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# The Stability and Complexity of Antibody Responses to the Major Surface Antigen of *Plasmodium falciparum* Are Associated with Age in a Malaria Endemic Area\*<sup>§</sup>

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Individuals that are exposed to malaria eventually develop immunity to the disease with one possible mechanism being the gradual acquisition of antibodies to the range of parasite variant surface antigens in their local area. Major antibody targets include the large and highly polymorphic *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) family of proteins. Here, we use a protein microarray containing 123 recombinant PfEMP1-DBL $\alpha$  domains (VAR) from Papua New Guinea to seroprofile 38 nonimmune children (<4 years) and 29 hyperimmune adults ( $\geq 15$  years) from the same local area. The overall magnitude, prevalence and breadth of antibody response to VAR was limited at <2 years and 2–2.9 years, peaked at 3–4 years and decreased for adults compared with the oldest children. An increasing proportion of individuals recognized large numbers of VAR proteins (>20) with age, consistent with the breadth of response stabilizing with age. In addition, the antibody response was limited in uninfected children compared with infected children but was similar in adults irrespective of infection status. Anal-

ysis of the variant-specific response confirmed that the antibody signature expands with age and infection. This also revealed that the antibody signatures of the youngest children overlapped substantially, suggesting that they are exposed to the same subset of PfEMP1 variants. VAR proteins were either seroprevalent from early in life, (<3 years), from later in childhood ( $\geq 3$  years) or rarely recognized. Group 2 VAR proteins (Cys2/MFK-REY+) were serodominant in infants (<1-year-old) and all other sequence subgroups became more seroprevalent with age. The results confirm that the anti-PfEMP1-DBL $\alpha$  antibody responses increase in magnitude and prevalence with age and further demonstrate that they increase in stability and complexity. The protein microarray approach provides a unique platform to rapidly profile variant-specific antibodies to malaria and suggests novel insights into the acquisition of immunity to malaria. *Molecular & Cellular Proteomics* 10: 10.1074/mcp.M111.008326, 1–12, 2011.

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Malaria caused by infection with *Plasmodium falciparum* is responsible for over 500 million clinical cases and at least 1 million deaths each year, predominantly in children under five years of age (1). After repeated exposure, individuals living in endemic areas develop naturally acquired immunity to malaria, which manifests as an age-associated decline in the prevalence of severe, then mild clinical episodes (reviewed in (2, 3)). Antibodies are important mediators of this naturally acquired immunity as shown by experiments involving passive transfer of immune sera to nonimmune children (4–6). Antibody targets include variant surface antigens (VSA)<sup>1</sup> that are expressed on the surface of the infected erythrocyte (7, 8). Malaria-exposed adults have antibodies against a wide range

<sup>1</sup> The abbreviations used are: VSA, variant surface antigens; DBL, Duffy binding like; MFI, Mean Fluorescence Intensity; Pf, *P. falciparum*; PfEMP1, *Plasmodium falciparum* Erythrocyte Membrane Protein 1; PNG, Papua New Guinea; RTS, Rapid Translation System.

of parasite clones expressing distinct VSA whereas young children have antibodies against a small number of parasite clones (8–10). Consequently, naturally acquired immunity is thought to develop after exposure to the range of VSAs in the parasite population of an endemic area (8, 11).

