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Plasmodium falciparum Malaria in the Peruvian Amazon, a Region of Low Transmission, Is Associated with Immunologic Memory

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The development of clinical immunity to *Plasmodium falciparum* malaria is thought to require years of parasite exposure, a delay often attributed to difficulties in developing protective antibody levels. In this study, we evaluated several *P. falciparum* vaccine candidate antigens, including apical membrane antigen 1 (AMA-1), circumsporozoite protein (CSP), erythrocyte binding antigen 175 (EBA-175), and the 19-kDa region of merozoite surface protein 1 (MSP1₁₉). After observing a more robust antibody response to MSP1₁₉, we evaluated the magnitude and longevity of IgG responses specific to this antigen in Peruvian adults and children before, during, and after *P. falciparum* infection. In this low-transmission region, even one reported prior infection was sufficient to produce a positive anti-MSP1₁₉ IgG response for >5 months in the absence of reinfection. We also observed an expansion of the total plasmablast (CD19⁺ CD27⁺ CD38^{high}) population in the majority of individuals shortly after infection and detected MSP1-specific memory B cells in a subset of individuals at various postinfection time points. This evidence supports our hypothesis that effective antimalaria humoral immunity can develop in low-transmission regions.

Individuals living in areas of intense *Plasmodium falciparum* transmission suffer from repeated malaria episodes, resulting in significant morbidity and mortality (10, 35). Even so, clinical immunity to symptomatic *P. falciparum* malaria can be acquired after repeated parasite exposures (4), and passive transfer studies indicate that IgG is a critical component of naturally acquired immunity (13, 31). A number of *in vitro* studies have shown that antibodies specific for *P. falciparum* blood-stage proteins, such as the merozoite surface protein-1 (MSP1), restrict parasite growth and erythrocyte invasion (5, 11). Animal model studies correlated the presence of these antibodies with protection from infection (12, 16), and many (2, 6, 22, 29), but not all (18), human epidemiological studies have corroborated these findings. However, longitudinal studies in humans residing in areas of high *P. falciparum* transmission have shown that antibody responses specific for MSP1 and other blood-stage antigens are relatively short-lived, lasting for as little as 1 month (1, 9, 19, 23, 25).

Determining whether transmission intensity influences the efficiency of acquiring malaria-specific immunologic memory remains an important question. Recent studies in areas of high *P. falciparum* transmission suggest that immune dysregulation of B cells account for the delayed acquisition and rapid loss of *P. falciparum*-specific antibodies. For example, studies in Kenya (19) and Mali (42) indicate that *P. falciparum*-specific memory B cells (MBCs) are acquired inefficiently despite intense parasite exposure. Other studies performed in high-transmission regions indicate that *P. falciparum* infection may decrease the total CD19⁺ B cell (24) and CD19⁺ IgD⁻ CD38⁻ MBC (3) compartments. In contrast, a recent study in Thailand demonstrated the acquisition of *P. falciparum*-specific MBCs despite low *P. falciparum* transmission (43). Perhaps the low-transmission setting provides an ideal environment for the normal development of humoral immunity to malaria infection.

Low-malaria-transmission regions, such as Iquitos, Peru, are

ideal for the longitudinal study of naturally acquired antimalarial immunity because malaria infections rarely overlap, making it easy to follow the immune response to a single infection (7, 39). In Iquitos, clinical malaria episodes are typically spaced by a year or more, and greater than 60% of malaria infections are asymptomatic (7, 30). In addition, because of the easily accessible health infrastructure, individuals living in and around Iquitos that do have symptomatic *P. falciparum* infections can reliably report the approximate number of prior symptomatic exposures to the parasite. This allows us to examine factors affecting the acquisition of natural immunity to malaria, particularly the influences of age versus amount of parasite exposure.

We propose that the antibody responses to conserved antigens, such as the 19-kDa region of MSP1 (MSP1₁₉), may be more efficiently acquired and durable in low- versus high-transmission settings. Our previous cross-sectional analysis of antibody responses during the wet versus dry season in Peru suggested that anti-MSP1₁₉ IgG responses persist throughout the 5-month dry season when there is negligible parasite transmission (39). In the present study, we performed longitudinal sampling for approximately 180 days following a documented *P. falciparum* infection to determine whether individuals exposed to infrequent *P. falciparum* infections acquire IgG and MBCs specific for MSP1. This time period was chosen because 180 days is longer than the duration of anti-

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MSP1₁₉ antibody responses observed in previous longitudinal studies. In addition, the likelihood of having another infection within 180 days in this region is very low.

Despite low exposure, we detected MSP1-specific IgG and MBCs in most individuals, even after only one prior *P. falciparum* infection. These results support our hypothesis that humoral immunity to *P. falciparum* may be more efficiently acquired in areas of low transmission. Thus, a protein-antigen vaccine may effectively eradicate malaria if transmission in regions where malaria is endemic can be reduced by fumigation campaigns, tightly controlled treatment protocols, and other control strategies.

MATERIALS AND METHODS

Study area and sample collection. The Malaria Immunology and Genetics in the Amazon (MIGIA) study began in 2003. This longitudinal cohort includes more than 2,000 individuals living in communities just south of Iquitos, Peru, in a region called Zungarococha, where the force of infection is less than 0.5 *P. falciparum* infection/person/malaria season (7). The malaria season typically lasts from February to July. More than 60% of *P. falciparum* infections in this region are thought to be asymptomatic with low parasite density (7, 30).

From 2003 to 2009, we performed cross-sectional surveys for *P. falciparum* infection at the beginning (January) and end (August) of each malaria season. We also conducted weekly active surveillance for *P. falciparum* infection, sampling a new group of approximately 150 individuals living near sentinel houses each month during the malaria season. This allowed us to collect blood samples before, during, and after *P. falciparum* infection. Passive case detection (PCD) for symptomatic malaria episodes was performed year round at the community health post and at two hospitals in Iquitos. The active case detection (ACD) and PCD methods used in this study are described in more detail elsewhere (7).

