Molecular profiling reveals features of clinical immunity and immunosuppression in asymptomatic \emph{P. falciparum} malaria

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Abstract

Clinical immunity to \emph{P. falciparum} malaria is non-sterilizing, with adults often experiencing asymptomatic infection. Historically, asymptomatic malaria has been viewed as beneficial and required to help maintain clinical immunity. Emerging views suggest that these infections are detrimental and constitute a parasite reservoir that perpetuates transmission. To define the impact of asymptomatic malaria, we pursued a systems approach integrating antibody responses, mass cytometry, and transcriptional profiling of individuals experiencing symptomatic and asymptomatic \emph{P. falciparum} infection. Defined populations of classical and atypical memory B cells and a \(T_{\text{H}2}\) cell bias were associated with reduced risk of clinical malaria. Despite these protective responses, asymptomatic malaria featured an immunosuppressive transcriptional signature with upregulation of pathways involved in the inhibition of \(T\)-cell function, and CTLA-4 as a predicted regulator in these processes. As proof of concept, we demonstrated a role for CTLA-4 in the development of asymptomatic parasitemia in infection models. The results suggest that asymptomatic malaria is not innocuous and might not support the induction of immune processes to fully control parasitemia or efficiently respond to malaria vaccines.

Keywords: asymptomatic infection; immunity; immunosuppression; malaria; \emph{P. falciparum}

Subject Categories: Immunology; Microbiology; Virology & Host Pathogen Interaction; Molecular Biology of Disease

Introduction

Malaria remains one of the most serious infectious diseases of humans with over 200 million clinical cases and 600,000 deaths estimated in 2020 (World Health Organization, 2021). \emph{Plasmodium falciparum} is the most virulent species of malaria parasites and is responsible for disease syndromes ranging from febrile illness to life-threatening complications including respiratory distress, hypoglycemia, renal failure, pulmonary edema, and cerebral malaria (White & Ho, 1992; Miller et al., 2002). A large body of data supports the concept that disease syndromes result from the sequestration of parasitized red blood cells (RBCs) in vascular beds of target organs (Miller et al., 2002). Inflammatory responses also contribute to severe disease, with high levels of TNF (Molyneux et al., 1993), IFN-\(\gamma\), IL-1\(\beta\) (Pongponratn et al., 2003), and CXCL10 (Wilson et al., 2011) found to be associated with severe \emph{P. falciparum} malaria. Immune mechanisms controlling parasite growth to below the threshold inducing clinical symptoms develop after repeated exposure over several years. Antibodies are important in clinical immunity to malaria with roles that include inhibition of parasite invasion into RBCs (Blackman et al., 1990) and opsonization for phagocytosis (Hill et al., 2013).
The acquisition of long-lived antibody-mediated immunity requires the establishment of germinal centers (GCs) in secondary lymphoid organs, where naïve B cells activated by cognate antigen undergo somatic hypermutation of their immunoglobulin (Ig) genes followed by selection of B-cell clones of high affinity for antigen. These processes require help from T follicular helper (TFH) cells (Vinuesa et al., 2005), which orchestrate GC responses and promote the differentiation of B cells into long-lived plasma cells and memory B cells (MBCs) (Crotty, 2014). Recent studies in infection models and human malaria revealed that inflammatory cytokines produced in response to acute malaria such as IFN-γ play an important role in modulating the development of these processes, by upregulating expression of the T helper 1 (Th1)-defining transcription factor T-bet in TFH cells and GC B cells. While T-bet expression in TFH cells impairs their differentiation (Ryg-Cornejo et al., 2016), thereby reducing the magnitude of the antibody response (Obeng-Adjei et al., 2015; Ryg-Cornejo et al., 2016), T-bet expression in B cells promotes the differentiation of cells with increased affinity for antigen, thereby improving the quality of the antibody response (Ly et al., 2019). Thus, the same inflammatory pathways mediating disease symptoms modulate the acquisition of antibody-mediated clinical immunity.

Clinical immunity to malaria is not sterilizing, and adults in endemic areas often experience asymptomatic infections. Field studies have shown that antibodies to P. falciparum antigens are rapidly lost in the absence of ongoing exposure to the parasite (Fruh et al., 1991; Kinyanjui et al., 2007; Weiss et al., 2010), suggesting that asymptomatic infections might be required to sustain clinical immunity (Kinyanjui et al., 2004). Aligned with that view, the presence of asymptomatic P. falciparum infections at the end of the dry season was found to reduce the risk of febrile illness in the ensuing malaria season (al-Yaman et al., 1997; Bereczky et al., 2007; Doumbo et al., 2014). Thus, despite perpetuating a parasite reservoir that deters malaria elimination efforts (Schneider et al., 2007; Sattabongkot et al., 2018), asymptomatic malaria has been long viewed as beneficial to help reduce the risk of severe disease. However, emerging evidence suggests that persistent asymptomatic malaria infections could be detrimental, with important health, developmental, and productivity consequences (Chen et al., 2016). Furthermore, recent studies revealed that treatment of asymptomatic individuals before the start of transmission season does not increase their risk of symptomatic malaria upon re-infection (Portugal et al., 2017), suggesting that subclinical infections might not always be required to maintain clinical immunity.

To date, the real impact that asymptomatic malaria has on the host and whether these infections should be treated or not remain controversial. To address these issues, we pursued a systems biology approach integrating antibody profiling, high-dimensional mass cytometry, and peripheral blood mononuclear cell (PBMC) transcriptomic analysis in individuals from a malaria-endemic area experiencing symptomatic and asymptomatic P. falciparum infection. Antibody responses to parasite invasion ligands, populations of classical and atypical MBCs as well as T helper 2 (Th2) cells were associated with a reduced risk of clinical malaria in asymptomatic individuals. Despite these protective responses, asymptomatic P. falciparum malaria was also characterized by a strong immunosuppressive transcriptional signature with the upregulation of several inhibitory pathways and featuring cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) as a predicted regulator in these processes. Thus, our results suggest that subclinical malaria infections are not benign and do not support the development of immune processes required for the thorough control of parasite replication. As proof of concept, we demonstrated a role for CTLA-4 in the development of asymptomatic recrudescent parasitemia in infection models.

### Results

#### Cohort characteristics

The study recruited symptomatic ($n = 30$) and asymptomatic ($n = 40$) $P. falciparum$-infected individuals, as well as light-microscopy and PCR parasite-negative healthy community controls ($n = 31$). Papuans from the Timika region reside both in the lowlands where malaria exposure is common and in the highlands where malaria is absent. Migration of non-immune adults from the highlands to lowlands means a first infection and symptomatic malaria can occur in all age groups. There were no significant differences in age or gender composition between the symptomatic, asymptomatic, and healthy controls (Fig 1A and B). As expected, mean parasitemia was higher in the symptomatic group compared with the asymptomatic group (Fig 1C). No significant differences were observed between hemoglobin or hematocrit levels (Fig 1D and E), but platelet counts were significantly lower in the symptomatic group compared with both the healthy control and asymptomatic malaria groups (Fig 1F).

Assessment of antibody responses to a $P. falciparum$ parasite lysate confirmed that not only infected participants but also healthy controls had been previously exposed to $P. falciparum$, with high parasite-specific IgG titers detected in all individuals from each group (Fig 1G). Erythrocyte-binding antigens (EBAs) and $P. falciparum$ reticulocyte binding protein-like homologs (PIRh2s), two protein families involved in invasion of the parasite into the RBC, are well-defined targets of naturally acquired immunity to malaria (Cowman & Crabb, 2006). Total IgG levels against EBA-175, EBA-140, PIHR2 (a/b common region), PfRh4, PfRh5, and PIHR5-interacting protein (PIRipr) were examined to identify associations between antibody responses and symptomatic malaria. In general, antibody titers to PfRh5 and PIRipr were lower across the cohort compared with the other invasion ligands and rarely detected among symptomatic individuals (Fig 1H). While IgG levels to EBA-140 increased with parasite levels, antibody titers to PfRh5 and PIRipr were negatively correlated with parasitemia (Fig 1I). Furthermore, logistic regression analysis revealed that IgG levels against PIHR5 and PIRipr in asymptomatic individuals and healthy immune controls significantly reduced the odds of symptomatic infection (Fig 1J). Thus, all participants in the cohort had been exposed to $P. falciparum$ malaria, and asymptomatic individuals and healthy immune controls generated circulating antibodies able to reduce the odds of symptomatic infection.

