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Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area[1]

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Abstract

Epidemiological observations in malaria endemic areas have long suggested a deficiency in the generation and maintenance of B cell memory to *Plasmodium falciparum* (*Pf*) in individuals chronically reinfected with the parasite. Recently, a functionally and phenotypically distinct population of FCRL4^{+} hypo-responsive memory B cells (MBCs) was reported to be expanded in HIV-infected individuals with high viral loads. Here we provide evidence that a phenotypically similar atypical MBC population is significantly expanded in *Pf*-exposed Malian adults and children as young as two years of age as compared to healthy U.S. adult controls. The number of these atypical MBCs was higher in children with chronic asymptomatic *Pf* infections compared to uninfected children suggesting that the chronic presence of the parasite may drive expansion of these distinct MBCs. This is the first description of an atypical MBC phenotype associated with malaria. Understanding the origin and function of these MBCs could be important in informing the design of malaria vaccines.

Keywords

B Cells; *Plasmodium falciparum* malaria; vaccination; memory; immunomodulation

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Introduction

Plasmodium falciparum (Pf), the most deadly of malaria parasites, imposes an enormous disease burden on much of the world's population (1). Malaria kills nearly one million children each year in Africa alone and there is little doubt that a malaria vaccine would play a central role in preventing these deaths. At present, the human immune response to this pathogen is poorly understood and the development of a highly effective vaccine would, in all likelihood, benefit from a better understanding of the interactions of this complex pathogen with the human immune system (2). Antibodies have been shown to have a crucial role in controlling the blood stage of *Pf* infection. The transfer of antibodies from malaria-immune adults to children with malaria resulted in a significant decrease in parasite levels and disease (3). However, epidemiological observations have long suggested that clinical immunity to malaria is slow to develop and short-lived (2). IgG antibody responses specific for *Pf* antigens are often inconsistently generated, unexpectedly short lived, and fail to consistently boost upon reinfection (4). Earlier analyses of *Pf*-specific memory B cells (MBCs) in adults and children in Kenya provided evidence that not all exposures to malaria result in the generation of MBCs (5). Recently, an increase in the total number of MBCs and a decrease in the number of naïve B cells in peripheral blood were reported for children with acute malaria (6). Collectively these observations suggest a deficiency in the generation and long-term maintenance of MBCs specific for *Pf*. An understanding of the cellular mechanisms responsible for this deficiency would inform the design of malaria vaccines that go beyond the traditional empiric approach and address *Pf*-specific modulation of the immune response.

Immunological memory, the ability to respond more rapidly and robustly to re-exposure to an antigen, is a hallmark of adaptive immunity. For antibody responses, memory is encoded, in part, in long lived MBCs (7). Most MBCs in humans express CD27, a member of the TNF family, and have somatically mutated V genes and switched Ig isotypes. Ehrhardt *et al.* (8) described a morphologically and functionally distinct human MBC population in tonsil defined by the expression of FCRL4, a member of a recently identified family of FcR like proteins. As the intracellular domain of FCRL4 contains three immunoreceptor based inhibition motifs, it is a potential B cell inhibitory receptor and recent studies *in vitro* showed that a chimeric protein containing the intracellular domain of FCRL4 and the extracellular domain of FcγRIIB blocked B cell activation when coligated to the B cell receptor (BCR) (9). The expression of the classical marker for human MBCs, CD27, is much reduced on these $FCRL4^+$ MBCs, but these B cells have undergone isotype switching and somatic hypermutation. $FCRL4^+$ MBCs were found almost exclusively in lymphoid tissues near epithelial surfaces. This MBC population expressed the activation markers CD69, CD80 and CD86 and was functionally distinct from $CD27^+$ $FCRL4^-$ MBCs, as $FCRL4^+$ MBCs proliferated and secreted high levels of immunoglobulins in response to cytokines and CD40 ligand (CD40L) but failed to proliferate in response to BCR crosslinking or treatment with Staphylococcus aureus Cowen (SAC). Recent transcriptome analyses of $FCRL4^+$ and $FCRL4^-$ MBCs showed that these two populations differentially express genes in several categories including cell-cycle regulators, adhesion molecules, homing receptors and signal transduction intermediates (10). Although a distinct function has not yet been attributed to $FCRL4^+$ MBCs *in vivo*, their exclusive location in epithelial associated lymphoid tissues and their activated phenotype suggest that they may play a role in musosal defense against invading pathogens.

Recently, Moir *et al.* (11) showed that in the peripheral blood of HIV patients with high viremia, an atypical population of memory B cells ($CD20^{hi}$/ $CD27^-$/ $CD21^{lo}$) with increased expression of FCRL4 was greatly expanded, representing on average 19% of total peripheral blood B cells, compared to less than 4% in healthy individuals. These atypical MBCs in HIV-infected individuals had undergone somatic hypermutation and class switching albeit to lower levels as compared to $CD27^+$ MBCs. Compared to naive B cells and classical memory B cells, the

atypical MBCs in the peripheral blood of HIV-infected individuals proliferated less to BCR-crosslinking and/or CD40L and the Toll-like receptor agonist, CpG, and showed a decreased ability to differentiate into antibody secreting cells in response to CpG and SAC. The atypical MBCs in HIV-viremic individuals expressed relatively high levels of inhibitory receptors and a profile of homing receptors similar to that described for tissue-based $FCRL4^+$ MBCs (10, 11) and for exhausted $CD8^+$ T cells during chronic viral infection (12). Because of the overall hypo-responsiveness of these atypical MBCs, their altered expression of inhibitory and homing receptors that together are signatures for virus-induced exhaustion of T cells (12-14), Moir *et al.* coined these atypical MBCs 'exhausted MBCs'. HIV-specific MBCs were found to be increased in the exhausted MBC compartment as compared to the classical MBC compartment, in contrast, influenza-specific MBCs were more prevalent in the classical MBC compartment. Importantly, exhausted MBCs were found in normal levels in peripheral blood of individuals treated to reduce viremia. These authors proposed that chronic HIV stimulation of B cells may lead to their premature exhaustion, contributing to the poor antibody responses in HIV-infected individuals. Here we report that B cells phenotypically and functionally similar to exhausted MBCs are expanded in individuals chronically exposed to *Pf*. We propose that the high prevalence of MBCs of this atypical population may contribute to the delayed acquisition and short-lived nature of malaria B cell immunity.