At each visit (ACD or PCD), a medical history and a focused physical exam were performed, and a finger-prick blood sample (500 μ l) was collected in a microtube containing EDTA anticoagulant, from which material for the blood smear slide, hematocrit capillary, plasma sample, and red blood cell sample was taken. If diagnosed with malaria, a 4- to 8-ml Vacutainer tube of venous blood was collected at presentation prior to treatment (day 0), and 500- μ l finger-prick blood samples were collected weekly for 2 months postinfection. Beginning in 2008, 4 to 8 ml of venous blood was collected at postinfection days 7, 28, 49, and 60 in addition to day 0. At least once a year, a demographic survey, GPS coordinates of home, nutritional questionnaire, weight and height, helminth examination, and self-report of lifetime *P. falciparum* malaria (matched with the Peruvian Ministry of Health [MINSA] malaria species-specific treatment records) were recorded for each individual. Considering the concordance of scoring the patient's self-report with the MINSA records, there was complete agreement in the 0, 1, 2 or >2 classification 56.3% of the time. When considering classification within one difference (e.g., a "0" in patient report versus a "1" in the health post records), there was concordance of 83.1%.

Diagnosis and treatment. Thick blood smears were stained with Giemsa and examined by oil-immersion microscopy. If >50 asexual *P. falciparum* parasites were detected after reading 200 leukocytes, parasitemia was calculated from these fields. Otherwise, at least 500 leukocytes were counted. Parasitemia was expressed as the number of parasites/ μ l of blood, assuming 6,000 leukocytes/ μ l. The blood smear was considered negative if no parasites were seen after examination of 200 fields.

Symptomatic malaria episodes were defined by parasites identified via blood smear and one of the following: (i) axillary temperature of $\geq 38.3^{\circ}\text{C}$ or a self-reported history of fever within 2 days prior to diagnosis, or (ii) a hematocrit of <30% PCV. During active surveillance, blood smears of asymptomatic individuals were not read immediately but within 7 days. All *P. falciparum*-infected individuals, irrespective of the presence of

symptoms, were treated with mefloquine and artesunate for *P. falciparum* infections and chloroquine and primaquine for *P. vivax*.

Human subject approval. All individuals enrolled in this study gave signed informed consent or assent. Ethical clearance was received from New York University (NYU Institutional Review Board [IRB] approval no. 08-982), the University of Alabama at Birmingham, and the Peruvian Ministry of Health and National Institutes of Health Internal Ethical Review Boards.

Antigens. The recombinant *P. falciparum* MSP1₁₉ strain, corresponding to the Ugandan-PA *P. falciparum* strain (with the MSP1₁₉ variant amino acid positions being E-K-N-G) was expressed in *Saccharomyces cerevisiae* (provided by David Kaslow) (28). Individuals in the MIGIA cohort respond to this MSP1₁₉ variant (E-K-N-G) and the Q-K-N-G variant (also detected in this geographic region) in a nearly identical manner (37). In addition, apical membrane protein 1 (AMA-1) (21), erythrocyte binding antigen 175 (EBA-175) (27), and circumsporozoite protein (CSP) were evaluated in this study. AMA-1, EBA-175, and CSP were a gift from David Lanar (WRAIR).

Measurement of antibody levels. Enzyme-linked immunosorbent assays (ELISAs) for total IgG antibodies were performed as described previously (39). Immunosorbent plates (Nunc) were coated with each antigen (at 25 ng/ml) in borate buffer solution (BBS) overnight at 4°C and then washed once with sodium phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T). The plates were blocked with BBS containing 1% bovine serum albumin (BSA) and washed three times with PBS-T. Sera were diluted 1:100 in AB washing solution (0.15 M Na₂HPO₄, 0.05% Tween 20, 0.05% BSA, 500 mM NaCl) containing 1.5% nonfat milk, added in duplicate to microtiter plates, and incubated at room temperature for 1 to 2 h. Unbound antibodies were removed by four washes with AB washing solution. Bound antibodies were detected with peroxidase-conjugated goat anti-human antibodies (Chemicon, Billerica, MA) diluted 1:5,000 for IgG. The secondary antibody was allowed to bind for 1 to 2 h, and then wells were washed three times with AB washing solution. To develop the plates, 50 μ l of the substrate 3,3',5,5'-tetramethylbenzidine (KPL, Gaithersburg, MD) was added to each well, and approximately 6.5 min later, the reaction was stopped with 25 μ l of 0.25 M hydrochloric acid per well. The plates were read at an A₄₅₀ with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad model 3550). Six negative control samples were obtained from healthy individuals residing in Peru who had not been exposed to malaria, and six were obtained from healthy individuals living in the United States who had not been exposed to malaria.

A standard curve with positive controls was made for each set of experiments to calibrate the system. Five samples IgG positive for AMA-1, CSP, EBA-175, and MSP1₁₉ were pooled and serially diluted at 1:50, 1:100, 1:400, 1:800, and 1:1,600. This positive pool was run each ELISA experiment day. To standardize the data among experiment days, we averaged the positive pool optical densities (ODs) at each dilution for all experiment days (overall average). The experiment day OD at each dilution minus the overall average resulted in a correction factor that standardized the results for each given dilution. In this manner, the correction specifically addressed the experimental day differences seen in the range of ODs observed. For each of the patient sample results, the correction factor corresponding to the dilution most similar to the patient sample OD result was then subtracted. This method gave final results standardized by experiment day.

The negative cutoff was determined for each experiment day by averaging the negative control samples and adding 2 standard deviations. For MSP1₁₉, AMA-1, CSP, and EBA-175 IgG, the mean values of the negative cutoff for each experiment day were as follows: 0.336, 0.144, 0.156, and 0.122. A positive result was considered an OD greater than the negative cutoff. To describe the intensity of a positive response, a result was called "low positive" if it was greater than the negative cutoff but less than twice the negative cutoff. A result greater than or equal to twice the negative

cutoff was called “high positive.” Anything less than or equal to the negative cutoff was called “negative.”

Cellular assay sample collection and preparation. Flow cytometric analysis of B cell surface markers was performed using 100 μ l of whole blood collected from patients on days 0, 7, 28, 45, and 60. The cell staining protocol for B cell surface markers performed on these samples is described below. The remaining blood was separated using Ficoll-Histopaque density gradient centrifugation (Amersham Biosciences, Pittsburgh, PA) to collect and freeze peripheral blood mononuclear cells (PBMCs). These cells were immediately frozen in 90% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) at -80°C for 24 h and then moved to a -130°C storage freezer. After being shipped from Peru to the United States, the PBMC samples were stored in liquid nitrogen until thawed for batch analysis in memory B cell enzyme-linked immunospot (ELISPOT) experiments, described below. After thawing, PBMCs were counted using an automatic cell counter (Cellometer Auto T4; Nexcelom Bioscience, Lawrence, MA), and the viability of each sample was assessed by trypan blue (BioWhittaker, Walkersville, MD) dye exclusion to be greater than 75% before stimulation.

