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The Regulation of Inherently Autoreactive VH4-34–Expressing B Cells in Individuals Living in a Malaria-Endemic Area of West Africa

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Plasmodium falciparum malaria is a deadly infectious disease in which Abs play a critical role in naturally acquired immunity. However, the specificity and nature of Abs elicited in response to malaria are only partially understood. Autoreactivity and polyreactivity are common features of Ab responses in several infections and were suggested to contribute to effective pathogen-specific Ab responses. In this article, we report on the regulation of B cells expressing the inherently autoreactive VH4-34 H chain (identified by the 9G4 mAb) and 9G4+ plasma IgG in adults and children living in a P. falciparum malaria-endemic area in West Africa. The frequency of 9G4+ peripheral blood CD19+ B cells was similar in United States adults and African adults and children; however, more 9G4+ B cells appeared in classical and atypical memory B cell compartments in African children and adults compared with United States adults. The levels of 9G4+ IgG increased following acute febrile malaria but did not increase with age as humoral immunity is acquired or correlate with protection from acute disease. This was the case, even though a portion of 9G4+ B cells acquired phenotypes of atypical and classical memory B cells and 9G4+ IgG contained equivalent numbers of somatic hypermutations compared with all other VHs, a characteristic of secondary Ab repertoire diversification in response to Ag stimulation.

Determining the origin and function of 9G4+ B cells and 9G4+ IgG in malaria may contribute to a better understanding of the varied roles of autoreactivity in infectious diseases. The Journal of Immunology, 2016, 197: 3841–3849.

Plasmodium falciparum malaria is a deadly infectious disease that takes the lives of nearly 600,000 individuals each year, almost all African children and pregnant women (1). There is no highly effective vaccine for malaria (2), and understanding the nature of naturally acquired malaria immunity would contribute to vaccine-development efforts (3). Abs play a central role in naturally acquired immunity to malaria, as demonstrated by the passive transfer of Abs from malaria-resistant adults to children with clinical malaria that resulted in a reduction in the levels of parasitemia and fever in these children (4). Individuals living in malaria-endemic areas acquire protective Abs, but the process is remarkably slow, requiring many years of repeated P. falciparum infections (5). The inefficient acquisition of protective Abs was attributed to the extensive genetic diversity of P. falciparum parasites (6) and to infection-mediated dysregulation of B cell responses (5, 7, 8). Understanding the mechanisms at play in the development of malaria immunity is limited by our incomplete understanding of the nature and specificities of the Abs elicited in response to malaria.

A common feature of protective Abs elicited during several viral infections is their autoreactivity and polyreactivity. High levels of autoreactive Abs were described in several viral infections in humans, including HIV, EBV, hepatitis viruses and chicken pox, and measles and mumps viruses (9). The relationship between autoreactivity and polyreactivity is perhaps best studied in Ab responses to HIV. The HIV envelope is highly diverse, and the vast majority of HIV-specific Abs elicited in infected individuals are incapable of neutralizing multiple HIV viral clades (10). Although rare, many broadly neutralizing (bN) HIV envelope–specific mAbs have been isolated (11–13), and a recent study showed that autoreactivity and polyreactivity are significantly more frequent in the bN mAbs compared with nonneutralizing HIV-specific mAbs (14). These data suggest that autoreactivity and polyreactivity of bN Abs reflect the nature of the specific neutralizing epitope and are not the result of the HIV infection per se. Moreover, the observation that HIV bN Abs are autoreactive and polyreactive...
supports the hypothesis that conserved neutralizing epitopes of HIV mimic host proteins to exploit immunological-tolerance mechanisms that would remove B cells expressing Abs specific for such epitopes from the B cell repertoire (15). Autoreactivity and polyreactivity of HIV bN Abs may be beneficial and contribute to enhanced virus neutralization by allowing bivalent heteroligation between a high-affinity HIV envelope–specific Ab binding site and a low-affinity interaction with other ligands on the viral surface (16).

