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Chronic Exposure to *Plasmodium falciparum* Is Associated with Phenotypic Evidence of B and T Cell Exhaustion

Joseph Illingworth,*† Noah S. Butler, ‡ Sophie Roeync,*,‡§ Jedida Mwacharo,* Susan K. Pierce,§ Philip Bejon,*† Peter D. Crompton,* Kevin Marsh,*† and Francis M. Ndungu,*†

Naturally acquired immunity to malaria develops slowly, requiring several years of repeated exposure to be effective. The cellular and molecular factors underlying this observation are only partially understood. Recent studies suggest that chronic *Plasmodium falciparum* exposure may induce functional exhaustion of lymphocytes, potentially impeding optimal control of infection. However, it remains unclear whether the “atypical” memory B cells (MBCs) and “exhausted” CD4 T cells described in humans exposed to endemic malaria are driven by *P. falciparum* per se or by other factors commonly associated with malaria, such as coinfections and malnutrition. To address this critical question we took advantage of a “natural” experiment near Kilifi, Kenya, and compared profiles of B and T cells of children living in a rural community where *P. falciparum* transmission is ongoing to the profiles of age-matched children living under similar conditions in a nearby community where *P. falciparum* transmission ceased 5 y prior to this study. We found that continuous exposure to *P. falciparum* drives the expansion of atypical MBCs. Persistent *P. falciparum* exposure was associated with an increased frequency of CD4 T cells expressing phenotypic markers of exhaustion, both programmed cell death-1 (PD-1) alone and PD-1 in combination with lymphocyte-activation gene-3 (LAG-3). This expansion of PD-1–expressing and PD-1/LAG-3–coexpressing CD4 T cells was largely confined to CD45RA* CD4 T cells. The percentage of CD45RA*CD27* CD4 T cells coexpressing PD-1 and LAG-3 was inversely correlated with frequencies of activated and classical MBCs. Taken together, these results suggest that *P. falciparum* infection per se drives the expansion of atypical MBCs and phenotypically exhausted CD4 T cells, which has been reported in other endemic areas. *The Journal of Immunology*, 2013, 190: 1038–1047.

L ongstanding and compelling evidence points to a critical role for Abs (1, 2) and CD4 T cells (3–5) in mediating naturally acquired immunity to the erythrocytic stages of *Plasmodium falciparum* malaria in humans. However, immunity to *P. falciparum* infection is relatively slow to develop and is probably never sterile (6–8). The mechanisms underlying these observations are only partially understood. Additionally, it is unclear why otherwise promising experimental malaria vaccine candidates (9–11) subsequently fail to protect, or only partially protect, residents of malaria-endemic areas (12). Although it is likely that antigenic variation and allelic diversity play an important role in these observations (13–15), recent studies suggest that chronic *P. falciparum* exposure induces qualitative changes in B and T cell responses that may also play a role in *P. falciparum* immune evasion.

Indeed, functional “exhaustion” (immune dysfunction) among T and B cell subsets is a well-described feature of chronic viral infections, such as hepatitis B and C and HIV viruses (16–21). T cell exhaustion was initially described for CD8 T cells in mice chronically infected with lymphocytic choriomeningitis virus clone 13 (21). In the lymphocytic choriomeningitis virus mouse model, repeated Ag stimulation through the T cell Ag receptor drives the sustained expression of T cell inhibitory receptors, including programmed cell death-1 (PD-1) and lymphocyte activation gene-3 (LAG-3), on virus-specific CD8 T cells. Sustained signaling through these inhibitory receptors directly and indirectly induces transcriptional changes that negatively regulate proliferation and the expression of proinflammatory cytokines by virus-specific CD8 T cells (22, 23). T cell exhaustion was subsequently described in humans in the context of chronic viral infections, such as HIV and hepatitis C virus (HCV) as well as in the context of cancer (16, 17, 19).

Regarding B cell exhaustion, Moir et al. (24) recently demonstrated that HIV viremia is associated with an expanded subset of memory B cells (MBCs) that had previously been defined by the
expression of the inhibitory receptor FcR-like-4 (FCRL4) (25), representing on average 19% of total peripheral B cells, compared with <4% in healthy individuals. B cells with a similar phenotype have also been identified in individuals infected with HCV (26). It was demonstrated that this subset of B cells in HIV-infected individuals had undergone isotype class switching and somatic hypermutation, but compared with naïve B cells and classical MBCs, FCRL4+ MBCs proliferated less well in response to BCR crosslinking and/or CD40L and TLR9 agonist CpG and showed a decreased ability to differentiate into Ab-secreting cells (12). Given that the function of lymphoid-homing receptors similar to what is expressed on exhausted CD8 T cells during chronic viral infections (27). Owing to the relative hyposresponsiveness of these MBCs and their altered expression of inhibitory and homing receptors that together are signatures for virus-induced exhaustion of T cells (27–29), Moir et al. (24) referred to this subset of MBCs as “exhausted.” Exhausted MBCs were disproportionately HIV-specific as compared with the classical MBC compartment; in contrast, influenza-specific MBCs were more prevalent in the classical MBC compartment. These authors proposed that chronic HIV stimulation of B cells leads to their premature exhaustion, contributing to the poor Ab responses in HIV-infected individuals (30). In contrast, Ehrhardt et al. (25), who first described FCRL4+ “tissue-like” MBCs in lymphoid tissues of healthy individuals, suggested that these cells might play a protective role during infection.