Materials and Methods

Subjects and clinical samples

Details of the study site and cohort have been published elsewhere (15). In May 2006, just prior to the malaria season, 225 individuals between the ages of 2 and 25 years were enrolled in an observational cohort study in the rural village of Kambila, Mali. Individuals were invited to participate after random selection from an age-stratified census of 1500 inhabitants. Enrollment exclusion criteria were hemoglobin <7 g/dL, fever ≥37.5°C, acute systemic illness, use of anti-malarial or immunosuppressive medications in the past 30 days, or pregnancy. Of note, asymptomatic *Pf* parasitemia at enrollment was not exclusionary, and was not treated with antimalarial drugs. For this analysis, an age-stratified subset (n=87) was randomly selected from the study cohort. *Pf* transmission is intense at this site and typically begins in June, peaks in October, and ends in December (16). During the eight month study period that pertains to this analysis (May – December 2006), subjects were instructed to report symptoms of malaria at the village health center, staffed 24 hours per day by a study physician (i.e. passive malaria surveillance). From those with signs or symptoms of malaria, thick blood smears were stained with Giemsa and counted against 300 leukocytes. Slide positive patients were treated with a standard 3-day course of artesunate plus amodiaquine. Children with severe malaria were referred to the District Hospital after an initial parenteral dose of quinine. For data analysis, malaria was defined as an axillary temperature ≥37.5°C, *Pf* asexual parasitemia ≥5000/μl, and a non-focal physical examination by the study physician. For each study participant, the malaria incidence and the time to the first malaria episode were determined. Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood collected before (May 2006) and at the peak of malaria transmission (October 2006) and stored in liquid nitrogen. Stool and urine were examined at enrollment for the presence of helminth infections. Hemoglobin was typed by high performance liquid chromatography (HPLC; D-10 instrument; Bio-Rad, Hercules, CA). Peripheral blood samples from 10 healthy, anonymous, adult blood bank donors in the US were also analyzed. Travel history for these individuals was not available, but prior exposure to *Pf* is unlikely.

This study was approved by the Ethics Committee at the Faculty of Medicine, Pharmacy and Odonto-Stomatology; and the Institutional Review Board at the National Institute of Allergy

and Infectious Diseases. Informed consent was obtained from adult participants, and from the parents or guardians of participating children.

Malaria slides

Thick blood smears were stained with Giemsa and counted against 300 leukocytes. *P. falciparum* densities were recorded as the number of asexual parasites/μl of whole blood, based on an average leukocyte count of 7500/μl. Each smear was evaluated separately by two expert microscopists blinded to the clinical status of study participants. Any discrepancies were resolved by a third expert microscopist.

Stool and urine exam for helminth infection

At enrollment, duplicate stool samples were examined for *Schistosoma mansoni* eggs and other intestinal helminths using the semi-quantitative Kato-Katz method. To detect *Schistosoma haematobium* eggs, 10 ml of urine were poured over Whatman filter paper. One or two drops of ninhydrine were placed on the filter and left to air dry. After drying, the filter was dampened with tap water and helminths eggs detected by microscopy.

Statistical analysis

Data was analyzed using STATA software (StataCorp LP, 2007, Release 10.0). The nonparametric Wilcoxon rank-sum and Wilcoxon matched pairs tests were used to compare continuous variables between unpaired and paired groups, respectively. Age adjusted Cox regression, logistic regression and poisson regression models were used to assess the association between the percentage of atypical MBC and malaria risk. For all tests, two-tailed p values were considered significant if ≤ 0.05.

Phenotype analysis

PBMCs were obtained by density-gradient centrifugation. All phenotypic analyses were performed using mouse mAbs specific for human B cell markers conjugated to fluorophores. The source of mAbs specific for the following markers conjugated to fluorophores is as follows: PECy7-CD19, PE-CD20, APC-CD10, APC-CD27 and PE-IgG, BD Biosciences (San Jose, CA); FITC-CD21, Beckman Coulter (Fullerton, CA); PE-CD85j, PE-CXCR3, PE-CCR6, PE-CCR7, PE-CXCR4, and PE-CXCR5, R&D Systems (Minneapolis, MN) and PE-CD11c, Invitrogen (Carlsbad, CA). The FCRL4-specific mAb was kindly provided by M.D. Cooper (Emory University School of Medicine, Atlanta, GA) (8). PE-conjugated rabbit antibodies specific for mouse IgG2a were purchased (Invitrogen) and used to detect the mouse FCRL4-specific mAb. A four color two-stain strategy was used to identify B cell subpopulations (stain 1: FITC-CD21, PE-CD20, PECy7-CD19, APC-CD10; stain 2: FITC-CD21, PE-IgG, PECy7-CD19, APC-CD27). Using this strategy we report naïve B cells as the number of $CD19^+$ $CD21^+$ $CD27^-$ cells from which was subtracted the number of $CD19^+$ $CD21^+$ cells that were $CD10^+$, namely immature cells. We determined that naïve germinal center cells, $CD19^+$ $CD21^+$ $CD10^+$ $CD27^+$ constitute approximately 2% of $CD19^+$ cells (unpublished observations) and this 2% was added to the number of naïve B cells. Plasma cells/blasts are reported as the number of $CD19^+$ $CD21^-$ $CD20^-$ $CD10^-$ cells. Immature B cells are reported as the number of $CD19^+$ $CD10^+$ cells. Classical MBCs are reported as the number of $CD19^+$ $CD27^+$ $CD21^+$ cells. Atypical MBCs are reported as the number of $CD19^+$ $CD21^-$ $CD27^-$ cells minus the number of $CD19^+$ $CD21^-$ $CD10^+$ immature B cells. Activated MBCs are reported as the number of $CD19^+$ $CD21^-$ $CD27^+$ cells minus the number of $CD19^+$ $CD21^-$ $CD10^-$ $CD20^-$ plasma cells. FACS analyses were performed on a FACSCalibur flow cytometer, BD Biosciences using FlowJo software, Tree Star (Ashland, OR).