The major VSA is the highly polymorphic *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1 (12, 13)), which is expressed on the surface of the infected erythrocyte (14, 15). One mechanism that parasites use to evade the host immune response is the switching of PfEMP1 variants through differential expression of ~60 distinct members of the *var* multi-gene family per genome (16–18). The slow development of naturally acquired immunity in endemic areas may be explained by the diversity found in the *var* genes (both within and among clones) with a few hundred to thousands of alleles predicted to circulate in endemic areas (19–22). PfEMP1 also mediates adhesion to molecules on the host vascular endothelium via domains named Duffy Binding Like (DBL) or Cysteine-Rich Interdomain. This sequesters infected erythrocytes in the peripheral vasculature to avoid being destroyed by the spleen (17). Adhesion of PfEMP1 to certain host receptors such as Complement Receptor 1 in the formation of rosettes (23) and Intercellular Adhesion Molecule 1 in cerebral malaria (24) is associated with symptoms of severe disease in children. Immunity against severe malaria develops after just a few infections (25) and is associated with antibodies against structurally and antigenically similar PfEMP1 variants (10, 26, 27). Parasites isolated from children with severe disease express relatively conserved subgroups of PfEMP1/*var* genes (e.g. group A and B/A) (21, 22, 28–33). These *var* gene subgroups are also expressed by parasites isolated from young children with limited anti-VSA antibody repertoires (34) and adults with no previous exposure to malaria (35). It is thought that a limited antibody response gives parasites that express relatively conserved and more efficiently binding variants the greatest growth advantage. Conversely, hosts with uncomplicated malaria and broad antibody responses harbor parasites that express more diverse variants (10, 21, 22, 28–30, 34). Recent evidence shows that this hierarchy of *var* gene expression is imprinted in the host antibody response, with antibodies against recombinant PfEMP1 domains from the *P. falciparum* reference strain, 3D7, showing a marked bias toward group A *var* genes in very young children (<1-year-old) compared with broader recognition of all subgroups by older children and adults (36, 37). Such PfEMP1 variants, if they could be isolated from natural parasite populations, may be ideal malaria vaccine candidates. However, the actual variants involved as well as the mechanisms underlying the acquisition of naturally acquired anti-PfEMP1 immunity are unclear.

Given the well-documented extreme diversity of PfEMP1 (19–21, 32, 38–40), we sought to develop a strategy to measure antibody responses to the large numbers of PfEMP1 variants present in natural parasite populations. We chose a protein microarray platform, which allows the parallel detec-

tion of antibodies to hundreds of proteins from small volumes of serum or plasma (<5  $\mu$ l). This methodology has previously been developed to measure antibody responses to entire pathogen proteomes (>3000 proteins) (41, 42) including *P. falciparum* (43–45). These studies demonstrated strong correlations between the antibody responses to immunodominant *P. falciparum* antigens as measured by traditional methods (e.g. ELISA) and by protein microarray (44, 46). Here, we describe the analysis of antibodies to PfEMP1 in Papua New Guinean (PNG) children and adults who are exposed to malaria, using a protein microarray fabricated with 123 recombinant PfEMP1-DBL $\alpha$  domains sampled from parasites infecting the same human population (20). By investigating antibody responses to large numbers of naturally circulating PfEMP1 variants in parallel, this study provides new and important insights into acquisition of immunity to malaria.

### EXPERIMENTAL PROCEDURES

**Ethics Statement**—Samples archived in a biobank at the PNG Institute of Medical Research were used in the study. The informed consent procedure for the initial study consisted of (1) the provision of general information about the aims of the study to the community through holding a village meeting known as a “tok save,” (2) information sessions on the study with parents of eligible children in their own household or hamlet, (3) formal meetings with village leaders to obtain their consent for their community to participate in the study. Except for the general information session (step (1)), where there was a complete translation from Melanesian pidgin into local language, all discussions, meetings and forms were held or written in pidgin with village reporters who could translate into the local language when necessary. The aims of the study were conveyed to the individuals in layman’s terms. Informed consent was given verbally on the basis of this explanation during enrolment for the survey, as this was the ethical expectation at the time (c.1999). All consenting members of selected populations were eligible for enrolment into the community surveys. People with concurrent or chronic illness that might impede their participation in the surveys were excluded. Enrolment in the study, possible only if consent was given, was recorded in a database. The use of these archived samples for the current study was approved by the PNG Institute of Medical Research Institutional Review Board (IRB) and the Medical Research Advisory Committee of PNG; the Alfred Research and Ethics Unit, Melbourne, Australia, and the University of California Irvine IRB, USA.