#### High-dimensional mass cytometry identifies subsets of memory B cells and T cells associated with protection from symptomatic $P. falciparum$ infection

To identify cellular responses associated with reduced risk of clinical $P. falciparum$ malaria, PBMCs from symptomatic and
Figure 1.
asymptomatic *P. falciparum*-infected individuals as well as healthy immune controls were stained with a panel of metal-labeled antibodies specific for a range of B-cell and T-cell markers (Reagents and Tools Table) and analyzed by mass cytometry. In the memory CD4⁺ T cell pool, expression of CXCR3 and CCR6 allows the identification of T_{H1}-like CD4⁺ T cells (CXCR3⁺CCR6⁻) and T_{H2}-like CD4⁺ T cells (CXCR3⁻CCR6⁺), whereas memory T_{H1} cells may be identified by expression of CXCR5 (CD4⁺CXCR5⁺) (Fig EV1A). To reveal the cellular complexity within these pools, FlowSOM clustering was applied within each gated population. This allowed the identification of six subsets of T_{H1}-like CD4⁺ T cells, T_{H2}-like CD4⁺ T cells, and T_{H3} cells, with different expression levels of CD27, CD127, and CD25 (Fig 2A–C). Most but not all CXCR3⁺T_{H1}-like CD4⁺ T cells expressed T-bet (Fig 2A). Within T_{H2}-like CD4⁺ T cells, PD-1 expression was in general low (Fig 2B). Two subsets of T-bet⁺ T_{H2} cells were detected, as well as multiple clusters of CXCR3⁻CCR6⁺ cells within the T_{H3} pool (Fig 2C).

Within the circulating MBC pool, expression of the CD21 and CD27 markers enables the identification of CD21⁺CD27⁺ classical, CD21⁺CD27⁻ atypical, and CD21⁻CD27⁺ activated MBCs (Fig EV1B). FlowSOM clustering was performed in each population, allowing for the identification of six sub-populations of classical, atypical, and activated MBCs (Fig 2D–F). Most classical MBC clusters expressed high levels of CD45RA, whilst expression of chemokine receptors (CXCR5 and CCR6), IgD, and IgM differed across clusters (Fig 2D). Several clusters of IgD⁺IgM⁺, class-switched MBCs were also identified. Downstream flow cytometry experiments revealed that most of these cells expressed IgG. IgM⁺IgD⁺ and class-switched cells were found within clusters of the three MBC sub-populations, with populations in the atypical and activated MBC pool co-expressing the transcription factor T-bet (Fig 2D–F).

Unsupervised identification of differentially abundant cell populations between clinical groups was performed using the CITRUS algorithm (FDR < 5%) (Bruggner et al., 2014). Populations of CD27⁺CD127⁺low T_{H1}-like CD4⁺ T cells were significantly reduced in symptomatic individuals, relative to asymptomatic counterparts (Fig 3A). In contrast, a population of CXCR3⁻CD4⁺ T cells, expressing high T-bet levels was significantly increased in symptomatic individuals compared to healthy immune controls (Fig 3B). Within the T_{H2} memory pool, a CD127⁺ cluster, expressing low levels of CD25 was enriched among healthy immune controls relative to symptomatic participants (Fig 3B). Two clusters of T_{H2} polarized T_{H1} cells expressing CD127 and CD27 were also increased in both healthy immune controls and asymptomatic individuals compared to symptomatic counterparts (Fig 3C). CITRUS analysis revealed four main populations of classical MBCs differentially abundant between clinical groups. Whereas CXCR5⁺CCR6⁺ class-switched and IgM⁺ MBCs were higher in symptomatic individuals, class-switched and IgD⁺IgM⁺low MBCs expressing low CXCR5 and CCR6 levels were significantly enriched in asymptomatic participants and healthy immune controls (Fig 3D). Two subsets of IgM⁺IgD⁺ MBCs among the classical and atypical compartments were also significantly higher in asymptomatic compared to symptomatic participants (Fig 3D and E).

To define associations between cell populations and risk of symptomatic malaria, logistic regression models were applied (Benjamini–Hochberg adjusted FDR<5%). Consistent with previous studies (Obeng-Adjei et al., 2015; Ryg-Cornejo et al., 2016), PD-1⁺CXCR3⁺T-bet⁺T_{H1} cells were associated with increased odds of *P. falciparum* symptomatic malaria (Fig 3F). Similarly, CXCR5⁺CCR6⁺ IgM⁺ classical and activated MBCs were associated with increased odds of symptomatic infection. In contrast, IgD⁺IgM⁺low classical MBCs and two populations of atypical MBCs (IgM⁺IgD⁺ and class-switched T-bet⁺ cells) expressing low or no CXCR5 and CCR6, along with CD127⁺CD25⁺low T_{H2}-like memory CD4⁺ T cells, were associated with reduced odds of symptomatic infection (Fig 3F). Thus, diverse MBCs expressing low chemokine receptor levels and a CD4⁺ T_{H2} cell bias were associated with reduced odds of clinical malaria.

**RNA sequencing of PBMCs segregates transcriptional profiles of symptomatic and asymptomatic *P. falciparum* malaria and healthy immune controls**

To identify molecular pathways associated with the acquisition of clinical immunity, selected *P. falciparum*-infected symptomatic and asymptomatic study participants, as well as healthy immune controls, were chosen for transcriptional profiling by RNA-seq (RNA-seq). Clinical parameters, parasite-specific antibody responses, and frequencies of CyTOF clusters in these samples were representative of those observed in the entire cohort (Fig EV2). As our mass cytometry analysis focused only on MBCs and CD4⁺ T cells, we used the cell-type deconvolution package dtan-gle (Hunt et al., 2019) with hematopoietic cell RNA-seq data (Choi et al., 2019) as a reference dataset to estimate the proportion of all PBMC populations in the blood (Fig 4A). No significant enrichment was observed in frequencies of total MBCs, CD4⁺ and CD8⁺ T cells, NK cells, monocyte subsets, and dendritic cells across the three clinical groups.
Multi-dimensional scaling of transcriptional profiles revealed a good level of segregation between symptomatic *P. falciparum* -infected individuals and asymptomatic participants, with a small degree of overlap between these individuals and healthy immune controls (Fig 4B). Clinical groups were then incorporated as a factor into linear modeling for gene expression estimation and

Figure 2. Identification of MBC and memory CD4+ T-cell sub-populations induced by *P. falciparum* symptomatic and asymptomatic infection.

PBMCs from *P. falciparum* symptomatic (n = 16) and asymptomatic (n = 24) infected individuals as well as healthy immune controls (n = 24) were stained with a panel of metal-labelled antibodies and analyzed by CyTOF. t-distributed Stochastic Neighbor Embedding (tSNE) analysis was performed and FlowsOM clustering was used to identify individual cell subpopulations within gated:

A. T\(_{H1}\)-like memory CD4+ T cells (CD19^+^CD3^+^CD4^+^CD45RA^+^CCR6^+^CXCR3^+^)
B. T\(_{H2}\)-like memory CD4+ T cells (CD19^+^CD3^+^CD4^+^CD45RA^-^CCR6^-^CXCR3^-^)
C. Circulating memory T\(_{FH}\) cells (CD19^+^CD3^-^CD4^-^CD45RA^-^CXCR5^-^)
D. Classical MBCs (CD3^-^CD19^-^CD20^-^CD10^-^CD27^-^CD21^-^)
E. Atypical MBCs (CD3^-^CD19^-^CD20^-^CD10^-^CD27^-^CD21^-^)
F. Activated MBCs (CD3^-^CD19^-^CD20^-^CD10^-^CD27^-^CD21^-^)

The tSNE plots in the top panel display cell density and represent the pooled data for each group, while the lower panel shows a projection of the FlowsOM clusters on a tSNE plot. Heatmaps show the median marker expression for each FlowsOM cluster.
Figure 3. High-dimensional mass cytometry identifies subsets of MBCs and T cells associated with reduced risk of symptomatic *P. falciparum* malaria.

Unsupervised identification of differentially abundant high-dimensional cell populations between clinical groups was performed using CITRUS (FDR < 5%).

A–E tSNE plots of clinical groups and viSNE plots depicting expression of selected surface markers, relative abundance and cellular phenotype of differentially abundant clusters identified among CXCR3+CCR6+ memory CD4+ T cells (A), CXCR3–CCR6+ memory CD4+ T cells (B), circulating memory T_{Mn} cells (C), classical MBCs (D), and atypical MBCs (E). The tSNE plots in the top or each panel display cell density and represent pooled data for each group as calculated in the clustering analysis shown in Fig 2A–E, while the viSNE plots on each top panel depict relevant marker expression on tSNE overlays. The lower panels from left to right show differentially abundant populations identified in purple on a tSNE overlay, whiskers showing the range (minimum to maximum), with lines representing the median of 16 (symptomatic), 24 (asymptomatic), and 24 (healthy immune controls) biological replicates, while the pink histograms illustrate marker expression in identified differentially abundant populations, relative to background expression, shown in lilac.