Immunophenotyping for B cell subsets. To evaluate B cell subsets, 100 μ l of whole blood was incubated for 15 min with 10 μ l CD27-fluorescein isothiocyanate (FITC), 2.5 μ l CD38-phycoerythrin (PE)/Cy5, and 5 μ l CD19-PE (BD Pharmingen, San Diego, CA). After staining, cells were fixed with 1 ml of BD lysis buffer for 10 min, washed 3 times with PBS, and then resuspended in 400 μ l of PBS and stored at 4°C until analysis within 7 days on a FACS scan fluorescence-activated cell sorting flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data were analyzed using CellQuestPro software (BD Immunocytometry Systems). For each sample, the percentages of total B cells (CD19⁺), plasma cells (CD19⁺ CD27⁺ CD38^{high}), and memory B cells (CD19⁺ CD27⁺ CD38^{low}) were recorded. A total of 100,000 events were collected for each analysis. Eight negative control samples taken from healthy Peruvian adults with no known history of *Plasmodium* infection were also analyzed.

Memory B cell ELISPOT. Memory B cell ELISPOTS were done as previously described (15). Briefly, PBMCs were stimulated with 2.5 μ g/ml of CpG oligodeoxynucleotide-2006 (Operon Technologies), SAC at 1/10,000 dilution (Sigma-Aldrich), pokeweed mitogen at 1/100,000 dilution (Sigma-Aldrich), and 25 ng/ml interleukin-10 (IL-10) (R&D Systems) in complete medium. Cells were kept at 37°C in a 5% CO_2 incubator for 6 days, washed twice with complete medium warmed to 37°C , counted, and incubated for 5 h on 96-well plates that had been prepared for the MBC ELISPOT assay (coated with IgG [polyvalent goat anti-human IgG, from Caltag Laboratories, at 10 μ g/ml in PBS], MSP1 [merozoite surface protein 1 42-kDa combination 1, from MVDB/NIAID, at 2.5 μ g/ml], or tetanus toxoid [from Biologic Laboratories, University of Massachusetts Medical School at Jamaica Plains, at 2.5 μ g/ml]). The MSP1 42-kDa antigen has previously been shown to be properly folded and free of contaminants (32). Plates were then incubated overnight with an anti-IgG Fc-alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed with the substrate BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium) (Calbiochem). ELISPOTS were quantified using a Cellular Technology Ltd., plate reader, and results were analyzed using Cellspot software (SmartTools Publishing). The limit of detection of the assay was five antibody-secreting cells (ASCs) per 10^6 PBMCs, based on the average number of ASCs on the bovine serum albumin (BSA) control. Assay failure was defined as fewer than 1,000 IgG⁺ ASCs per 10^6 PBMCs after the 6-day culture. For individuals with a limited number of PBMCs, priority was given to performing the ELISPOT assay for MSP1 and then tetanus toxoid.

Statistical analysis. Fisher’s exact test was used to compare the magnitude of antibody responses over the course of an infection. To compare multiple groups of data (such as antibody optical densities), the nonparametric Kruskal-Wallis one-way analysis of variance was used. Dunn’s multiple comparison posttest was used to compare one group of data to

another within multiple groups of data after analysis via the Kruskal-Wallis test. The nonparametric Wilcoxon rank sum (Mann-Whitney) test was used to test for differences in continuous variables between two groups.

Kaplan-Meier curves were used to estimate the cumulative probability of remaining MSP1 seropositive after infection over 180 days. The probability of seropositivity by age group and number of prior infections were compared by applying the log rank (Mantel-Cox) test. MSP1₁₉ IgG seropositivity was defined by an OD that is ≥ 2 standard deviations (SD) above the negative cutoff.

All statistical analyses were done using the GraphPad Prism version 5 for Windows, GraphPad Software, San Diego, CA.

RESULTS

Organization of study cohort. There are three distinct groups of data included in this article (Fig. 1). First we evaluated IgG responses to AMA-1, CSP, EBA-175, and MSP-1₁₉ in 150 individuals, using plasma samples collected between 2003 and 2007 (Fig. 2). Each of these individuals had at least three available plasma samples: one collected within 30 days prior to infection, one collected during infection, and one collected within 30 days postinfection. Next we evaluated the longevity of MSP-1 IgG responses in 260 individuals using plasma samples collected between 2003 and 2009 (Fig. 3 and 4). Each of these individuals had available plasma samples taken within 30 days before infection and during infection, as well as at least three postinfection samples separated by approximately 30 days. These individuals were stratified into “adult” (≥ 14.5 years old) and “child” (< 14.5) age groups. The demographic characteristics for these 260 individuals and descriptive information about their infection types are shown in Table 1. Finally, we evaluated B cell subsets (Table 2) and MSP-1-specific MBC responses (Table 3) during infection and postinfection time points using PBMC samples collected between 2007 and 2009.

MSP1₁₉ is the most immunogenic of the four antigens tested. Using the 150 individuals mentioned above, we measured IgG levels specific for AMA-1, CSP, EBA-175, and MSP-1₁₉ before, during, and after *P. falciparum* infection (Fig. 2).

MSP1₁₉, AMA-1, and EBA-175 showed similar percentages of positive responders before infection (47.7%, 50.8%, and 56.9%, respectively). However, during infection and within 30 days after infection, more individuals had positive MSP1₁₉ responses. For instance, during infection, the following percentages of individuals responded positively to each antigen: 71.8% to MSP1₁₉, 56.8% to AMA-1, 45.9% to CSP, and 66.2% to EBA-175. Thus, we focused our attention on MSP1₁₉ to explore the question of antibody-level longevity in our Peruvian cohort.