These results raise the question: Are autoreactive or polyreactive Abs a component of immune responses in malaria? A variety of autoantibodies have been described in the serum of malaria-exposed individuals (17); conversely, in at least one case, sera from individuals with a systemic autoimmune disease (i.e., systemic lupus erythematosus [SLE]) reacted against *P. falciparum* parasites (18). Individuals with SLE have elevated levels of Abs encoded by the VH4-34 IgG gene detected by the 9G4 idiotype-specific mAb (19, 20). 9G4+ Abs have an intrinsic autoreactivity that is primarily determined by the VH4-34 H chain (21) and recognize N-acetyl-lactosamine epitopes expressed on a variety of glycoproteins (22–25). Indeed, 9G4+ Abs from individuals with SLE bind to a variety of self-antigens, including the N-acetyl-lactosamine on the ligand expressed on the surface of RBCs, the same glycan on the abundant B cell surface protein B220, according to distinct structural features of the 9G4+ Abs (21). It was recently shown that HIV-infected individuals had elevated 9G4+ Abs and that these 9G4+ Abs were HIV envelope specific but lacked many of the key self-reactive properties of 9G4+ Abs in individuals with SLE (26).

Indeed, a link was suggested between the control of autoimmunity in Africans and malaria exposure. Greenwood et al. (27) observed a low incidence of rheumatoid arthritis and other systemic autoimmune diseases in tropical Africa and suggested that the African environment plays a role in protection from autoimmune disease, particularly the multiple parasitic infections that individuals are exposed to from childhood, among which malaria is key. Taken together with the frequent occurrences of SLE in African Americans compared with Americans of European descent (28) this observation suggests that the African genome may encode autoimmuno-susceptibility genes that are suppressed by malaria infection. This hypothesis was tested by Greenwood et al. (29) in animal models of spontaneous autoimmune disease. Results showed that infections with the nonlethal rodent parasite *Plasmodium chabaudi* had protective effects against the development of autoimmune disease in autoimmune-susceptible (New Zealand Black × New Zealand White) F1 mice. Conversely, we provided evidence that a genetic susceptibility to autoimmunity in mice due to a deficiency inFcγRIIB or overexpression of TLR7 protected against lethal cerebral malaria caused by infection with the rodent parasite *Plasmodium berghei* ANKA (30). Despite enormous advances in the field of malaria immunology in recent years (31–33), the cellular and molecular mechanisms underlying these observations remain incompletely understood.

To address the role of autoreactivity in malaria, we undertook an analysis of the expression of 9G4+ B cells and 9G4+ Ig in a cohort of malaria-exposed Malian children and adults. Our results provide evidence that, compared with United States adults, more 9G4+ B cells acquire the phenotypes of classical and atypical memory B cells (MBCs) and that 9G4+ VH contain similar numbers of somatic hypermutations (SHMs), characteristic of Ag-driven diversification, compared with all other VH. Although the levels of 9G4+ Ig increase following acute febrile malaria, levels of 9G4+ Ig did not increase with age or correlate with protection from acute disease. Determining the origins and function of 9G4+ B cells and 9G4+ Ig in malaria may further our understanding of the possible roles of autoreactivity in infectious diseases.

**Materials and Methods**

**Study approval**
The Ethics Committee of the Faculty of Medicine, Pharmacy, and Dentistry at the University of Sciences, Techniques, and Technologies of Bamako and the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health approved this study. Written informed consent was received from participants prior to inclusion in the study. Written informed consent was obtained from parents or guardians of participating children prior to inclusion in the study.

**Human samples**

United States adult PBMC and plasma samples were obtained from the National Institutes of Health blood bank, where the medical history is unknown, but prior *P. falciparum* exposure is unlikely. The Malian samples were collected from children and adults enrolled in one of two multyear longitudinal studies of the acquisition of immunity to malaria in two rural villages, Kambila (34) and Kalifabougou (35), in Mali. In Fig. 1, adult peripheral blood samples were obtained from individuals enrolled in the Kambila study in 2009, and Malian children (6-y olds) peripheral blood samples were obtained from individuals enrolled in the Kalifabougou study in 2014. In Fig. 3B–E samples were from the Kambila study in 2009. Clinical malaria was defined as ≥2500 asexual parasites per microliter, an axillary temperature ≥ 37.5˚C or self-reported fever within 24 h, and no other cause of fever discernible by physical examination. For the Kambila and Kalifabougou cohort studies, individuals with clinical signs and symptoms of malaria and parasitemia detected by microscopy were treated with antimalarials, according to the Malian National Malaria Control Program guidelines.