F Odds ratios as determined by logistic regression showing associations between cell frequencies and the risk of symptomatic *P. falciparum* infection. Symbols represent the odds ratio estimated using 16 (symptomatic, SM), 24 (asymptomatic, AM), and 24 (healthy immune controls, HC) biological replicates, and vertical lines depict the 95% confidence interval. *P < 0.05, **P < 0.01, ***P < 0.001.
RNA sequencing of PBMCs segregates transcriptional profiles of symptomatic and asymptomatic *P. falciparum* malaria and healthy immune controls.

PBMCs from *P. falciparum* symptomatic (n = 6, SM) and asymptomatic (n = 5, AM) infected individuals as well as healthy immune controls (n = 6, HC) were selected for RNA-seq analysis.

A Estimated proportions of PBMC subpopulations determined from cell-type deconvolution from study participant transcriptional profiles.

B Unsupervised multidimensional scaling of the top 1,000 most variably expressed genes across all samples.

C Venn diagram of the number of differentially expressed genes between clinical groups determined at a false discovery rate (FDR) of 15%.

D Blood transcriptional module (BTM) analysis showing significant BTMs differentially enriched for pairwise comparisons (FDR<15%).
identification of differentially expressed genes, whereby the greatest difference was found between symptomatic malaria and healthy immune controls (Fig 4C).

To identify immunological processes underlying differences in transcriptional profiles, differential enrichment was assessed with tmod (preprint: Weiner & Domaszewska, 2016) using pairwise comparisons between clinical groups and blood transcriptional modules (BTMs) (Li et al, 2013) as gene sets (Fig 4D). Multiple modules involved in cell division, cell development, and cell cycle were enriched in symptomatic individuals compared with both asymptomatic participants and healthy immune controls. Conversely, B-cell differentiation and nuclear receptor subfamily 4 modules were significantly reduced in symptomatic individuals compared to asymptomatic counterparts and healthy immune controls. Interestingly, enrichment of complement, cell adhesion, and chemokine cluster modules was observed in symptomatic malaria only when compared against asymptomatic individuals, suggesting that persistent infections of low parasitemia have a regulatory effect on the induction of those pathways (Fig 4D).

**Symptomatic *P. falciparum* malaria infection drives transcriptional profiles supporting inflammatory processes and fatty acid metabolism**

Unsupervised hierarchical clustering of the 922 genes differentially expressed between *P. falciparum* symptomatic participants and healthy immune controls revealed clear segregation of transcriptional profiles (Fig 5A). Gene set enrichment using gene ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and Ingenuity Pathway Analysis (IPA) showed significant upregulation of terms involved in cell proliferation in symptomatic malaria (Fig 5B–E), including genes encoding cyclins such as CCNB1 and CCNB2, MKI67, replication factors such as CDT1 as well as mitotic checkpoint kinases like BUB1 (Fig 5B). In addition, multiple stress response pathways were upregulated during symptomatic infection (Fig 5C–E), along with terms involved in cholesterol and fatty acid metabolism. Various genes associated with inflammatory responses were upregulated in symptomatic *P. falciparum* malaria, including SLAMF7, SLAMF8, CXCL9, IFNG, JAK3, C1QC, C1QB, and LAG3 (Fig 5B).

Upstream regulator analysis identified increased activation of the transcription regulator Non-POU domain-containing octamer-binding protein (NONO) and the cytokines IFN-γ and IL-13 in symptomatic individuals compared to healthy immune controls (Fig 5F). Whereas several genes involved in stress response and inflammatory processes were predicted as targets of NONO and IFN-γ-mediated pathways, the TOP2A-cytokine IL-13 was predicted to control genes involved in cholesterol biosynthesis and fatty acid metabolism (ACADVL, DHCR24, ENPP2, FADS2, and HSD11B1). Interestingly, many IL-13 and IFN-γ-predicted target genes upregulated in symptomatic individuals were significantly correlated with each other (Fig 5G and H), suggesting that IL-13-polarized responses become activated during acute malaria with the potential to help meet the energy demands posed by the inflammatory response to symptomatic infection.

Among the pathways downregulated during symptomatic infection were regulation of gene expression, metabolic processes, and signaling (Fig 5B–E). These included genes encoding DNA-binding elements and transcription factors such as NAB2, SP3, FOXN3, as well as the nuclear receptors NR4A3, NR4A2, and NR1D2, known to modulate immune cell activation, cell migration, apoptosis, and cell metabolism (Fig 5B). Thus, concomitant with a proliferative/inflammatory transcriptional signature, symptomatic malaria appears to impact the control of cellular processes by altering the expression of transcriptional regulators.

**Asymptomatic *P. falciparum* malaria drives a transcriptional profile supporting immunosuppressive processes**

IPA of the 418 genes differentially expressed between *P. falciparum* symptomatic and asymptomatic individuals showed significant upregulation of cell proliferation canonical pathways in response to symptomatic infection (Fig 6A). In contrast, cell cycle checkpoint control, together with p53 signaling, were among the pathways downregulated in symptomatic relative to asymptomatic *P. falciparum* malaria (Fig 6A). As in Fig 5, upstream regulator analysis predicted activation of IFN-γ and IL-13-mediated pathways in symptomatic malaria (Fig 6B). Notably, the only upstream regulator significantly upregulated by asymptomatic infection was CTLA-4 (Fig 6B). All genes in the dataset predicted to be controlled by CTLA-4 were involved in the control of cell proliferation. To further define transcriptional signatures preferentially activated by asymptomatic or symptomatic infection, hierarchical clustering was performed (Fig 6C). The inclusion of healthy immune controls in this analysis allowed visualization of 4 gene clusters. Two of them had genes either upregulated (cluster 1) or downregulated (cluster 2) in response to symptomatic infection compared to both asymptomatic infection and healthy immune controls (Fig 6C). These groups featured genes involved in response to stress, similar to those described in Fig 5. In contrast, clusters 3 and 4 consisted of genes in which expression profiles were either enriched or diminished by symptomatic or asymptomatic infection relative to homeostatic transcriptional levels from healthy immune controls (Fig 6C–F). A group of integrins (ITGA2B, ITGB3, and ITGB8), proteosome-encoding genes (PSMA4, PSMB5, PSMC2, PSMD1), and cytoskeleton regulators (KIF23, KIF2C, KIF18A) involved in antigen presentation, along with various genes encoding protein products involved in immune defense to microorganisms (LCN2, PRDX1, SUCNR1, BATF2, ALPK1, and IDO2), were upregulated in response to symptomatic malaria but underrepresented in asymptomatic malaria carriers, suggesting that persistent asymptomatic infection may have a suppressive effect in the induction of those responses (Fig 6D–F). Similarly, genes involved in cell proliferation (CCNA2 and TOP2A) predicted to be controlled by CTLA-4 (Fig 6B) were also reduced in asymptomatic individuals (Fig 6E and F). Furthermore, linear regression analysis identified 18 additional genes involved in cell cycle control downregulated by asymptomatic malaria, with transcription patterns correlated with CCNA2 expression (Fig 6G).