**Study Population, Parasites and Serum Samples**—The study was conducted in the local region of Amele, Madang Province on the north coast of PNG and included eight villages distributed over a 10 km<sup>2</sup> area. In this region, intense transmission of *P. falciparum* malaria occurs year round (47) with inoculation rates ranging from 68–526 infective bites per person per year (48). Blood samples were obtained from asymptomatic volunteers of all ages in November to December of 1999, and separated into plasma (stored at –80°C), buffy coat, and erythrocyte fractions by centrifugation (both stored in guanidine hydrochloride at 4°C) (49). *P. falciparum* infection was diagnosed by microscopy. Samples from 30 *P. falciparum* positive children (6 months to 11 years) were subject to genomic DNA extraction and used for *var* gene sequencing (see below, (20)). Plasma samples from 40 children (5 months to 4 years) and 40 adults ( $\geq$ 15 years) were selected for anti-PfEMP1 antibody screening by protein microarray. Plasma was also obtained from 10 adults residing in the USA with unknown exposure to *P. falciparum* and were used as naïve (background) controls.

**Var gene sampling**—Var gene sequencing and population genetic analysis were done as described in detail elsewhere (20). Briefly, DBL $\alpha$  domains were amplified from *P. falciparum* genomic DNA using degenerate primers specific for conserved amino acid blocks B or D (forward primer) and H (reverse primer). PCR products were then cloned and sequenced. Aligning all sequences with  $\geq 96\%$  DNA sequence identity identified distinct sequence types. Among the 30 *P. falciparum* isolates from Amele that were analyzed, a total of 185 unique DBL $\alpha$  sequence types were identified with isolates sharing  $\sim 7\%$  of their var genes (20). A total of 128 of these unique DBL $\alpha$  types were selected for inclusion on the protein microarray.

**Microarray Production**—Cloned DBL $\alpha$  sequence types (20) were used as templates for homologous recombination using sequence-specific primers incorporating an N-terminal HIS or C-terminal-HA tag. Products were expressed in an *E. coli in vitro* cell-free transcription/translation system (rapid translation system (RTS); Roche, Indianapolis, IN), and printed onto microarray chips as previously described (41, 45).

For quality control, each of the RTS reactions was spotted on nitrocellulose, air dried, blocked with 5% nonfat milk powder in Tris-buffered saline containing 0.05% Tween-20, stained with mouse anti-polyHIS mAb (clone HIS-1; Sigma) and rat anti-HA mAb (clone 3F10; Roche) and detected with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Bio-Rad) or goat anti-rat IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) secondary antibodies respectively. Blots were visualized with nitroblue tetrazolium (NBT) developer according to the manufacturer's instructions (Thermo Fisher Scientific). The presence of protein was confirmed for 123 of the 128 PfEMP1-DBL $\alpha$  domains (VAR proteins) included on the microarray. The following controls were also included: (1) three "no DNA" negative control RTS reactions with an empty plasmid vector to provide a baseline signal for fluorescence readout as a measure on nonmalaria induced reactivity; (2) serially diluted human IgG as a positive control to confirm reactivity of secondary antibodies and account for potentially nonviable hybridization steps (secondary and tertiary antibody binding, washing, etc.); and (3) serially diluted Epstein-Barr Virus nuclear antigen-1 (EBNA1) as a methodological control given the high prevalence of latent Epstein-Barr Virus infection in human populations but which vary in reactivity between subjects (supplemental Fig. S1).

**Serological Screening**—Human plasma samples were pre-absorbed against *E. coli*-lysate at 1:100 dilution in blocking buffer (to block anti-*E. coli* antibodies which could potentially mask protein-specific responses when using whole RTS reactions), for 1 h at room temperature as described previously (41, 45). Slides were rehydrated in blocking buffer for at least 30 min at room temperature before diluted plasma was added to each pad and incubated overnight at 4 °C with a gentle constant speed on a platform rocker. Bound human IgG was detected with biotin-conjugated goat anti-human IgG secondary antibody (1:1000 dilution; Jackson ImmunoResearch) for 1 h at room temperature with a gentle constant speed (and visualized with streptavidin P-3-conjugated secondary antibodies (1:200 dilution, Columbia Biosciences), then air-dried. Slides were scanned in a ScanArray 4000 laser confocal scanner (GSI Lumonics, Billerica, MA; or similar). Spot-specific background calculated from a region surrounding the spot was subtracted and fluorescence intensities of each spot quantified using the Scanarray Express version 4 (Perkin Elmer).

