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Plasmodium falciparum Gametocyte-Specific Antibody Profiling Reveals Boosting through Natural Infection and Identifies Potential Markers of Gametocyte Exposure

Jeff Skinner,[a] Chiung-Yu Huang,[b] Michael Waisberg,[a*] Philip L. Felgner,[c,d] Ogobara K. Doumbo,[e] Aissata Ongoiba,[c] Kassoum Kayentao,[c] Boubacar Traore,[e] Peter D. Crompton,[a] Kim C. Williamson[f,g]

Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland, USA[a]; Division of Biostatistics and Bioinformatics, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, Maryland, USA[b]; Division of Infectious Diseases, Department of Medicine, University of California, Irvine, Irvine, California, USA[c]; Antigen Discovery, Inc., Irvine, California, USA[d]; Mali International Center of Excellence in Research, University of Sciences, Techniques and Technologies of Bamako, Bamako, Mali[e]; Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA[f]; Department of Biology, Loyola University Chicago, Chicago, Illinois, USA[g]

Malaria elimination efforts would benefit from vaccines that block transmission of *Plasmodium falciparum* gametocytes from humans to mosquitoes. A clear understanding of gametocyte-specific antibody responses in exposed populations could help determine whether transmission-blocking vaccines (TBV) would be boosted by natural gametocyte exposure, and also inform the development of serologic tools to monitor gametocyte exposure in populations targeted for malaria elimination. To this end, plasma was collected from Malian children and adults before and after the 6-month malaria season and probed against a microarray containing 1,204 *P. falciparum* proteins. Using publicly available proteomic data, we classified 91 proteins as gametocyte specific and 69 as proteins not expressed by gametocytes. The overall breadth and magnitude of gametocyte-specific IgG responses increased during the malaria season, although they were consistently lower than IgG responses to nongametocyte antigens. Notably, IgG specific for the TBV candidates Pfs48/45 and Pfs230 increased during the malaria season. In addition, IgGs specific for the gametocyte proteins Pfmdv1, Pfs16, PF3D7_1346400, and PF3D7_1024800 were detected in nearly all subjects, suggesting that seroconversion to these proteins may be a sensitive indicator of gametocyte exposure, although further studies are needed to determine the specificity and kinetics of these potential serologic markers. These findings suggest that TBV-induced immunity would be boosted through natural gametocyte exposure, and that antibody responses to particular antigens may reliably indicate gametocyte exposure.

The 2014 Malaria World Report indicates that malaria morbidity and mortality continued to decline in 2013, renewing optimism for malaria elimination efforts (1). However, malaria control remains a major challenge in many countries, and in high-risk areas of Africa the incidence of febrile malaria continues to be ≥1 case/1,000 persons (1). Current malaria control strategies include diagnosis and treatment of cases and vector control measures, such as insecticide-treated bed nets and indoor residual spraying. However, these labor-intensive measures are constantly threatened by the emergence of drug-resistant parasites and insecticide-resistant mosquitoes (1). In addition, blood-stage gametocytes—the sexual parasite stages responsible for transmission from humans to the mosquito vector—are not affected by many of the antimalarial drugs in common use (2, 3), and the prevalence of submicroscopic and asymptomatic infections is often high (4). Clearly, new tools such as transmission-blocking vaccines (TBV) are needed to decrease malaria transmission and eventually eradicate the parasite (5).

The sexual stages of the *Plasmodium* parasite that are transmitted from the human host to the mosquito vector begin development in human red blood cells (RBCs). In the case of *P. falciparum*, differentiation into mature stage V gametocytes requires 10 to 12 days, whereas the gametocytes of other *Plasmodium* species reach maturation in only one to 2 days (6). If not taken up in the blood meal of a mosquito, *Plasmodium* gametocytes die and are cleared by the human host (7). Once taken up in the blood meal, conditions in the mosquito midgut stimulate gametocytes to emerge from RBCs as extracellular male and female gametes that fertilize and form an oocyst, thus initiating sporogonic development of the parasite in the mosquito (6, 8).

TBVs that target gametocyte and gamete antigens could play a critical role in the effort to eliminate and eventually eradicate malaria (5). TBV-induced antibodies taken up by the mosquito could

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Address correspondence to Peter D. Crompton, pcrompton@niaid.nih.gov, or Kim C. Williamson, kwilli4@luc.edu.

* Present address: Michael Waisberg, Department of Pathology, School of Medicine, University of Virginia, Charlottesville, Virginia, USA.

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interfere with parasite development in the mosquito midgut. Indeed, it has been demonstrated that murine-derived monoclonal antibodies that bind to the surface of extracellular gametes block parasite development in the mosquito and disrupt transmission (9–12). To date, four *Plasmodium* antigens have been identified as targets of transmission-blocking monoclonal antibodies: P25, P28, P230, and P48/45. The translation of genes encoding the rodent malaria orthologues Pbs25 and Pbs28 does not begin until the parasite enters the mosquito midgut (13), and accordingly, there is no evidence that IgG specific for Pfs25 (PF3D7_1031000) or Pfs28 (PF3D7_1030900) is generated through natural *P. falciparum* infection in humans (14, 15). In contrast, Pfs230 (PF3D7_0209000) and Pfs48/45 (PF3D7_1346700) are expressed by gametocytes within RBCs in the human host, and naturally acquired antibodies specific for these proteins have been described (16, 17). However, P230 and P48/45 are located on the parasite surface within RBCs and therefore not accessible to circulating antibodies until a mosquito takes up the gametocyte and it emerges from the RBC.