Adults and children produce robust anti-MSP1₁₉ IgG responses. To determine the magnitude of the antibody level over the course of infection, we examined MSP1₁₉-specific antibody responses more closely, using samples from 260 individuals with at least 150 days of sampling follow-up. As expected, if antibody responses persist from prior parasite exposure, adults were more frequently high positive responders before *P. falciparum* infection (48.1% of adults versus 21.1% of children respond in a high positive manner; $P < 0.001$, Fisher’s exact test) (Fig. 3A). Significantly more adults than children responded high positively at day 0 and at each subsequent postinfection time point ($P < 0.001$, Fisher’s exact test), except at day 135, where, although there was no significant difference between the numbers of adults and children responding high positively, significantly more adults responded positively in general ($P < 0.001$, Fisher’s exact test; 63% of adults

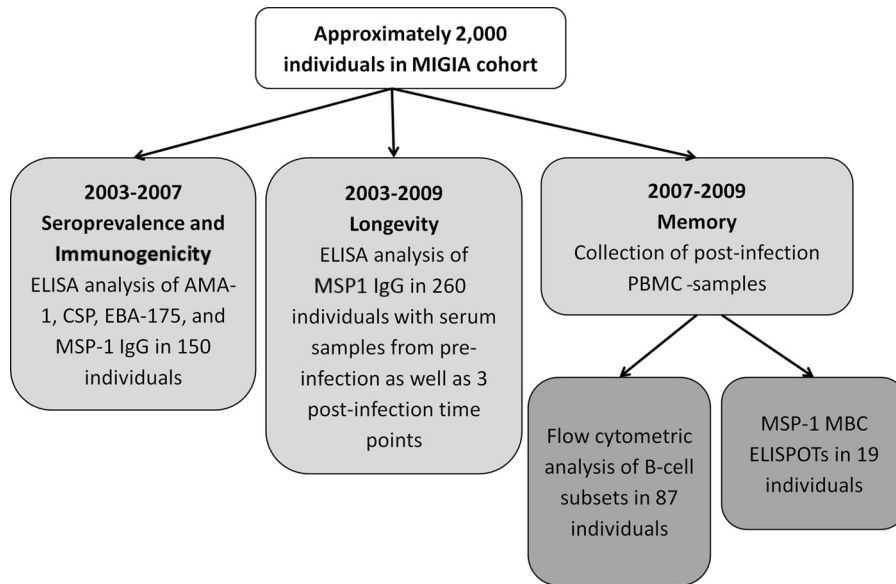


FIG 1 Study design. Shown is a flowchart depicting the three subcohorts used in the study.

and 40% of children remained positive for MSP1₁₉ IgG at the day 135 time point).

Subsequently we compared anti-MSP1₁₉ IgG levels between adults and children (Fig. 3B). Adults had significantly higher IgG levels 30 days before *P. falciparum* infection. Just after infection, at the day 30 time point, we did not observe significant differences between the anti-MSP1₁₉ IgG levels of adults and children. However, at day 75 and day 135, adults showed significantly higher anti-MSP1₁₉ IgG levels than children.

Anti-MSP1₁₉ IgG levels are maintained for at least 5 months in adults and children. We examined the longevity of anti-MSP1₁₉ IgG levels up to 180 days postinfection via Kaplan-Meier plots (Fig. 4A). Adults had significantly longer-lasting IgG levels

than did children ($P < 0.013$, log rank test [Mantel-Cox]). The median seropositivity time was 162 days for children, versus an “undefined” time (indicating that the median seropositivity time exceeded 180 days) for adults.

Prior number of *P. falciparum* infections influences duration of the anti-MSP1₁₉ response. Although age is a logical indicator of past parasite exposure, in this Peruvian population, there are adults who report zero prior *P. falciparum* exposures and children who report many prior exposures. We stratified both adult and child groups by their reported level of prior exposure. For those we can most likely conclude did not have prior exposure to malaria (children reporting 0 prior infections), the median seropositivity time was 86 days. All four adult groups (adults reporting

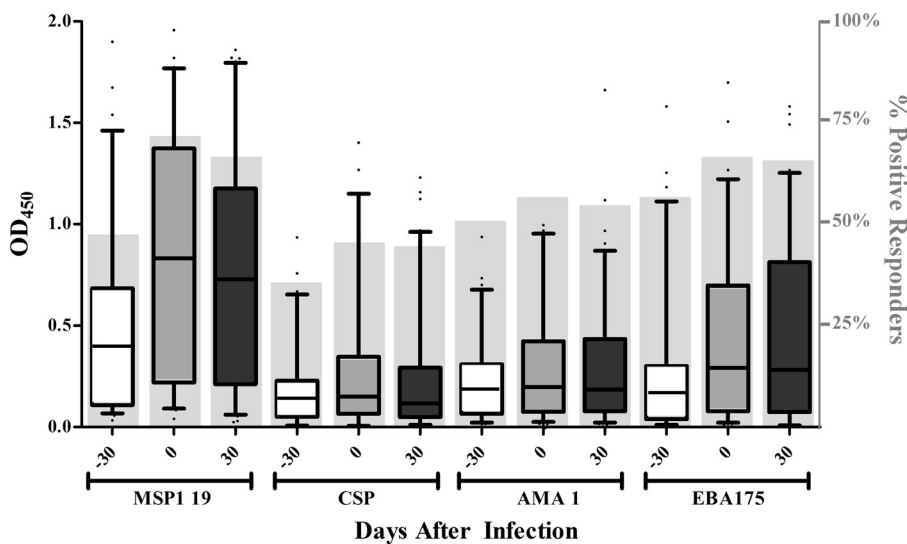


FIG 2 Antigen comparison of immunogenicity over the course of *P. falciparum* infection. Preinfection (within 30 days before infection [“-30”]), $n = 65$; during infection, $n = 73$ (“0”); and postinfection (7 to 45 days after infection [“30”]), $n = 88$. Box-and-whisker plots correspond to the left x axis. Whiskers indicate the 10 to 90th percentiles, and outliers are indicated by dots above and below the boxes. A horizontal line within the boxes represents the mean value. Light gray bars (corresponding to the right y axis) indicate the proportions of positive responders for each antigen at the respective time point.