B cell fractionation

Mature ($CD10^-$) B cells were isolated from PBMCs by negative magnetic bead-based selection using a B cell enrichment cocktail supplemented with tetrameric CD10-specific mAb, Stem Cell Technologies (Vancouver, B.C., Canada). Mature B cells were separated into $CD19^+$, $CD27^+/CD21^{hi}$ and $CD27^-/CD21^{lo}$ fractions using a two step magnetic bead selection process as detailed elsewhere (11). The subpopulations were cultured as previously described (17) in complete media alone or complete media plus a cocktail of polyclonal activators which included 2.5 μg/ml of CpG oligonucleotide ODN-2006 (18) from Eurofins MWG/Operon (Huntsville, AL), Protein A from *Staphylococcus aureus* Cowan at 1/10,000 dilution from Sigma-Aldrich (St. Louis, MO), pokeweed mitogen at 1/100,000 dilution from Sigma-Aldrich, and IL-10 at 25 ng/ml from BD Biosciences. Cells were kept at 37°C in a 5% CO_2 incubator for 5 days, washed twice with complete media warmed to 37°C, counted and distributed onto 96 well ELISPOT plates coated with human IgG-specific goat antibodies to detect all IgG-secreting cells as described (19).

Results

To determine the impact of *Pf* infection on the generation and maintenance of B cell memory we initiated a longitudinal study of children and adults in Mali in May 2006, just prior to the six-month malaria transmission season as described earlier (15). Baseline characteristics of the study subjects are shown in Table I. Several of the variables listed are known to be associated with decreased risk of malaria including age (20), red blood cell polymorphisms (21), and asymptomatic parasitemia (22). Of the variables listed, we determined that only three were associated with decreased risk of clinical malaria in our Mali cohort, namely greater age, sickle cell trait (HbAS), and asymptomatic *Pf* parasitemia at the time of enrollment, just before the malaria season (15). Asymptomatic parasitemia at the time of enrollment implies that the individual has been stably infected with *Pf* parasites for at least six months as there is little or no malaria transmission during the six month dry season prior to enrollment (16). Malaria outcomes by age group are shown in Table II and, as noted, risk of clinical malaria decreased with age, as measured by either malaria incidence or time to the first malaria episode.

The B cells in the peripheral blood of the volunteers at the peak of the malaria transmission season in October were characterized by flow cytometry using a panel of fluorophore conjugated antibodies specific for CD19, CD27, CD21, CD10 and CD20 that allowed the identification of naïve B cells ($CD19^+$ $CD27^-$ $CD21^+$ $CD10^-$), plasma cells or plasma blasts ($CD19^+$ $CD27^+$ $CD21^-$ $CD20^-$), immature B cells ($CD19^+$ $CD10^+$), classical MBCs ($CD19^+$ $CD27^+$ $CD21^+$ $CD10^-$), atypical MBCs ($CD19^+$ $CD27^-$ $CD21^-$ $CD20^+$ $CD10^-$) and activated MBCs ($CD19^+$ $CD27^+$ $CD21^-$ $CD20^+$ $CD10^-$). Fig. 1 shows the gating strategy used to identify these B cell subpopulations and a representative example of an individual from the U.S. as compared to an individual in Mali.

A comparison of the proportion of B cells in each subpopulation in the peripheral blood of ten healthy U.S. adults to 87 Malian adults and children is given (Figs. 2A-F) as a percent of total $CD19^+$ B cells. The percent of $CD19^+$ B cells per PBMC did not vary significantly between individuals before and at the peak of the malaria transmission season (before season: mean 12.16% [95% CI, 11.36-12.97] vs. peak season: mean 11.71% [95% CI, 10.86-12.57] p=0.451). The relative proportions of all the B cell subpopulations analyzed per total B cells for each age group are also shown as stacked plots in Fig. 2G. The percent of naïve B cells (Fig. 2A) was similar in both U.S. and Malian adults and children, as was the percent of plasma cells/plasma blasts (Fig. 2B). The proportion of immature B cells appeared to be lower in Malian adults as compared to either U.S. volunteers or Malian children (Fig. 2C) although a more complete phenotypic analysis will be necessary to better characterize these cells. As compared to U.S. adults, Malian adults had a similar percent of classical MBCs (Fig. 2D). Malian children in

both the 2-7 and 8-10 year age groups had a smaller percent of classical MBCs as compared to either U.S. or Malian adults, likely an age-related phenomenon unrelated to malaria. Strikingly, the percent of atypical MBCs was significantly higher in Malian adults and children compared to U.S. adults, with a trend of increasing atypical MBCs with increasing age among the Malian donors (Malian adults: mean 15.5% [95% CI, 9.7-21.2] vs. U.S. adults: mean 1.6% [95% CI, 1.0-2.2]; $p<0.001$; Malian children: mean 9.8% [95% CI, 8.2-11.3]; $p<0.001$ vs. U.S. adults) (Fig. 2E). The percent of B cells with an activated MBC phenotype was significantly higher in Malian adults as compared to Malian children and US adults (Malian adults: mean 3.7% [95% CI, 2.5-5.0] vs. U.S. adults: mean 1.3% [95% CI, 0.6-2.1]; $p=0.001$; Malian children: mean 1.9% [95% CI, 1.4-2.4]; $p<0.001$ vs. Malian adults) (Fig. 2F). As with the atypical MBCs, there was a trend of increased activated MBCs with increased age among the Malian donors. For both atypical MBCs and activated MBCs the largest differences were between U.S. and Malian adults. We verified that the expansion of atypical MBCs in this population was not an artifact of freezing and thawing by analyzing fresh PBMC from 16 adults at the same study site (mean percentage of atypical MBCs from fresh PBMC: 14.1% [95% Cl, 8.1-20.1]).

