The Plasmodium falciparum-Specific Human Memory B Cell Compartment Expands Gradually with Repeated Malaria Infections

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Abstract

Immunity to Plasmodium falciparum (Pf) malaria is only acquired after years of repeated infections and wanes rapidly without ongoing parasite exposure. Antibodies are central to malaria immunity, yet little is known about the B-cell biology that underlies the inefficient acquisition of Pf-specific humoral immunity. This year-long prospective study in Mali of 185 individuals aged 2 to 25 years shows that Pf-specific memory B-cells and antibodies are acquired gradually in a stepwise fashion over years of repeated Pf exposure. Both Pf-specific memory B cells and antibody titers increased after acute malaria and then, after six months of decreased Pf exposure, contracted to a point slightly higher than pre-infection levels. This inefficient, stepwise expansion of both the Pf-specific memory B-cell and long-lived antibody compartments depends on Pf exposure rather than age, based on the comparator response to tetanus vaccination that was efficient and stable. These observations lend new insights into the cellular basis of the delayed acquisition of malaria immunity.

Citation: Weiss GE, Traore B, Kayentao K, Ongoiba A, Doumbo S, et al. (2010) The Plasmodium falciparum-Specific Human Memory B Cell Compartment Expands Gradually with Repeated Malaria Infections. PLoS Pathog 6(5): e1000912. doi:10.1371/journal.ppat.1000912

Introduction

To date, most successful vaccines have targeted pathogens that induce long-lived protective antibodies after a single infection, such as the viruses that cause smallpox, measles and yellow fever [1]. It has proved more difficult to develop highly effective vaccines against pathogens that do not induce sterile immunity such as the human immunodeficiency virus type-1 (HIV-1), Mycobacterium tuberculosis (Mtb), and Plasmodium falciparum malaria [2]. However, unlike HIV-1 and Mtb, clinical immunity to malaria can be acquired, but only after years of repeated Pf infections [3]. Passive transfer studies indicate that antibodies ultimately play a key role in protection from malaria [4], yet several studies show that antibodies to Pf antigens are inefficiently generated and rapidly lost in the absence of ongoing exposure to the parasite (reviewed in [5]). Elucidating the cellular basis of the inefficient acquisition of malaria immunity may ultimately prove critical to the design of an effective malaria vaccine.

Despite the key role that antibodies play in protection from a variety of infectious diseases, remarkably little is known about the cellular basis of acquiring humoral immunity in response to natural infections in humans. This gap in our knowledge is due in large part to the difficulty in studying natural infections in humans when we cannot predict who within a population will be infected with a given pathogen at a given time. Thus, our current understanding of the acquisition of immunity is largely derived from animal models and studies of humans after vaccination. These studies have established that long-lived, antibody-based immunity requires the generation and maintenance of memory B cells (MBCs) and long-lived plasma cells (LLPCs) (reviewed in [6,7]). This process begins when naive B cells bind antigen near the interface of B and T cell areas of secondary lymphoid organs. Several studies suggest that high-affinity binding drives naive B cells to differentiate into short-lived, isotyped switched plasma cells (PCs) within the extra-follicular region which contributes to the initial control of infection. In contrast, lower affinity binding selects for entry of naive B cells into follicles where germinal centers are formed. After a period of 7–10 days, through the CD4+ T-cell dependent process of somatic hypermutation, the germinal center reaction yields MBCs and LLPCs of higher affinity than the initial wave of short-lived plasma cells (SLPCs). MBCs recirculate and mediate recall responses after re-exposure to their cognate antigen by rapidly expanding and differentiating into PCs, whereas LLPCs residing in the bone marrow constitutively secrete antibody and provide a critical first line of defense against re-infection.
Memory B Cell Response to *P. falciparum* Malaria

Author Summary

*Plasmodium falciparum* (*Pf*) is a mosquito-borne parasite that causes over 500 million cases of malaria annually, one million of which result in death, primarily among African children. The development of an effective malaria vaccine would be a critical step toward the control and eventual elimination of this disease. To date, most licensed vaccines are for pathogens that induce long-lived protective antibodies after a single infection. In contrast, immunity to malaria is only acquired after repeated infections. Antibodies play a key role in protection from malaria, yet several studies indicate that antibodies against some *Pf* proteins are generated inefficiently and lost rapidly. The cells that are responsible for the maintenance of antibodies over the human lifespan are memory B-cells and long-lived plasma cells. To determine how these cells are generated and maintained in response to *Pf* infection, we conducted a year-long study in an area of Mali that experiences a six-month malaria season. We found memory B-cells and long-lived antibodies specific for the parasite were generated in a gradual, step-wise fashion over years despite intense *Pf* exposure. This contrasts sharply with the efficient response to tetanus vaccination in the same population. This study lends new insights into the delayed acquisition of malaria immunity. Future studies of the cellular and molecular basis of these observations could open the door to strategies for the development of a highly effective malaria vaccine.

The mechanisms by which antibody responses are maintained over the human life-span remains an open question. In one model, LLPCs survive indefinitely in the bone marrow and independently maintain steady-state antibody levels [8]. Alternative models predict that PCs are replenished by MBCs that proliferate and differentiate in response to persistent [9] or intermittent exposure to antigen, and/or through non-specific bystander activation (e.g. cytokines or TLR ligands) [10]. Unlike PCs, which are terminally-differentiated, MBCs may be maintained through homeostatic proliferation [11], possibly through exposure to polyclonal stimuli [10]. To address fundamental questions related to the generation and maintenance of MBCs and Abs specific for *Pf* malaria in children in malaria endemic areas, we conducted a year-long prospective study in a rural village of Mali that experiences an intense, sharply-demarcated six-month malaria season annually. We determined whether *Pf* infection generates MBCs specific for *Pf* blood stage antigens, and if so, whether they accumulate with age and cumulative *Pf* exposure, and also whether their frequency correlates with protection from malaria. In addition, we determined whether acute, symptomatic *Pf* infection resulted in an increase in the number of *Pf*-specific MBCs and the levels of specific antibodies, and if so, whether this increase remained stable over a six-month period of markedly reduced *Pf* transmission. By taking advantage of the tetanus immunization schedule in Mali in which infants and women of child-bearing age are vaccinated, we compared the relative efficiencies of the acquisition of tetanus toxoid (TT)- and *Pf*-specific MBCs and Ab, and also tested three hypotheses: 1) that growth of the MBC compartment depends on immunological experience rather than age, 2) that *Pf* infection induces non-specific activation of bystander B cells [12,13], and 3) that polyclonal activation during heterologous immune responses is a general mechanism for maintaining MBCs and LLPCs [10].