It is conceivable that chronic *P. falciparum* exposure in malaria-endemic areas would also be associated with T and B exhaustion and thus contribute to the protracted acquisition of malaria immunity, but few studies have explored this possibility. Butler et al. (31) recently reported that natural *P. falciparum* infection in Malian children resulted in higher expression of the inhibitory receptor PD-1 on CD4 T cells. In the same report it was shown that nonlethal *Plasmodium yoelii* infection induces CD4 T cell exhaustion and that in vivo blockade of the PD-1 ligand (PD-L1) and the inhibitory receptor LAG-3 restored CD4 T cell function, amplified the number of follicular helper T cells and germinal center B cells and plasmablasts, and enhanced protective Abs and rapid clearance of blood-stage malaria in mice (31), thus suggesting that the increase in PD-1–expressing CD4 T cells in *P. falciparum–exposed* Malian children may reflect functional exhaustion, but that has yet to be determined. Recent studies in Mali, Gambia, and Peru also indicate that *P. falciparum* exposure is associated with an expansion of a phenotypically distinct population of MBCs identified by the cell surface markers CD19+CD20+CD21*−*CD27−CD10+ (32–36), similar to the exhausted MBCs described in individuals infected with HIV (24) and HCV (26). Similar to HIV, this MBC subset in the context of malaria expresses high levels of inhibitory receptors and a profile of lymphoid-homing receptors (35). Given that the function of FCRL4+ MBCs in *P. falciparum–exposed* individuals remains unknown, this B cell subset has been referred to as “atypical” rather than exhausted in the context of malaria (35).

Importantly, in the absence of *P. falciparum* Ag–specific B and T cell data, it remains unclear whether the atypical MBCs and putatively exhausted CD4 T cells reported from *P. falciparum–endemic* areas are driven by *P. falciparum* infection per se or by other factors commonly associated with *P. falciparum* transmission, such as infections, malnutrition, or genetic polymorphisms (34, 35). To address this critical question, we took advantage of a “natural” experiment near Kilifi, Kenya, and compared B and T cell profiles of children living in a rural community where *P. falciparum* transmission is ongoing to those of age-matched children, living under similar conditions in a nearby community where *P. falciparum* transmission ceased 5 y prior to this study. We observed a higher frequency of atypical MBCs and PD-1/LAG-3–coexpressing CD4 T cells in children exposed to ongoing *P. falciparum* transmission, thus providing the strongest evidence yet that *P. falciparum* infection per se drives the phenotypic changes in B and T cells that may be indicative of functional exhaustion.

Materials and Methods

Ethics

This study was approved by the Kenyan Medical Research Institute National Ethics Committee. Written informed consent was obtained from the parents/guardians of the children as required.

Study site

The study took place at the Kenya Medical Research Institute, Centre for Geographical Medicine Research (Coast) situated at Kilifi District Hospital, Kilifi, Kenya. The hospital serves ~500,000 people living in Kilifi district. The children investigated were resident of two villages, located within 30 km of each other, with Junju lying on the southern side and Ng'enerya on the northern side of an Indian Ocean creek. These study sites are inhabited by predominantly Mijikenda people, who share similar beliefs and customs and are described in detail elsewhere (37, 38).

Study population

Although there has been a gradual decline of *P. falciparum* transmission in the Kilifi district (39, 40), Junju remains stably endemic with two high transmission seasons (May to August and October to December) and high a parasite prevalence of 25% (41, 42). In contrast, *P. falciparum* transmission has dramatically reduced in Ng’enerya, which was endemic with a parasite prevalence of 40% and a transmission intensity of 10 infective bites per person per year in 1998 (43, 44). *P. falciparum* prevalence had declined to negligible levels by 2005 and has remained so ever since. Children are recruited into the cohorts at birth and actively followed weekly (41) for detection of malaria episodes until the age of 13 y. We maintain extensive and detailed records of the number and dates of malaria experiences for each child, either from birth or at the time of recruitment.

PBMCs

Venous blood samples (5 ml) were collected and blood smears were performed in preseason cross-sectional surveys in May 2009, 2010, and 2011, a time preceded by 4 mo minimal *P. falciparum* transmission in Junju. PBMCs were harvested and stored in liquid nitrogen until the time when the assays were performed.

Determination of parasitemia

Thick and thin blood smears were stained with Giemsa, and *P. falciparum–infected* red cells were counted against 500 leukocytes and 1000 RBCs, respectively, by expert microscopists.