To assess class-switching in the MBC subpopulations we analyzed cell surface IgG expression of atypical and classical MBC in our Malian cohort. Overall the pattern of IgG-expression was similar for classical and atypical MBCs (Fig. 3) with the proportion of IgG^+ MBCs increasing with age in both classical and atypical MBCs. The proportion of atypical MBCs that were IgG^+ was somewhat higher than the proportion of classical MBCs that were IgG^+. We conclude that the istotype switching history is similar in the different MBC subpopulations and that the high proportion of IgG^+ atypical MBCs indicates that it is likely these cells have undergone somatic hypermutation.

The atypical MBCs, classical MBCs and naïve B cells from peripheral blood of a randomly selected subset of Malian adults (n=6) and children age 2-4 (n=6) were further analyzed to determine the expression level of several inhibitory and homing receptors which are characteristic of both tissue-based (10) and exhausted MBCs (11) (Fig. 4). FCRL4, the cell-surface marker that defines tissue-based MBC and is a characteristic of exhausted MBCs was expressed at significantly higher levels on atypical MBCs compared to classic MBCs and naïve B cells. The expression pattern of inhibitory and homing receptors on atypical MBC was similar for Malian children and adults, and comparable to that observed in HIV viremic individuals and in $FCRL4^+$ tonsilar MBCs (8,10,11). Atypical MBCs showed increased expression of the inhibitory receptors CD85j and CD22. No differences were observed between activated MBCs and atypical MBCs in the expression of the inhibitory receptor LAIR1, although as compared to naïve B cells both subpopulations expressed less LAIR1. CD11c and CXCR3 levels were increased on atypical MBCs as compared to either classic MBCs or naïve B cells. CXCR4, CD62L, CXCR5 and CCR7 expression was decreased on atypical MBCs and little difference between subpopulations was observed in the expression of CCR6 (Fig. 4) or CD72 (data not shown). The phenotypes of both the naïve B cells and classical MBCs was similar to that described for U.S. individuals.

Exhausted MBCs in HIV-viremic individuals were hypo-responsive in their ability to differentiate into antibody secreting cells (ASC) *in vitro* in response to polyclonal stimulation with a combination of CpG and SAC (11). In preliminary studies, we separated peripheral blood B cells from Malian adults into atypical MBCs ($CD19^+$ $CD27^-$ $CD21^{lo}$), classical MBCs ($CD19^+$ $CD27^+$ $CD21^{hi}$) and naïve B cells ($CD19^+$ $CD27^-$ $CD21^{hi}$) as described (11). When stimulated with CpG and SAC atypical MBCs failed to produce any ASC (n=1; classic MBC $34{,}900/10^6$ at end of culture [SD ± 2,000]; atypical MBC $0/10^6$; naïve B cells $1{,}300/10^6$ [SD ± 200]). As compared to classical MBCs, atypical MBCs responded poorly to the combination of pokeweed mitogen, SAC, CpG and IL-10 a combination that is more efficient in inducing

the differentiation of MBCs into ASCs (unpublished data) (n=2; classic MBC 102,800/10^6 at end of culture [SD ± 18,200]; atypical MBC 2,600/10^6 [SD ± 1,200]; naïve B cells 8,250/10^6 [SD ± 2,000]). Naïve B cells, as predicted (7), responded weakly to both stimulation cocktails. By this criteria, the atypical MBCs in Malian volunteers are hypo-responsive to stimuli that activate classical MBCs, and thus both phenotypically and functionally resemble exhausted MBCs.

To determine if the increase in atypical MBCs is related to exposure to *Pf*, we compared the percentage of atypical MBCs in children with or without asymptomatic *Pf* parasitemia at the end of the six-month dry season, during which little or no parasite transmission occurs (16). *Pf* parasitemia at this timepoint reflects a chronic infection persisting from the previous year's transmission season; conversely, aparasitemic individuals at this time have likely been aparasitemic for months (16). The percent of asymptomatic parasitemic individuals was similar across all age groups and ranged between 2-5%. There was a trend toward a higher percentage of atypical MBCs in children age 2-10 years with asymptomatic *Pf* infection (n=9) as compared to those without infection (n=62) (Fig. 5A; with *Pf* parasitemia: mean 14.7% [95%CI, 2.7-21.0]; without*Pf* parasitemia: mean 9.9% [95%CI, 8.1-11.7]; p=0.055). In multivariate regression analysis that included age as a covariate, this association did not reach statistical significance. Intestinal helminth infection was not associated with a significant change in the percentage of atypical MBCs in children aged 2-10 years (Fig. 5B); with helminth infection: n=8 mean 13.4% [95%CI, 5.2-21.6]; without helminth infection: n=56 mean 9.9% [95%CI, 8.1-11.7]; p=0.291), although the sample size may be too small to detect a significant difference. Of note, neither the percentage of atypical MBCs before the malaria season nor the percentage at the peak of the malaria season was associated with risk of clinical malaria, as defined by malaria incidence or time to the first malaria episode (not shown).