Results

Malaria immunity is acquired gradually despite intense exposure to the *Pf* parasite

In May 2006 we initiated an observational cohort study in Mali to investigate the mechanisms underlying naturally-acquired malaria immunity. A detailed description of the study site and cohort has been reported elsewhere [14]. The study population was an age-stratified, random sample representing 15% of all individuals living in a small, rural, well-circumscribed, non-migratory community where antimalarial drugs were provided exclusively by the study investigators. During a two-week period one month prior to the abrupt onset of the six-month malaria season, we enrolled 225 individuals in four age groups: 2–4 years (n = 73), 5–7 years (n = 52), 8–10 years (n = 51), and 18–25 years (n = 49). Attendance at scheduled follow-up visits was >99% for children (2–10 years) and 82% for adults (18–25 years) during the one-year study period indicating a high degree of study awareness and participation. For the MBC analysis reported here, a subset of 185 individuals was randomly selected within each of the four age categories. All subsequent data and analysis refer to these 185 individuals. The baseline demographic and clinical characteristics of this subset are shown in Table 1, according to age group. As previously reported [14], only three of the characteristics shown in Table 1 were associated with decreased malaria risk in multivariate analysis—increasing age, sickle cell trait (HbAS), and asymptomatic *Pf* parasitemia at study enrollment. During the one-year study period there were 380 unscheduled clinic visits, during which 219 cases of malaria were diagnosed, five of which met the WHO criteria for severe malaria [15]. Malaria episodes were defined as an axillary temperature ≥37.5°C, *Pf* asexual parasitemia ≥5000 parasites/μL, and a non-focal physical exam by the study physician. As expected in this region of Mali, all malaria cases were confined to a six-month period that began in July, peaked in October, and ended by January (Fig. 1A). The incidence of malaria and the proportion of individuals experiencing at least one malaria episode decreased with age, whereas the time to the first malaria episode increased with age (Table 2 and Fig. 1B). Thus, despite intense annual *Pf* transmission at this study site, malaria immunity is acquired slowly.

Analysis of *Pf*-specific and TT-specific MBCs and Abs in *Pf*-uninfected children and adults before the malaria season

We first established baseline levels of IgG* Pf*-naïve individuals [16]. This afforded the opportunity to compare the acquisition of B cell memory to the same antigens after vaccination versus natural *Pf* infection. We express MBC data as ‘MBCs per 10⁶ PBMCs’, where ‘MBCs’ refers to the number of antibody secreting cells derived from MBCs during the six-day culture, and ‘10⁶ PBMCs’ refers to the number of PBMCs after culture. In the present study, the mean
frequency of AMA1-specific MBCs per 10^6 PBMCs increased with age (Fig. 2A; 2–4 yr: 1.2 [95% CI: 0.55–1.9]; 5–7 yr: 3.2 [95% CI: 2.9–9.0]; 8–10 yr: 5.9 [95% CI: 6.3–14.3]; P<0.001). Likewise, the proportion of individuals who had detectable MSP1-specific MBCs (2–4 yr: 9.1%; 5–7 yr: 27.8%; 8–10 yr: 34.3%; 18–25 yr: 47.6%; P = 0.001) was similar to that for AMA1. Similarly, MSP1-specific Ab levels and the proportion of individuals seropositive for AMA1-specific Abs increased with age (Fig. 2A; P<0.001 for both comparisons). There was a positive correlation between the frequency of AMA1-specific MBCs and Ab levels (Spearman’s correlation coefficient = 0.33; P = 0.005; Fig. S1).

We observed a similar age-associated increase in the frequency of MSP1-specific MBCs, although the overall frequency was lower than that for AMA1-specific MBCs (Fig. 2B; 2–4 yr: 1.2 [95% CI: 0.55–1.9]; 5–7 yr: 3.2 [95% CI: 1.2–5.2]; 8–10 yr: 5.9 [95% CI: 2.9–9.0]; 18–25 yr: 10.3 [95% CI: 6.3–14.3]; P<0.001). Likewise, the proportion of individuals who had detectable MSP1-specific MBCs (2–4 yr: 9.1%; 5–7 yr: 27.8%; 8–10 yr: 34.3%; 18–25 yr: 47.6%; P = 0.001) was similar to that for AMA1. MSP1-specific Ab levels and the proportion of individuals seropositive for MSP1-specific Abs also increased gradually with age (Fig. 2B; P<0.001 for both comparisons). There was a positive correlation between the frequency of MSP1-specific MBCs and Ab levels (Spearman’s correlation coefficient = 0.34; P = 0.004; Fig. S1). Remarkably, despite exposure to 50–60 infective mosquito bites per month at the peak of each malaria season in this area [17], only approximately half of adults had detectable MBCs specific for AMA1 and MSP1, even though most had detectable AMA1- and MSP1-specific antibodies. Of the 72 individuals without detectable AMA1-specific MBCs before the malaria season, 64 (88.9% [95% CI 79.3–95.1]) did not have detectable MSP1-specific MBCs, suggesting that failure to generate MBCs to one Pf antigen is associated with failure to generate MBCs to other Pf antigens.