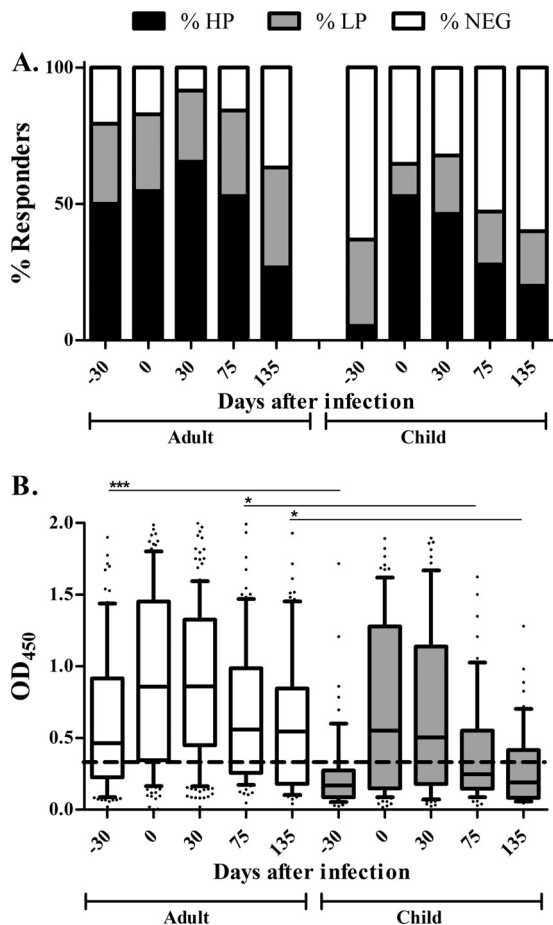


FIG 3 Magnitude and longevity of the MSP1₁₉ IgG responses in adults and children. Postinfection time points are represented by the “Days after infection” groups on the x axis. “-30” represents values for samples taken within 30 days before detection of parasitemia at day 0, “0” represents day 0 values, “30” represents values for samples taken 7 to 45 days after day 0, “75” represents values for samples taken 46 to 105 days after day 0, and “135” represents values for samples taken 106 to 165 days after day 0. For adults, $n = 93, 133, 149, 85,$ and 71 for the -30, 0, 30, 75, and 135 groups, respectively. For children, $n = 62, 88, 88, 65,$ and 56 for the -30, 0, 30, 75, and 135 groups, respectively. (A) Seroprevalence. NEG (white bars), negative ($<$ negative cutoff, 0.336); LP (gray bars), low positive ($>$ negative but $<$ $2\times$ negative cutoff); and HP (black bars), high positive ($\geq 2\times$ negative cutoff). (B) ELISA OD comparison. The dotted line indicates the negative cutoff. Whiskers indicate the 10 to 90th percentiles, and outliers are indicated by dots above and below the boxes. The horizontal line within each bar indicates the mean value. Comparisons were made using Dunn’s multiple comparison test: *, $P < 0.05$; ***, $P < 0.001$.

0, 1, 2, or >2 prior exposures) as well as children reporting 1 or 2 prior exposures showed seropositivity times lasting as long as we measured (up to 180 days postinfection) (Fig. 4B and C). Interestingly, in the high-recent-exposure group, children reporting >2 prior infections, the median seropositivity time was only 72 days. No significant differences were observed between exposure groups via Kaplan-Meier survival analysis.

B cell surface marker analysis shows evidence of functional memory responses. In 2008 and 2009, we collected whole blood samples for cellular assays as well as plasma samples for antibody experiments. For flow cytometric analysis, we collected samples from 87 individuals (68 adults [≥ 14.5 years old] and 19 children [<14.5 years old]), contributing 252 patient samples. Eighty-

seven of these were day 0 samples, 28 were day 60 samples, and the remaining 137 were follow-up samples taken between day 0 and day 60.

To evaluate B cell population dynamics and possible dysregulation post-*P. falciparum* infection, we compared CD19, CD27, and CD38 cell surface markers using flow cytometry from samples taken at day 0 and at various postinfection time points (Fig. 5). Adult CD19⁺ CD27⁺ CD38^{high} cells (plasmablasts) were significantly increased at day 0 versus recovery (greater than 11 days after infection) (Table 2). Adult CD19⁺ CD27⁺ CD38^{low} population (memory B cells) levels were higher but not significantly different comparing day 0 to recovery time points, likely indicating maintenance of previously established memory B cell pool.

Children demonstrated significantly fewer CD19⁺ B cells at day 0 versus recovery, with significantly higher populations of CD19⁺ CD27⁺ CD38^{low} cells (memory B cells) and CD19⁺ CD27⁺ CD38^{high} cells (plasmablasts) (Table 2). Child memory B cell populations at the recovery time point were significantly lower than negative control values (control samples taken from 8 healthy, seronegative, Peruvian adults), which is likely because the control samples were from adults. However, we observed no significant differences at any time point when adult cells were compared to the corresponding child cell population.

MSP1-specific B cell memory is detectable in our Peruvian cohort. A subset of individuals in this study had PBMCs available for antigen-specific memory B cell (MBC) analysis by ELISPOT. PBMC samples from 34 individuals (29 adults and 5 children) were thawed and cultured for MBC ELISPOT. Samples from 19 individuals (4 children [average age, 9.7 years] and 15 adults [average age, 33.2 years]) were cultured successfully; the remaining samples had fewer than 1,000 IgG antibody-secreting cells (ASCs) per million PBMCs after culture and thus were considered assay failures.

We assessed the MSP1-specific MBC response in these individuals at various time points, either at presentation (day 0) or during follow-up (from day 10 to 56). Although we tested multiple time points, we only used the time point with the highest MSP1 MBC level in our analysis (each individual showed similar MBC frequency at each time point tested). Assays were considered positive if they yielded >5 MSP1 MBC spots per million PBMCs (Table 3).

We detected MSP1-specific MBC responses in the majority of samples tested. Twelve of 15 adults as well as 2 of 4 children showed an MSP1-specific MBC response. We observed MSP1-specific MBCs even in adults having their first infection (9 MSP1 MBC-positive adults reported zero prior infections). The mean frequency of MSP1-specific MBCs among responders was 71 MSP1-specific MBCs per million PBMCs; range, 7 to 352). The frequencies of MSP1-specific MBCs were similar on the various days of sampling (day 0 versus follow-up days). Amount of prior exposure (Table 3) did not predict the MSP1-specific MBC response magnitude, likely due to low sample numbers. All individuals with detectable MSP1-specific MBCs had positive antibody titers for MSP1. As expected, MSP1-specific MBCs were not detected in Peruvian or American volunteers with no history of *P. falciparum* exposure.

