Parasite Immunol. 32, 296–304. https://doi.org/10.1111/j.1365-3024.2009.01193.x (2010).
7. Changrob, S. et al. Persistence of long-lived memory B cells specific to Duffy binding protein in individuals exposed to Plasmodium vivax. Sci. Rep. 8, 8347. https://doi.org/10.1038/s41598-018-26677-x (2018).
8. Dorfman, J. R. et al. B cell memory to Plasmodium falciparum blood-stage antigens in a malaria-endemic area. J. Infect. Dis. 191, 1623–1630. https://doi.org/10.1086/429671 (2005).
9. Ndungu, F. M. et al. Long-lived Plasmodium falciparum specific memory B cells in naturally exposed Swedish travelers. Eur. J. Immunol. 43, 2919–2929. https://doi.org/10.1002/eji.201343630 (2013).
10. Weiss, G. E. et al. The Plasmodium falciparum-specific human memory B cell compartment expands gradually with repeated malaria infections. PLoS Pathog. 6, e1000912. https://doi.org/10.1371/journal.ppat.1000912 (2010).
11. Wipasa, J. et al. Long-lived antibody and B cell memory responses to the human malaria parasites, Plasmodium falciparum and Plasmodium vivax. PLoS Pathog. 6, e1000770. https://doi.org/10.1371/journal.ppat.1000770 (2010).
12. Knox, J. J., Myles, A. & Cancro, M. T-bet+ memory B cells: generation, function, and fate. Immunol. Rev. 288, 149–160. https://doi.org/10.1111/imr.12736 (2019).
13. Lazarevic, V., Glimcher, L. H. & Lord, G. M. T-bet: a bridge between innate and adaptive immunity. Nat. Rev. Immunol. 13, 777–789. https://doi.org/10.1038/nri3536 (2013).
14. Peng, S. L., Szabo, S. J. & Glimcher, L. H. T-bet regulates IgG class switching and pathogenic autoantibody production. Proc. Natl. Acad. Sci. USA 99, 5545–5550. https://doi.org/10.1073/pnas.021148999 (2002).
15. Wang, N. S. et al. Divergent transcriptional programming of class-specific B cell memory by T-bet and ROAs. Nat. Immunol. 13, 604–611. https://doi.org/10.1038/nature12294 (2012).
16. Knox, J. J. et al. T-bet+ B cells are induced by human viral infections and dominate the HIV gp140 response. JCI Insight 2, e92943. https://doi.org/10.1172/jci.insight.92943 (2017).
17. Obeng-Adjei, N. et al. Malaria-induced interferon-y drives the expansion of Tbet+ atypical memory B cells. PLoS Pathog. 13, e1006576. https://doi.org/10.1371/journal.ppat.1006576 (2017).
18. Barnett, R. E. et al. Cutting Edge: B Cell-Intrinsic T-bet expression is required to control chronic viral infection. J. Immunol. 197, 1017–1022. https://doi.org/10.4049/jimmunol.1500368 (2016).
19. Rubtsova, K., Rubtsov, A. V., van Dyk, L. F., Kappler, J. W. & Marrack, P. T-box transcription factor T-bet, a key player in a unique type of B-cell activation essential for effective viral clearance. Proc. Natl. Acad. Sci. USA 110, E3216–E3224. https://doi.org/10.1073/pnas.1312348110 (2013).
20. Ambeagaonkar, A. A., Nagata, S., Pierce, S. K. & Sohn, H. The differentiation in vitro of human tonsil B cells with the phenotypic and functional characteristics of T-bet+ atypical memory B cells in malaria. Front. Immunol. 10, 852. https://doi.org/10.3389/fimmu.2019.00852 (2019).
21. Portugal, S. et al. Malaria-associated atypical memory B cells exhibit markedly reduced B cell receptor signaling and effector function. Elife 4, e07218. https://doi.org/10.7554/elife.07218 (2015).
22. Jenks, S. A. et al. Distinct effector B cells induced by unregulated toll-like receptor 7 contribute to pathogenic responses in systemic lupus erythematosus. Immunity 49, 725–739.e726. https://doi.org/10.1016/j.immuni.2018.08.015 (2018).
23. Johnson, J. L. et al. The transcription factor T-bet resolves memory B cell subsets with distinct tissue distributions and antibody specificities in mice and humans. Immunity 52, 842–855.e846. https://doi.org/10.1016/j.immuni.2020.03.020 (2020).
24. Sullivan, R. T. et al. FCLR5 delineates functionally impaired memory B cells associated with Plasmodium falciparum exposure. PLoS Pathog. 11, e1004894–e1004894. https://doi.org/10.1371/journal.ppat.1004894 (2015).
25. Sundling, C. et al. B cell profiling in malaria reveals expansion and remodelling of CD11c+B cell subsets. JCI Insight. doi:https://doi.org/10.1172/jci.insight.126492 (2019).
26. Sutton, H. J. et al. Atypical B cells are part of an alternative lineage of B cells that participates in responses to vaccination and infection in humans. Cell Rep. 34, 108684–108684. https://doi.org/10.1016/j.celrep.2020.108684 (2021).
27. Marqueso, E. et al. IFNγ induces epigenetic programming of human T-bet(b) B cells and promotes TLR7/8 and IL-21 induced differentiation. Elife 8, e41641. https://doi.org/10.7554/elife.41641 (2019).
28. Austin, J. W. et al. Overexpression of T-bet in HIV infection is associated with accumulation of B cells outside germinal centers and poor affinity maturation. Sci. Transl. Med. 11, eaax0904. https://doi.org/10.1126/scitranslmed.aax0904 (2019).
29. Chang, L. Y., Li, Y. & Kaplan, D. E. Hepatitis C virus RNA reversibly maintains subset of antigen-specific T-bet+ tissue-like memory B cells. J. Viral. Hepat. 24, 389–396. https://doi.org/10.1111/jvh.12659 (2017).
30. Hao, Y., O'Neill, P., Naradikian, M. S., Scholz, J. L. & Cancro, M. P. A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice. *Blood* **118**, 1294–1304. https://doi.org/10.1182/blood-2011-01-330530 (2011).