Flow cytometry

PBMCs were isolated at the time of blood collection by centrifugation over a Ficoll-Paque PLUS barrier (GE Healthcare, catalog no. 17-1440-02). Cells were cryopreserved in FCS containing 10% DMSO. After thawing, cells were stained with two panels of Abs. PBMCs were analyzed during a 4-wk period and with equal numbers of case control samples analyzed on each day. The B cell panel consisted of anti–CD10–allophycocyanin (BD Biosciences, catalog no. 332777), anti–CD20–allophycocyanin.H7 (BD Biosciences, catalog no. 641396), anti–CD19-PerCP.Cy5.5 (eBioscience, catalog no. 45-0198-42), anti–CD21-PE (eBioscience, catalog no. 12-0219-42), and anti–CD27-PE.Cy7 (eBioscience, catalog no. 25-0279-42). The T cell staining panel consisted of anti–PD-1-PE (BD Pharmingen, catalog no. 560795), anti–CD45RA-FITC (BD Pharmingen, catalog no. 560609), anti–CD4-PerCP.Cy5.5 (BD Biosciences, catalog no. 341654), and anti–LAG-3 conjugated with biotin (R&D Systems, catalog no. BAF2319). The primary anti–LAG-3-biotin conjugate was detected by generating a quaternary complex. The secondary detection reagent was streptavidin conjugated to allophycocyanin (Invitrogen, catalog no. 5868). This signal was in turn amplified using
anti-APC conjugated to biotin to produce a tertiary complex (BioLegend, catalog no. 408004) followed by a second incubation with the streptavidin-allophycocyanin conjugate (see Supplemental Fig. I for validation of LAG-3 staining strategy). Flow cytometry was performed on the Beckman Coulter CyAn ADP, and data analysis was done using FlowJo software (Tree Star).

Statistical analyses

Calculations were performed using GraphPad Prism. The Ngerenya naive group was considered the main control group for comparison with the two groups of Junju persistently exposed children and the Ngerenya historically exposed children. Statistical significance between various paired groups was determined separately using the Wilcoxon rank-sum test. Bivariate correlations between T cell and B cell subsets were performed using a Spearman rank nonparametric correlation test. For all tests, statistical significance was considered at the 5% level.

Results

Characteristics of study subjects

Frequencies of atypical MBCs and phenotypically exhausted CD4 and CD8 T cells were determined in children from two cohorts, Junju and Ngerenya, who have been under active weekly surveillance for detection and recording of febrile malaria episodes since 2005 and 1989, respectively. The reduction in \( P. \) falciparum malaria infection according to our records due to the dramatic transmission intensity from a parasite prevalence of 40 to 0% during 7 y in Ngerenya is contrasted with sustained transmission in Junju, where the parasite prevalence remains >25% (39, 44). We selected 40 children from Junju who had had at least one recorded clinical episode of malaria in the past and who remained at risk for infection because of ongoing \( P. \) falciparum transmission (Junju-persistently exposed) and split them into two groups: those with asymptomatic parasitemia (Junju-parasitic) and those without parasitemia (Junju-nonparasitic) at sampling (Table I). Although they had \( P. \) falciparum parasitemia by microscopy at sampling, the Junju-parasitic children were afebrile and healthy. We matched the Junju-parasitic and Junju-nonparasitic groups by age and sex to 24 children from Ngerenya, who had no history of malaria infection according to our records due to the dramatic reduction in \( P. \) falciparum transmission in Ngerenya (Ngerenya-naive). Additionally, we added two extra control groups: 8 children from Junju with no recorded history of \( P. \) falciparum infection (Junju-naive) and 21 children from Ngerenya, whose last recorded episodes were well >5 y before the sampling date (Ngerenya-historically exposed). Of note, Junju-parasitic, Junju-nonparasitic, and Ngerenya-historically exposed children had comparable numbers of previous malaria episodes and similar age distributions. Junju-naive children are younger than their Ngerenya-naive counterparts because \( P. \) falciparum transmission intensity, and thus the risk of experiencing clinical malaria at a younger age, is higher in Junju. For these reasons, our analyses are focused on comparing B and T cell phenotypes of persistently exposed Junju children with both Ngerenya-naive children as the main naive control group.

**Table I. Characteristics of study subjects**

<table>
<thead>
<tr>
<th>Group</th>
<th>Junju-Parasitic</th>
<th>Junju-Nonparasitic</th>
<th>Junju-Naive</th>
<th>Ngerenya-Naive</th>
<th>Ngerenya-Historically Exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>21</td>
<td>19</td>
<td>8</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>Age (y), median (IQR)</td>
<td>7.6 (4.0–9.5)</td>
<td>7.2 (4.0–8.8)</td>
<td>2.1 (2.0–2.5)</td>
<td>6.2 (3.3–8.0)</td>
<td>9.2 (8.8–9.6)</td>
</tr>
<tr>
<td>Previous episodes, mean (y)</td>
<td>3.2</td>
<td>3.6</td>
<td>0</td>
<td>0</td>
<td>3.9</td>
</tr>
<tr>
<td>Time from last episode (y)</td>
<td>1.26</td>
<td>1.13</td>
<td>NA</td>
<td>NA</td>
<td>&gt;5</td>
</tr>
</tbody>
</table>

IQR, Interquartile range; NA, not applicable.