**PBMC and plasma processing**

In Mali, citrate-containing cell-preparation tubes (BD, Franklin Lakes, NJ) were used to collect venous blood from subjects. Samples were transported 45 km to the Malaria Research and Training Centre in Bamako, where PBMCs and plasma were isolated per the manufacturer’s instructions. PBMCs were frozen in PBS (Life Technologies, Grand Island, NY) containing 7.5% DMSO (Sigma, St. Louis, MO), kept at −80˚C for 24 h, and then stored in liquid nitrogen. Plasma was frozen in 1-ml aliquots at −80˚C. These samples were shipped to the United States on dry ice for analysis. PBMCs were thawed and slowly diluted in complete media (RPMI 1640 + 10% FBS) by drop-wise addition. Cells were counted using a hemacytometer and stained for flow cytometry analysis.

**Abs and flow cytometry**

For cell surface staining, PBMCs were washed in PBS and in PBS incubated with LIVE/DEAD fixable Aqua (Invitrogen, Carlsbad, CA) and fluoroscently labeled Ab as indicated (Supplemental Table I). To identify 9G4+ B cells, B cells incubated with biotinylated 9G4 mAb were washed in PBS, and the biotinylated 9G4 mAb was detected using Streptavidin–Alexa Fluor 680. FACS analyses were performed on a BD LSR II flow cytometer (BD) and analyzed using FlowJo software (TreeStar, Ashland, OR).

**ELISAs**

9G4 ELISAs were carried out as described, with some modifications (36). Briefly, Nunc PolySorp plates were coated with purified 9G4+ mAb (10 μg/ml) overnight at 4˚C. Plates were washed three times with PBS–TWEEN 0.1% and blocked with Super Blocking Solution (Thermo Scientific, Waltham, MA) for 45 min at room temperature. Three serial 2-fold dilutions of samples in diluent (three parts PBS, one part SuperBlock solution, plus 0.1% Tween 20), starting at 1:4000, were incubated for 1 h at room temperature. Recombinant VH4-34 Ab (88F7) was used as a standard to allow quantification of 9G4+ IgG levels. After washing, plates were incubated with goat Abs specific for human IgG labeled with alkaline phosphatase (Sigma) at a dilution of 1:30,000 for 1 h at room temperature. Plates were washed, developing solution BluePhos substrate (KPL, Gaithersburg, MD) was added following the manufacturer’s guidelines, and OD was measured at 650 nm using a microplate reader.

9G4-IgM ELISAs were as described above for 9G4-IgG ELISAs, with the exceptions that samples were at a starting concentration of 1:1000, and goat Abs specific for human IgM labeled with alkaline phosphatase (Sigma) were used at a dilution of 1:20,000.
For total IgG and IgM ELISA, Nunc Polystrep plates were coated with human anti-IgG or human anti-IgM (both from Jackson ImmunoResearch, West Grove, PA) in PBS at 2 μg/ml and incubated overnight at 4°C. Plates were washed and incubated with SuperBlock solution for 45 min at room temperature. Three serial 2-fold dilutions of samples in diluent, starting at 1:64,000 for IgG and 1:5,000 for IgM, were incubated for 1 h at room temperature. Eleven serial 2-fold dilutions of IgG whole molecule or IgM whole molecule (both from Jackson ImmunoResearch) were used as standards, respectively. After washing, plates were incubated with goat anti-human IgG Ab labeled with alkaline phosphatase or goat anti-human IgM Ab labeled with alkaline phosphatase for 1 h at room temperature. Plates were washed, and developing was performed as described above.

AMA-1 and MSP-142 ELISAs were performed as described previously (37). AMA-1 was produced in Pichia pastoris and characterized as described (37). MSP-142 was produced in Escherichia coli, refolded, and purified as described (38). Briefly, a mixture of FVO and 3D7 P. falciparum AMA-1 and MSP-142 was used to coat the ELISA plates. The limit of detection for the ELISA was defined as the unit value at the lowest point on the standard curve multiplied by the dilution factor for plasma used. Eleven and thirty-three ELISA units were the minimal detection levels for MSP1 and AMA-1, respectively. All data below the limit of detection were assigned 6 U for MSP1 and 17 U for AMA-1 (approximately half the limit of detection).