Discussion

The finding of an expanded, atypical MBC subpopulation in Malian individuals is the first description of a phenotypic alteration of MBCs in individuals exposed to *Pf*. At present, the factors that cause the expansion of the atypical MBCs are not known. In HIV infections the virus appears to play a role in driving B cells into the exhausted MBC subpopulation. In individuals with untreated HIV infections, the majority of HIV-specific MBCs were present in the exhausted MBC subpopulation (11) and in patients whose viral loads were reduced to levels below detection by antiretroviral therapy, the number of exhausted MBCs decreased to about half over a period of six months. However, the exhausted MBC subpopulation in treated patients remained statistically greater than that in healthy donors, presumably due to viral spiking (unpublished observation). Due to the small cell numbers we were able to obtain in our Malian cohort, especially from young children, we were unable to carry out similar assays to directly determine if *Pf*-specific MBCs were differentially represented in the atypical MBC population. Finding that persistent *Pf* infection may be associated with a greater degree of expansion of atypical MBCs, suggests that parasite antigens or other parasite products may be responsible for driving the B cells into the atypical MBC subpopulation. Other factors could account for the expanded atypical MBC in Malian individuals, such as genetic background or environmental factors associated with *Pf* transmission that were not assessed in our study, for example, malnutrition. Although we did not test for HIV, it is unlikely that HIV is responsible for the expanded atypical MBC compartment in our study population since the prevalence of HIV in Mali is extremely low (1.5%) and based on demographic data we would expect HIV prevalence be lower than the country average in our study population (23).

The role of atypical MBCs in the context of malaria remains unclear. It has been suggested that $FCRL4^+$ MBCs resident in mucosal lymphoid tissue play a role against invading pathogens, possibly through their influence on other cells, either directly or indirectly through

the secretion of cytokines (8,10). Moir *et al.* (11) concluded that the HIV-associated exhaustion of B cells may play a role in the diminished HIV-specific antibody responses in infected individuals. By analogy it may be that atypical MBCs in malaria are the end product of a defective pathway that normally functions to yield differentiated MBCs. Indeed, our preliminary results suggested that atypical MBC in malaria exposed individuals were hypo-responsive to a combination of polyclonal activators that are known to activate classical MBCs. It is possible that atypical MBCs are responsive to other stimuli and contribute to the short-lived nature of the antibody response observed in malaria endemic areas (2) by giving rise to short-lived plasma cells. We recently observed that *Pf*-specific antibodies rose significantly during the six month malaria transmission season in the children in the cohort analyzed here, however these antibodies were short lived and were undetectable by the beginning of the next malaria transmission season (unpublished observation). This phenomenon could reflect the activation of atypical MBCs to give rise to short-lived plasma cells or the interference of atypical MBCs in the normal generation of long-lived plasma cells in response to *Pf* infection.

It is also possible that the expansion of atypical MBCs in *Pf* infection may in some way benefit the host, reflecting the unique relationship between the parasite and the host that allows the asymptomatic persistence of the parasite within an otherwise functional immune system in individuals who have acquired clinical immunity. To this point, we observed that adults, the majority of whom are immune to clinical malaria showed more atypical MBCs in peripheral blood as compared to children. Given that the human and *Plasmodium* genomes have co-evolved (24), it is possible the *Pf* has shaped immune mechanisms which allows chronic and recurrent infections to occur. Presumably the persistence of the parasite has some benefit to the host as indicated, for example, by our finding that asymptomatic *Pf* infection is associated with protection against clinical disease (15). It is possible that atypical MBCs play a beneficial role in protecting the host from clinical disease by modulating immune responses, for example, through the secretion of cytokines to control inflammation.

It will be important to determine if individuals with expanded atypical MBCs can be effectively vaccinated to produce long term *Pf*-specific memory responses. We have observed that compared to U.S. adults, Malian adults appear to respond less well to the same candidate malaria vaccine in Phase I clinical trials as measured by the generation of antigen-specific MBC ((17) and unpublished observations). Future studies will be needed to determine whether the expansion of atypical MBCs represents a protective response or an immune evasion strategy of *Pf*, and if the latter, whether it can be overcome by vaccination that specifically addresses this mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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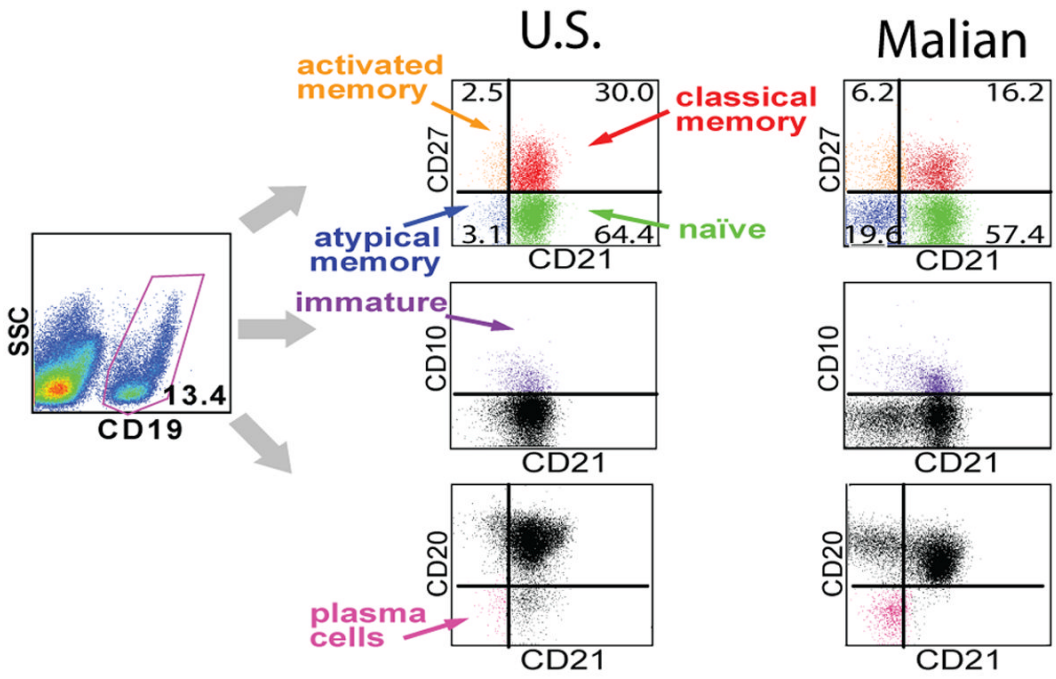

FIGURE 1.