DISCUSSION

A commonly accepted tenet in malaria research is that clinical immunity to symptomatic *P. falciparum* infection is achievable but requires several years of constant parasite exposure. Although

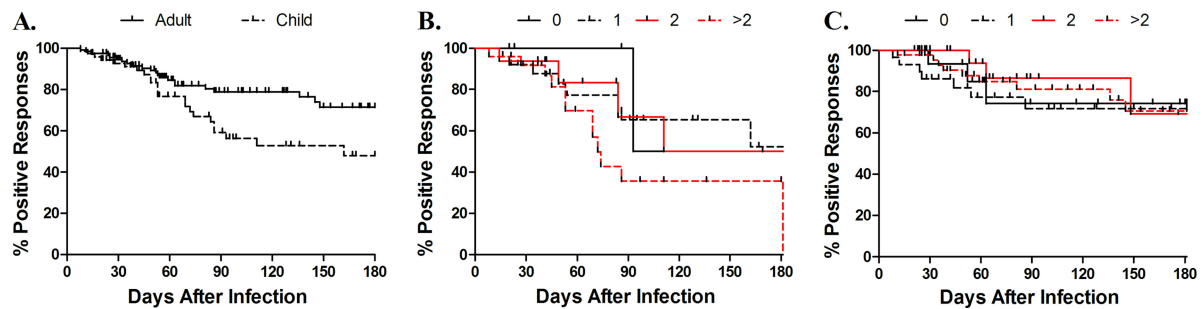


FIG 4 Anti-MSP₁₁₉ IgG duration. Kaplan-Meier curves comparing time (in days) of decline to below negative cutoff. Only individuals with a day 0 OD of >0.500 were included in this analysis. (A) Adults versus children. The median seropositivity time for children ($n = 70$) is 162 days, and the median seropositivity time for adults ($n = 113$) is >180 days. Log rank (Mantel-Cox) test, $P < 0.028$. (B) Children stratified by reported prior *P. falciparum* exposures. The median seropositivity times for children reporting 0, 1, 2, and ≥ 2 prior exposures ($n = 5, 25, 15,$ and 25 , respectively) are 86, >180, >180, and 72 days, respectively. (C) Adults stratified by reported prior *P. falciparum* exposures. The median seropositivity time for adults reporting 0, 1, 2, or >2 prior exposures ($n = 17, 28, 25,$ and 43 , respectively) is >180 days postinfection.

humoral responses are thought to be the mechanism driving immunity to malaria infection, most prior epidemiologic studies performed in high-transmission areas demonstrate short-lived antimalaria antibody responses and irregularities in cellular memory responses (3, 19, 36, 40). These findings help to explain the delayed acquisition of protective immunity in high-transmission areas, which is likely due to multiple factors that impair effective immune response development, including high infection rate, diverse parasite genetic characteristics, host genetics, and/or concomitant infections.

However, as most prior studies evaluating the humoral response to malaria infection were performed in high-transmission areas, they were limited in their ability to consider postinfection antibody dynamics by the rapidity of reinfection that defines these regions (1, 9, 19, 25, 33). Interestingly, several studies performed at low-malaria-transmission sites suggest more rapid development of clinical immunity (4) and stable anti-MSP₁₁₉ responses (6, 43), although these studies were complicated by widely spaced sampling time points. Other groups have reported robust antimalarial antibody responses to low levels of parasite exposure (26, 33). Thus, we hypothesized that antibody responses to malaria infection could develop more effectively in low-transmission areas, even after only one or two documented *P. falciparum* infections.

The communities around Iquitos, Peru, are an ideal setting for testing this hypothesis. The malaria transmission rate in this region is less than 0.5 infection/person/year (7), leaving little possibility of overlapping infections. We used a unique longitudinal sampling strategy that enabled us to capture pre- and postinfection antibody dynamics to evaluate our Peruvian cohort. In addition,

by comparing personal histories to health post records, we were able to estimate each individual's number of prior *P. falciparum* infections.

In this study, we first evaluated the seroprevalence of IgG to four different malaria vaccine candidate antigens (AMA-1, CSP, EBA-175, and MSP-1₁₉) at time points before, during, and after infection. Individuals in our cohort had a more robust response to MSP₁₁₉ than to any of the other antigens tested (Fig. 2), and thus we chose to focus on the MSP₁₁₉ antigen for the remainder of the study. We sampled our study subjects for at least 180 days after infection, during which time, we used blood smear microscopy to establish that there was no continuing *P. falciparum* infection after treatment. The majority of individuals (>60% of adults and 40% of children) maintained a positive anti-MSP₁₁₉ response for more than 5 months postinfection (Fig. 3A and B), indicating that most adults as well as a large percentage of children are able to maintain an appropriate antibody response to infection. This finding supports the results of prior studies in which adults were shown to maintain a stronger antibody response than children; however, those studies did not explore the idea that this phenomenon is due to prior *P. falciparum* exposure rather than the physiologic changes of aging (1, 17, 20, 26, 33). Due to the low malaria transmission rate and highly regulated regional malaria drug treatment policies, we were able to consider previous parasite exposure separate from age.

Our study region contains both adults and children with zero or very few prior symptomatic *P. falciparum* infections in their lifetime. Even among groups with various levels of exposure (0, 1, 2, or >2 prior infections), adult MSP₁₁₉ antibody responses had a median seropositivity time of >180 days (Fig. 4C). With respect to

TABLE 1 MSP₁₁₉ IgG longevity study participants and *P. falciparum* infection history

	No. of individuals ($n = 260$) by:							No. of infections ($n = 452$) by:							
	Sex		No. of prior exposures					Clinical infection type ^b			No. of prior exposures				
Age group ^a	Male	Female	0	1	2	>2	NA ^c	SYM	ASY	CD	0	1	2	>2	NA
Adults	84	65	36	37	20	26	30	189	62	4	34	63	52	75	31
Children	56	55	24	26	28	20	23	150	42	5	25	44	42	60	26

^a Adults were defined as ≥ 14.5 years of age and children as < 14.5 years of age.

^b "Clinical infection type" is the classification of *P. falciparum* infection based on clinical symptoms experienced at time of infection. SYM, symptomatic infection; ASY, asymptomatic infection; CD, infection type undeterminable.

^c NA, not available.