IgH sequence analysis

Next-generation sequencing of the IgH repertoire from three B cell populations, naive, classical MBCs, and atypical MBCs, purified from the peripheral blood of four Malian adults was as previously described (39) (Sequence Read Archive accession number SRP087640, https://trace.ncbi.nlm.nih.gov/Traces/sra_sub/sra_sub.cgi?acc=SRP087640&from=list&action=show;STUDY). For the IgH sequence analysis of PBMCs of Malian infants and toddlers, a molecular identifier, clustering-based immune repertoire sequencing method was used to increase sequencing accuracy and minimize bias introduced during PCR amplification (D. Wu, B.S. Wendel, M. Qu, C. He, E. Liu, P.D. Crompton, S.K. Pierce, Q. Li, P. Ren, K. Chen, and N. Jiang, submitted for publication). The use of molecular identifiers to reduce the PCR and sequencing errors is similar to previously published methods (40). Consensus sequences were aligned to the IMGT Ab germline allele families, and possible novel germline alleles were detected via TIgGER (41). Sequences that mapped to possible novel germline alleles were excluded from these analyses. Mutations were counted as the Hamming Distance to the best-aligned IMGT germline allele, excluding the CDR3 due to the difficulty determining the germline sequence. The average number of mutations was calculated weighted by the number of consensus sequences and reported for the V segment alone or for the V and J segments together (Sequence Read Archive accession number phs001209, http://www.ncbi.nlm.nih.gov/gap).

Results

Characterization of 9G4+ B cells in United States adults and Malian adults and children

We compared the percentage of CD19+ B cells that expressed BCRs containing the VH4-34 H chain recognized by the 9G4 mAb (9G4+ B cells) in peripheral blood of adults living in the United States and adults and 6-y-old children living in P. falciparum malaria-endemic Mali. The Malian adults and children were enrolled in one of two multiyear longitudinal studies of the acquisition of immunity to malaria (34, 35). 9G4+ CD19+ B cells were detected by flow cytometry using the VH4-34 idiotype-specific mAb, 9G4, by the gating strategy shown in Supplemental Fig. 1A. The percentage of 9G4+ CD19+ B cells was similar in peripheral blood from United States adults and Malian adults and children (~4%) (Fig. 1A). Because 9G4+ B cells were shown to be restricted to naïve B cells in healthy United States individuals but increased in the MBC compartment in individuals with SLE (42, 43), we determined which B cell subsets contained 9G4+ B cells. To do so, we used the gating strategy shown in Supplemental Fig. 1A to analyze the distribution of all CD19+ B cells from United States adults and Malian adults and children in four B cell subsets: naive B cells (IgD+ CD27−), switched CD27+ B cells (IgD+ CD27+), classical switched MBCs (IgD− CD27+), and switched CD27− B cells (IgD− CD27−). The greatest difference between the United States and Malian B cells was in the percentage of all CD19+ B cells that were in the switched CD27+ B cell (IgD− CD27+) subset (Fig. 1B). In United States individuals, the percentage of cells in this subset was low (~5%). In contrast, switched CD27+ B cells represented between 15 and 20% of the CD19+ B cells in the peripheral blood of Malian adults and children. We also observed a small, but significant, decrease in the percentage of CD19+ B cells that were naïve and an increase in the unswitched and switched CD27+ B cells in Malian adults compared with Malian children.

We then determined the percentage of 9G4+ B cells in each subset. The majority of 9G4+ B cells in United States adults and Malian adults and children were naïve B cells (Fig. 1C). Malian children had a significantly larger proportion of 9G4+ naïve B cells compared with United States and Malian adults. Of particular interest, Malian adults and children had a larger percentage of switched CD27+ and switched CD27− B cells that were 9G4+ compared with United States adults. As predicted from the B cell subset distribution, the majority of 9G4+ B cells in United States and Malian adults were IgD+ and IgM+, and a greater number of 9G4+ B cells were isotype switched to IgG in Malian adults compared with United States adults (Fig. 1D).

We previously described a population of CD27− CD21− B cells that was greatly expanded in the peripheral blood of Malian adults and children but had a low frequency in United States adults, which we termed atypical MBCs (39, 44). Following the gating strategy in Supplemental Fig. 1C, we determined the percentage of all CD19+ cells that were atypical MBCs (CD19+ IgD− CD27− CD21−) and the percentage of 9G4+ cells that were atypical MBCs (Fig. 1E, 1F). Among United States adults, only ~20% of IgD− CD27− B cells were CD21− atypical MBCs, and ~30% of 9G4+ B cells were atypical MBCs (9G4+ IgD− CD27+ CD21−). In contrast, among Malian adults, ~50% of IgD− CD27− B cells were CD21− atypical MBCs, and ~45% of 9G4+ B cells were atypical MBCs (9G4+ IgD− CD27+ CD21−). In addition, a greater percentage of 9G4+ IgD− CD27− B cells were CD21− compared with all of the IgD− CD27+ B cells (Fig. 1F).

