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

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

*J Immunol* published online 21 December 2012
http://www.jimmunol.org/content/early/2012/12/21/jimmunol.1202438

Supplementary Material http://www.jimmunol.org/content/suppl/2012/12/31/jimmunol.1202438.DC1

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Author Choice Freely available online through *The Journal of Immunology*
Author Choice option

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2012 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Chronic Exposure to *Plasmodium falciparum* Is Associated with Phenotypic Evidence of B and T Cell Exhaustion

Joseph Illingworth,*† Noah S. Butler, ‡ Sophie Roetynck,*§ 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+CD27+ 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: 000–000.

L

*Kenya Medical Research Institute, Centre for Geographical Medicine Research (Coast), Kilifi, Kenya; †Centre for Clinical Vaccinology and Tropical Medicine, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7HL, United Kingdom; ‡Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104; Division of Parasitology, National Institute for Medical Research, London NW7 1AA, United Kingdom; and §Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20852.

Received for publication August 30, 2012. Accepted for publication November 29, 2012.

This work was supported by Wellcome Trust Grant 89RTI0. F.M.N. is a Postdoctoral Fellow under the Malaria Vectorized Vaccines Consortium, funded by the European and Developing Countries Clinical Trials Partnership. J.I. is supported by a Wellcome Trust Ph.D. Studentship. P.B. is funded by the Medical Research Council (U.K.). This manuscript is published with permission from the Director of the Kenya Medical Research Institute.

F.M.N., J.I., N.S.B., P.D.C., S.K.P., P.B., and K.M. designed research; J.I., S.R., J.M., and F.M.N. performed research; J.I., S.R., and F.M.N analyzed research; and J.I. and F.M.N. wrote the manuscript. All authors reviewed and approved the manuscript.

Address correspondence and reprint requests to Dr. Francis M. Ndungu, Kenya Medical Research Institute, Centre for Geographic Medicine Research Coast, P.O. Box 230–80108, Kilifi, Kenya. E-mail address: FNdungu@kenmi-welcome.org

The online version of this article contains supplemental material.

Abbreviations used in this article: FCR-L4, Fre-l-like-4; HCV, hepatitis C virus; LAG-3, lymphocyte activation gene-3; MBC, memory B cell; PD-1, programmed cell death-1; PD-L1, programmed cell death-1 ligand; TCM, T central memory; TEM, T effector memory; TEMEMEM, T effector memory plus effector.

This article is distributed under The American Association of Immunologists, Inc., Reuse Terms and Conditions for Author Choice articles.
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 in response to CpG and the polyclonal activator Staphylococcus aureus Cowan (24). FCRL4+ MBCs in HIV-viremic individuals (24) also express high levels of inhibitory receptors and a profile of lymphoid-homing receptors similar to what is expressed on exhausted CD8 T cells during chronic viral infections (27). Owing to the relative hyporesponsiveness 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, Ehrrhardt 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 coinfections, 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 Geographic 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 Ngereya 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 Ngereya, which was endemic with 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. 323777), anti–CD20-allophycocyanin. (BD Biosciences, catalog no. 641396), anti–CD19-PerCP/Cy.5.5 (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/Cy.5.5 (BD Biosciences, catalog no. 560609), and anti–LAG-3-PE.Cy7 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

Customs and are described in detail elsewhere (37, 38).
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. 1 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 either of 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* transmission intensity from a parasite prevalence of 40% to 0% during 7 y in Ngerenya is contrasted with sustained transmission intensity from a parasite prevalence of 40 to 0% during 7 y in Junju. Although they had previously experienced malaria episodes, the Junju-parasitemic children were afebrile and healthy. 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-parasitemic) and those without parasitemia (Junju-nonparasitemic) at sampling (Table I). Although they had *P. falciparum* parasitemia by microscopy at sampling, the Junju-parasitemic children were afebrile and healthy. We matched the Junju-parasitemic and Junju-nonparasitemic 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 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-parasitemic, Junju-nonparasitemic, 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.