To understand if the expansion of Pf-specific MBCs with age was driven by repeated exposure to Pf antigens or simply a function of age, we determined the frequency of MBCs specific for an unrelated antigen, tetanus toxoid (TT), with age. In Mali, a single TT vaccine is administered to infants less than six months of age and a second TT vaccine is administered to females around 15 years of age to prevent neonatal tetanus. Thus, we measured TT-specific antibody and MBC responses at least 18 months after TT vaccination, a point at which the TT-specific response is likely to be at steady state. In contrast to what was observed for AMA1- and MSP1-specific MBCs, the frequency of TT-specific MBCs among males did not change significantly from age 2 to 25 years (Fig. 2C) [2–4 yrs: 10.8 [95% CI 7.4–29.0]; 5–7 yrs: 7.3 [95% CI 0.7–13.9]; 8–10 yrs: 8.0 [95% CI 3.1–12.8]; 18–25 yrs: 4.7 [95% CI 1.4–8.1]; P = 0.80). Similarly, the proportion of male adults who were positive for TT-specific MBCs did not differ significantly from male children (2–4 yrs: 25.0%; 5–7 yrs: 33.3%; 8–10 yrs: 40.9%; 18–25 yrs: 28.6%; P = 0.80). The slightly higher frequency of TT-specific MBCs in male versus female children was not statistically significant. However, the frequency of TT-specific MBCs was significantly higher in female adults compared to female children (Fig. 2C; mean frequency of TT-specific MBCs per million PBMC by age group [2–4 yrs: 2.9 [95% CI 0.2–6.1]; 8–10 yrs: 3.4 [95% CI 1.1–5.7]; 18–25 yrs: 58.7 [95% CI 34.2–83.3]; P<0.001) presumably the result of booster vaccination. Likewise, the proportion of female adults who were positive for TT-specific MBCs was significantly higher as compared to female children (2–4 yrs: 28.1%; 5–7 yrs: 25.0%; 8–10 yrs: 27.3%; 18–25 yrs 88.0%; P<0.001). For both females and males, TT-specific Ab levels mirrored MBC frequencies (Fig. 2C)—clearly increasing from female children to female adults (P<0.001), while not changing significantly by age in males (P = 0.44). Overall, TT-specific Ab levels and MBC frequencies correlated (Spearman’s correlation coefficient = 0.48;
To track the B-cell response to acute malaria, and after a period of prolonged period of decreased Pf exposure

The observation that Pf-specific MBCs increased with age while TT-specific MBCs in individuals who received no booster vaccine did not increase and tended to decrease slightly with age indicates that the increase in Pf-specific MBCs is driven by repeated antigen exposure and is not simply a function of age. Of note, the size of the total IgG MBC compartment, as reflected in the peripheral blood, increased with age (Fig. 3; P<0.001), consistent with the maturation of the total MBC compartment with immunological experience.

Longitudinal analysis of the Pf- and TT-specific MBC and Ab responses two weeks after acute malaria and after a prolonged period of decreased Pf exposure

To assess the Pf-specific MBC and Ab responses to acute malaria, and to determine the stability of this response during a period of little to no Pf transmission, we measured the frequencies of MBCs and Ab levels specific for AMA1 and MSP1 14 days after the first episode of malaria (convalescence), and in a cross-sectional survey at the end of the following dry season (month 12), and compared these frequencies to the pre-malaria season baseline (month 0; as detailed above). Malaria episodes were defined as an axillary temperature ≥37.5°C, Pf asexual parasitemia ≥5000 parasites/μL, and a non-focal physical exam by the study physician. Because few adults experienced malaria (Table 2), this analysis only included children aged 2–10 years (see Fig. 4 for sample sizes at each time point). The mean frequency of AMA1-specific MBCs in children aged 2–10 years increased from month 0 to convalescence (Fig. 4A; month 0: 4.7 [95% CI: 2.8–6.6]; convalescence: 13.4 [95% CI: 2.7–24.1; P=0.006] and then decreased from convalescence to month 12 (Fig. 4A; month 12: 5.9 [95% CI: 2.4–9.4]; P=0.93 versus convalescence) to a point just above the frequency at month 0 (Fig. 4A; P=0.021, month 0 vs. month 12). Likewise, the level of AMA1-specific Ab increased from month 0 to convalescence (Fig. 4A; month 0: 422.8 [95% CI: 2.4–6.6]; convalescence: 797.2 [95% CI: 460.0–1134.7; P=0.006], consistent with the maturation of the total MBC compartment with immunological experience.

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Figure 2. The Pf-specific MBC and long-lived antibody compartments expand gradually with age. Shown are the MBC frequencies (bars, left axis) and antibody levels (lines, right axis) specific for AMA1 (A) and MSP1 (B) by age category; and TT (C) by age category and gender; before the malaria season in Pf-uninfected individuals. The frequency of AMA1- and MSP1-specific MBCs increased with age (P<0.001 for both trends), as did the level of AMA1- and MSP1-specific antibodies (P<0.001 for both trends). There were no significant differences by gender for the AMA1- and MSP1-specific responses (not shown). To determine if the expansion of Pf-specific MBCs with age was driven by exposure to antigen or simply a function of age, we measured the TT-specific MBC and antibody response with age. In Mali, infants are vaccinated with TT, and females receive a TT booster around the age of 15 years to prevent neonatal tetanus. In contrast to AMA1 and MSP1, the frequency of TT-specific MBCs and the level of TT-specific antibodies for males did not change significantly from age 2 to 25 years (P = 0.80 and P = 0.44, respectively). However, the frequency of TT-specific MBCs and the level of TT-specific antibodies was higher in female adults compared to female children (P<0.001 for both comparisons). MBC frequencies were determined by ELISPOT and are expressed per million PBMC. The number of individual samples assayed and the percent of individual samples that exceeded the limit of detection (i.e. those considered positive) is indicated below the graph. The discrepancy in the sample size for ELISA data among 2–4 year olds is due to technical error during the performance of the ELISA. P values were obtained by the Kruskal-Wallis test. Data are shown as mean ± s.e.m.
doi:10.1371/journal.ppat.1000912.g002