Flow cytometry gating strategies for B cell phenotyping. FACS plots of B cell subsets of a representative malaria naïve US and a *Pf*-exposed Malian volunteer. Within the $CD19^{+}$ gate the B cell subpopulations are defined as follows: activated memory (orange) ($CD27^{+}CD21^{lo}CD20^{hi}CD10^{-}$), classical memory (red) ($CD27^{+}CD21^{hi}$), atypical memory (blue) ($CD27^{-}CD21^{lo}CD20^{hi}CD10^{-}$), naïve (green) ($CD27^{-}CD21^{hi}CD10^{-}$), immature (purple) ($CD10^{+}$) and plasma cells or plasma blasts (pink) ($CD27^{hi}CD21^{lo}CD20^{-}$). A four-color two-stain strategy was used as detailed in Materials and Methods to quantify the number of B cells in each subpopulation.

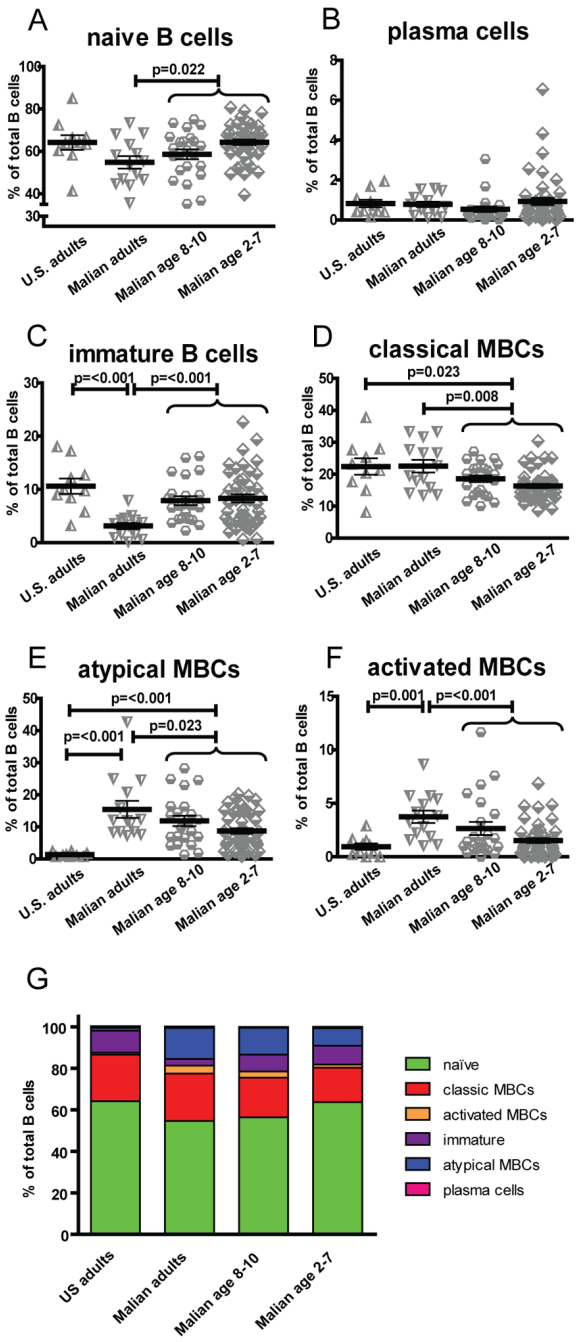

FIGURE 2.

Atypical MBCs are significantly increased in Malian as compared to U.S. volunteers. (A-F) The percent of B cell subsets was determined by flow cytometry with phenotypic analysis of subsets as defined in Fig. 1 and detailed in the Materials and Methods section. B cell subpopulations are expressed as a percent of total $CD19^+$ B cells for U.S. adults (n=10), Malian adults (n=14) and Malian children ages 8-10 (n=25) and 2-7 (n=36). The Wilcoxon rank-sum test was used to compare continuous variables between groups. (G) The relative proportions of all the B cell subpopulations as analyzed per total $CD19^+$ B cells for each age group are given in stacked plots.