TABLE 2 Comparison of B cell phenotypes detected at different time points after *P. falciparum* infection^a

Age group and cell type ^b	Median % (25th to 75th percentile) of cell type			Significance ^c		
	Day 0 (A)	Posttreatment (P)	Recovery (R)	A vs P	A vs R	P vs R
Adults						
CD19 ⁺	9.515 (5.273–13.53)	7.660 (4.105–14.11)	8.425 (3.345–13.19)	0.400	0.476	0.815
CD38 ⁻ CD27 ⁺ (MCs)	21.22 (11.02–34.67)	15.90 (9.280–34.68)	14.77 (8.708–28.74)	0.291	0.067	0.590
CD38 ⁺ CD27 ⁺ (plasmablasts)	2.075 (0.3600–1.953)	1.600 (2.450–5.570)	0.875 (0.3600–1.953)	0.235	0.014	0.219
Children						
CD19 ⁺	5.170 (1.190–10.23)	7.640 (5.193–12.63)	13.670 (6.513–16.56)	0.155	0.013	0.132
CD38 ⁻ CD27 ⁺ (MCs)	23.54 (13.55–35.59)	13.42 (8.410–17.27)	11.09 (8.413–16.80)	0.011	0.007	0.624
CD38 ⁺ CD27 ⁺ (plasmablasts)	3.450 (2.450–5.570)	1.255 (0.9525–1.910)	0.780 (0.3925–1.453)	0.017	0.001	0.052

^a Shown are the median percentages of lymphocyte subsets at presentation with noncomplicated malaria (A), 4 to 10 days postinfection (P), and 11 days or greater postrecovery (R). All percentages of memory cell (MC) and plasmablast populations represent percentages of the total CD19⁺ population. A total of 100,000 events were collected for each analysis.

^b For adults, the sample sizes were $n = 40$ for all day 0 (A) cell populations, $n = 37$ for postinfection (P), and $n = 68$ for recovery (R). For children, the sample sizes were $n = 11$, 14, and 19, respectively. For negative controls, $n = 8$ (healthy Peruvian adults). Control populations were 8.680% (25th to 75th percentile, 8.288 to 11.08) CD19⁺, 18.47% (25th to 75th percentile, 13.95 to 25.32) CD38⁻ CD27⁺, and 0.9900% (25th to 75th percentile, 0.3725 to 3.143) CD38⁺ CD27⁺.

^c Significant differences ($P < 0.05$, Mann-Whitney test) are in bold. Significant differences were found when comparing child day 0 versus control CD19⁺ cell populations ($P = 0.063$).

the adults reporting 0 prior infections, the fact that their anti-MSP1₁₉ responses last for >180 days indicates that either they mount a robust response to their first infection or they had previously been exposed to *Plasmodium* but were not diagnosed at the health post. In addition, more than 60% of children reporting either 1 or 2 prior infections had median antibody seropositivity times of at least 180 days postinfection, suggesting that only moderate *P. falciparum* exposure (1 or 2 prior infections) is necessary to maintain a robust anti-MSP1₁₉ IgG response. Individuals who had antibody responses that lasted <180 days were frequently children with no history of infection (this being their first infection) or children reporting >2 prior infections (Fig. 4B). Initial antibody level did not explain the shorter-duration response, as individuals included in the Kaplan-Meier analysis did not have significantly different levels of anti-MSP1₁₉ antibody at the time of infection. (The analysis only included individuals who had an IgG OD of ≥ 0.500 at day 0.) These individuals may have a diminished capacity to develop a lasting response due to experiencing more than the average number of prior infections early in life.

The presence of serum antibody in the absence of infection can be considered secondary evidence of plasma cell maintenance (34,

38). Thus, we examined cellular immunologic memory in this Peruvian cohort by evaluating the dynamics of B cell subsets during and just after infection. Upon antigenic stimulation, both naive and memory B cells proliferate and differentiate into antibody-secreting plasmablasts and eventually become nondividing, highly productive plasma cells (38). In this study, we used whole blood samples taken shortly after *P. falciparum* diagnosis, as well as follow-up samples from these patients, to evaluate changes in the dynamics of certain B cell subsets, namely, CD19⁺ CD27⁺ CD38^{high} plasmablasts and CD19⁺ CD27⁺ CD38^{low} memory B cells over time. We observed a larger population of CD19⁺ CD27⁺ CD38^{high} plasmablasts at day 0 compared to the recovery phase 11 or more days later in both adults and children (Table 2), indicating that these individuals are successfully generating pathogen-initiated, antibody-producing plasma cells at the time of infection. While this plasmablast increase following infection could reflect polyclonal B cell activation with accompanying hypergammaglobulinemia, that is more characteristic of chronic malaria infections occurring in high-transmission regions. We also observed a larger population of CD19⁺ CD27⁺ CD38^{low} memory B cells in children at day 0, but not in adults, perhaps because adults already have an established malaria-specific memory B cell pool. We did not observe the disruption in B cell memory and plasmablast responses that Asito et al. described in high-transmission settings (3), which could be due to the lower transmission setting of our study. Because of the small number of individuals with >2 prior infections in our study, we could not directly test the association between B cell population dynamics and prior *P. falciparum* exposure. Such a test may provide further insight into the discrepancy between our findings and those of Asito et al.

To determine whether the memory B cells we detected via flow cytometry were *Plasmodium* specific, we conducted malaria antigen-specific memory B cell ELISPOT assays. Although memory B cells are a rare population in the peripheral blood, especially as time increases after infection, we found that a subset of individuals demonstrated MSP1-specific memory B cells at day 0 as well as at various postinfection time points (Table 3). This indicates that individuals in a low-transmission setting generate *Plasmodium*-specific memory B cells, even after a single or few *P. falciparum*

TABLE 3 Assessment of MSP-1-specific MBC ELISPOT results by age and reported number of prior *P. falciparum* exposures

MSP1 MBC ELISPOT result by age ^a	No. of prior exposures	<i>n</i>	Median no. of MSP1 MBCs/10 ⁶ PBMCs	Avg % of total ASC population (SE)
Positive				
Child	0	5	41	0.206 (0.130)
Adult	0	5	20	0.045 (0.015)
Child	>0	2	16	0.050 (0.300)
Adult	>0	3	40	1.181 (0.775)
Negative				
Child	0	0	Negative	
Adult	0	2	Negative	
Child	>0	1	Negative	
Adult	>0	1	Negative	

^a Adults were defined as ≥ 14.5 years of age and children as < 14.5 years of age.