Taken together, these results indicate that, although the percentage of all CD19+ B cells that are 9G4+ is similar in individuals living in the United States and in malaria-endemic Mali, the frequency of 9G4+ B cells in the classical and atypical MBC compartments is larger in malaria-exposed individuals compared with individuals in the United States.

Characterization of 9G4+ IgG in United States and Malian adult plasma

We quantified 9G4+ IgG in adult plasma samples and determined that Malian plasma contained significantly more 9G4+ IgG compared with plasma from United States individuals (Fig. 2A). However, because the total amount of IgG was greater in Malian plasma (Fig. 2B), the mean proportion of plasma IgG that was 9G4+ was slightly less in adults in Mali compared with adults in the United States (Fig. 2C). This result indicates that, although the levels of 9G4+ IgG are elevated in Malians compared with United States individuals, 9G4+ IgG is not preferentially enriched in adult Malian plasma.

The effect of febrile malaria on 9G4+ B cells and 9G4+ IgG

We next determined the impact of acute febrile malaria on the subset distribution of 9G4+ B cells and on the levels of 9G4+ IgG. CD19+ 9G4+ B cells were quantified in the four B cell subsets in PBMCs collected from 6-y-old children at the end of the 6-mo dry season in Mali, during which there is negligible malaria transmission, as well as 7 d after their first episode of acute febrile
malaria during the ensuing transmission season. We observed a small, but statistically significant, increase in the mean percentage of 9G4+ B cells in the switched CD27+ MBC subset following acute malaria (Fig. 3A). The mean percentage of B cells in the switched CD27+ B cell subset, which contained ~60% atypical MBCs (Fig. 1F), also appeared to increase, but this was not statistically significant.

We also determined the effect of acute malaria on the levels of 9G4+ IgG in the plasma in Malian children and adults (age 2–25 y). Our previous studies showed that Abs specific for a large number of P. falciparum Ags, as measured using protein microarrays, increase during the 6-mo transmission season (45). However, in children, by the end of the subsequent dry season the Ab levels drop to slightly above those of the previous dry season. This pattern continued year after year in children, ultimately resulting in the acquisition of P. falciparum–specific Abs by early adolescence that were maintained through the dry season in adults. Assuming that the increase in stable Ag-specific Abs reflected increases in Ag-specific long-lived plasma cells, we concluded that most P. falciparum–specific Abs following acute malaria are produced by short-lived plasma cells; however, with age, long-lived P. falciparum–specific plasma cells accumulate (5). In similar studies, we did not observe increases in the levels of IgG specific for an irrelevant Ag (i.e., tetanus toxoid) during the

FIGURE 1. Characterization of 9G4+ B cells in the peripheral blood of United States adults and Malian adults and children. (A) Percentage of CD19+ B cells that are 9G4+ in the PBMCs of United States adults and Malian adults and 6-y-old children. Mean and SD are shown. (B) Percentage of all CD19+ B cells that are naive (IgD+ CD27+), unswitched CD27+ (IgD+ CD27+), classical switched MBCs (IgD+ CD27+), or switched CD27+ B cells (IgD+ CD27+). Mean and SEM are shown. (C) Percentage of B cells in each subset in (B) that are 9G4+. Mean and SEM are shown. (D) Percentage of 9G4+ B cells that stained using anti-IgM, anti-IgD, anti-IgG, or anti-IgA or were not stained by these reagents (Other) as shown in Supplemental Fig. 1B. Mean and SEM are shown. Percentage of all CD19+ B cells in each subset (All) or percentage of 9G4+ B cell in each subset (9G4+) that were CD21− for United States adults (E) and Malian adults (F) as shown in Supplemental Fig. 1C. Mean and SD are shown. *p < 0.05, **p < 0.005, ***p < 0.0005, Student t test.