Figure 3. The size of total IgG+ MBC compartment expands gradually with age. The frequency (bars) and level (lines) of total IgG+ MBCs measured before the malaria season increased with age (P<0.001). The number of individuals in each age category is indicated. The P value was obtained by the Kruskal-Wallis test. Data are shown as mean ± s.e.m.
doi:10.1371/journal.ppat.1000912.g003
To determine if malaria induces non-specific activation of ‘bystander’ MBCs, we compared the frequencies of TT-specific MBCs and Ab levels before the malaria season (month 0) to that 14 days after acute malaria (convalescence). We observed a small, but statistically significant increase in the frequency of TT-specific MBCs from month 0 to convalescence (Fig. 4C; month zero: 7.1 [95% CI: 3.1–11.2]; convalescence: 8.4 [95% CI: 5.0–11.8]; P = 0.012) that did not change significantly at month 12 (month 12: 9.1 [95% CI: 3.2–15.4]; P = 0.974 versus convalescence). In contrast, TT-specific Ab levels decreased slightly from month 0 to convalescence, and again from convalescence to month 12, although neither decline was statistically significant (Fig. 4C; month 0: 0.58 [95% CI: 0.5–0.7]; convalescence: 0.57 [95% CI: 0.5–0.7]; P = 0.063; month 12: 0.54 [95% CI: 0.4–0.6]; P = 0.525 versus convalescence). Collectively these results indicate that malaria infection results in an increase in the frequencies of both Pf-specific, and bystander MBCs. However, malaria selectively induces Pf-specific Ab production but does not appear to drive the differentiation of bystander naïve and memory B cells into PCs.

B cell subsets in Pf-uninfected children and adults before the malaria season

By FACS we determined the proportion of B cell subsets in individuals (2–4 yrs [n = 38], 5–7 yrs [n = 21], 8–10 yrs [n = 23], 18–25 yrs [n = 27]) before the malaria season (Fig. 5A). With increasing age, and as a percentage of total CD19+ B cells we observed a decrease in immature B cells (CD19+CD10+; P < 0.001) and naïve B cells (CD19+CD27−CD21+CD10+; P = 0.047) and an increase in resting IgG+ MBCs (CD19+CD27+CD21−CD20+; P < 0.001) and activated IgG+ MBCs (CD19+CD27+CD21−CD20+CD10−; P < 0.001). The increase with age of classical MBCs is consistent with the increase in total IgG+ MBCs we observed using the MBC ELISPOT assay (Fig 3).

In a subset of 87 individuals from this same study cohort, we previously reported that Pf exposure is associated with an expanded subset of ‘atypical’ MBCs that express FCRL4 and are hyporesponsive to in vitro stimuli [18], similar to the ‘exhausted’ MBCs described in viremic, HIV-infected individuals [19]. Atypical MBCs are defined as CD19+CD27−CD21−CD20+ and typically represents <4% of circulating CD19+ B cells in healthy U.S. adults [19]. Here, analyzing a larger number of individuals in the cohort, we confirmed that this subset of MBCs is expanded in Malian children and adults compared to malaria-naïve U.S. adults (U.S. adults: 1.4% [95% CI: 0.9–1.8]; Malian children aged 2–10 years: 10.2% [95% CI: 8.7–11.8]; P < 0.001 versus U.S. adults; Malian adults aged 18–25 years: 14.0% [95% CI: 11.0–19.1]; P < 0.001 versus U.S. adults). Thus, in addition to the increase in classical MBCs, an ‘atypical’ MBC subset is expanding with age in this study population.
were CD19+ 14 days after acute malaria. Within the CD19+ B cell population there were no significant changes in the percent of immature B cells, naïve B cells, or resting MBCs, after acute malaria. Moreover, there was no change in the proportions of resting and atypical MBCs that were IgG+.

For this analysis a malaria episode was defined as an axillary temperature ≥37.5°C, Pf asexual parasitemia ≥5000 parasites/µL, and a non-focal exam by the study physician. Because the incidence of malaria was very low in adults during the study period (Table 2), they were excluded from this analysis. Three measures of malaria risk were analyzed: 1) whether or not malaria was experienced, 2) the incidence of malaria, and 3) the time to the first malaria episode. In the corresponding multivariate regression models (logistic, Poisson, and Cox regression) which controlled for malaria episode. In the corresponding multivariate regression models (logistic, Poisson, and Cox regression) which controlled for malaria risk and AMA1- or MSP1-specific Ab levels or MBC frequencies measured just prior to the six month malaria season were associated with the subsequent risk of malaria. For this finding was not unexpected based on the observation that the AMA1- and MSP1-specific MBC frequencies and Ab levels and malaria risk

We investigated the impact of acute malaria on the relative proportion of B cells in each subset in children aged 2–10 years. Compared to the pre-malaria season baseline (month 0), there were no significant changes in the percent of lymphocytes that
and molecular mechanisms at play in the generation of MBCs under these very different conditions of exposure of children versus adults as these could have significance with regard to vaccine development. Moreover, recent studies in mouse models are revealing multiple, phenotypically and functionally distinct populations of MBCs [23,24] and it will be of interest to further characterize Pf-specific MBCs in different malaria endemic settings.

The study described here provides a rare view of the acquisition and maintenance of human B cell memory. Most prospective studies of human B and T cell immunological memory have evaluated responses to vaccination rather than natural infection, in part because of the difficulty of predicting who among a population will be infected with a given pathogen at a given time. In response to a single vaccination, several studies have described an expansion and contraction of vaccine-specific MBCs [25,26] and CD8+ memory T cells [27]. In one of the few longitudinal studies of the populations of MBCs [23,24] and it will be of interest to further development. Moreover, recent studies in mouse models are under these very different conditions of exposure of children versus and molecular mechanisms at play in the generation of MBCs.