Relatively little is known about the dynamics of naturally acquired antibody responses to gametocyte or gamete proteins and how this compares to antibody responses to asexual blood stage antigens, which have been more extensively studied (18). The few seroepidemiological studies conducted in areas of malaria endemicity that have focused on antibody responses to gametocytes are inherently biased in that they have been constrained to measuring antibodies by enzyme-linked immunosorbent assay (ELISA) to the relatively few *P. falciparum* proteins identified by traditional cloning methods, <0.5% of the 5,400+ *P. falciparum* proteome (15, 17, 19, 20), or to parasite surface proteins amenable to radiolabeling (21, 22). Prior seroepidemiological studies have focused primarily on the TBV candidates Pfs230 and Pfs48/45 or the gametocyte-specific (GS) antigens Pfs16 and Pfg27. Although several cross-sectional studies have shown that individuals in areas of malaria endemicity can generate antibodies specific for Pfs230, Pfs48/45, Pfg27 (PF3D7_1302100), and Pfs16 (PF3D7_0406200) (19, 23, 24), IgGs specific for these proteins have been reported to not increase with age or malaria exposure, a finding that stands in contrast to antibody responses specific for asexual blood-stage antigens, which are generally boosted after malaria exposure (15, 19, 22, 25). These observations have led to the suggestion that the immune response to sexual blood-stage antigens differs from responses to asexual stage antigens, possibly due to the low number of circulating gametocytes relative to asexual parasites, the context of the initial exposure, or reduced T-cell help (19, 22, 26). However, to our knowledge, no studies have compared antibody responses to large sets of gametocyte-specific and non-gametocyte-specific (NGS) antigens. A clearer understanding of gametocyte-specific antibody responses in exposed populations could help determine whether TBV-induced immunity would be boosted by natural gametocyte exposure and could also inform the development of serologic tools to monitor gametocyte exposure in populations targeted for malaria elimination.

To obtain a broader and less-biased view of the seroprevalence and kinetics of gametocyte-specific IgG responses in relation to IgG responses to antigens expressed during other stages of the parasite life cycle, we probed plasma collected from Malian children and adults before and after the 6-month malaria season against a protein microarray containing 1,204 *P. falciparum* antigens, i.e., ~23% of the predicted *P. falciparum* proteome (27). Using publicly available proteomic data (www.plasmodb.org), we identified 91 of these proteins as gametocyte specific and 69 proteins as not expressed in gametocytes (28) and then compared their respective antibody profiles.

MATERIALS AND METHODS

Ethics statement. The Institutional Review Board at the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and the Ethics Committee of the University of Sciences, Technique and Technology of Bamako approved this study. Written informed consent was obtained from adult participants and from the parents or legal guardians of participating children.

Study site and participants. This study was conducted in Kambila, Mali, a small rural village with a population of ~1,500 persons where *P. falciparum* transmission is seasonal and intense (29). The site was selected because of the sharp demarcation between the 6-month rainy season and the 6-month dry season, during which there is either intense or negligible *P. falciparum* transmission, respectively, thus allowing for comparisons of immunological parameters in the presence or absence of malaria exposure. In May 2006, during a 2-week period just before the malaria season, 225 individuals between the ages of 2 and 10 years ($n = 176$) and 18 and 25 years ($n = 49$) were enrolled in the cohort study after random selection from an age-stratified census of the entire village population. Enrollment exclusion criteria were a hemoglobin level of <7 g/dl, an axillary temperature of ≥37.5°C, acute illness discernible on examination, or the use of antimalarial or immunosuppressive medications in the past 30 days. Blood smears and venous blood samples were collected during the 2-week enrollment period and during a 2-week period at the end of the 6-month malaria season. Of the 225 subjects enrolled in the study, microarray data were available for 220 before the malaria season and 194 after the malaria season. A detailed description of the study site and design is reported elsewhere (29).

Blood samples. Venous blood was drawn into sodium citrate-containing tubes (BD Biosciences, Vacutainer CPT). Samples were transported 20 km to the laboratory for processing within 3 h of collection. Plasma was isolated and stored at −80°C.

Detection of *P. falciparum* infection. Thick blood smears were stained with Giemsa and counted against 300 leukocytes, and *P. falciparum* densities were recorded as the number of asexual parasites per microliter of whole blood based on a mean leukocyte count of 7,500 cells per μl. Each smear was evaluated separately by at least two expert microscopists.