**Data Analysis**—Raw signal intensities were adjusted using a one-parameter normalization based on the negative (no DNA) controls. Because distinct groups were being compared, these signals were transformed using the *vsn* method (*asinh* transformation, similar to log for higher intensities) (50) which has been shown to effectively calibrate array measurements through shifting and scaling and also to stabilize the variance in DNA microarray and two-dimensional gel

electrophoresis (51) data. From the transformed signal intensities, we calculated the mean and standard deviation (SD) per VAR protein per age and/or infection category for the PNG samples and for the US adults (naive controls). To obtain the background-corrected mean fluorescence intensity (MFI) per VAR protein, these values were reverse-transformed using the formula:

$$\sinh(x) - \sinh(\text{meancontrols})/SD\text{controls} \quad (\text{Eq. 1})$$

Where  $x$  = the mean signal per VAR protein for a group of PNG samples, and *controls* = naïve control samples. Negative values were converted to zero to avoid artificial deflation of mean signal. To obtain the background corrected signals for individual PNG samples the normalized signal intensities were reverse-transformed using the formula:

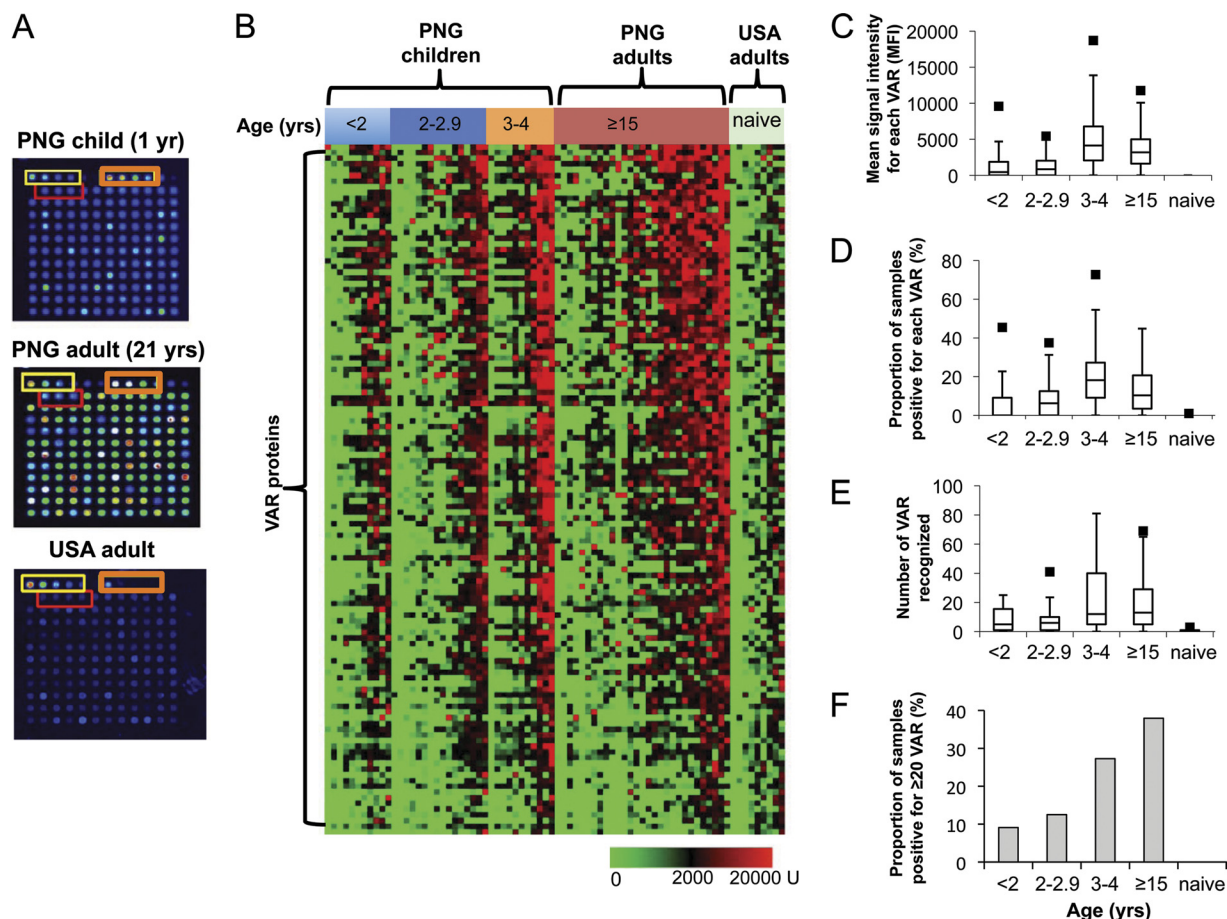
$$\sinh(y) - \sinh(\text{meancontrols})/SD\text{controls} \quad (\text{Eq. 2})$$