In one of the few longitudinal studies of the memory B cell response to Pf infection, Harris et al. [27] examined antigen-specific MBC responses of patients presenting with acute *Vibrio cholerae* infection, a pathogen that elicits long-term protective immunity against subsequent disease [28]. In contrast to our results, they observed that the majority of patients acquired IgA and IgG MBCs specific for two *Vibrio cholerae* antigens and that these persisted up to one year after infection.

Whereas MBCs mediate recall responses to reinfection by rapidly expanding and differentiating into PCs, LLPCs residing in the bone marrow constitutively secrete antibody in the absence of antigen and thus provide a critical first line of defense against reinfection [6]. Logistical constraints precluded the direct measurement of circulating PCs in this study. However, we took advantage of the discrete six-month dry season, a period of little to no Pf transmission, to infer the relative contributions of SLPCs and LLPCs to the Pf-specific IgG response based on a serum IgG half-life of ~21 days [29]. Two weeks after acute malaria, AMA1- and MSP1-specific Ab levels increased significantly and then decreased over a six-month period to a point just above pre-infection levels, indicating that the majority of PCs generated in response to acute Pf infection were short-lived. This observation is consistent with previous studies that described rapid declines in Pf-specific Ab within weeks of an acute malaria episode [30,31]. We infer that the small net increase in Pf-specific Ab at the end of the six-month dry season represents the acquisition of Pf-specific LLPCs. Because Pf transmission resumes after the six-month dry season, we cannot estimate the long-term decay rate of Pf-specific Ab in the absence of reinfection. It remains to be seen whether long-term decay rates of Pf-specific Ab are comparable to rates of Ab decay after exposure to common viral and vaccine antigens such as mumps and measles, for example, which elicit Ab with half-lives exceeding 200 years [32]. The small incremental gains in AMA1- and MSP1-specific Abs in response to acute malaria mirrors the gradual exposure-related increase in Pf-specific MBCs, consistent with the long-lived Abs being the products of LLPCs derived from MBCs. Unlike the response to some other pathogens, such as measles, which induce long-lived protective Abs after a single exposure, it may be that repeated exposure to the Pf parasite is necessary to ‘fill’ the Pf-specific LLPC compartment to the point where basal levels of circulating Abs to any given Pf antigen reach a protective threshold. In a separate study of this cohort, we observed a similar pattern of transient increases during the malaria season of Abs specific for a large number of Pf antigens using protein microarrays [33] suggesting that malaria induces a relatively high SLPC-to-LLPC ratio that is not exclusively a function of the inherent qualities of any given antigen per se.

In contrast to the highly efficient immune response to a single smallpox vaccination, which generates long-lived (>50 years) MBCs in nearly all vaccinees [34], a remarkably high proportion of adults in the present study did not have detectable AMA1- or MSP1-specific MBCs despite annual exposure to 50–60 infective mosquito bites per person per month at the height of the malaria season [17], similar to what Dorfman et al. observed in a cross-sectional study in Kenya [35]. Importantly, most female adults in the present study had detectable TT-specific MBCs three to ten years after a single TT booster vaccine in adolescence. We previously reported that AMA1- and MSP1-specific MBCs were reliably generated in Pf-naive U.S. adults following just two vaccinations [16]. Taken together, these observations indicate that the relatively inefficient generation and/or maintenance of Pf-specific MBCs in response to natural Pf infection cannot be ascribed entirely to inherent deficiencies in the antigens themselves. Collectively, these observations raise a central question: if AMA1 and MSP1-specific MBCs and Abs can be efficiently generated by vaccination of Pf-naive adults, and TT-specific MBCs and Abs can be efficiently generated by vaccination of Pf-exposed individuals in this cohort, what underlies the inefficient acquisition and/or maintenance of AMA1 and MSP1-specific MBCs and Abs in response to natural Pf infection? One simple answer, in addition to parasite antigenic variation [36,37], might be that the enormous number of antigens encoded by the over 5,400 Pf genes overwhelms the immune system’s capacity to select for and commit a sufficient number of MBCs and LLPCs specific for any given Pf antigen to a long-lived pool [38]. If immunity to clinical malaria requires high levels of antibodies to a large number of Pf proteins, then the inability to commit large numbers of MBCs and LLPCs specific for any given Pf antigen during any given infection, as shown here, may explain, in part, why malaria immunity is acquired slowly. In this scenario the Pf-infected individual is capable of the normal generation and maintenance of MBCs and LLPCs, but acquiring a sufficient number of MBCs and LLPCs to a large number of antigens may simply take years.

It is also possible that Pf infection disrupts the immune system’s ability to generate or maintain MBCs or LLPCs. The differentiation of B cells into long-lived MBCs depends to a great extent on the affinity of their BCRs for antigen. Recently, evidence was presented that affinity maturation of B cells may fail to occur in the absence of adequate Toll-like receptor (TLR) stimulation [39]. We recently reported that Malian adults were relatively refractory to Cpg, a TLR9 agonist incorporated into two subunit malaria vaccine candidates [40], raising the possibility that the slow acquisition of MBCs observed here may be due to a failure of B cells to undergo affinity maturation during Pf infection. Although our data do not directly address the role of apoptosis in the gradual acquisition of Pf-specific MBCs, it is worth noting that we found no evidence of Pf-induced ablation of *Plasmodium*-specific MBCs, as was observed in mice four days after *Plasmodium yoelii* infection [41]. The relatively inefficient response to natural Pf infection also does not appear to be due to a persistent, Pf-induced general immunosuppression as the frequency of TT-specific MBCs increased significantly in most adult females in response to a single TT booster vaccination, an increase that appeared to be maintained for years. In an experimental model of lymphocytic choriomeningitis virus (LCMV) infection, a high antigen-to-B cell ratio disrupted germinal center formation and the establishment of B cell memory [42]. It is plausible that a similar mechanism is at play during the blood stage of Pf infection when the immune system encounters high concentrations of parasite proteins. Indeed, germinal center disruption is observed in mice infected with *P. berghei ANKA* [43] and *P. chabaudi* [44]. It is also possible
that specific parasite products selectively interfere with the regulation of B cell differentiation [45] or with the signals required for sustaining LLPCs in the bone marrow [46]. It is also conceivable that the disproportionately high level of class-switched SLPCs we observed in response to Pf infection arises from pre-diversified IgM+IgD+CD27+ (marginal zone) B cells—alogous to the rapid protective response against highly virulent encapsulated bacteria that do not elicit classical T-dependent responses [47]. These and other hypotheses could be tested by applying systems biology methods [48] and targeted ex vivo and in vitro assays to rigorously conducted prospective studies of Pf-exposed populations.