Genes in cluster 4 (Fig 6C), underrepresented in symptomatic malaria and upregulated by asymptomatic infection, included several genes encoding protein products mediating negative regulation of immune processes, such as the anti-proliferative molecules BTG1 and NDRG2, as well as the checkpoint receptor CD83 (Fig 6D and E). PLD4 encoding phospholipase D4 and WNT10B implicated in anti-inflammatory responses (Trischler et al, 2016) were also upregulated in this group (Fig 6D–F). Two nuclear receptors described to negatively modulate transcriptional profiles of a range
of immune responses were also overrepresented in asymptomatic individuals: IRF2BP2 encoding the transcriptional repressor IFN regulatory factor 2 binding protein 2 that suppresses CD4+ T-cell proliferation (Secca et al., 2016) and the nuclear receptor NR1D1, a circadian clock gene that controls the transcription of several inflammatory cytokines (Chang et al., 2019; Liu et al., 2020) (Fig 6E and F). Correlation analysis of this immunoregulatory signature with parasitemia levels identified two groups of genes. NR1D1, NDRG2, and PLD4 showed significant negative correlations with parasitemia, suggesting that downregulation of transcription could be driven by high parasite burden and/or the concomitant inflammation associated with symptomatic infection (Fig 6H). In contrast, integrins, cytoskeleton regulators, and anti-microbial genes along with IRF2BP2, CD83, BTG1, and WNT10B with anti-proliferative and anti-inflammatory activity were not a function of high parasitemia (Fig 6H), raising the possibility that low parasitemia asymptomatic P. falciparum infections activate a blood transcriptional profile that drives immunosuppressive processes.
Figure 6.
To further define molecular processes influenced by asymptomatic *P. falciparum* infection, transcriptional profiles of asymptomatic individuals and healthy immune controls were compared (Fig 7A). Within the pathways uniquely downregulated by asymptomatic malaria were response to endogenous stimulus, regulation of cell proliferation, and cell-cell adhesion (Fig 7B). These included genes encoding 7-dehydrocholesterol reductase (*DHCR7*) involved in vitamin D metabolism, myeloperoxidase (*MPO*) with important antimicrobial function, as well as the secreted protein acidic and rich in cysteine (*SPARC*) and vascular cell adhesion protein 1 (*VCAM1*) that play an important role in cell adhesion under inflammatory conditions. *MAP3K7CL* encoding the mitogen-activated protein kinase 7 and *PDE5A* encoding a phosphodiesterase implicated in the control of T-cell function were also significantly downregulated in asymptomatic individuals compared to healthy controls (Fig 7C). Gene set enrichment analysis showed significant upregulation of retinol metabolism, terms involved in negative regulation of T-cell co-stimulation, and nitric oxide synthesis (Fig 7B and C) in response to asymptomatic malaria. These included *RDH13*, encoding retinol dehydrogenase 13, genes involved in NFKB regulation such as *CRIP2* and *PDCD2L*, as well as *NOS3*, encoding nitric oxide synthase. The expression of *ILSRA* encoding the receptor for the *T-helper* cytokine IL-5 was also upregulated in asymptomatic individuals (Fig 7C). Various genes encoding protein products involved in the negative control of B cell (such as *TRPM5*) and T-cell effector function (such as *CD160*), as well as the transcription regulators *EGR3* (Saford et al, 2005) and *FOSB* (Baumann et al, 2003), were also upregulated in *P. falciparum* asymptomatic malaria compared to healthy individuals (Fig 7C). Except for *DHCR7*, which negatively correlated with high parasitemia, transcription profiles of immunoregulatory genes differentially expressed between asymptomatic infected individuals and healthy controls were not correlated with parasitemia levels (Fig 7D). Furthermore, various genes such as *CRIP2*, *TRPM5*, *EGR3*, *PDE5A*, *MAP3K7C*, and *SPARC*, among others, displayed similar levels of upregulation or downregulation in asymptomatic individuals compared to both symptomatic counterparts and healthy controls (Fig 7E). Thus, these results support the notion that asymptomatic *P. falciparum* infections drive a blood transcriptional profile that supports anti-inflammatory and immunosuppressive processes.

**Asymptomatic* P. falciparum* malaria-immunosuppressive transcriptional profiles are not correlated with protective responses to infection**

To integrate immune responses associated with increased or reduced risk of malaria identified by mass cytometry and blood transcriptional signatures, Spearman correlations were applied (Benjamini–Hochberg adjusted FDR < 5%). In general, cell populations overrepresented in asymptomatic malaria were positively correlated with the transcriptional signature, with T-bet+ memory CD4+ T cells and activated IgM+ MBCs featuring the highest

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**Figure 6.** Asymptomatic *P. falciparum* malaria upregulates transcription of pathways involved in immunosuppressive processes. Gene expression profiles of PBMCs from *P. falciparum* symptomatic (SM) and asymptomatic (AM) infected participants were compared.

A. IPA canonical pathway analysis scaled by –log10(P-value) using the 418 differentially expressed genes (DEGs) between symptomatic and asymptomatic *P. falciparum* malaria. Pathways with a positive z-score in red are activated, and pathways with a negative z-score in blue are inhibited in symptomatic compared with asymptomatic *P. falciparum* malaria. The ratios of DEGs found in each pathway over the total number of genes in the pathway are listed on the right. The red line corresponds to a P-value of 0.05.

B. Upstream regulator analysis of the 418 DEGs between *P. falciparum* symptomatic and asymptomatic infected participants. The red lines represent a significant activation z-score of ± 2.

C. Heatmap of the 418 DEGs in *P. falciparum* symptomatic and asymptomatic infected participants as well as in healthy immune controls.

D. Bar plots showing significantly enriched gene ontology (GO) terms scaled by log10(P-value) in clusters 3 and 4 from the heatmap in C. Red GO terms are upregulated and blue GO terms are downregulated in asymptomatic compared to symptomatic *P. falciparum* malaria.

E. Volcano plot displaying selected genes clusters 3 and 4 from heatmap in C scaled by log2-fold-change and -log10(P-value) differentially expressed between *P. falciparum* symptomatic and asymptomatic infected individuals. Genes in red are overrepresented in symptomatic malaria and genes in blue are overrepresented in asymptomatic malaria.

F. Mean Reads Per Kilobase of transcript per Million mapped reads (RPKM) ± SEM of selected genes in *P. falciparum* malaria symptomatic (n = 6) and asymptomatic (n = 5) individuals. Dotted red lines depict transcriptional levels of healthy immune controls, Mann–Whitney test of biological replicates, *P < 0.05.

G. Hierarchical clustering heatmap of genes associated with CCNA2 identified by linear regression analysis (FDR < 5%).

H. Spearman correlation matrix (Benjamini–Hochberg adjusted FDR < 5%) between parasitemia levels and immunoregulatory genes differentially expressed between symptomatic and asymptomatic *P. falciparum*-infected individuals. Significant positive correlations are shown in blue and significant negative correlations are shown in red.

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**Figure 7.** Asymptomatic *P. falciparum* malaria upregulates transcription of pathways involved in immunosuppressive processes. Gene expression profiles of bulk PBMCs from *P. falciparum*-infected asymptomatic individuals (AM) and healthy immune controls (HC) were compared.

A. Hierarchical clustering heatmap of map 171 of the 171 significantly expressed genes (DEGs) in *P. falciparum* asymptomatic and healthy immune control participants.

B. Bar plots showing significantly enriched gene ontology (GO) terms scaled by log10(P-value) within clusters 1 and 2 from the heat map shown in A. Red GO terms are upregulated and blue GO terms are downregulated in asymptomatic *P. falciparum* malaria compared to healthy immune controls.

C. Mean-difference plot displaying DEGs between asymptomatic *P. falciparum* malaria and healthy immune controls. Each gene is plotted as a single point determined by log-fold-change and average transcript abundance. Red genes are overexpressed, and blue genes are underexpressed in asymptomatic malaria.

D. Spearman correlation matrix (Benjamini–Hochberg adjusted FDR < 5%) between parasitemia levels and immunoregulatory genes differentially expressed between asymptomatic *P. falciparum*-infected individuals and healthy immune controls. Significant positive correlations are shown in blue and significant negative correlations are shown in red.

E. Mean RPKMs ± SEM of selected genes in *P. falciparum* malaria asymptomatic individuals (n = 5) and healthy immune controls (n = 6). Dotted red lines depict transcriptional levels of *P. falciparum*-infected symptomatic individuals, Mann–Whitney test of biological replicates, *P < 0.05.
number of associations with gene expression profiles (Fig EV3). Whereas expression of genes involved in cell cycle progression, inflammatory and stress responses positively correlated with frequencies of T-bet+ memory CD4+ T cells (Figs 8A and EV3A and B), genes involved in cell proliferation and telomerase recruitment were associated with the expansion of the activated IgM+ MBC.
compartment (Figs 8B and EV3C and D). Frequencies of activated IgM + MBCs, associated with increased odds of symptomatic infection, were also positively correlated with classical IgM + MBCs and with CXCR3 + TFH cells (Figs 8C and EV4A and B). Together, these findings suggest that the highly inflammatory milieu during symptomatic infection supports a TH1-polarized TFH cell compartment, which induces activation and expansion of this MBC pool (Fig 8C).

Populations of class-switched and IgD + IgM low MBCs associated with reduced odds of symptomatic infection, that were overrepresented in asymptomatic individuals and healthy immune controls, were highly correlated with each other and were also positively associated with a population of CD27 + Tbet-polarized Tfh cells. This suggests that help from this Tfh cell lineage sustains the expansion of MBCs contributing to clinical immunity (Figs 8D and EV4A and C). Transcription levels of most genes enriched in asymptomatic infection were positively associated with each other (Fig 8D). Unlike symptomatic malaria, where blood transcriptional profiles showed coherent correlations with key immune cell populations expanding in response to infection, virtually no associations were found between the asymptomatic transcriptional signature and cells.
Figure 9.
Figure 9. CTLA-4 is upregulated in memory CD4+ T cells in asymptomatic malaria and contributes to the development of clinically silent parasitemia.

A. Estimated proportions of PBMC subpopulations determined from cell-type deconvolution featuring transcriptional profiles upregulated in asymptomatic individuals.