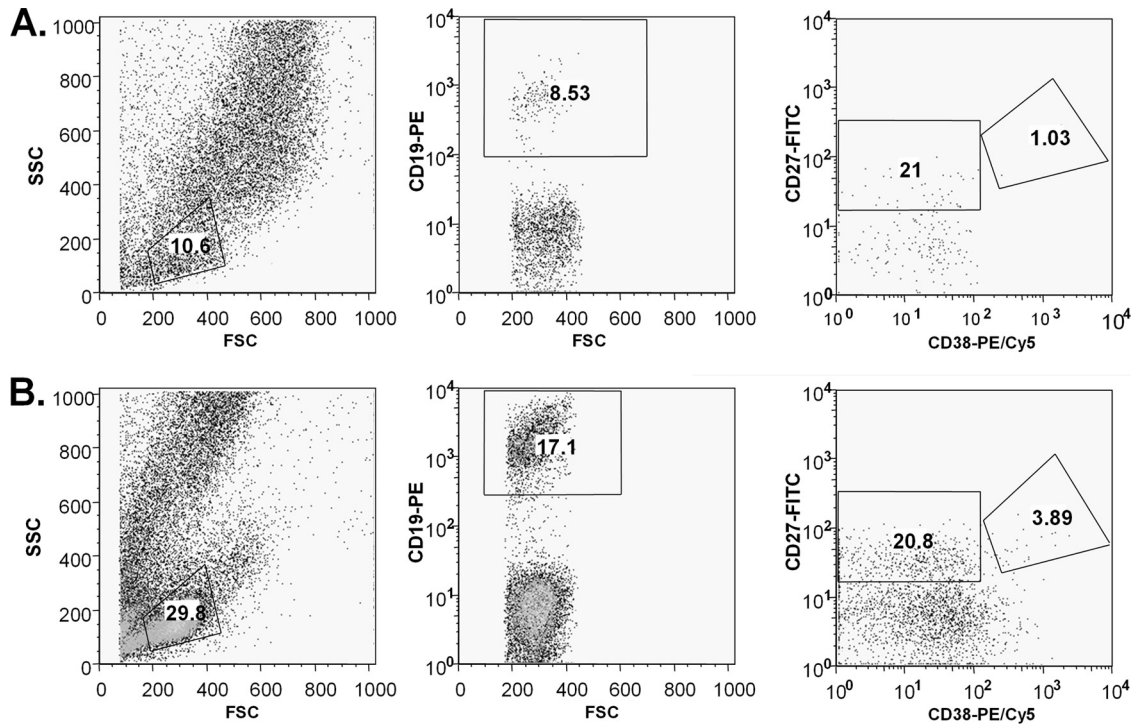


FIG 5 Gating strategy for flow cytometric analysis of B cell subsets. Expression of CD38 and CD27 was used to identify naive and memory B cell subpopulations in the peripheral blood. In the left-hand panel, a sample gate for lymphocytes is outlined in black. In the middle panel, a sample gate for CD19⁺ cells is outlined in black. In the right-hand panel, a sample gate for CD27⁺ CD38^{low} cells (memory B cells) is represented by the black-bordered shape on the left, and a sample gate for CD27⁺ CD38^{high} cells (plasmablasts) is represented by the black-bordered shape on the right. (A) Negative control sample (uninfected, North American control). (B) Day 0 sample from an adult. SSC, side scatter; FSC, forward scatter.

arum infections. The combined observations of robust MSP1₁₉ antigen-specific antibody responses, infection-specific plasmablast population expansion, and MSP1-specific memory B cells suggest that immunologic memory is achievable in individuals living in this low-transmission region.

Few studies have identified malaria-specific memory B cells (19, 40, 42, 43), perhaps due to the relatively recent availability of assays that make it possible to test antigen-specific memory B cell populations. However, Weiss et al. recently reported that an FCRL4(+) hypo-responsive subset of memory B cells, coined “atypical” memory B cells, are increased in individuals exposed to high transmission rates in Mali (42). These authors proposed that this newly defined memory population could represent a clue into the delayed acquisition of immunity to malaria. Recently, we directly compared the memory B cell responses of our Peruvian cohort to those of a high-transmission population in Mali (41). We found that, compared to *P. falciparum*-naive controls, “atypical” memory B cells were increased in Peruvian adults exposed to low *P. falciparum* transmission, but to a lesser degree than that observed in Malian adults exposed to intense *P. falciparum* transmission (41), suggesting that our low-transmission setting provides the appropriate stimulus for developing B cell memory to malaria. The data from our current study further support the hypothesis that antimalarial immune responses are more stable in regions of low transmission because they are not disrupted by extreme parasite exposure.

The exact mechanism(s) that occur in high-transmission areas to produce a dysfunctional humoral response remain unclear. In areas of high transmission, increased parasite genetic diversity re-

sults in infections being mixtures of many different genetic types. In a study in western Kenya, we found that individuals having mixed-genotype infections (antigenically complex infections) were more rapidly reinfected. We suggested that complex infections could result in mediocre immune response development to conserved antigens, such as MSP1₁₉ (8). In addition, Crompton et al. hypothesized that this poor response could be related to the host immune system being overwhelmed by the large number of parasite antigens presented to it during infection (14). Thus, infection spacing, parasite antigenic diversity/quantity, and geographic differences might together explain why many prior studies have not found evidence for functional immunologic memory to malaria.

There are several limitations to our study. We were unable to demonstrate an independent measure of prior *P. falciparum* exposure. Although this variable may always remain inextricably tied to age, perhaps with a larger sample size, more definitive differences in exposure groups could be demonstrated. In addition, our study involved a very long sample collection period, during which sample degradation could have occurred. During this sample collection period, we did not begin to collect cellular data until 2009, and thus our cellular data cannot be directly compared to our antibody data.

In this study, we present a model of effective immunity against *P. falciparum* malaria that may be the result of human genetic or environmental factors specific to this low-transmission region or perhaps to the presence of fewer and less antigenically complex infections. These results may signify that, as vaccine strategies and other malaria-blocking interventions

work to decrease the malaria burden, the resulting reduced transmission intensity would pave the way for the rapid development of naturally acquired immunity, further reducing malaria pathology and transmission.

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