We previously reported that Pf exposure is associated with a functionally and phenotypically distinct population of FCRL4+ hypo-responsive atypical MBCs [18], similar to the ‘exhausted’ MBCs described in HIV-infected individuals [19]. In this study, with a larger sample size, we confirmed that Pf exposure is associated with an expansion of FCRL4+ MBCs. The accumulation of atypical MBCs could be linked to the slow acquisition of Pf-specific MBCs, as naive B cells in response to Pf infection could have a propensity to differentiate into atypical rather than classical MBCs. We also observed that the FCRL4+ MBC population decreased in the peripheral circulation two weeks after acute malaria suggesting that these MBCs are directly involved in the response to Pf infection, possibly trafficking to secondary lymphoid tissues. Although the function of FCRL4+ MBCs is not established, Moir et al. [19] suggested that FCRL4+ ‘exhausted’ MBCs contribute to the B cell deficiencies observed in HIV-infected individuals. In contrast, Ehhardt et al. [49], who first described FCRL4+ ‘tissue-like’ MBCs in lymphoid tissues associated with epithelium, suggested that these cells may play a protective role during infections. At present, the factors that underlie the expansion of atypical MBCs in this study population are not known. Genetic or environmental factors that are associated with Pf transmission but not accounted for in this study could explain this observation. It will be of interest to understand the origin, antigen-specificity, and function of FCRL4+ MBCs in the context of Pf infection and the potential impact of these MBCs on the ability of children to respond to malaria vaccines.

In multivariate analysis we found no correlation between the frequency of MBCs and levels of Abs specific for AMA1 or MSP1 and malaria risk. This is not necessarily unexpected in light of recent clinical trials that showed that vaccination with either AMA1 or MSP1 did not confer protection [20,21]. Furthermore, we suspect that the frequency of MBCs per se may not reliably predict clinical immunity to malaria regardless of antigen specificity. Malaria symptoms only occur during the blood stages of Pf infection and can begin as early as three days after the blood stage infection begins [50]. Because the differentiation of MBCs into PCs peaks ~6–8 days after re-exposure to antigen [10], there may not be sufficient time for MBCs specific for Pf blood stage antigens to differentiate into the antibody-secreting cells that would prevent the onset of malaria symptoms. In contrast, the longer incubation period of other pathogens allows MBCs to differentiate into protective antibody-secreting cells before symptoms develop. For example, follow-up studies of hepatitis B vaccinees have shown that protection can persist despite the decline of hepatitis B-specific antibodies to undetectable levels [51], presumably due to the recall response of persistent MBCs. Thus, protection against the blood stages of malaria may depend on achieving and maintaining a critical level of circulating antibody that can rapidly neutralize the parasite. MBCs may contribute to the gradual acquisition of protective immunity by differentiating into LLPCs with each Pf infection.

Here we also provide evidence concerning the mechanism by which MBCs and LLPCs are maintained. We observed a modest but statistically significant increase in TT-specific MBCs two weeks after acute malaria, in support of the hypothesis that MBCs are renewed by polyclonal or ‘bystander’ activation [10]. The stable frequency of TT-specific MBCs with age suggests that the small increases associated with Pf-induced polyclonal activation are matched by the rate of loss of senescent TT-specific MBCs. It has also been proposed that non-specific polyclonal stimulation maintains long-lived Ab responses by driving MBCs to differentiate into SLPCs or LLPCs [10]. Similarly, it has been hypothesized that Plasmodium infection generates large amounts of non-specific Ig [32] through polyclonal B cell activation [12,13]. However, despite the presence of TT-specific MBCs and their expansion following Pf infection, we did not observe a concomitant increase in TT-specific IgG. This finding is consistent with recent human studies that demonstrate a lack of bystander IgG production after heterologous vaccination or viral infection [32,53]; as well as studies in mice that demonstrate PC persistence after MBC depletion [54], and the failure of MBCs to differentiate into PCs in vivo upon TLR4 and 9 activation [55]. This finding does not represent an overt inability of TT-specific MBCs to differentiate into PCs, since adult females in this study had a sharp increase in tetanus IgG after a single tetanus booster. It is possible that bystander MBCs specific for antigens other than TT differentiate into PCs after Pf infection, but based on the results of this study we hypothesize that the predominance of IgG produced in response to malaria is specific for the ~2400 Pf proteins expressed during the blood-stage of infection [56], and that increases in ‘non-specific’ IgG reflect boosting of cross-reactive B cells [57,58]. From a basic immunology perspective, these data support a model in which non-specific stimuli contribute to MBC self-renewal, but not to the maintenance of LLPCs. Studies of other Ab specificities and isotypes before and after malaria and other infections would test this hypothesis further. Although a recent mouse study showed that MBCs do not proliferate in vivo after immunization with an irrelevant antigen [59], this may reflect the difference in requirements for MBC maintenance in mammals with relatively short life spans.

It is of general interest to determine which parasite products are responsible for the polyclonal activation of MBCs observed here. Studies in vitro suggest that Pf drives polyclonal MBC activation by the cysteine-rich interdomain region 1α (CIDR1α) of the Pf erythrocyte membrane protein 1 (PfEMP1) [13,60], but it is conceivable that Pf-derived TLR agonists [61,62] or bystander T cell help [63,64,65] also contribute to MBC proliferation in the absence of BCR triggering [66].