Classifying *P. falciparum* proteins as gametocyte and non-gametocyte specific. Proteins for which there is evidence of exclusive expression in gametocytes or no expression in gametocytes were identified using publicly available proteomic data accessed through www.plasmodb.org (28). To our knowledge, the study by Florens et al. is the only published protein expression data that include sporozoites, merozoites, trophozoites, and stage V gametocytes (28). Using these data, we compared the total number of peptide spectra identified in stage V gametocytes versus the intraerythrocytic asexual and sporozoite stages for each protein or protein fragment represented on the microarray (see Table S1 in the supplemental material). Proteins with more than three times as many spectra in the gametocyte stage relative to all other stages combined were classified as "gametocyte specific" (GS), whereas proteins with more than six times as many spectra in the nongametocyte stages versus the gametocyte stage were classified as "non-gametocyte-specific" (NGS) proteins. A larger ratio was used to classify nongametocyte proteins because samples from three different stages were included (sporozoites, merozoites, and trophozoites).

Antibody profiling. As described elsewhere (27), protein microarrays were constructed in four steps: (i) PCR amplification of complete or partial *P. falciparum* open reading frames, (ii) *in vivo* recombination cloning, (iii) *in vitro* transcription/translation, and (iv) microarray chip printing. Fluorescently labeled anti-IgG was used to detect IgG in plasma samples

that reacted to proteins on the microarray. A microarray containing ~23% of the *P. falciparum* 5,400-protein proteome was used to probe plasma samples.

Statistical analysis. Protein microarray data were analyzed using the R Project for Statistical Computing (30), version 3.1.2. A median foreground intensity of 635 nm and a mean background intensity of 635 nm were imported separately from 150 raw data files generated by the ProScanArray Express software. Foreground and background intensities were both $\log_2$-transformed separately, with random white noise added to any spots with zero intensity values (uniformly distributed integers from 1 to 20 for slide background zeros and uniformly distributed integers from 41 to 100 for foreground zeros). The protein arrays included no-DNA negative-control spots used to estimate cross-reaction with the *Escherichia coli* vector used to print the antigen spots on the array. Slide background noise and cross-reaction noise were subtracted from the foreground intensity before normalization using a robust linear model (RLM) method described previously (31). Box plots, histograms, density plots, and principal component analysis (PCA) plots of the data were made for quality control assessments immediately after import and after RLM normalization to assess the impact on any batch effects or specific groups of spots. Any target spot with a $\log_2$ intensity value >2 standard deviations (SD) above the mean of the similarly transformed no DNA control spots was considered reactive. The percentage of reactive peptides on the array (*X*/2,320 peptides, where *X* is the number of reactive peptides) per subject represents each subject's antibody "breadth," while the percentage of reactive plasma samples (*X*/418 samples, where *X* is the number of reactive samples) per antigen represents the seroprevalence of that peptide. Differences in percent breadth, percent seroprevalence, and the average "magnitude" of antibody response per sample were tested using a linear mixed model with the malaria season (before and after), the antigen type (GS or NGS), and their interaction term as covariates. Additional comparisons also examined infection status (smear positive or negative) and patient age categories (ages 2 to 5, ages 6 to 10, and ages 18 to 25). Comparisons of breadth, magnitude, and seroprevalence were made among the antigen spots identified as gametocyte-specific (GS) or non-gametocyte-specific (NGS) antigens. The complete list of 2,320 peptides was filtered to remove any peptides with a seroprevalence less than 20% of all samples prior to a comparison of reaction magnitudes for individual antigen fragments using empirical Bayes moderated linear models (32). False discovery rate (FDR)-adjusted *P* values were computed using the p.adjust function from the stats package library in R (33, 34).

RESULTS

Classification of *P. falciparum* proteins as gametocyte specific or non-gametocyte specific. We chose to classify proteins as gametocyte specific (GS) or non-gametocyte specific (NGS) on the basis of publicly available stage-specific proteomic/mass spectrometry data (www.plasmodb.org) (28) rather than RNA expression evidence, since mRNA transcript abundances only partially correlate with protein abundances (35, 36). Peptides corresponding to 2,415 of the 5,268 predicted *P. falciparum* proteins (http://www.sanger.ac.uk/resources/downloads/protozoa/plasmodium-falciparum.html) were detected by mass spectroscopy and represented the following parasite life cycle stages: sporozoites, merozoites, trophozoites, and stage V gametocytes (28). Of these, 114 proteins (4.7%) had >3 times the number of peptide spectra in gametocytes versus sporozoites, merozoites, and trophozoites combined (28; see also Table S1 in the supplemental material) and were thus considered GS, while 108 proteins (4.5%) had >6 times the number of peptide spectra in nongametocyte stages relative to gametocytes and were thus considered NGS. GS and NGS proteins are listed in Table S1 in the supplemental material. Of the 2,320 polypeptides on the protein microarray (representing 1,204 unique proteins), 194 peptides corresponded to 91 of the 114 proteins identified as GS, including the well-characterized gametocyte proteins Pfs230, Pf11-1 (PF3D7_1038400), and Pfs16 (37), whereas 126 peptides on the array corresponded to 69 of the 108 NGS proteins (see Table S2 in the supplemental material). Although the protein microarray was not designed to be equally representative of GS and NGS proteins, there were similar proportions of GS (7.5%) and NGS (5.7%) proteins on the array. Of note, GS proteins identified in this way were also found to be enriched in gametocytes in the proteomic analyses of Lasonder et al. (38) and Silvestrini et al. (39). Among the 2,320 peptides on the array, 2,221 peptides were found to be reactive based on a cutoff value of 2 SD above the negative (no DNA) control in at least 20% of all samples, and these peptides were individually tested for differences in reactivity before and after the malaria season using FDR-adjusted results from the empirical Bayes moderated *t* test. Of the 320 peptides (160 unique proteins) that were classified as either GS or NGS, 304 peptides were reactive in at least 20% of all samples based on the same cutoff value of 2 SD above the negative (no DNA) control—181 GS peptides and 123 NGS peptides (see Table S2 in the supplemental material).