FIGURE 2. 9G4+ IgG and total IgG in United States and Malian adult plasma. Quantification of IgG in the plasma of the individuals in Fig. 1. (A) 9G4+ IgG (mg/ml) in plasma of United States and Malian adults. (B) Total IgG (mg/ml) in United States and Malian adult plasma. (C) Ratio of 9G4+ IgG/total IgG (9G4+ IgG mg/ml/total IgG mg/ml) for each individual. Mean and SD are shown. *p < 0.05, ***p < 0.0005, Student t test.
transmission season, indicating a lack of obvious nonspecific, polyclonal malaria-induced activation of B cells (46).

Consistent with these published data, we observed that the levels of total IgG in plasma significantly increased in individuals following acute febrile malaria compared with IgG levels in the same individuals at baseline before the malaria-transmission season (Fig. 3B). In addition, the levels of total IgG at baseline increased with age, as shown by a linear regression in the baseline total IgG levels with age (p < 0.0001). Similarly, the amount of 9G4+ IgG increased following acute febrile malaria in children 3–10 y of age but not significantly in adults (Fig. 3C). However, in contrast to the increase in total IgG with age, the amount of 9G4+ IgG in plasma at baseline before malaria did not increase with age (Fig. 3C). The ratios of 9G4+ IgG/total IgG indicate that, for most individuals, 9G4+ IgG before malaria did not increase with age (Fig. 3C). The ratios of the levels of 9G4+ IgG in (C) to the levels of total IgG in (B). (D) The ratio of the levels of 9G4+ IgG in (C) to the levels of total IgG in (B). (E) Arbitrary ELISA units (AU) for MSP-142–specific and AMA-1–specific IgG with age. Linear regressions and 95% confidence interval are shown. Both slopes are significantly > 0 (p < 0.005). (F) 9G4+ IgG levels with age in the subjects in (D). Linear regression is shown with 95% confidence interval. The slope is not significantly different from zero. *p < 0.05, ***p < 0.0005, Student t test.

Rate of SHM in 9G4+ Ig in Malian children and adults

SHM is an important characteristic of Ab repertoire secondary diversification due to Ag stimulation (47). To determine whether 9G4+ IgGs accumulated similar numbers of SHM compared with all other VH, we analyzed two data sets of high-coverage IgVH repertoire sequences. The first was an analysis of SHM in B cells obtained from PBMCs of Malian infants (< 12 mo old) and toddlers (13–42 mo old) before and after their first episode of acute febrile malaria (D. Wu et al., submitted for publication). The second was an analysis of SHM in three subpopulations of B cells in PBMCs of four Malian adults: naive B cells, conventional MBCs, and atypical MBCs (39). For both data sets, we determined the number of SHMs in the VH4-34 sequences and compared these with the number of SHMs in all other VH sequences (Fig. 4).

The average number of SHMs in VH4-34 for both infants and toddlers was similar to that for all other VH for all isotypes, with IgMs expressing fewer SHMs than IgG and IgA for VH4-34 and all other VH (Fig. 4A). For VH4-34 and all other VH, the number of SHMs was higher in toddlers compared with infants before the malaria-transmission season and after acute febrile malaria. The only exception was for VH4-34 IgM prior to the malaria-transmission season; the number of SHMs was not significantly different in infants and toddlers. Given that the number of IgM SHMs in toddlers was higher than in infants following acute febrile malaria, these data suggest that toddler VH4-34-IgM-expressing B cells that acquired SHMs following acute malaria did not detectably persist through the 6-mo dry season, in contrast to IgG and IgA VH4-34 and IgM, IgG, and IgA of all other VH.

The number of SHMs in VH4-34 expressed by B cells in adults was similar to the number of SHMs in adult B cells expressing all
other VH for each of the three B cell subpopulations analyzed (i.e., naive, classical MBCs, and atypical B cells) (Fig. 4B). The number of SHMs in atypical and classical MBCs was greater than that in naive B cells. Thus, although SHMs acquired in the VH4-34 IgM following acute malaria in young children appeared short-lived compared with SHMs in VH4-34 IgG and IgA and in all other VH following acute malaria, B cells expressing VH4-34 IgM with acquired SHMs appear to eventually enter the long-lived B cell pool.