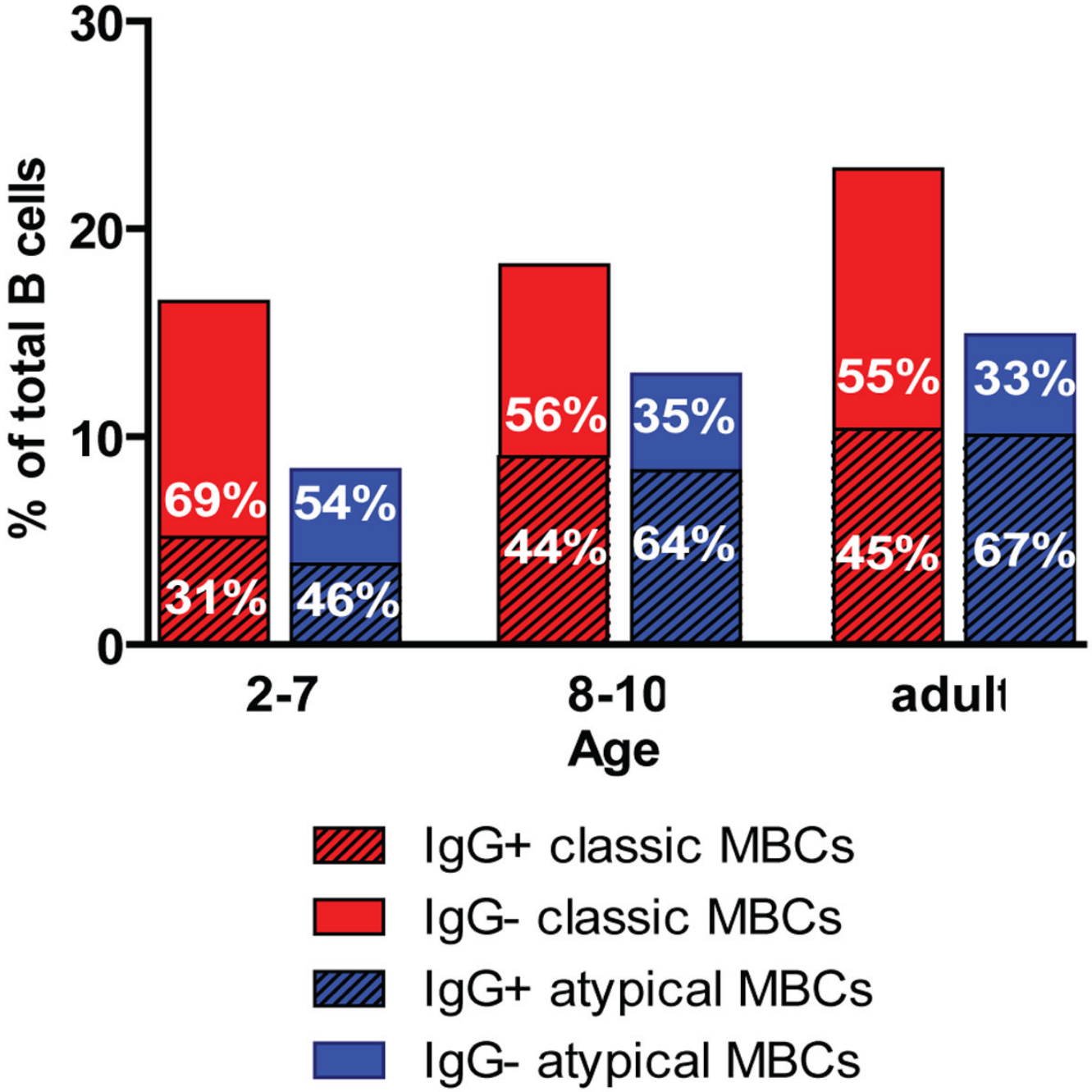

FIGURE 3.

The IgG expression of atypical and classical MBCs is similar. Stacked plots showing the proportion of IgG^+ and IgG^- MBC for both the classical MBC subpopulation and the atypical MBC subpopulation. The phenotyping was as in Fig. 1, detailed in Materials and Methods.

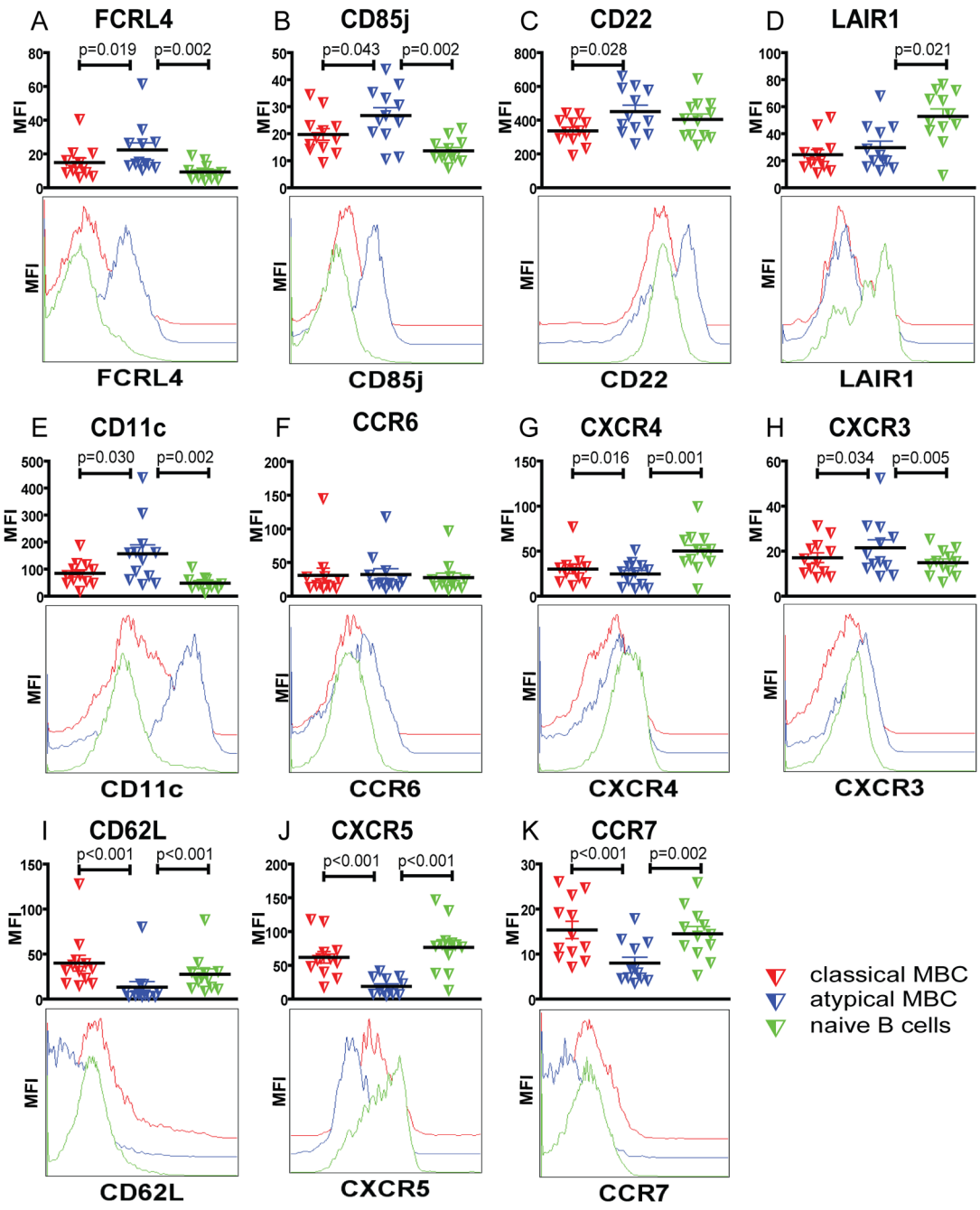

FIGURE 4.