Gametocyte-specific IgG responses generally increase with malaria transmission but are lower than responses to nongametocyte proteins. We first sought to understand the relative immunogenicity of GS and NGS proteins by examining both the breadth and magnitude of GS and NGS IgG responses at two time points: after the 6-month dry season (a period of negligible *P. falciparum* transmission) and after the 6-month malaria season (a period during which nearly all study subjects are infected with *P. falciparum* in this area of Mali) (40). We defined the "breadth" for each plasma sample as the number of proteins to which the level of IgG reactivity exceeded 2 SD above the negative (no DNA) control. Because the numbers of GS and NGS proteins differed, we present the data as the percentages of GS and NGS proteins that were reactive in each plasma sample. We found that the average percentage of reactive GS proteins increased from before to after the malaria season (Fig. 1A; GS mean before: 21.7% [SD 15.6%]; GS mean after: 30.0% [SD 16.7%]; $P < 0.001$), and likewise, the average percentage of reactive NGS proteins increased from before to after the malaria season (Fig. 1A; NGS mean before: 30.2% [SD = 17.8%]; NGS mean after: 41.2% [SD 18.2%]; $P < 0.001$). On average, the percentage of GS proteins that were reactive before the malaria season was significantly lower than the percentage of NGS proteins that were reactive at the same time point ($P < 0.001$) (Fig. 1A). Similarly, the average percentage of GS proteins that were reactive after the malaria season was significantly lower than the percentage of NGS proteins that were reactive at the same time point ($P < 0.001$) (Fig. 1A).

Individuals with *P. falciparum* infection by blood smear before the malaria season ($n = 15$ samples, or 7.2%) had significantly higher IgG breadth for both GS and NGS proteins compared to blood smear-negative subjects (see Fig. S1A in the supplemental material). Both smear positive and smear negative patients displayed higher percent breadth to NGS proteins than to GS proteins, supporting our previous result (Fig. 1A). Stratifying patients by age revealed higher percent breadth after malaria season and higher breadth to NGS proteins in all three age categories (see Fig. S2 in the supplemental material), supporting our previous result (Fig. 1A).

Next, we compared the overall levels of IgG reactivity to GS and NGS proteins before and after the 6-month malaria season. We