31. Ratliff, M., Alter, S., Frasca, D., Blomberg, B. B. & Riley, R. L. In senescence, age-associated B cells secrete TNFα and inhibit survival of B-cell precursors. *Aging Cell* **12**, 303–311. https://doi.org/10.1111/acel.12055 (2013).

32. Wang, S. et al. IL-21 drives expansion and plasma cell differentiation of autoreactive CD11c(bi)T-bet(+) B cells in SLE. *Nat. Commun.* **9**, 1738. https://doi.org/10.1038/s41467-018-03750-7 (2018).

33. Braddock, A. E., Batugedara, G., Bol, S. & Bunnik, E. M. Potential functions of atypical memory B cells in *Plasmodium*-exposed individuals. *Int. J. Parasitol.* **50**, 1033–1042. https://doi.org/10.1016/j.ijpara.2020.08.003 (2020).

34. Kurosaki, T., Kometani, K. & Ise, W. Memory B cells. *Nat. Rev. Immunol.* **15**, 149–159. https://doi.org/10.1038/nri3802 (2015).

35. Portugal, S., Obeng-Adjei, N., Moir, S., Crompton, P. D. & Pierce, S. K. Atypical memory B cells in human chronic infectious diseases: an interim report. *Cell. Immunol.* **321**, 18–23. https://doi.org/10.1016/j.cellimm.2017.07.003 (2017).

36. Kim, C. C., Baccarella, A. M., Bayat, A., Pepper, M. & Fontana, M. F. PCRL5(+) memory B cells exhibit robust recall responses. *Cell Rep.* **27**, 1446–1460.e1444. https://doi.org/10.1016/j.celrep.2019.04.019 (2019).

37. Rivera-Correa, J. et al. *Plasmodium* DNA-mediated TLR9 activation of T-bet+ B cells contributes to autoimmune anemia during malaria. *Nat. Commun.* **8**, 1282. https://doi.org/10.1038/s41467-017-01476-6 (2017).

38. Kang, K. Y. et al. Impact of interleukin-21 in the pathogenesis of primary Sjögren’s syndrome: increased serum levels of interleukin-21 and its expression in the labial salivary glands. *Arthritis Res. Ther.* **13**, R179. https://doi.org/10.1186/ar3504 (2011).

39. Oke, V. et al. High levels of circulating interferons type I, type II and type III associate with distinct clinical features of active systemic lupus erythematosus. *Arthritis Res. Ther.* **21**, 107–107. https://doi.org/10.1186/s13075-019-1878-y (2019).

40. Nasr, A., Allam, G., Hamid, O. & Al-Ghamdi, A. IFN-gamma and TNF associated with severe falciparum malaria infection in Saudi pregnant women. *Malar. J.* **13**, 314. https://doi.org/10.1186/1475-2875-13-314 (2014).

41. Solaymani-Mohammadi, S., Eckmann, L. & Singer, S. M. Interleukin-21 in inflammation and immunity during parasitic diseases. *Front. Cell. Infect. Microbiol.* **9**, 401–401. https://doi.org/10.3389/fcimb.2019.00401 (2019).

42. Baird, J. K., Schwartz, E. & Hoffman, S. L. Prevention and treatment of vivax malaria. *Curr. Infect. Dis. Rep.* **9**, 39–46. https://doi.org/10.1007/s11908-007-0021-4 (2007).

43. Krootski, W. A. et al. Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium vivax* infection. *Am. J. Trop. Med. Hyg.* **31**, 1291–1293. https://doi.org/10.4269/ajtmh.1982.31.1291 (1982).

44. Imwong, M. et al. Relapses of *Plasmodium vivax* infection usually result from activation of heterologous hypnozoites. *J. Infect. Dis.* **195**, 927–933. https://doi.org/10.1086/512241 (2007).

45. Handayani, S. et al. High deformability of *Plasmodium vivax*-infected red blood cells under microfluidic conditions. *J. Infect. Dis.* **199**, 445–450. https://doi.org/10.1093/infdis/jiv0048 (2009).

46. Lim, C. et al. Reticuloocyte preference and stage development of *Plasmodium vivax* isolates. *J. Infect. Dis.* **214**, 1081–1084. https://doi.org/10.1093/infdis/jiv303 (2016).

47. Hopp, C. S. et al. Atypical B cells upregulate co-stimulatory molecules during malaria and secrete antibodies with T follicular helper cell support. Preprint (bioRxiv), 2021.2012.2007.471601. https://doi.org/10.1101/2021.12.07.471601 (2021).

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Author contributions
P.K. and P.C. designed the study. P.K. and K.W. performed experiments. P.K., C.L., L.K., and P.C. analyzed and interpreted the data. P.K., P.T. and S.C. drafted and wrote the manuscript. J.H.A. and P.C. reviewed the final draft. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to P.C.

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