Inhibitory and tissue-homing receptor expression is increased and lymph node homing receptor expression is decreased on atypical MBCs relative to classical MBCs. FACS analysis of the expression of inhibitory and homing receptors on naïve B cells (green), atypical MBCs (blue) and classical MBCs (red) on a subset of 12 Malian individuals: 6 children age 2-4 and 6 adults. For each panel (A-F) the top plots show individual MFI values for each cell subpopulation of each individual, as well as the average MFI and standard deviation. Underneath are histograms of the MFI from a representative individual of each subpopulation. The expression of inhibitory receptors (A-D), tissue homing receptors (E-H) and lymph node homing receptors (I-K) is given for classical MBCs, atypical MBCs and naïve B cells (as defined in Fig. 1) using

appropriately labeled antibodies specific for CD19, CD27, CD21 and the particular inhibitory and homing receptors indicated. The data for different subsets of B cells from individual donors were paired and the Wilcoxon matched pair test was used for the comparison.

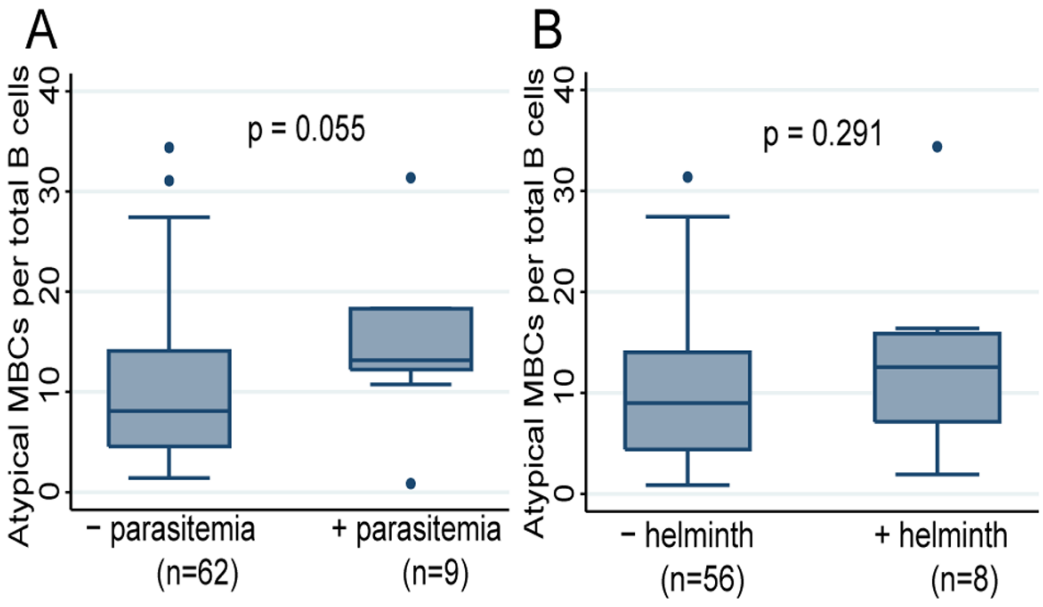

FIGURE 5.

The percent of atypical MBCs is larger in children with persistent asymptomatic *P.falciparum* parasitemia as compared to parasite-free children. Shown is the percent of atypical MBCs per total B cells in children aged 2-10 years (A) with (n=9) or without (n=62) *Pf* parasitemia at the end of the dry season or (B) with (n=8) or without (n=56) helminth infection. The Wilcoxon rank-sum test was used to compare continuous variables between groups.

Table I

Baseline characteristics by age group.

	Age group, years				
	2-4 (n=35)	**5-7 (n=11)**	**8-10 (n=25)**	**18-25 (n=16)**	**All (n=87)**
Gender, % female (no.)	71.4 (25)	54.6 (6)	32.0 (8)	50.0 (8)	54.0 (47)
Ethnicity, % (no.)					
Bambara	62.9 (22)	27.3 (3)	56.0 (14)	68.8 (11)	57.5 (50)
Sarakole	31.4 (11)	72.7 (8)	28.0 (7)	25.0 (4)	34.5 (30)
Fulani	2.9 (1)	0.0 (0)	12.0 (3)	6.3 (1)	5.7 (5)
Malinke	2.9 (1)	0.0 (0)	4.0 (1)	0.0 (0)	2.3 (2)
Hemoglobin AS, % (no.)[a]	15.2 (5)	0.0 (0)	4.0 (1)	12.5 (2)	9.4 (8)
P. falciparum smear positive at enrollment, % (no.)[b]	8.6 (3)	18.2 (2)	16.0 (4)	12.5 (2)	12.6 (11)
Parasitemia if smear positive at enrollment, parasites/microliter [geometric mean (95% CI)]	952 (33– 27878)	2862 (155 – 52838)	426 (80– 2265)	491 (0.31-765982)	770 (356– 1664)
GI helminth, % positive at enrollment (no.)[c]	12.9 (4)	18.2 (2)	9.1 (2)	0 (0)	10.5 (8)
Urine schistosomiasis, % positive at enrollment (no.)[d]	0 (0)	0 (0)	8.3 (2)	41.7 (5)	9.5 (7)
Distance lived from clinic, meters (mean ±SD)	397.4 (±125)	334 (±64)	385 (±85)	386 (±74)	384 (±99)
Bed net use, % (no.)[e]	29.0 (9)	30.0 (7)	16.7 (4)	33.3 (5)	28.7 (25)

[a] Data available for 85 subjects.

[b] All subjects were asymptomatic at enrollment.

[c] Data available for 76 subjects; GI=gastrointestinal.

[d] Data available for 74 subjects.

[e] Nightly bednet use self-reported at the end of the malaria season.