Persistent exposure to \( P. \) falciparum infection is associated with expansion of atypical MBCs

We determined whether persistent exposure to \( P. \) falciparum infection drives expansion of atypical MBCs and other B cell subsets by comparing their relative proportions in the peripheral blood between the two Junju-persistently exposed and different control groups: Ngerenya-naive, Junju-naive, and Ngerenya-historically exposed children. B cell subsets were defined as: naive B cells, CD19+CD27-CD21+CD10; plasma cells, CD19+CD27+CD21-CD10; immature B cells, CD19+CD10+; classical MBCs, CD19+CD27+CD21-CD10; atypical MBCs, CD19+CD27+CD21+CD10+; and activated MBCs, CD19+CD27+CD21+CD20+CD10+.

Fig. 1 illustrates the flow cytometric gating strategies used in the determination of the relative proportions of B cell subsets and a representative comparison of the Junju-nonparasitemic group with Ngerenya-naive children.

The proportion of atypical MBCs was significantly higher in the PBMCs of either of the two Junju-persistently exposed groups compared with Ngerenya-naive children (Fig. 2A). The Junju-naive children, who live in the same environment as the Junju-persistently exposed children, had a similar frequency of atypical MBCs as did the Ngerenya-naive group (Fig. 2A), providing additional evidence that the expansion of atypical MBCs in Junju-persistently exposed children is malaria-driven. The proportion of atypical MBCs among the Ngerenya-historically exposed children was significantly lower than among either of the two Junju-persistently exposed groups, but not different from that of the Ngerenya-naive children, further suggesting that the expansion of the atypical MBC compartment is maintained by continuous \( P. \) falciparum exposure (Fig. 2A).

In contrast to atypical MBCs, the proportion of naive B cells among the Junju-parasitic and Junju-nonparasitic groups was lower than among the Ngerenya-naive children (Fig. 2F). The Ngerenya-historically exposed children had a similar percentage of naive B cells to the Ngerenya-naive children, suggesting that the reduction in frequency of naive B cells in the Junju-persistently exposed children may be driven by persistent \( P. \) falciparum exposure. Similar observations were made by Bejon et al. (41) and Scott et al. (45), whereby general \( P. \) falciparum exposure rather than concurrent parasitemia per se were associated with reduced T cell responses to vaccination and with \( Salmonella \) bacteremia, respectively. Moreover, the expansion of atypical MBCs in the two Junju-persistently exposed groups appears to be at the expense of naive B cells (Fig. 2G), suggesting that \( P. \) falciparum–activated naive B cells may preferentially differentiate into atypical MBCs in this environment.

There were no significant differences between either of the two Junju-persistently exposed groups and the different control groups for activated B cells, classical MBCs, plasma cells, and immature B cells (Fig. 2B, 2C, 2D, and 2E, respectively).
Taken together, these data indicate that ongoing *P. falciparum* exposure drives the expansion of atypical MBCs, perhaps at the expense of the naive B cell compartment, without affecting the relative frequencies other B cell subsets. Additionally, the expansion of *P. falciparum*-associated atypical MBCs appears to depend on persistent *P. falciparum* exposure. However, asymptomatic *P. falciparum* infection did not affect the frequency of atypical MBCs, suggesting that they were sustained at stable levels during the 4-mo dry period preceding the cross-sectional bleed.

**Persistent exposure to *P. falciparum* infection is associated with upregulation of PD-1 and LAG-3 on CD4 T cells**

We next compared the distribution of the major CD4 T cell subsets, representing different stages of differentiation. Additionally, we evaluated the expression of PD-1 and LAG-3 on CD4 T cells between Junju-persistently exposed and Junju-naive children, as well as Ngerenya-naive and Ngerenya-historically exposed control groups. We defined CD4 T cell subsets by their expression of CD45RA and CD27 (46, 47). CD27 expression distinguishes between central and effector memory T cells, with the later being brighter (48–50). Although CD45RA expression is historically associated with naive T cells, there is substantial evidence showing that late memory (Ag-experienced) CD4 (50–52) and CD8 (51, 52) T cells do re-express CD45RA. CD45RA re-expressing T cells have a highly differentiated effector-like phenotype (51, 53). Taking this into account, we defined four T cell subsets: naive/long-lived (late) central memory (T_{CM-late}) (CD45RA^−CD27^+), nascent (early) central memory (T_{CM-early}) (CD45RA^-CD27^+), effector memory (T_{EM}) (CD45RA^-CD27^-), and effector cells (T_{EM-FF}) (CD45RA^-CD27^-) (54, 55). Fig. 3 illustrates the gating strategy for defining the CD4 T cell subsets, as well as the analysis of PD-1 and LAG-3 expression on CD4 T cells, in a representative comparison between the Junju-persistently exposed and Ngerenya-naive children. There were no significant differences in the distribution of the four major CD4 T cell subsets across the four groups of children (Supplemental Fig. 2A), suggesting that persistent *P. falciparum* exposure does not affect the general profile of CD4 T cell differentiation as phenotypically defined in this study.