B-G. PBMCs from P. falciparum symptomatic (n = 6, SM) and asymptomatic (n = 6, AM) infected individuals as well as healthy immune controls (n = 6, HC) were stained with a panel of antibodies and analyzed by flow cytometry. tSNE plots (B) display cell density of the pooled data for each group, (C) shows a projection of the FlowSOM clusters on a tSNE plot, and (D) is a heatmap depicting the cellular phenotype of clusters of CTLA-4+CD3+CD19+ cells. Bar plots representing mean relative abundance of biological replicates ± SEM of relevant clusters among clinical groups (E), Mann–Whitney test, *P < 0.05. The panel on the left shows the average CTLA-4 mean fluorescent intensity (MFI) or 6 biological replicates in cluster 4 ± SEM, Mann–Whitney test, **P < 0.01, while representative histograms of CTLA-4 expression in cluster 4 are shown on the right (F). Marker intensity within clusters 1 and 6 (G).

H. C57BL/6 mice (n = 10) were infected with P. chabaudi chabaudi. Percentage parasitemia of infected mice was determined every 2–3 days. Symbols represent average parasitemia ± SEM.

I-Q. Splenocytes from P. chabaudi chabaudi infected mice were stained with fluorescent antibodies and analyzed by flow cytometry. (I) Representative contour plots showing CTLA-4-expressing cells among Treg and CD4+CD44+CD62Llow T cells. (J, K) Percentage of CTLA-4+ cells among CD4+FoxP3+ Treg cells (J) and CD4+ activated CD4+ T cells (K) at peak (day 8 p.i.) and recrudescence (day 20 p.i.) parasitemia and after infection resolution (day 40 p.i.). Bar plots represent mean percentage of 6 biological replicates ± SEM (unpaired t-test, ***P < 0.005, *P < 0.05). (L–N) tSNE plots (L) display cell density of the pooled data for each group, (M) shows a projection of the FlowSOM clusters on a tSNE plot, and (N) is a heatmap depicting the cellular phenotype of CTLA-4+ conventional CD4+ T cells during peak and recrudescent parasitemia. (O) Bar plots represent the average relative abundance of 6 biological replicates in relevant clusters ± SEM (unpaired t-test, **P < 0.01). (P and Q) Representative contour plots (P) and absolute number (Q) of CTLA-4+CD4+CCR7+CD62Llow central memory (CM) and CD4+CD44+CCR7+CD62Llow effector memory (EM) cells during peak and recrudescent parasitemia. Symbols represent the mean cell number of 6 biological replicates ± SEM (unpaired t-test, ***P < 0.05).

R. C57BL/6 mice (n = 6) were infected with P. chabaudi chabaudi and treated with anti-CTLA-4 or isotype control every 3 days from day 13 p.i. as indicated by the red arrows. Parasitemia was determined every 2–3 days. Bar plots represent mean parasitemia of biological replicates ± SEM (unpaired t-test, **P < 0.01, *P < 0.05).

associated with reduced risk of clinical malaria (Figs 8D and EV3E). Thus, these results suggest that two independent processes with presumably different etiologies operate during asymptomatic malaria: an antibody-dependent immune response that reduces the risk of symptomatic infection, and an immunoregulatory signature capable of reducing immune cell effector function.

CTLA-4 is upregulated in memory CD4+ T cells in asymptomatic malaria and contributes to the development of clinically silent parasitemia

To infer potential cellular sources of the immunoregulatory transcriptional signature featured by asymptomatic P. falciparum malaria, cell-type-specific marker genes were examined using tangle. This analysis predicted CD4+ T cells as the main sources of asymptomatic malaria transcriptional profiles (Fig 9A). We then sought to provide proof of concept for the regulatory pathways identified by our bioinformatic analysis in the control of asymptomatic infection. Since CTLA-4 was predicted as a potential regulator in these processes (Fig 6) and is expressed by CD4+ T cells, this pathway was selected for further analysis. Flow cytometry followed by high-dimensional analysis of CD3+CTLA-4+ cells identified specific populations expressing this receptor in response to symptomatic and asymptomatic P. falciparum malaria. Although similar frequencies of CTLA-4+CD4+FoxP3+ regulatory cells (Treg) were found between groups, CTLA-4 expression in this compartment was twice as high in symptomatic compared to asymptomatic infection (Fig 9A–F). CTLA-4+CD4+ T cells expressing high CD25 levels were also increased in symptomatic infection, but conventional CD45RA− memory CD4+ T cells became important sources of CTLA-4 expression during asymptomatic infection compared to symptomatic counterparts (Fig 9A–G). To extend these observations and provide mechanistic evidence for the role of CTLA-4 in the control of asymptomatic parasitemia, C57BL/6 mice were infected with P. chabaudi chabaudi, which results in a peak of parasitemia around day 8 post-infection (p.i.) that is resolved after two weeks and followed by asymptomatic recrudescences of low parasitemia (Fig 9H). Although these recrudescences differ from persistent clinically silent infections seen in human malaria, they provide a valuable tool to investigate the role of regulatory pathways in asymptomatic parasitemia. Frequencies of CTLA-4-expressing cells were then examined among gated FoxP3+CD25+CD4+ Treg cells and CD4+ activated CD4+ T cells by flow cytometry. When animals were experiencing peak parasitemia on day 8 p.i., the percentage of CTLA-4+ cells significantly increased in both Treg and CD4+ T-cell compartments (Fig 9I–K). Whereas frequencies of CTLA-4+ Treg cells returned to normal levels after resolution of peak parasitemia, they remained significantly higher than uninfected controls among CD4+ T cells when mice were experiencing asymptomatic recrudescence on day 20 p.i. (Fig 9I–K). tSNE and FlowSOM analysis of gated CTLA-4+CD4+ T cells revealed clear differences in the composition of the CTLA-4+ pool expanding during peak parasitemia and asymptomatic recrudescence, with high frequencies and absolute numbers of CD4+CCR7+CD62Llow effector memory phenotype, coinciding with the onset of recrudescent infection (Fig 9L–Q). Thus, CTLA-4 expression remains elevated among memory CD4+ T cells in both human and mouse asymptomatic malaria. Furthermore, administration of anti-CTLA-4 blocking antibodies to P. chabaudi chabaudi-infected mice from day 13 p.i. resulted in a 10-fold reduction in recrudescent parasitemia levels compared to controls (Fig 9R), providing proof of concept for CTLA-4 as an immunoregulatory pathway contributing to the development of persistent asymptomatic parasitemia after malaria infection.

Discussion

This study pursued a systems biology approach integrating P. falciparum-specific antibody responses, multidimensional mass cytometry, and blood transcriptional profiling in adults from a malaria-endemic area of Indonesia to understand cellular and molecular processes operating during asymptomatic P. falciparum infection. Aligned with the concept of clinical immunity, our findings identified antibody responses to parasite invasion ligands along with specific populations of classical and atypical MBCs associated with reduced odds of symptomatic infection in asymptomatic individuals. Notably, asymptomatic P. falciparum malaria was also
characterized by an important immunoregulatory blood transcriptional signature with the upregulation of various pathways involved in the inhibition of CD4+ T-cell function. Thus, our findings suggest that although these sub-clinical infections allow the development of antibody responses that reduce parasitemia levels to below the threshold required to induce clinical symptoms, they might not entirely support the effective induction of cellular immune processes required for the thorough control of parasite replication.

Aligned with previous RNA-seq analyses (Tran et al., 2016; Lee et al., 2018; Bediako et al., 2019) and a well-defined role for IFN-γ in acute malaria (Pongponratn et al., 2003), transcriptional profiles of *P. falciparum* symptomatic malaria predicted this cytokine as an upstream regulator of the stress response induced during infection. Concomitant with this inflammatory response, symptomatic malaria was also characterized by the upregulation of enzymes required for fatty acid metabolism. Our bioinformatic approach predicted the T<sub>12</sub> cytokine IL-13 as the regulator responsible for these processes. Recent studies revealed that IL-13 plays a key role in driving metabolic conditioning during endurance exercise by preserving glycolgen in favor of fatty acid oxidation (Knudsen et al., 2020). Thus, although upregulation of type-2 cytokines in response to malaria has been historically proposed to ameliorate the effects of excessive inflammatory responses to infection, our findings here are consistent with a model in which T<sub>12</sub> pathways might become activated during acute malaria to help meet the energy demands posed by the inflammatory response to symptomatic infection.