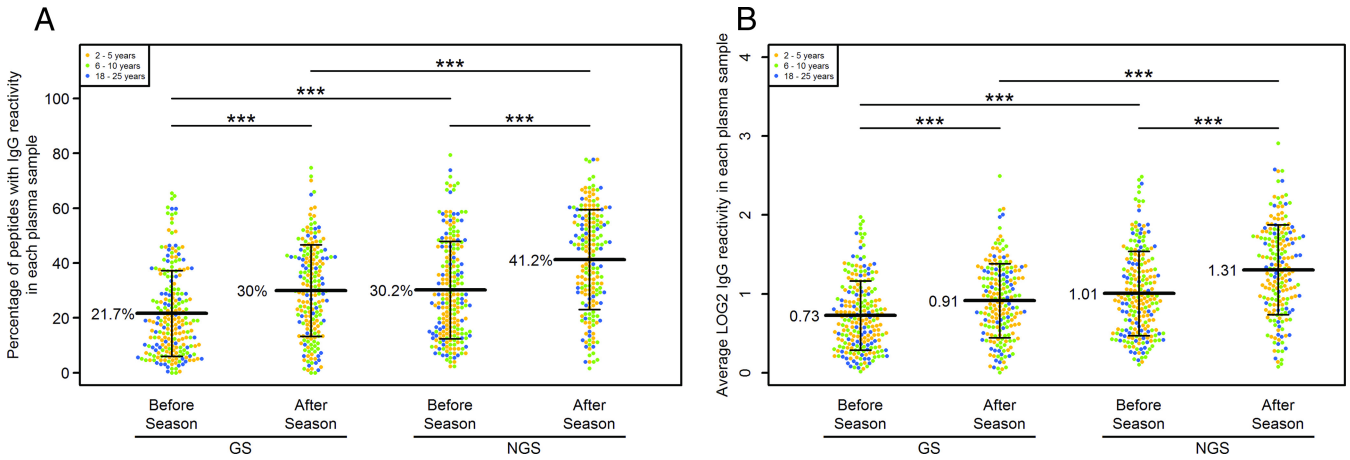

FIG 1 Breadth and magnitude of IgG responses to GS and NGS peptides before and after the malaria season. (A) Each symbol represents the percentage of peptides with IgG reactivity (breadth) in an individual plasma sample ($n = 223$ subjects before season; $n = 195$ subjects after season). (B) Each symbol represents the average IgG reactivity (magnitude) against 194 GS peptides or 126 NGS peptides in individual plasma samples. Symbol color indicates the subject's age group. Lines and whiskers represent means and standard deviations, respectively. *P* values determined by a linear mixed model with Bonferroni-adjusted contrasts. ***, $P < 0.001$.

calculated average IgG reactivities by adding the positive IgG $\log_2$-transformed intensity values for GS and NGS proteins separately and dividing by the number of peptides in each group. We found that the average level of IgG reactivity to GS proteins was significantly lower than reactivity to NGS proteins before the malaria season (Fig. 1B; GS mean before: 0.726 [SD 0.439]; NGS mean before: 1.007 [SD 0.535]; $P < 0.001$) and after the malaria season (Fig. 1B; GS mean after: 0.914 [SD 0.468]; NGS mean after: 1.306 [SD 0.570]; $P < 0.001$). We then sought to determine whether the overall magnitude of IgG reactivity to GS and NGS proteins changed within individuals in response to the 6-month malaria season. We observed that the average IgG reactivity increased from before to after the malaria season for both GS proteins ($P < 0.001$) (Fig. 1B) and NGS proteins ($P < 0.001$) (Fig. 1B).

Smear-positive individuals showed significantly higher IgG reactivity than smear negative individuals before the malaria season, while reactivity to NGS peptides remained higher than reactivity to GS peptides before the malaria season among the infection stratified results (see Fig. S1B). Age-stratified analysis of the same data suggested that IgG reactivity was higher for NGS than GS proteins at all age groups, while IgG reactivity to GS and NGS proteins rose more consistently in young children than in older children or adults (see Fig. S2 in the supplemental material). All three age groups yielded significant boosting in the NGS proteins.

Together, these data suggest that the overall IgG response to GS proteins is boosted during the malaria season, particularly in children, and may be maintained by ongoing infection during the dry season, although in general the IgG responses to GS proteins appeared to be lower than the IgG responses to NGS proteins.

Malaria transmission boosts IgG specific for the TBV candidates Pfs48/45 and Pfs230 but not Pfs25. Although the overall GS IgG response clearly increased with malaria transmission, it remained possible that antigens constituting current TBV candidates do not elicit IgG responses in the context of natural *P. falciparum* infection. The protein microarray included peptides corresponding to the TBV candidates Pfs25, Pfs48/45, and Pfs230. Due to its large size, Pfs230 was represented on the microarray as four peptides (s1 to s4), the amino-terminal peptide of which (s1) contains the site reported to be a target for transmission-blocking antibodies (Fig. 2A) (41, 42). For each of these proteins/peptides, we determined the seroprevalence as well as average IgG reactivity before and after the 6-month malaria season.

We found that IgG reactivity specific for Pfs25 exceeded 2 SD above the negative control in only one (0.5%) sample before the malaria season and in five (2.6%) samples after the malaria season and, accordingly, there was no significant difference in the average $\log_2$ Pfs25-specific IgG reactivity between the two time points (Fig. 2B; mean before: −0.30 [SD 0.66]; mean after: −0.26 [SD 0.80]; FDR-adjusted $P = 0.63$). This result is consistent with the absence of Pfs25 expression in the parasite until it is taken up by the mosquito (14, 15). In contrast, IgG reactivity specific for Pfs48/45 exceeded 2 SD above the negative control in 107 samples (48%) before the malaria season and in 127 samples (65%) after the malaria season (see Table S3 in the supplemental material). Accordingly, the average $\log_2$ reactivity of Pfs48/45-specific IgG increased from before to after the malaria season (Fig. 2C; mean before: 1.16 [SD 1.12]; mean after: 1.53 [SD 1.17]; FDR-adjusted $P = 0.003$).