The proportion of total CD4 T cells expressing PD-1 was higher among the Junju-persistently exposed children than among the controls, but this difference was only statistically significant between Junju-parasitic and Ngerenya-historically exposed children (Fig. 4A). Further analysis revealed increased PD-1 expression within the CD45RA^-CD27^- and CD45RA^-CD27^- CD4 T cells among the Junju-parasitic and Junju-nonparasitic children relative to the Ngerenya-naive and Ngerenya-historically exposed controls (Fig. 4B and 4C, respectively). There were no significant differences in PD-1 and LAG-3 expression on CD45RA^-CD27^- and CD45RA^-CD27^- CD4 T cell phenotypes between any groups (data not shown). However, the CD45RA^-CD27^- CD4 T cells comprised most of the PD-1^- CD4 T cells among all the groups (Supplemental Fig. 3).

The proportion of LAG-3^-expressing CD4 T cells tended to be higher among the Junju-nonparasitic children than the Ngerenya-naive and Ngerenya-historically exposed children, but these differences were only statistically significant for the CD45RA^-CD27^- CD4 T cells in comparison with Ngerenya-naive controls (Fig. 4D–F). Although there was a general trend across the CD4 T cell compartment for a reduction in the proportion of LAG-3^-expressing CD4 T cells among the Junju-parasitic compared with the nonparasitic children, these differences were not statistically significant. There were no significant differences in PD-1 and LAG-3 expression on CD45RA^-CD27^- and CD45RA^-CD27^- CD4 T cell phenotypes between any groups (data not shown).

The proportion of PD-1^- and LAG-3^-double-positive CD4 T cells was higher in both Junju-persistently exposed groups compared with the controls, but this difference was only significant between Junju-parasitic and Ngerenya-naive children (Fig. 4G). Additional analyses revealed increases in the proportion of PD-1^- and LAG-3^-double-positive CD4 T cells within the CD45RA^-CD27^- and CD45RA^-CD27^- subsets among the Junju-parasitic and Junju-nonparasitic children relative to the Ngerenya-naive and Ngerenya-historically exposed controls (Fig. 4H, 4I). We also found no significant differences in PD-1^- and LAG-3^-double-positive analyses detailed above, CD45RA^-CD27^- CD4 T cells comprised most of the PD-1^- and LAG-3^-double-positive CD4 T cells among all of the groups (Supplemental Fig. 3).

Collectively, these data indicate that persistent *P. falciparum* exposure may not alter the relative distribution of the four major CD4 T cell subsets as defined by CD27 and CD45 expression. However, our data show that persistent malaria exposure is associated with increased expression of both inhibitory receptors PD-1 and LAG-3 on CD4 T cells, especially in CD45RA^-CD27^- (naive/
Persistent malaria exposure is associated with upregulation of PD-1 on CD8 T cells

We then compared the distribution of the major CD8 T cell subsets as well as the expression of PD-1 and LAG-3 by CD8 T cells between the Junju-parasitemic children and the Junju-naive, Ngerenya-naive, and Ngerenya-historically exposed children. For CD4 T cells, we defined four different CD8 T cell subsets based on their expression of CD45RA and CD27 as follows: naive/TCM-late (CD45RA<sup>+</sup>CD27<sup>+</sup>), nascent central memory (TCM-early) (CD45RA<sup>+</sup>CD27<sup>+</sup>), effector memory (TEM) (CD45RA<sup>+</sup>CD27<sup>+</sup>), and effector cells (TEM<sup>+</sup>EFF) (CD45RA<sup>+</sup>CD27<sup>+</sup>). Supplemental Fig. 4 illustrates the gating strategies used to define CD8 T cell subsets and to measure the expression of PD-1 and LAG-3 on CD8 T cells (representative plots of Junju-persistently exposed and Ngerenya-naive children). Similar to CD4 T cells, the relative distribution of CD8 T cell subsets was not significantly altered by persistent P. falciparum exposure (Supplemental Fig. 2B). However, the frequency of total CD8 T cells expressing PD-1 was elevated among the Junju-persistently exposed children compared to the control groups, but these differences were only significant for the comparison between Junju-parasitemic and Ngerenya-naive as well as with the Ngerenya-historically exposed children (Fig. 5A). The presence of asymptomatic parasitemia was associated with increased PD-1 expression, and this increase was significant among CD45RA<sup>+</sup>CD27<sup>+</sup> CD8 T cells (Fig. 5B). There were no significant differences between the different groups when considering central and effector CD8 T cell phenotypes (data not shown).