The high-dimensional unsupervised single-cell mass cytometry approach used in this study allowed us to dissect distinct T<sub>H1</sub> and T<sub>H2</sub>-polarized memory CD4+ T cells, T<sub>REG</sub> cells, and specific subpopulations of classical and atypical MBCs associated with either increased or reduced odds of *P. falciparum* symptomatic malaria. Similar to *P. vivax* malaria (Ioannidis et al., 2021), class-switched T<sup>bet</sup>CCR6<sup>-</sup> atypical MBCs were associated with reduced odds of *P. falciparum* malaria, suggesting that specific subsets within this cell lineage, previously viewed as a predictor of poor infection outcomes (Obeng-Adjei et al., 2017), might play a beneficial role in the control of parasite burden. Unlike *P. vivax* malaria, in which only class-switched MBCs were associated with reduced odds of symptomatic infection (Ioannidis et al., 2021), populations of both classical and atypical IgD<sup>-</sup>IgM<sup>-low</sup> MBCs were found to be associated with protection from symptomatic *P. falciparum* infection. Parasite-specific IgM<sup>-</sup> MBCs have been detected in *P. falciparum*-exposed individuals (Krishnamurty et al., 2016) and IgM antibodies specific for blood-stage antigens have been found to be associated with protection from symptomatic malaria (Arama et al., 2015; Boyle et al., 2019). Unlike class-switched MBCs, IgD<sup>-</sup>IgM<sup>-</sup> MBCs have the capacity to differentiate into GC B cells upon re-stimulation (Seifert et al., 2015). Consistently, mouse malaria studies found that IgM<sup>-</sup> MBCs adopt a GC B-cell phenotype upon secondary infection (Pietrzak et al., 2020), while sequencing studies revealed that IgM<sup>-</sup> MBCs acquire further mutations upon *P. falciparum* re-infection in children (Wendel et al., 2017), suggesting a role for unswitched MBCs in B-cell receptor repertoire expansion and remodeling during malaria.

 Whereas several transcriptional profiles of individuals presenting with symptomatic *P. falciparum* malaria have been successfully described (Tran et al., 2016; Bediako et al., 2019; Boldt et al., 2019; Nallandhigbal et al., 2019), blood transcriptional activity induced in response to asymptomatic infection is still underreported. A longitudinal study following *P. falciparum*-asymptomatic individuals through the dry season found transcriptional changes in several metabolic pathways (Andrade et al., 2020). Whole-blood microarray analysis of children with *P. falciparum* asymptomatic malaria found chromatin remodeling-driven gene regulation to contribute to the maintenance of an asymptomatic status (Boldt et al., 2019). Similarly, regulatory genes involved in chromatin remodeling were differentially expressed in monocytes from malaria-infected men living in Burkina Faso, with lower susceptibility to *P. falciparum* malaria than sympatric tribes (Quin et al., 2017). Whole-blood transcriptional profiling of children who differed in their capacity to control parasitemia and fever following *P. falciparum* infection revealed a signature of p53 activation at baseline in participants who remained asymptomatic upon re-infection (Tran et al., 2019), thereby identifying a critical pathway in clinical immunity. Despite the development of protective MBCs and antibody responses in our study, transcriptional profiling revealed several integrins, proteosome-encoding genes, and cytoskeleton regulators involved in antigen presentation underrepresented in asymptomatic malaria carriers relative to symptomatic counterparts, suggesting that persistent asymptomatic low-parasitemia infection might have a detrimental effect in the induction of those pathways. Furthermore, our analysis identified significant downregulation of genes involved in the control of cell proliferation in asymptomatic malaria and predicted the inhibitory receptor CTLA-4 as the upstream regulator responsible for these processes. Upregulation of CTLA-4 in T<sub>REG</sub> cells and CD4<sup>+</sup> T cells has previously been shown in human (Goncalves-Lopes et al., 2016; Mackroth et al., 2016) and mouse malaria infection models (Haque et al., 2010; Hafalla et al., 2012), and its expression has been associated with restricting pathogenic cellular responses during acute infection, at the expense of inhibiting cell proliferation (Mackroth et al., 2016) and antibody responses to infection (Kurup et al., 2017). While CTLA-4 blockade improved control of acute parasitemia in mice, it was also found to worsen experimental cerebral malaria disease outcomes (Jacobs et al., 2002; Lepenies et al., 2007), suggesting a protective role for this receptor in the development of T-cell-mediated organ-specific syndromes during acute infection. In all the above-mentioned settings, upregulation of CTLA-4 was always documented in response to acute malaria. Using human studies and mouse malaria infection models, our results revealed a novel and previously unexplored role for CTLA-4, showing that low parasitemia levels in clinically silent persistent infections are also capable of activating this suppressive pathway, thereby preventing full control of parasite replication. Further work is required to determine if upregulation of CTLA-4 in these settings arises to prevent pathogenic responses potentially caused by persistent low parasitemia infections.

Concomitant with the downregulation of genes promoting cell proliferation, our approach identified various transcriptional regulators with immunoregulatory and anti-inflammatory functions arising in the blood in response to asymptomatic *P. falciparum* malaria. These included B-cell translocation gene 1 (BTG1), a member of the BTG/transducer of Erb family known to inhibit cell proliferation (Rouault et al., 1992), and the early growth response gene 3 (EGR3) that has recently emerged as a regulatory molecule that is able to suppress excessive CD4<sup>+</sup> T-cell responses (Singh et al., 2017). Expression levels of the transcriptional repressor IFN regulatory
factor 2 binding protein 2 (IRF2BP2), that restrains CD4+ T-cell activation (Secca et al., 2016), were also upregulated by asymptomatic infection. More mechanistic work is now needed to examine if activation of these pathways during asymptomatic infection also modulates the control of low parasitemia infection.

Without the deployment of a highly effective vaccine, there has been an increased focus on interventions to reduce malaria transmission, including administration of artemisinin combined therapies and long-lasting insecticide-treated bed nets (Smithson et al., 2015). A central issue in the malaria eradication agenda is the challenge posed by asymptomatic infection, as this clinically silent parasite reservoir is known to perpetuate transmission (Bousema et al., 2004). Strategies of antimalarial mass drug administration to at-risk populations or mass screening and treatment of asymptomatically infected individuals have been considered. However, this has raised concerns about whether these strategies will have a negative impact on the immune status of the population and increase the risk of severe malaria upon re-infection. To date, this idea remains controversial, with epidemiological evidence showing shifts to older age in malaria-related hospital admissions in previously eliminated areas (Ceesay et al., 2008), to no increased risk of clinical malaria upon re-infection after treatment of asymptomatic infections right before the start of the wet season (Portugal et al., 2017). Furthermore, increasing evidence suggests that asymptomatic malaria results in detrimental effects for the host including splenomegaly, anemia, diminished learning and school performance in children, and increased incidence of invasive bacterial infections requiring hospitalization, suggesting a reduced capacity to fight infection in individuals carrying asymptomatic malaria infections (Chen et al., 2016). Our findings here have uncovered immunosuppression as another potentially deleterious consequence of asymptomatic malaria, which has critical implications for the administration of malaria vaccines, to populations carrying subclinical infections. In line with this concept, attenuated sporozoite vaccine formulations were found to be significantly less immunogenic in malaria-exposed African adults than malaria-naive adults (Ishizuka et al., 2016; Jongo et al., 2019; Sissoko et al., 2017), suggesting that chronic exposure to malaria limits vaccine efficacy. Thus, our results suggest that asymptomatic infections are not necessarily benign and provide a framework to consider screening and treatment of asymptomatic *P. falciparum* malaria.

### Materials and Methods

**Reagents and Tools Table**

| Reagent or resource | Reference or source | Identifier or catalog number |
|---------------------|---------------------|-----------------------------|
| **Biological samples and experimental models** | | |
| Melbourne unexposed healthy control | Walter and Eliza Hall Institute of Medical Research Volunteer Blood Donor Registry | N/A |
| Timika healthy community control | Pigapu and Hirapau villages, Timika | HC |
| Timika asymptomatic *P. falciparum malaria* | Pigapu and Hirapau villages, Timika | AM |
| Timika symptomatic *P. falciparum malaria* | Rumah Sakit Mitra Masyarakat Hospital, Timika | SM |
| C57Bl6/J (M. musculus) | Jex Laboratory, Walter and Eliza Hall Institute | N/A |
| **Antibodies** | | |
| **ELISA antibodies** | | |
| Fc-HRP-conjugated mouse anti-human IgG | Southern Biotech | Clone H2; Cat# 9042-05 |
| **Surface marker antibodies for CYTOF** | | |
| 141Pr-conjugated mouse anti-human CD196/CCR6 | Fluidigm | Clone 31A9; Cat# 3140104A |
| 144Nd-conjugated mouse anti-human CD45RA | Fluidigm | Clone H100; Cat# 314006B |
| 146Nd-conjugated mouse anti-human IgD | Fluidigm | Clone IA6-2; Cat# 3140058 |
| 147Sm-conjugated mouse anti-human CD20 | Fluidigm | Clone 2H7; Cat# 3147001B |
| 151Eu-conjugated hamster anti-human ICOS | Fluidigm | Clone 0X29; Cat# 3151020B |
| 152Sm-conjugated mouse anti-human CD21 | Fluidigm | Clone B13; Cat# 3152010B |
| 153Eu-conjugated rat anti-human CXCR5 | Fluidigm | Clone RF58B2; Cat# 3153020B |
## Reagents and Tools table (continued)