The seroprevalences of IgG specific for Pfs230 s1, s2, s3, and s4 before the malaria season were 45, 9, 18, and 31%, respectively (see Table S3 in the supplemental material), whereas the seroprevalences for the same peptides after the malaria season were 46, 13, 24, and 43%, respectively. Moreover, only the average reactivity of Pfs230 s3- and s4-specific IgG increased from before to after the malaria season (Fig. 2F; s1 mean before: 1.24 [SD 1.20]; s1 mean after: 1.37 [SD 1.11], FDR-adjusted $P = 0.35$; s2 mean before: 0.08 [SD 0.97], s2 mean after: 0.06 [SD 1.03], FDR-adjusted $P = 0.23$; s3 mean before: 0.31 [SD 1.03], s3 mean after: 0.54 [SD 1.00], FDR-adjusted $P = 0.04$; s4 mean before: 0.87 [SD 0.91], s4 mean after: 1.14 [SD 1.00], FDR-adjusted $P = 0.01$). Stratifying the samples by patient age, we find IgG responses to both Pfs48/45 and Pfs230 (see Fig. S3 in the supplemental material) are boosted (although not statistically significant) in young children (age 2 to 5) and older children (age 6 to 10) but not in adults (age 18 to 25). Collectively, these findings suggest that IgG responses to the TBV candidates Pfs48/45 and Pfs230 could be boosted through natural

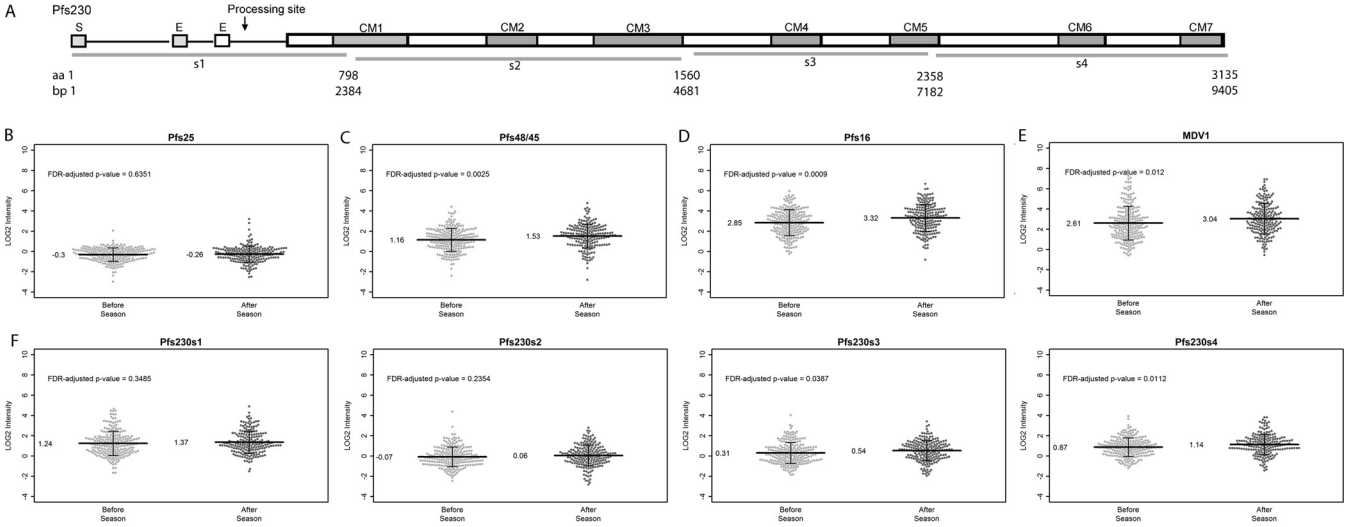

FIG 2 IgG reactivity to TBV candidates Pfs25, Pfs48/45 and Pfs230 before the malaria season. (A) Schematic of Pfs230 indicating the location of the peptides (s1 to s4) used on the microarray in relation to the secretory signal sequence (S), the glutamate repeat regions (E), the proteolytic processing site (arrow), and the seven 6-cysteine domains (CM1 to CM7). The amino acids (aa) and base pairs (bp) corresponding to the start and end of peptides s1 to s4 are indicated. (B to F) Symbols represent the level of IgG reactivity against Pfs25 (B), Pfs48/45 (C), Pfs16 (D), Pfmdv1 (E), and Pfs230 (F) s1 to s4 in individual plasma samples (n = 223 subjects before season; n = 195 subjects after season). Lines and whiskers represent means and standard deviations, respectively. FDR-adjusted P values determined by eBayes moderated t tests of 2,221 antigen fragments recognized in at least 20% of all samples.

P. falciparum infection, whereas boosting of Pfs25 vaccine-induced responses would not be expected.

Identifying candidate serologic markers of gametocyte exposure. Efforts to reduce the prevalence of gametocytes and thus malaria transmission would benefit from serologic markers that reflect gametocyte exposure (43–45). GS antigens with a high seroprevalence are likely to be sensitive indicators of gametocyte exposure and thus could be further evaluated as potential serologic markers. We defined the seroprevalence of each antigen as the percentage of plasma samples with IgG reactivity that exceeded 2 SD above the negative control. Figure 3 shows the distribution of seroprevalence for each of the GS and NGS antigens at the end of the dry season and after the malaria season. The average seroprevalence of GS proteins before the malaria season was significantly lower than the average seroprevalence of NGS proteins at the same time point (Fig. 3; GS mean before: 21.7% [SD 20.7%]; NGS mean before: 30.2% [SD 24.5%]; P = 0.01). Similarly, the average seroprevalence of GS proteins after the malaria season was significantly lower than the seroprevalence of NGS proteins at the same time point (Fig. 3; GS mean after: 30.0% [SD 26.7%]; NGS mean after: 41.2% [SD 29.8%], $P < 0.001$). We also found that the average seroprevalence of GS proteins increased from before to after the malaria season ($P < 0.0001$) (Fig. 3). Likewise, the average seroprevalence of NGS proteins increased from before to after the malaria season ($P < 0.0001$) (Fig. 3).