Although there were no significant differences in the frequencies of LAG-3 single-positive cells in the total CD8 T cells across groups, the proportion of LAG-3 single-positive CD45RA<sup>+</sup>CD27<sup>+</sup> CD8 T cells was significantly larger among the Ngerenya-historically exposed group compared with both Junju-persistently exposed and Ngerenya-naive groups (Fig. 5E). With respect to naive/TCM-late and TCM-early CD8 T cells, there were no significant differences in LAG-3 or PD-1 expression between groups (data not shown).

Finally, we found that CD45RA<sup>+</sup>CD27<sup>+</sup> cells coexpressing PD-1 and LAG-3 were significantly more abundant among circulating CD8 T cells from Junju-parasitemic children compared with those from Ngerenya-naive and Ngerenya-historically exposed controls (Fig. 5I). Asymptomatic parasitemia was again associated with increased proportions of PD-1 and LAG-3 double-positive CD8 T cells, but more significantly for CD45RA<sup>+</sup>CD27<sup>+</sup> and CD45RA<sup>+</sup>CD27<sup>+</sup> subsets, among the Junju-persistently exposed children (Fig. 5H, 5I). There were no significant differences between the different groups for TCM-early and TEM CD8 T cell phenotypes (data not shown).

Taken together, these data show that the increased frequency of PD-1 single-positive and PD-1 and LAG-3 double-positive CD8 T cells among the Junju-persistently exposed children is mainly associated with asymptomatic parasitemia, suggesting that T cell inhibitor receptor expression in this group is associated with active infection and not maintained after parasite burdens are reduced to levels below the limits of detection.

**Frequencies of CD45RA<sup>+</sup>CD27<sup>+</sup> CD4 T cells coexpressing PD-1 and LAG-3 were independently inversely associated with proportions of activated B cells and classical MBCs among the persistently malaria-exposed Junju children but not among the Ngerenya-naive children**

The enhanced and improved B cell response observed in Plasmodium-infected mice following treatment with anti–PD-L1 and LAG-3 blocking Abs (31) suggested that CD4 T cell exhaustion might contribute to the inefficient acquisition of Ab-mediated immunity to malaria in humans. We therefore explored the relationship between the frequencies of CD4 and CD8 T cells coexpressing PD-1 and LAG-3 with B cell subsets among the Junju-persistently exposed (n = 38) and Ngerenya-naive children (n = 40). Frequencies of CD45RA<sup>+</sup>CD27<sup>+</sup> (naive/TCM-late) CD4 T cells expressing both PD-1 and LAG-3 correlated negatively with the frequencies of activated B cells (Spearman r = -0.57, p = 0.0002), and classical MBCs (Spearman r = -0.59, p = 0.0001) among the...
Junju-persistently exposed children but not among the Ngerenya-naive children (Fig. 6). However, a similar relationship was not observed between PD-1+/LAG-3+CD45RA+CD27+ CD4 T cells and plasma cells (Spearman $r = 0.28$, $p = 0.095$, for the Junju-persistently exposed group) (Fig. 6). There was no association between frequencies of all PD-1+ and all LAG-3+ CD4 T cells (as well as all the CD8 T cell subsets) and the different B cell subsets (data not shown). Taken together, these data suggest that an increase in CD4 T cells expressing markers of exhaustion may contribute to suboptimal B cell responses as measured in this study by decreased numbers of activated and classical memory B cells.

**Discussion**

This study aimed to determine whether exposure to *P. falciparum* infection drives the expansion of atypical MBCs and the expres-
FIGURE 5. CD8 T cells expressing the inhibitory PD-1 and LAG-3 molecules are significantly increased in children exposed to persistent *P. falciparum* infections. Comparison of the frequencies of CD8 T cells and CD8 T cell subsets expressing PD-1 (A–C), LAG-3 (D–F), and both PD-1 and LAG-3 (G–I) are shown. Each dot is an individual child, and the solid horizontal lines indicate the median values for the respective groups. Dot plots compare the expression of the inhibitory receptors across the different groups of children for total CD8 T cells (left column), CD45RA+CD27 (T_{EM}) (middle column), and CD45RA+CD27 (T_{EM,eff}) CD8 T cells (right column). Statistical significance between pairs of groups was determined with the Wilcoxon rank-sum test. *p < 0.05, ** p < 0.01, ***p < 0.001.