Levels of 9G4+ IgG in children resistant to or susceptible to febrile malaria

We determined the levels of 9G4+ IgG in children who were protected or not from febrile malaria. We focused on 7–9-yr-old children because children in this age group in our cohorts undergo a transition from malaria susceptible to malaria resistant (45). We analyzed 9G4+ IgG in plasma samples taken at the end of the dry season before the malaria-transmission season. Because we previously showed that *P. falciparum* PCR positivity in asymptomatic children at the end of the dry season correlated with a decreased risk for febrile malaria during the ensuing transmission season compared with susceptible children (Fig. 5A). This was only the case in children who were uninfected at the end of the dry season. However, the total IgG levels were also higher in protected PCR− children compared with children who were not protected from febrile malaria (Fig. 5B). We then used logistic regression to test whether levels of 9G4+ IgG at the end of the dry season predicted whether a child would be resistant to acute febrile malaria in the ensuing malaria-transmission season (Fig. 5C). First, 9G4+ IgG, 9G4+ IgM, and total IgG and IgM levels were tested individually in four simple logistic regressions, but none of the four models was significant. A multiple logistic-regression model was built using step-wise model selection to determine whether any of the 9G4+ IgG, 9G4+ IgM, or total IgG and IgM levels were significant after adjustments for patient age, gender, Hb type, and asymptomatic infections (PCR+) at the end of the dry season. The step-wise procedure did not identify any model with a significant relationship between malaria resistance and Ab levels. Model coefficients and their p values are reported for the best-fitting model (Fig. 5C), even though none of its coefficients were significant, and its total R² was low (R² = +0.0855).

**FIGURE 4.** SHM analysis of Malian young children and adult VH. (A) IgH sequence analysis for PBMCs acquired from six infants (3–11 mo) and nine toddlers (13–42 mo) prior to the malaria-transmission season (Pre) and 7 d following acute febrile malaria (Acute). Average number of mutations in IgM–, IgG–, and IgA–VH4-34 (upper panels). Average number of mutations in IgM, IgG, and IgA for all other VH genes (lower panels). (B) Number of nongap VH mutations for IgM and both IgG and IgA for naive B cells, classical MBCs, and atypical MBCs purified from the PBMCs acquired from four Malian adults for VH4-34 and all other VH. n.s: *p > 0.05, **p ≤ 0.05, ***p ≤ 0.01, Mann–Whitney U test.
Taken together with the observation that 9G4+ IgG levels did not increase with age as resistance to acute febrile malaria was acquired, these data suggest that pre-existing levels of 9G4+ IgG are not protective in malaria.

**Expression of the apoptosis-inducing receptor CD95 on B cells**

Having observed the failure of 9G4+ IgG to increase with age, even though more 9G4+ B cells appeared to have differentiated into switched CD27+ classical and CD27− atypical MBCs, we analyzed B cells for the expression of CD95, the apoptosis-inducing receptor for Fas ligand (Fig. 6). We observed in Malian adults that the percentage of B cells that express CD95 is significantly higher in 9G4+ IgD− CD27+ B cells and in 9G4+ IgD− CD27− MBCs compared with the same subsets of all CD19+ B cells. We did not observe any differences in CD95 expression in the same sub-populations in United States adults. This result suggests that 9G4+ B cells may be more sensitive to apoptosis and may, as a consequence, be short-lived.

**Discussion**

To better understand the regulation of autoimmune Abs in individuals living in malaria-endemic areas in Africa, we followed the expression of the inherently autoreactive VH4-34, recognized by the mAb 9G4, in adults and children enrolled in two different longitudinal studies of the acquisition of immunity to *P. falciparum* malaria. The regulation of 9G4+ B cells and 9G4+ Ig is of particular interest because 9G4 autoantibodies expand in SLE patients in a disease-specific fashion and recognize a variety of self-antigens (21). The expansion of 9G4+ Abs in SLE appears to be due to a defect in the exclusion of autoreactive B cells from germinal centers (42, 43). In healthy individuals, 9G4+ B cells are excluded from germinal center reactions at an early stage, in contrast to SLE patients in whom 9G4+ B cells successfully participate in germinal center reactions and expand in IgG MBC and plasma cell compartments. In individuals living with HIV, 9G4+ Abs are also prevalent and encode HIV-specific neutralizing Abs; however, these 9G4+ Abs appear to be distinct from those in SLE, and their expansion in HIV-infected individuals does not appear to be due to a general breakdown in tolerance (26).

We report that, although the frequency of 9G4-CD19+ B cells in the peripheral blood of adults and children living in malaria-endemic Mali are similar to those in healthy individuals living in the United States, 9G4+ B cells in malaria-exposed individuals expand into what appear to be classical MBC and atypical MBC compartments. We would like to understand what factors drive the differentiation of 9G4+ atypical and classical MBCs and what function they might provide. The observation that VH4-34 accumulated SHMs in classical and atypical MBCs in adults to similar extents as all other VHs suggests that 9G4+ atypical and classical MBCs arise in response to antigenic stimulation, although it is not known whether these cells respond to self-antigens or are cross-reactive.