Next, we sought to identify the particular GS antigens that may

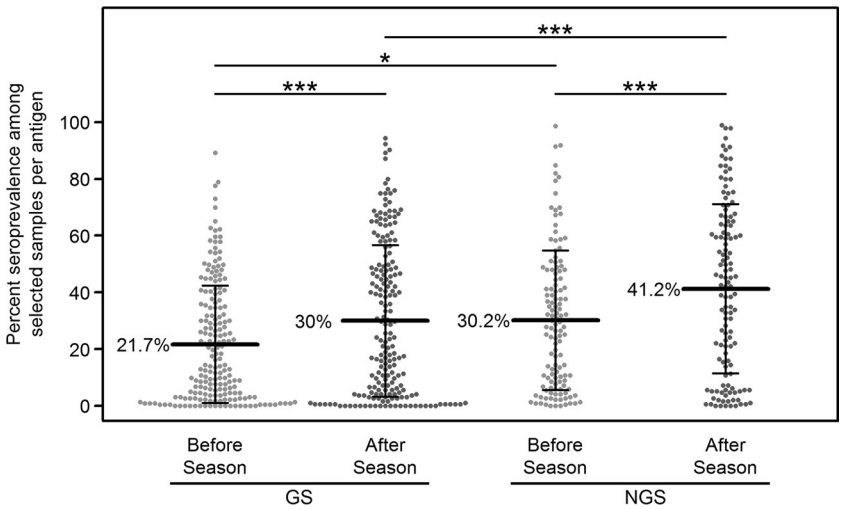

FIG 3 Seroprevalence of GS and NGS proteins before and after the malaria season. Each symbol represents an individual antigen (194 GS and 126 NGS). Lines and whiskers represent means and standard deviations, respectively. P values determined by a linear mixed model with Bonferroni-adjusted contrasts. *, $P < 0.05$; ***, $P < 0.001$.

be potential serologic markers of gametocyte exposure. Table S3 in the supplemental material lists GS proteins ranked by their seroprevalence at both time points. Among GS proteins, Pfs16 had the highest seroprevalence: 89% at the end of the dry season and 94% after the malaria season. Three other GS proteins—PF3D7_1346400 (e1s3 and e1s4; MAL13P1.234), Pfmdv1-e1s1 (PF3D7_1216500) (Fig. 3E), and PF3D7_1024800-e2s2 (PF10_0242)—had a seroprevalence of >70% at the end of the dry season and >87% after the malaria season. Not unexpectedly, two of these proteins—Pfs16 (Fig. 2D) and Pfmdv1 (Fig. 2E)—had the highest average IgG reactivity among GS proteins. Of note, 97% of plasma samples had detectable IgG reactivity to at least one of these four proteins at the end of the malaria season, suggesting that serology to these four proteins should be further tested to determine whether they could be used as sensitive markers of gametocyte exposure.

DISCUSSION

This study demonstrates that the breadth and intensity of IgG responses to a panel of GS proteins increase during the malaria season and also identifies four GS proteins/peptides—Pfs16, PF3D7_1346400 (e1s3 and e1s4), PF3D7_1024800 (e2s2), and PfMDV1(e1s1)—with high seroprevalence that may be sensitive markers of gametocyte exposure. It will be of interest in future studies to validate these preliminary findings with independent assays (e.g., ELISA) and in different malaria transmission settings. Prior seroepidemiological studies focused on a limited number of GS proteins (<5), which precludes analyses of IgG breadth, as well as the ranking of GS proteins by their relative immunogenicities (15, 17, 19, 20). By using a protein microarray containing peptides representing 1,204 unique proteins, 91 of which were GS proteins, including TBV candidates Pfs48/45, Pfs230, and Pfs25, we attained a less-biased view of the overall kinetics and quality of the IgG response to the sexual gametocyte stages that are responsible for transmission. In contrast to previous studies (19, 22, 26), we found that the IgG response to GS proteins increased from before to after the malaria season. However, both the breadth and the intensity of IgG responses to GS proteins were lower than responses to NGS proteins, possibly because GS proteins are intrinsically less immunogenic or because the ratio of circulating gametocytes to asexual parasites is low or a combination thereof. Despite the lower antibody responses to GS proteins, all but one study subject had antibodies that recognized at least one GS protein, suggesting widespread exposure to gametocytes in this population. This is consistent with studies that report a high prevalence of submicroscopic gametocytemia (46) and underscores the need for malaria transmission-blocking strategies with broad coverage of the community.