Animal models have provided important insights into the immunobiology of Plasmodium infection [67], but ultimately, despite obvious experimental limitations, it is critical to investigate the human immune response to Pf in longitudinal studies since findings from animal models do not always mirror human biology or pertain to the clinical context [68,69]. Key challenges for future studies will be to determine the molecular basis of the inefficient generation of MBCs and LLPCs in response to Pf infection and to determine the longevity of these cells in the absence of Pf transmission over longer periods of time. Greater insight into the molecular and cellular basis of naturally-acquired malaria immunity could open the door to strategies that ultimately prove useful to the development of a highly effective malaria vaccine.
Materials and Methods

Ethics statement
The ethics committee of the Faculty of Medicine, Pharmacy, and Odonto-Stomatolog, and the institutional review board at the National Institute of Allergy and Infectious Diseases, National Institutes of Health approved this study (NIAID protocol number 06-I-N147). Written, informed consent was obtained from adult participants and from the parents or guardians of participating children.

Study site
This study was carried out in Kambila, a small (<1 km²) rural village with a population of 1300, located 20 km north of Bamako, the capital of Mali. Pf transmission is seasonal and intense at this site from July through December. The entomological inoculation rate measured in a nearby village was approximately 50–60 infective bites per person per month in October 2000 and fell to near zero during the dry season [17]. A detailed description of this site and the design of the cohort study has been published elsewhere [14].

Sampling strategy, study participants, and malaria case definition
In May 2006, during a two-week period just prior to the malaria season, 225 individuals aged 2–10 years and 18–25 years were enrolled after random selection from an age-stratified census of the entire village population. 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. All analysis in the present study pertains to an age-stratified subset of individuals (n = 185) randomly selected from those who had complete sets of PBMC samples over the entire study period. From May 2006 through May 2007, participants were instructed to report symptoms of malaria at the village health center, staffed 24 hours per day by a study physician. For individuals with signs or symptoms of malaria, blood smears were examined for the presence of Pf parasites by diagnostic microscopy. Patients with positive smear results (i.e., any level of parasitemia) were treated with a standard 3-day course of artesunate plus amodiaquine, and all positive malaria cases were reported to the district health authorities. Children with severe malaria were referred to the nearest health center or hospital for treatment with a standard 3-day course of artesunate plus amodiaquine, and all positive malaria cases were reported to the district health authorities. Children with severe malaria were referred to the nearest health center or hospital for treatment with a standard 3-day course of artesunate plus amodiaquine, and all positive malaria cases were reported to the district health authorities.

PBMC and plasma collection
Blood samples (8 ml for children and 16 ml for adults) were drawn by venipuncture into sodium citrate-containing cell preparation tubes (BD, Vacutainer CPT Tubes) and transported 20 km to the laboratory where they were processed within three hours of collection. Plasma and PBMCs were isolated according to the manufacturer’s instructions. Plasma was stored at −80°C. PBMCs were frozen in fetal bovine serum (FBS) (Gibco, Grand Island, NY) containing 7.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), kept at −80°C for 24 hours, and then stored at −196°C in liquid nitrogen. For each individual, PBMC and plasma samples from all time points were thawed and assayed simultaneously.

Measurement of peripheral blood Pf parasitemia
Thick blood smears were stained with Giemsa and counted against 300 leukocytes. Pf 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.

Hemoglobin typing
Hemoglobin was typed by high performance liquid chromatography (HPLC; D-10 instrument; Bio-Rad, Hercules, CA) as previously described [14].

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 were eggs detected by microscopy.

Geographic information system data collection
Latitude and longitude coordinates of study subjects’ households were measured by a handheld global positioning system receiver (GeoX; Trimble) and reported earlier [14].

Antibody detection by ELISA
ELISAs were performed by a standardized method as described previously [70]. For both AMA1 and MSP1, a 1:1 mixture of FVO and 3D7 AMA1 and MSP1 isotypes was used to coat the ELISA plates. The limit of detection for the AMA1 and MSP1 ELISA is based on the range of values that gives reproducible results at the Malaria Vaccine and Development Branch at NIAID where the assay is routinely performed. More specifically, the limit of detection is the ELISA unit value at the lowest point on the standard curve, multiplied by the dilution factor at which samples are tested. The minimal detection levels for the MSP1 and AMA1 ELISA assays were 11 and 33 ELISA units, respectively. For analysis, all data below the minimum detection level were assigned a value of one half the limit of detection (i.e., 6 units for MSP1, 17 units for AMA1). The limit of detection for the TT ELISA was not determined because we did not have access to TT-naïve serum.

Memory B cell analysis
Antigen-specific MBCs were quantified by a modified version of the method developed by Crotty et al [71]. We found that adding IL-10 to the cocktail of polyclonal activators resulted in a six-fold increase in the efficiency of the assay (Weiss et al., unpublished observation). Briefly, PBMCs were thawed and cultured in 24 well plates at 37°C in a 5% CO2 atmosphere for six days in media