Where  $y$  = the signal per VAR protein for a given sample and again negative values were converted to zero. A seropositive result was defined as  $\geq 2$  SDs above the MFI for controls thus indicating a significant antibody titer.

To quantify the anti-PfEMP1 antibody response the normalized and background corrected data was summarized into three different outcomes: the *magnitude* of response, determined by calculating the mean fluorescence intensity (MFI) per VAR protein; the *seroprevalence*, determined by calculating the proportion of donors seropositive for each VAR protein; and the *breadth* of response, determined by counting the number of seropositive VAR proteins per donor. All response outcomes were treated as numerical with an asymmetric distribution. The associations between these anti-PfEMP1 response outcomes and continuous variables were assessed by Spearman's Rho ( $\rho$ ), and between categorical variables by Chi Squared or Fishers exact test where appropriate. To investigate the association of anti-PfEMP1 response outcomes with age, individuals were categorized into four groups (<2, 2–2.9, 3–4, and  $\geq 15$ -year-old) according to epidemiological patterns of naturally acquired immunity (52, 53). For the analysis of age together with parasitemia, the <2-year-old and 2–2.9-year-old children were combined and 3–4-year-old children were not analyzed because of limited sample size. Differences in anti-PfEMP1 response outcomes between pairs of categories (e.g. age group or infection status) over all VAR proteins were assessed by Mann Whitney *U* test and for individual VAR proteins using a Bayes regularized *t* test adapted from Cyber-T for protein arrays (54, 55), which has been shown to be more effective than other differential expression techniques (56). To investigate the antibody response to six clinically relevant DBL $\alpha$  subgroups as defined by Bull and colleagues (21, 57), the afore-mentioned analysis was stratified by subgroup. For this analysis, the <2-year-old children were divided further into <1-year-old and 1–1.9-year-old categories to capture early responses (36, 37). Statistical analysis was performed using SPSS version 17 and R 2.10 (www.r-project.org).

## RESULTS

**Profiling the Anti-PfEMP1 Antibody Repertoire with Protein Microarrays**—Protein microarrays fabricated with 128 recombinant PfEMP1-DBL $\alpha$  domains (VAR proteins) of which 123 were successfully expressed, were used to measure antibody reactivity in human plasma samples (supplemental Fig. S1, supplemental Table S1). Antibody profiling by VAR protein microarray was highly reproducible as indicated by a significant correlation between 12 experiments conducted in duplicate ( $R^2 = 0.92$ ,  $p = 1.2 \times 10^{-6}$ , supplemental Fig. S2).