**FIGURE 5.** Correlation of 9G4+ IgG levels and protection from acute febrile malaria. Seventy-four 7–9- y-old children (9 7-y olds, 51 8-y olds, and 14 9-y olds) were grouped as protected or not from acute malaria and either PCR− for malaria parasites or not before the malaria season. (A) 9G4+ IgG levels in May at the end of the dry season prior to the malaria-transmission season. Medians are shown. (B) Total IgG levels in May at the end of the dry season prior to the malaria-transmission season. Medians are shown. (C) Multiple logistic-regression testing of whether 9G4+ IgG or 9G4+ IgM levels at the end of the dry season predicted resistance to acute febrile malaria during the ensuing malaria-transmission season. Logistic regression was fit using JMP11. *p < 0.05, Mann–Whitney U test.

**FIGURE 6.** Expression of CD95 in B cell subsets in United States and Malian adults. Percentage of total (All) CD19+ B cells in each subset or percentage of 9G4+ B cells in each subset (9G4+) that were CD95+ (as shown in Supplemental Fig. 1D) in the peripheral blood of United States adults (A) and Malian adults (B) for individuals shown in Fig. 1A. Mean and SD are shown. ***p < 0.0005, Student t test.
with malarial Ags. Atypical MBCs were described in several chronic infections, including HIV (48, 49) and tuberculosis (50), and in autoimmune diseases (51), common features of which are chronic exposure to Ag and immunization. Clearly, inflammation could play a role in driving 9G4+ atypical MBCs in malaria. Concerning function, our recently published results indicate that atypical MBCs are not able to be activated to produce Abs or cytokines and, thus, are predicted not to contribute to sustained Ab levels (39). Although we observed increased numbers of 9G4+ B cells with phenotypic markers of classical MBCs in Malian adults and children compared with United States adults, the failure to develop stable levels of 9G4+ IgG suggests that the 9G4+ B cells may, in fact, not be expanding into functional long-lived MBCs or did not increase with age as protection is acquired, and the levels of 9G4+ IgG increased. Importantly, the levels of 9G4+ IgG appear to be selectively increased because the levels of total IgG of 9G4+ IgG may be differentially regulated to prevent the detrimental effects of the accumulation of high levels of atypical IgG. Clearly, determining the origins and function of the 9G4+ B cells and 9G4+ Ig in malaria may contribute to a better understanding of atypical autoreactive Abs in infectious diseases.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Gating strategy for assessing bulk and 9G4+ B cell subsets. Antibodies for the analysis are listed in supplemental table 1. A. Lymphocytes were first gated on by forward side scatter – area and side scatter – area. Next doublet cells were excluded by using the side scatter width 'low' gated population. Living cells were then gated as low for the live/dead dye. Next CD3 and CD19 antibodies were used to gate on bulk B cells as CD3-, CD19+. Bulk B cells were separated into naïve and memory B cell subsets by using IgD and CD27. Separately, the bulk B cells were gated on for 9G4+ity, and then the 9G4+ cells were separated by IgD and CD27 as well. B. Both bulk and 9G4+ B cells were further gated as IgD+, IgM, IgD-, or switched (IgD-, IgM-). The switched population was then further separated by IgG and IgA to decipher IgG+ (IgA-), IgA+ (IgG-), or ‘other’ unlabeled cells. C. Both bulk B cells or 9G4+ B cells were further gated on for being positive or negative for CD21 for the IgD, CD27 naïve/memory B cell subsets. D. Both bulk and 9G4+ B cells were assessed for CD95 by gating on IgD and CD27 memory subsets and using a histogram positive gate for CD95.
**Supplemental Table 1.**

**Adult Panel**

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**6 year old panel**

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Biolegend: San Diego, CA  
Beckman Dickinson (BD) Franklin Lakes, NJ  
Invitrogen Carlsbad, CA  
MiltenyiBiotec San Diego, CA

**Supplemental Table 1. Antibody panels used in cellular flow cytometric analysis.**

Two B cell flow panels with markers, fluorochrome colors, companies, catalog numbers, and company locations listed.