Expression of phenotypic markers of T cell exhaustion by comparing B and T cell profiles of children with differential *P. falciparum* exposure but who otherwise live under similar conditions in rural Kenya. From these well-characterized cohorts we were able to compare profiles of children who were persistently exposed, previously but no longer exposed, and malaria naive. We observed that persistent *P. falciparum* exposure is associated with an increased frequency of atypical MBCs, and we provided evidence that *P. falciparum*-associated atypical MBCs are expanded at the expense of naive B cells. We also showed that persistent *P. falciparum* exposure increases the proportion of CD4 T cells that are PD-1 single-positive, PD-1/LAG-3 coexpressing, and to a lesser extent LAG-3 single-positive. The expansion of PD-1+ and PD-1 and LAG-3 double-positive CD4 T cells was largely confined to the CD45RA+ cells (naiveT_{EM}-late and T_{EM,eff}). Of particular interest, we observed that the percentage of CD45RA+CD27+ CD4 T (naiveT_{EM}-late) cells coexpressing PD-1/LAG-3 was inversely correlated with the frequency of activated and classical MBCs (independently of each other) among the Junju-persistently exposed children but not the Ngerenya-naive children. We also observed increases in PD-1 and PD-1/LAG-3 coexpressing CD8 T cells among the total and CD45RA+CD27+ CD8 T (T_{EM,eff}) cells, respectively, in the presence of asymptomatic parasitemia.

Our study design controlled for other factors, including genetics, other cumulative and/or concurrent infections (e.g., helminths, HIV, and common viruses and bacteria), and malnutrition that may also drive expansion of atypical MBCs and phenotypically exhausted T cells. Indeed, various chronic viral infections of humans including HIV and HCV are known to induce increased frequencies of atypical MBC (24, 26) and exhausted T cell (16, 17, 19) phenotypes. It is also conceivable that genetic factors and nutritional states associated with increased susceptibilities to such chronic infections may also be associated with increased frequencies of these cells. Thus, the expansion of atypical MBCs among the Junju-persistently exposed children, relative to the Ngerenya-naive and historically exposed children, provides the strongest evidence yet that *P. falciparum* infection drives the expansion of atypical MBCs. These results are in agreement with Weiss et al. (34, 35), who found increased frequencies of atypical MBCs in individuals living in malaria endemic areas relative to malaria-naive American adults. Additionally, the reciprocal reduction in the proportion of naive B cells among the persistently exposed children relative to the naive controls suggests that atypical MBCs are expanded at the expense of naive B cells.

There were no differences in the proportions of various B cell subsets between Junju-parasitemic and Junju-nonparasitic children, suggesting that changes in the distribution of B cell phenotypes that arise from cumulative *P. falciparum* exposure over time are relatively stable and not perturbed significantly as children transition from uninfected to asymptomatically infected. This contrasts with the alterations in B cell subsets observed in children during febrile malaria in which the frequency of circulating naive B cells (defined as CD19+IgD+) was lower than that observed in children with asymptomatic infection (56). This difference may reflect increased trafficking of naive B cells to lymphoid tissues as a result of the chemokine response associated with febrile malaria (57).

The increased proportions of PD-1 and LAG-3 single-positive, as well as PD-1 and LAG-3 double-positive, CD4 T cells among the persistently exposed Junju children relative to the malaria naive and previously exposed Ngerenya children provides evidence that chronic parasitemia or regular reinfections elicit exhaustion-related phenotypes on CD4 T cells. However, in the case of CD8 T cells, the increased frequency of PD-1 single-positive and PD-1 and LAG-3 double-positive CD8 T cells among the persistently exposed Junju children was mainly associated with asymptomatic parasitemia, suggesting that it was driven by active infection and not maintained after parasite burdens are reduced to levels below the limits of detection. Taken together, these data are in agreement with the findings of Butler et al. (31) and are consistent with the idea that prolonged *P. falciparum* infections induce characteristics of T cell exhaustion among both CD8 and CD4 T cells.

The negative correlation between increasing percentages of CD45RA+CD27+ CD4 T cells coexpressing PD-1 and LAG-3 and...
CD45RA+ T cells also include Ag-experienced cells that have a phenotype, historically associated with naive T cells. However, antibody (Ab) titers were largely confined to the CD45RA+CD27+ CD4 T cell subset, which was recently found to be comprised of Ag-experienced naive T cells, this subset is also comprised of Ag-experienced and memory-experienced individuals, suggesting that they could have an alternative, but as yet unidentified, role that may be associated with naturally acquired immunity to malaria. However, the emergence of exhausted B and T cells in HIV-infected adults continues to be associated with increased viral loads and uncontrolled viral replication, and hence loss of immune function (63).

Collectively, these observations are of particular interest given the longstanding and strong evidence for a critical role of Abs in parasite control. Larger studies will reveal whether there are significant correlations between T cell exhaustion with quantitative as well as qualitative features of the B cell response to malaria Ags.