| Reagent or resource | Reference or source | Identifier or catalog number |
|---------------------|---------------------|-----------------------------|
| 156Gd-conjugated mouse anti-human CXCR3 | Fluidigm | Clone GD25H7; Cat# 3160004B |
| 158Gd-conjugated mouse anti-human CD10 | Fluidigm | Clone H10A; Cat# 3180011B |
| 165Ho-conjugated mouse anti-human CD19 | Fluidigm | Clone H1B19; Cat# 3165025B |
| 167Er-conjugated mouse anti-human CD27 | Fluidigm | Clone L128; Cat# 3167006B |
| 170Er-conjugated mouse anti-human CD3 | Fluidigm | Clone UCHT1; Cat# 3170001B |
| 172Yb-conjugated mouse anti-human IgM | Fluidigm | Clone MHM-88; Cat# 3172004B |
| 174Yb-conjugated mouse anti-human CD4 | Fluidigm | Clone SK3; Cat# 3174004B |
| 175Lu-conjugated mouse anti-human PD-1 | Fluidigm | Clone EH12.2H7; Cat# 3175008B |
| 176Yb-conjugated mouse anti-human CD127 | Fluidigm | Clone A019D5; Cat# 3176004B |
| APC-conjugated rat anti-human IgG | Biolegend | Clone M1310G05; Cat# 410711 |
| PE-conjugated mouse anti-human FcRL5 | Biolegend | Clone 509f6; Cat# 340304 |
| 145Nd-conjugated mouse anti-human PE | Fluidigm | Clone PE001; Cat# 3145006B |
| 162Dy-conjugated mouse anti-human APC | Fluidigm | Clone APC003; Cat# 3162006B |
| 161Dy-conjugated mouse anti-human T-bet | Fluidigm | Clone 4B10; Cat# 3161014B |
| **Infection model antibodies** | | |
| Syrian hamster anti-mouse CTLA-4 | Walter and Eliza Hall Institute | Clone 9H10 |
| Ultra-LEAF™ Purified Armenian Hamster IgG Isotype Control | Biolegend | Clone 400959; Cat# 400969 |
| **Flow cytometry antibodies** | | |
| AF700-conjugated mouse anti-human CD3 | Biolegend | Clone UCHT1; Cat# 300424 |
| BV786-conjugated mouse anti-human CD4 | BD Biosciences | Clone SK3; Cat# 664528 |
| BV421-conjugated mouse anti-human CD19 | Biolegend | Clone H1B19; Cat# 302234 |
| PE/Cy7-conjugated mouse anti-human CD25 | Biolegend | Clone M-A251; Cat# 356108 |
| BV711-conjugated mouse anti-human PD-1 | Biolegend | Clone EH12.2H7; Cat# 329928 |
| BV650-conjugated mouse anti-human CD45RA | Biolegend | Clone H100; Cat# 304136 |
| APC/Fire750-conjugated mouse anti-human CD8 | Biolegend | Clone SK1; Cat# 980914 |
| PE/Dazzle594-conjugated mouse anti-human CTLA-4 | Biolegend | Clone BN13; Cat# 369616 |
| BB700-conjugated mouse anti-human FoxP3 | BD Biosciences | Clone 236A/E7; Cat# 565626 |
| Rat anti-mouse CD16/CD32 Fc Block | BD Biosciences | Clone 2.4G2; Cat# 553142 |
### Reagents and Tools table (continued)

| Reagent or resource                                           | Reference or source | Identifier or catalog number |
|---------------------------------------------------------------|---------------------|------------------------------|
| FITC-conjugated hamster anti-mouse TCR β chain                | BD Biosciences      | Clone H57–597; Cat# 553170   |
| PE/Cy7-conjugated rat anti-mouse CD4                         | Biolegend           | Clone RM4–5; Cat# 100528     |
| BV785-conjugated anti-mouse CD44                              | Biolegend           | Clone IM7; Cat# 103041       |
| PerCp-Cy5.5-conjugated rat anti-mouse CXCR5                   | BD Biosciences      | Clone 2C8; Cat# 560528       |
| BV421-conjugated Armenian hamster anti-mouse CD44             | Biolegend           | Clone CXCR3-173; Cat# 126521 |
| APC/Cy7-conjugated rat anti-mouse CD62L                       | Biolegend           | Clone MEL-14; Cat# 104428    |
| Biotin-conjugated rat anti-mouse CCR7                         | Biolegend           | Clone 4B12; Cat# 120103      |
| Pacific Blue™-conjugated rat anti-mouse FoxP3                  | Biolegend           | Clone MF-14; Cat# 126409     |
| APC-conjugated Armenian hamster anti-mouse CTLA-4             | Biolegend           | Clone UC10–489; Cat# 106309  |

### Chemicals, Enzymes, and other reagents

- **P. falciparum lysate** (5 µg/ml) | Generated in-house | N/A |
- **P. falciparum recombinant protein** (0.5–2 µg/ml) | Generated in-house | N/A |
- **1x10⁷ P. chabaudi chabaudi AS** | Generated in-house | N/A |
- **Tetramethyl-benzidine/H₂O₂** | KPL Inc. | Cat# 50-76-03 |
- **Cell-ID Cisplatin** (5 µM) | Fluidigm | Cat# 201064 |
- **Dulbecco’s phosphate-buffered saline (DPBS)** | Gibco | Cat# 14190144 |
- **Human TruStain FcX** | Biolegend | Cat# 422302 |
- **Bovine serum albumin (0.5%) (w/v)** | Sigma-Aldrich | Cat# A7906-100G |
- **Sodium azide 0.02% (w/v)** | Sigma-Aldrich | Cat# S8032-2SG |
- **Maxpar nuclear antigen staining buffer set** | Fluidigm | Cat# 201063 |
- **Maxpar fix and perm buffer** | Fluidigm | Cat# 201067 |
- **Cell-ID intercalator (125nM)** | Fluidigm | Cat# 201192A |
- **EQ Four Element beads** | Fluidigm | Cat# 201078 |
- **Fixable Viability Dye efluor-506** | eBioscience | Cat# 65-0866 |

### Software

- **CyTOF software v7.0.8493** | Fluidigm | N/A |
- **FlowJo v10** | BD Biosciences | https://www.graphpad.com/ |
- **Cytobank** | (Kotecha et al, 2010) | https://www.beckman.com/flow-cytometry/software/cytobank-enterprise |
- **GraphPad Prism v8.0** | GraphPad | https://www.graphpad.com/ |
- **Rstudio v4.0.3-4.1.2** | (R Core Team, 2021) | https://www.R-project.org/ |
- **Rsubread v2.0.1** | (Liao et al, 2019) | http://bioconductor.org/packages/release/bioc/html/Rsubread.html |
- **limma v3.46.0** | (Ritchie et al, 2015) | http://bioconductor.org/packages/release/bioc/html/limma.html |
- **Ingenuity Pathway Analysis** | QIAGEN | http://pages.ingenuity.com/rs/ingenuity/images/PA_data_sheet.pdf |

### Reagent kits and Instruments

- **QIAamp DNA Blood Mini Kit** | QIAGEN | Cat# 51104 |
- **Chameleon plate reader** | Hidex | N/A |
- **Helios mass cytometer** | Fluidigm | N/A |
Reagents and Tools table (continued)

| Reagent or resource | Reference or source | Identifier or catalog number |
|---------------------|---------------------|-----------------------------|
| Isolate II RNA Mini Kit | Bioline | Cat# BIO-52072 |
| Illumina TruSeq RNA Library Prep Kit (< 100 ng) | Illumina | Cat# RS-122-2001 |
| FoxP3/Transcription Factor Staining Buffer Set | eBioscience | Cat# 00-5521-00 |
| BD LSR Fortessa X20 | eBioscience | N/A |
| Agilent TapeStation 2200 | Agilent | N/A |
| Illumina NextSeq 500 | Illumina | N/A |