**FIG. 1. Measurement of the acquisition of anti-PfEMP1 antibodies with age by VAR protein microarray.** *A*, Microarray images showing anti-PfEMP1 antibody response profiles of a nonimmune child, a naturally immune adult, and a naive US adult. Protein microarrays fabricated with 123 VAR proteins (from 30 PNG field isolates, (20)) were probed with plasma and scanned for fluorescence activity. Positive controls are on the *top* row and include 0.03, 0.01, 0.003, 0.001, and 0.0003  $\mu\text{g}/\mu\text{l}$  of human IgG (*yellow* box on the left) or 1, 0.3, 0.1, 0.03, and 0.01  $\mu\text{g}/\mu\text{l}$  of EBNA1 (*orange* box on the right). Negative controls are on the second row and include three “no DNA” spots (*red* box). The remaining spots on the *top* row are PBS buffer (no box). The remaining spots on rows 2–11 are VAR proteins (see [supplemental Fig. S1](#)). The VAR protein-specific antibody titer is indicated by the signal intensity with reference to positive and negative controls *i.e.* white (strongest) > red > green > blue (weakest, no reactivity). *B*, Heatmap showing the anti-PfEMP1 antibody response profiles of 38 non- or semi-immune children (aged <2, 2–2.9 or 3–4 years) and 29 naturally immune adults (aged  $\geq 15$  years) all resident in Amele, Madang Province, PNG, and 9 nonimmune USA adults (naïve controls). Each row shows the responses to a single VAR protein (listed from *top* to *bottom* in [supplemental Table S2](#)), and each column showing the responses of an individual (plasma sample) to each of these VAR proteins. Signal intensity (fluorescence units, U) is indicated by the key below the heatmap. *C–E*, Box and whisker plots for the three parameters of the anti-PfEMP1 response were plotted for each of the age groups and naïve controls. Boxes show the interquartile range, midline the median, whiskers the 95% confidence interval, black dots the outliers (95–99%). Pairwise values calculated using the Mann Whitney test are presented in the manuscript text. *F*, The proportion of individuals (%) that recognized 20 or more VAR proteins was plotted for each of the age groups and naïve controls.

Correlated antibody responses were also observed for highly similar VAR proteins ([supplemental Text S1](#), [supplemental Fig. S3](#)) demonstrating the reliability of the *in vitro* expression system. Plasma from a total of 90 individuals including 40 children (5 months to 4 years old) and 40 adults ( $\geq 15$  years) resident in Amele, Madang Province, PNG, and 10 USA adults (naïve controls) were screened with the VAR protein microarrays. High quality anti-PfEMP1 antibody profiles were obtained for 38 PNG children and 29 PNG adults and nine USA adults ([supplemental Text S1](#)). Fluorescent images for each microarray illustrated the variable recognition of the 123 VAR by individual samples (Fig. 1A,

[supplemental Fig. S1A, B](#)). A heatmap of the normalized and background subtracted signals for 123 VAR summarizes the results (Fig. 1B; numerical data can be found in [supplemental Table S1](#)). The heatmap depicts a high degree of diversity among antibody profiles of the different individuals and with age, with high levels of recognition for some variants. There was a gradient of reactivity across the population with some individuals displaying stronger signals and broader recognition of PfEMP1 variants than others, including some children with stronger responses than some of the low-responding adults.

*Anti-PfEMP1 Antibodies Are Associated with Age*—If naturally acquired immunity to *P. falciparum* is the result of a