IgGs specific for the TBV candidates Pfs230 and Pfs48/45 were detected before and after the 6-month malaria season. The magnitude of this response, as well as the seroprevalence, was highest for Pfs48/45, followed by Pfs230 peptide s1, which corresponds to the amino-terminal 778 amino acids of Pfs230. Pfs230 peptide s1 contains glutamate repeat regions, as well as the new amino-terminal region that is generated after Pfs230 is proteolytically processed in the mosquito midgut (47). The new N-terminal region of Pfs230 is exposed on the surface of the gamete and is the target of transmission-blocking IgG (41, 42). Even after the 6-month dry season (a period of negligible malaria transmission), the seroprevalence of IgG specific for Pfs48/45 and Pfs230 peptide 1 exceeded 45%, a finding consistent with prior exposure to these TBV antigens and at least partial maintenance of IgG titers through the dry season. After the intense 6-month malaria season, the seroprevalence of IgG specific for Pfs48/45, as well as the Pfs230 peptides s3 and s4, increased significantly, suggesting that natural exposure to gametocytes would boost TBV-induced IgG. Interestingly, prior studies that assayed for competition with transmission blocking monoclonal antibodies (MAbs) that recognize Pfs48/45 or Pfs230 estimated a much lower seroprevalence and did not demonstrate IgG boosting to these antigens in response to the malaria transmission (19, 23).

In contrast to the IgG responses to Pfs48/45 and Pfs230 peptides observed in the present study we did not detect significant IgG reactivity to the TBV candidate Pfs25. This finding is consistent with Pfs25 protein expression being limited to the mosquito midgut (13, 48) and underscores the concern that Pfs25-specific IgG induced through vaccination would not be boosted by natural *P. falciparum* infection (15). Although we confirmed that full-length recombinant Pfs25 was expressed in the high-throughput transcription/translation system, it remains possible that our inability to detect Pfs25-specific IgG in this population was due to protein misfolding or polymorphisms that resulted in false negatives. Further work is needed to differentiate these possibilities.

Analysis of the IgG response to the 91 GS proteins on the protein microarray also identified four highly immunogenic proteins/peptides: Pfs16, PF3D7_1346400 (e1s3 and e1s4), PF3D7_1024800 (e2s2), and PfMDV1 (e1s1). Their combined seroprevalence exceeded 97% in the study population, suggesting that IgG to these proteins may serve as a sensitive marker of gametocyte exposure at the population level. The ideal serologic marker of gametocyte exposure would not only be sensitive (high seroprevalence in gametocyte-exposed population) but also specific (no cross-reactivity with nongametocyte antigens). However, in observational field studies of natural *P. falciparum* infection, such as the one reported here, it is not possible to study antibody responses to gametocytes in isolation without "contamination" from asexual stages. Ideally, this would be tested by experimental infection of human subjects with purified *P. falciparum* sexual versus asexual stages; however, a more practical alternative is to immunize animals with recombinant protein and assay the stage specificity of the serum. Pfs16 and Pfmdv1 have already been shown to induce gametocyte-specific antibodies in mice (49, 50), but the stage specificity of PF3D7_1024800 and PF3D7_1346400 proteins remains to be tested.

The characteristics that underlie the immunogenicity of these proteins is also not known, although the high expression levels of Pfs16, Pfmdv1, and PF3D7_1024800 (51) and the large size of PF3D7_1346400 (697,058 kDa) may play a role. The transcript levels of Pfs16, Pfmdv1, and PF3D7_1024800 are among the top 10% in midstage gametocytes (51) and contain predicted secretory signal sequences (www.plasmodb.org). Pfs16 and Pfmdv1 have been localized to the parasitophorous vacuole (PV) membrane and PV, respectively, while PF3D7_1024800 was detected in the membrane fraction of the gamete proteome (36). The subcellular location of PF3D7_1346400 has not yet been determined. It is possible that these proteins are readily recognized by the immune system when terminally differentiated gametocytes die and degrade in the human host.

In future studies it will also be of interest to determine whether the high seroprevalence of IgG specific for these four proteins is

generalizable to other areas of malaria endemicity, and if so, to systematically evaluate these proteins as potential serologic tools to monitor the efficacy of interventions targeting gametocytes. To be useful in this regard, the candidate markers would need to be sensitive to gametocyte exposure (high seroprevalence after gametocyte exposure) and then decline in the absence of ongoing gametocyte exposure (short-lived antibody response); therefore, future studies will need to track the kinetics of gametocyte-specific antibody responses longitudinally in the absence of ongoing gametocyte exposure (e.g., during dry season after gametocytocidal therapy or in emigrants from areas of malaria endemicity).

In summary, these findings indicate that IgG specific for GS proteins generally increases in response to *P. falciparum* transmission, suggesting that TBV-induced immunity could be boosted through natural gametocyte exposure. In addition, we observed a high seroprevalence of IgG specific for several gametocyte proteins that have the potential to be developed as surveillance tools to track gametocyte exposure in populations targeted for malaria elimination.

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