**FIGURE 6.** Relationships between the expansion of PD-1 and LAG-3 double-positive CD45RA−CD27+ CD4 T cells and frequencies of different B cell subsets. *Left column,* Pooled data points from the Junju exposed-parasitic group (blue) and the exposed-nonparasitic group (red). *Right column,* Malaria-naive children (Ngerenya-naive). The correlation coefficients and associated p values were determined by Spearman rank correlation analysis.

frequencies of activated and classical MBCs suggests that *P. falciparum*–associated exhaustion of CD4 T cells may be linked to a failure to develop a fully differentiated B cell response. This observation is also consistent with the work of Butler et al. (31), who found that in vivo blockade of PD-L1 and LAG-3 restored not only CD4 T cell function, but also amplified the numbers of follicular helper T cells and germinal center B cells and plasmablasts, enhanced protective Ab levels, and rapidly cleared blood-stage malaria in mice (31). In that study, coadministration of anti-PD-L1 (a major ligand for PD-1) and anti-LAG-3 blocking Abs produced a synergistic effect, consistent with the idea that the numbers and intensity of the coexpression of inhibitory receptors are directly linked to the degree of T cell exhaustion (23, 27). Collectively, these observations are of particular interest given the longitudinal and strong evidence for a critical role of Abs in naturally acquired immunity to malaria.

The expansion of PD-1+ and PD-1 and LAG-3 coexpressing T cells was largely confined to the CD45RA−CD27+ CD4 T cell phenotype, historically associated with naive T cells. However, CD45RA+ T cells also include Ag-experienced cells that have reverted back from CD45RO expression (51, 52, 58–60). Thus, although the CD45RA−CD27+ T cell subset may include many naive T cells, this subset is also comprised of Ag-experienced and long-lived memory T cells. In agreement, Chehino et al. (61) recently found that >30% of CD4 T cells from malaria-exposed children secreting IFN-γ in response to stimulation with MSP142 were CD45RA−CD62L+.

Importantly, none of the commonly used combinations of surface markers with CD45 isotypes, including CCR7 and CD28, is wholly specific for any one functional subset (62). These caveats underscore the importance of future studies to both determine the Ag specificity and relative functional capacities of the immune cell subsets we have identified in this study.

Taken together, our data confirm that persistent *P. falciparum* infection can drive the expansion of atypical MBCs and the expression of phenotypic markers of T cell exhaustion. It remains to be determined whether atypical MBCs in the context of malaria are functionally exhausted as initially suggested in HIV (24). In the Mali malaria study (35), frequencies of atypical MBCs increased with age, and hence with immunity, among malaria-exposed individuals, suggesting that they could have an alternative, but as yet unidentified, role that may be associated with naturally acquired immunity to malaria. However, the emergence of exhausted B and T cells in HIV-infected adults continues to be associated with increased viral loads and uncontrolled viral replication, and hence loss of immune function (63). Further functional studies are therefore required to determine their function and hence the biological relevance for their expansion in malaria infection. Nevertheless, the induction of phenotypically exhausted T cells and the negative association of exhausted CD45RA−CD27+ CD4 T cells coexpressing PD-1 and LAG-3 with markers of humoral immunity suggest that induction of T cell exhaustion may also be an immune evasive strategy for *P. falciparum*, as suggested elsewhere (31).

Loss of CD4 T cell function may deny normal costimulation to B cells, resulting in poor Ab induction and impaired memory responses. Mechanistically, this could be related to recently described Plasmodium proteins that may play a role in suppressing an effective inflammatory response (64) and subverting the development of T cell memory (65). Future approaches should combine immunodevelopment and molecular parasitology to shed more light on the relationship between the parasite and the host immune system. Larger studies will reveal whether there are significant correlations between T cell exhaustion with quantitative as well as qualitative features of the B cell response to malaria Ags.

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**Disclosures**

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**References**


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Figure S1: Validation for LAG-3 staining. Because the LAG-3 staining protocol involved complexing an α-LAG-3 antibody conjugated to biotin with streptavidin-APC and an anti-APC monoclonal conjugated to biotin, we designed an experiment to determine whether this staining was specific. Shown are histograms showing either an isotype control (centre panels) or no primary antibody (right panels) and the effect of leaving out the tertiary and quaternary amplification (bottom panels). The complete staining is the top left plot.
Figure S2: The relative percentages of the various T-cell subsets out of the total A) CD4 and B) CD8 T-cells for each of the study group.
Figure S3: The relative contribution of CD4 T-cell subsets to the total PD-1⁺, LAG-3⁺ and PD-1⁺/LAG-3⁺ populations for each of the study groups. The total height of each bar was calculated with reference to the median frequency of the stated inhibitory phenotype across the CD4 T-cell compartment. The division of each bar into subsets was performed by calculating the mean proportion of each subset, and scaling that to the median across the CD4 compartment.
Figure S4: Gating strategy for flow cytometric phenotyping of CD8 T-cells. CD8 T-cells were identified and selected from the rest of PBMCs, as shown for representatives of the malaria-naive (top panel) and exposed (bottom panel) groups. CD8 T-cells were then phenotyped into four different subsets based on CD45RA and CD27 expression. Subsequently, the percentages of total CD8 T and the associated CD8 T-cell subsets expressing PD-1 and LAG-3 were determined.