PLoS Pathogens | www.plospathogens.org 10 May 2010 | Volume 6 | Issue 5 | e1000912
alone (RPMI 1640 with L-Glutamine, Penicillin/Streptomycin 100 IU/ml, 10% heat-inactivated FBS, 50 μM β-Mercaptoethanol) or media plus a cocktail of polyonal activators: 2.5 μg/ml of CpG oligonucleotide ODN-2006 (Eurofins MWG/Operon, Huntsville, AL). Protein A from Staphylococcus aureus Cowan (SAC) at a 1/10,000 dilution (Sigma-Aldrich, St. Louis, MO), pokeweed mitogen at a 1/100,000 dilution (Sigma-Aldrich), and IL-10 at 25 ng/ml (BD Biosciences). Cells were washed and distributed on 96-well ELISPOT plates (Millipore Multiscreen HTS IP Sterile plate 0.45 um, hydrophobic, high-protein binding) to detect antibody-secreting cells (ASCs). ELISPOT plates were prepared by coating with either: a 10 mg/ml solution of polyclonal goat antibodies specific for human IgG (Caltag) to detect all IgG-secreting cells; a 1% solution of bovine serum albumin (BSA) as a non-specific protein control; or 5 μg/ml solutions of either tetanus toxoid (TT), AMA1, or MSP1 to detect antigen-specific ASCs. For AMA1 and MSP1, a 1:1 mixture of FVO and 3D7 isotypes was used to coat the ELISPOT plates. Plates were blocked by incubation with a solution of 1% BSA. For the detection of antigen-specific ASCs, cells were plated in duplicate in eight serial dilutions beginning with 5 × 10^4 cells/well. For detection of total IgG ASCs cells were plated at six serial dilutions beginning at 4 × 10^5 cells/well. After a five hour incubation of the cells in the ELISPOT plates, plates were washed four times each in PBS and PBS-Tween 20 0.05% (PBST), and incubated overnight with a 1:1000 dilution of alkaline phosphatase-conjugated goat antibodies specific for human IgG (Zymed) in PBST/1% FCS. Plates were washed four times each in PBST, PBS, and ddH²O; developed using 100 μl/well BCIP/NBT for 10 minutes; washed thoroughly with ddH²O and dried in the dark. ELISPOTS were quantified using Cellular Technologies LTD plate-reader and results analyzed using Cellspot software. Results are reported as frequencies of MBCs per 10^6 PBMCs after the six-day culture. The limit of detection of the MBC ELISPOT assay for this analysis was five ASCs per 10^6 PBMC based on the average number of ASCs on the BSA control. Assay failure was defined as fewer than 1000 IgG ASCs per 10^6 PBMCs after the six-day culture which resulted in the exclusion of 15% of individuals at month 0, 13.2% 14 days after the first malaria episode, and 7.3% at month 12. For individuals with a limited number of PBMCs, priority was given to performing the ELISPOT assay for MSP1, then TT, and then AMA1.

Phenotypic analysis of B cell subsets

All phenotypic analyses were performed using mouse mAbs specific for human B cell markers conjugated to fluorophores as previously reported [18]. Fluorophore-conjugated mAbs specific for the following markers were used: PE-G7-19B1, PE-CD20, APC-CD10, APC-CD27, PE-IgG (BD Biosciences, San Jose, CA) and FITC-CD21 (Beckman Coulter, Fullerton, CA). A four-color, two-stain strategy was used to identify B cell subsets as follows: plasma cells/blasts (CD19^+ CD21^- CD27^+), naive B cells (CD19^+ CD27^- CD10^-), immature B cells (CD19^+ CD10^+), classical MBCs (CD19^+ CD27^+ CD21^-), atypical MBCs (CD19^+ CD21^- CD27^- CD10^+ ) and activated MBCs (CD19^- CD21^- CD27^- CD10^-). FACS analyses were performed on a FACS Calibur flow cytometer (BD Biosciences) using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Data were analyzed using STATA (StataCorp LP, Release 10.0) and GraphPad Prism for Windows (GraphPad Software, version 5.01). The Kruskal-Wallis test was used to compare continuous variables between groups, and the Fisher’s exact test was used to compare categorical variables. The Wilcoxon matched-pairs signed-rank test was used to compare measurements of the same parameter at two time points for the same individual. The correlation between different continuous measures was determined by using the Spearman correlation coefficient. The malaria-free probability over the twelve-month study period was estimated by the Kaplan-Meier curve, and the time to the first malaria episode was compared by the log-rank test. Cox’s proportional hazards model was used to assess the effect of the following factors on the hazard of malaria: age, gender, weight, ethnicity, distance lived from study clinic, self-reported bednet use, hemoglobin type, antigen-specific MBC frequencies and Ab levels. The same list of variables was included in logistic and Poisson regression models to determine their impact on the odds and incidence of malaria episodes, respectively. For all tests, two-tailed p values were considered significant if ≤0.05.

Supporting Information

Figure S1 Correlative analysis of antibody levels and memory B cell frequencies specific for AMA1, MSP1, and tetanus toxoid. Shown are scatterplots of antibody levels versus memory B cell frequencies specific for (A) AMA1 (n = 64), (B) MSP1 (n = 67) and (C) tetanus toxoid (n = 128). Data are derived from venous blood samples drawn before the malaria season. Only individuals with both antibody and memory B cell data are included. For AMA1 and MSP1 the plots include individuals with antibody levels at or above the limit of detection of the ELISA. Individuals with ‘failed’ ELISPOT assays are not included. As described in ‘Materials and Methods’, assay failure was defined as fewer than 1000 IgG ASCs per 10^6 PBMCs after the six-day culture. The Spearman’s correlation coefficient is given for each plot.

Acknowledgments

We sincerely thank the residents of Kambila, Mali for their ongoing support of this study. We also thank Tonkoro Diarra for helping to prepare at the study site, Bakary Coulibaly and Daouda Kane for helping to prepare the study site, and Julie Kim, Dr. Richard Sakai and the Mali Service Center for logistic support. We also thank the staff at Biologic Laboratories, University of Massachusetts Medical School at Jamaica Plains, MA for generously providing purified tetanus toxoid.

Author Contributions

Conceived and designed the experiments: G. Weiss, B. Traore, K. Kayentao, A. Onogiba, L. Miller, O. Dounbo, S. Pierce, P. Crompton. Performed the experiments: G. Weiss, S. Dousmbo, D. Dountable, Y. Kone, S. Dia, A. Guindo, A. Traore, M. Mircetic, S. Li, A. Baughman, P. Kayentao, A. Ongoiba, L. Miller, O. Doumbo, S. Pierce, P. Crompton. Analyzed the data: G. Weiss, P. Crompton. Contributed reagents/materials/analysis tools: C. Huang, K. Miura, D. Narum. Wrote the paper: G. Weiss, S. Pierce, P. Crompton. Provided medical care for study participants and collected clinical data at the field site: K. Kayentao, A. Onogiba, Y. Kone, S. Dia. Processed biological specimens and performed clinical assays: S. Dounbo, D. Dountable, Y. Kone, A. Traore.

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