**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-CD20-; immature B cells, CD19+CD10+; classical MBCs, CD19+CD27+CD21-CD10-; atypical MBCs, CD19+CD27+CD21-CD20-; 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-parasitemic and Junju-nonparasitemic 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).

### Table I. Characteristics of study subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Junju-Parasitemic</th>
<th>Junju-Nonparasitemic</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</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), mean</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* during the 4-mo dry period preceding the cross-sectional bleed.

...rons represent the percentage of the parent gate.

**FIGURE 1.** Gating strategy for flow cytometric phenotyping of B cells. Total B cells were identified by CD19 expression and then subsets were identified by the expression of CD10, CD20, CD21, and CD27. Shown are plots representative of the malaria-naive controls (top panels) and the exposed groups (bottom panels). All numbers represent the percentage of the parent gate.

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+EFF}) (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-parasiticemia and Ngerenya-historically exposed children (Fig. 4A). Further analysis revealed increased PD-1 expression within the CD45RA+CD27+ and CD45RA-CD27- T cell compartments among the Junju-parasiticemia and Junju-nonparasiticemia 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- 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-nonparasiticemia 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-parasiticemia compared with the nonparasiticemia 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-parasiticemia 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-parasiticemia and Junju-nonparasiticemia 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 expression on CD45RA+CD27+ and CD45RA+CD27- CD4 T cell phenotypes between any groups (data not shown). However, and similar to the PD-1 single-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/
The relative percentages of the various B cell subsets out of the total CD19+ B cells for each of the study groups. Statistical significance between various paired groups was determined with the Wilcoxon rank-sum test. *p < 0.05, **p < 0.01, ***p < 0.001. (G) The relative percentages of the various B cell subsets out of the total CD19+ B cells for each of the study groups.

**FIGURE 2.** Atypical MBCs are significantly expanded in the presence of persistent *P. falciparum* transmission. (A–F) Comparison of the proportions (of total CD19+ B cells) of different B cell subsets as defined in Fig. 1 between the different study groups. Each dot is an individual child, and the solid horizontal lines indicate the median values for the respective groups. Statistical significance between various paired groups was determined with the Wilcoxon rank-sum test. *p < 0.05, **p < 0.01, ***p < 0.001.

Persistence of 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-persistently exposed children and the Junju-naive, Ngerenya-naive, and Ngerenya-historically exposed children. As 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+CD27+), nascent central memory (TCM-early) (CD45RA+CD27-), effector memory (TEM) (CD45RA-CD27+), and effector cells (TEM+EFF) (CD45RA-CD27-). Supplementary 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 (Supplementary Fig. 2B). However, the frequency of total CD8 T cells expressing PD-1 was elevated among the Junju-persistently exposed children relative to the control groups, but these differences were only significant for the comparison between Junju-parasiticemic 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+CD27- 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+CD27- 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+CD27- CD8 T cells coexpressing PD-1 and LAG-3 were significantly more abundant among circulating CD8 T cells from Junju-parasiticemic 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+CD27- and CD45RA+CD27- subsets, among the Junju-persistently exposed children (Fig. 5H, I). 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+CD27- 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+CD27- (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+ (TMP) (middle column), and CD45RA+CD27+ (TEM+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.

...
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 immunoepidemiology 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.

**Acknowledgments**

We thank the families that participated, as well as Drs. John Harty and Eunice Nduati for support, advice, and reviews.

**Disclosures**

The authors have no financial conflicts of interest.

**References**

4. Pombo, D. J., G. Lawrence, C. Hirunpetcharat, C. Rzepczyk, M. Bryden, N. Cloonan, K. Anderson, Y. Mahakunkijcharoen, L. B. Martin, D. Wilson, et al. 2005. 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 immunoepidemiology 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.

**References**


lovirus infection induces the accumulation of short-lived, multifunctional CD4+CD45RA+CD27+ T cells: the potential involvement of interleukin-7 in this process. *Immunology* 132: 326–339.