Methods and Protocols

Study population

A retrospective study was conducted in the Timika region of Papua, Indonesia. Papuans reside both in the Timika lowlands where malaria exposure is common and the highlands where malaria is absent (Karyana et al., 2008). Migration of non-immune adults from the highlands to lowlands means a first malaria infection can occur in all age groups. Consenting participants (aged between 5 and 45 years) donated a venous blood sample at enrollment, and peripheral blood mononuclear cells (PBMCs) and plasma were frozen. Parasite densities were determined by blood smears and participants with light-microscopy confirmed malaria infections received first-line anti-malarial treatment according to the Indonesian Ministry of Health guidelines. In addition, 30 individuals presenting with symptoms of a probable malaria infection, as defined as individuals with an axillary fever temperature ≥ 37.5°C, chills, malaise, headache, or vomiting at the time of examination or up to 24 h prior to the examination and the presence of a P. falciparum positive blood smear and no other cause of fever discernible by physical exam. All individuals included in the immunity study were Papuan. Symptomatic individuals included in the immunity study had > 500 parasite/μl blood, whereas individuals with a P. falciparum-positive blood smear and no clinical symptoms were classified as asymptomatic infections. Healthy immune controls had a negative light-microscopy and PCR diagnosis. Previous exposure to malaria in these individuals was confirmed by ELISA against a P. falciparum parasite lysate. This study was approved by the Human Research Ethics Committees of the Iijman Institute for Molecular Biology, the Walter and Eliza Hall Institute of Medical Research, the Northern Territory Department of Health & Families, and the Menzies School of Health Research. Written informed consent was obtained from all study participants prior to their inclusion in the study. All the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

ELISA

Ninety-six well plates (Corning) were coated with 5 μg/ml of P. falciparum lysate or 0.5–2 μg/ml of P. falciparum recombinant protein in carbonate buffer pH 9.6 and incubated at 4°C overnight. After washing, plates were blocked with 5% skim milk in phosphate-buffered saline (PBS) for 1 h at 37°C. Blocked plates were washed with 0.05% Tween-20 in PBS and incubated with serial two-fold dilutions of plasma for 1 h at 37°C. After washing, plates were incubated with an HRP-conjugated mouse anti-human IgG antibody (Southern Biotech, USA) for 1 h at 37°C. Bound antibodies were detected by reaction of tetramethylbenzidine and H₂O₂ (KPL Inc., USA). The reaction was stopped by the addition of 1 M H₃PO₄ and absorbance read at 450 nm on a CHAMELEON plate reader (Hidex). Plasma from malaria-naïve Melbourne blood donors were included as background controls. Antibody titers were calculated as the serum dilution with an optical density (OD) value higher than that observed for negative controls at a 1/100 dilution.

CyTOF

PBMCs (2 × 10⁶) from P. falciparum symptomatic and asymptomatic infected individuals as well as healthy immune controls were stained with 5 μM Cell-ID Cisplatin (Fluidigm) in PBS (Gibco) for 5 min at room temperature. Cells were then blocked with Human TruStain FcX (Biolegend) and stained with a cocktail of surface marker antibodies (Reagents and Tools Table) in CyTOF staining buffer (PBS with 0.5% bovine serum albumin [BSA; Sigma] and 0.02% sodium azide [Sigma]) for 30 min at room temperature. After surface staining, cells were fixed and permeabilized with a Maxpar nuclear antigen staining buffer set (Fluidigm) and then stained with a 161Dy-conjugated anti-T-bet (clone 4B10; Fluidigm) antibody for 45 min at room temperature. Cells were then washed twice and stored in Maxpar fix and perm buffer (Fluidigm) with 125 nM Cell-ID iridium intercalator (Fluidigm) for a minimum of 18 h. Prior to data acquisition, cells were washed twice by centrifugation in ultrapure water then resuspended in a 1/10 dilution of 4-Element EQ normalization beads (Fluidigm) in ultrapure water. Cells were analyzed on a Helios model mass cytometer (Fluidigm) at ~300 events/s. Data were normalized using the signal from 4-Element EQ Beads (Fluidigm) as previously described (Finck et al., 2013). Manual gating was performed using FlowJo version 10 (BD Biosciences) to exclude doublets and dead cells before individual cell populations were selected and exported for further analysis in Cytobank (Kotecha et al., 2010). Individual cell populations were then visualized using viSNE (Amir et al., 2013), while FlowSOM (Van Gassen et al., 2015) was used to examine the composition of the MBC and memory CD4⁺ T cell pools. The following parameters were included in the viSNE analysis for classical, atypical, and activated MBCs: IgD, IgM, CXCR5, CXCR3, CCR6, CD45RA, PD-1, and T-bet. For CXCR3⁺ CCR6⁺ and CXCR3⁻ CCR6⁻ memory CD4⁺ T cells,
the following parameters were included: CXCR5, PD-1, CD27, CD25, CD127, and T-bet. For T\textsubscript{FH} cells, the following parameters were included: CXCR3, CCR6, PD-1, CD27, CD25, CD127, and T-bet. Self-organizing maps (SOMs) were then generated for each cell population using hierarchical consensus clustering on the tSNE axes. CITRUS (Bruggner et al., 2014) was used to identify differentially abundant cell populations using the same parameters as the viSNE analysis at a 5% FDR. Preliminary analysis revealed that a minimum of eight samples per group was required for identification of differentially abundant populations between groups.

RNA preparation and sequencing
RNA was extracted from 2 × 10\textsuperscript{5} PBMCs from selected samples with sufficient material to allow assessment of multiple endpoints using the ISOLATE II RNA Mini Kit (Bioline) following the manufacturer’s instructions, with the final elution step repeated once and RNA eluted into 40 µl total RNase-free water. RNA was quantified with RNA Screen Tape on the Agilent TapeStation 2200 System. Libraries were prepared with either 50 ng or 25 ng total RNA using the Illu-

Transcriptional analysis
Raw sequence reads in FASTQ file format were aligned to the human reference genome GRCh38/hg38 using the align() function in Bioconductor package RsuRead version 2.0.1 with default param-

Flower cytometry
Human PBMCs were blocked with Human TruStain FcX (Bio-

Statistical analysis
Characteristics of clinical groups were compared using one-way ANOVA with Tukey’s multiple comparisons for continuous data that were normally distributed, and the Kruskal–Wallis test for data that did not follow normal distribution. The Mann–Whitney test was used to compare paired data and the Chi-squared test for
nominal data. Logistic regression models were fitted for pairwise comparisons between groups to determine the odds ratio for antibody titers and cell populations. Hierarchical clustering heatmaps were calculated using the complete method and Euclidian distance matrix using the pheatmap package version 1.0.12 in R. Linear regression models and differential abundance of cell populations were assessed using limma and a robust empirical Bayes procedure (Phipson et al., 2016). When performing differential expression analysis, a 15% FDR was considered significant. The FDR was otherwise controlled to below 5% using the method of Benjamini and Hochberg. Correlations were determined using Spearman’s rank correlation coefficient in the psych package version 2.1.9 in R. Statistical analyses were performed in GraphPad Prism version 8 and R versions 4.0.3-4.1.2.

Data availability

Processed bulk RNA-seq data generated for this study are available as GEO series GSE181179 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181179). Raw data are available upon request, subject to approval by the Walter and Eliza Hall Institutional Data Access Committee (dataaccess@wehi.edu.au) to ensure preservation of patient confidentiality.

Expanded View for this article is available online.

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Author contributions

Stephanie I Studniberg: Formal analysis; Validation; Investigation; Visualization; Methodology; Writing—original draft; Project administration; Writing—review and editing. Lisa J Ioannidis: Formal analysis; Investigation; Methodology; Writing—original draft; Project administration. Retno A S Utami: Formal analysis; Investigation. Leily Trianty: Formal analysis; Investigation. Yang Liao: Formal analysis. Waruni Abeysekera: Formal analysis. Connie S N Li-Wai-Suen: Formal analysis. Halina M Pietrzak: Investigation; Methodology. Jullie Healer: Supervision; Methodology. Agatha M Puspitasari: Investigation. Dwi Apriliyanti: Investigation. Farah Coutier: Investigation. Jean R Poepopprojo: Resources; Supervision; Project administration. Enny Kenangalem: Resources; Project administration. Benediktus Andries: Resources; Project administration. Pak Prayoga: Resources. Novita Sariyanti: Resources; Project administration. Gordon K Smyth: Supervision. Alan F Cowman: Supervision. Ric N Price: Supervision; Project administration. Rintis Noviyanti: Conceptualization; Resources; Supervision; Investigation; Project administration. Wei Shih: Resources; Software; Formal analysis; Supervision; Investigation; Visualization; Methodology. Diana S Hansen: Conceptualization; Formal analysis; Supervision; Funding acquisition; Investigation; Methodology; Writing—original draft; Project administration; Writing—review and editing.

In addition to the CRediT author contributions listed above, the contributions in detail are:

SIS, LJI, and DSH conceived and designed the study. AMP, DA, FC, JRP, EK, BA, PP, NS, LT, RNP, and RN conducted the field sample collection. SIS, LJI, HMP, and RASU performed the experiments. JH and AFC provided reagents. SIS, LJI, RASU, YL, WA, CSNL-W-S, GKS, and DSH performed the analysis. SIS, LJI, and DSH wrote the manuscript. WS, ALG, and DSH supervised the study.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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