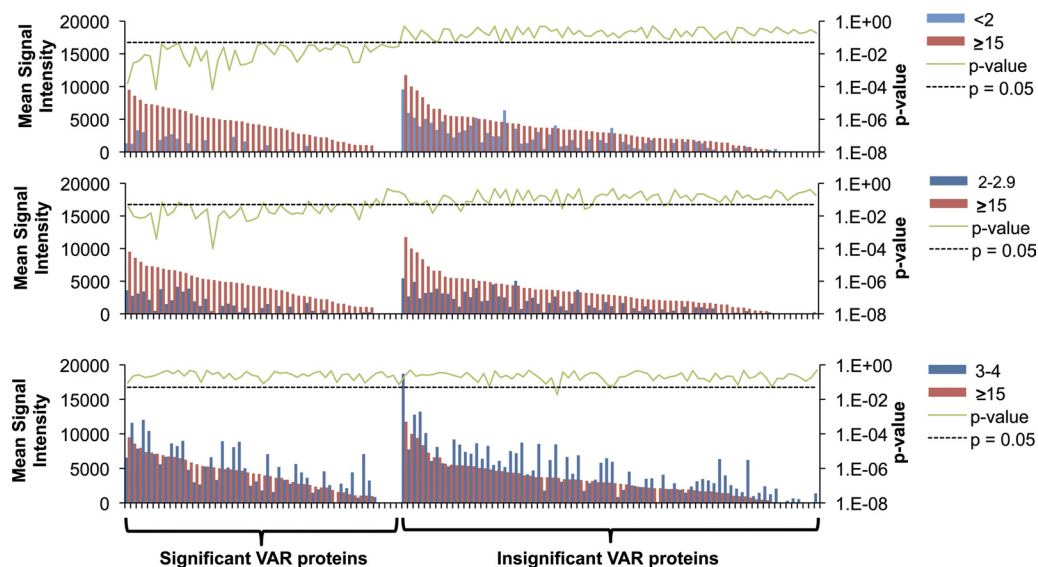


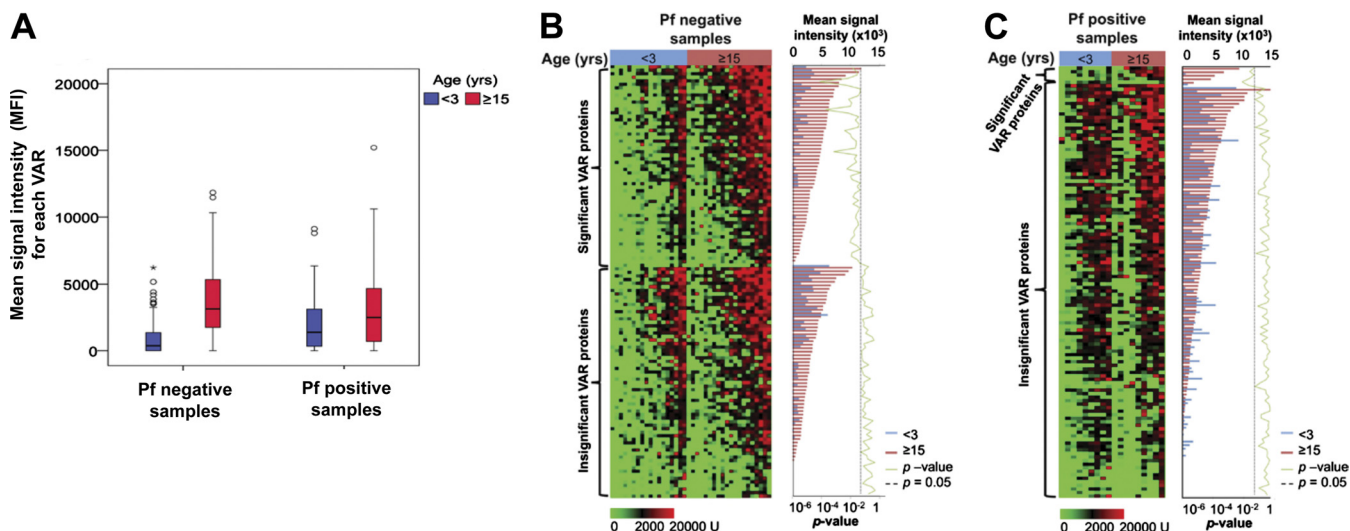
FIG. 2. Acquisition of antibodies to 123 distinct PfEMP1 variants with age. Mean antibody signals (MFI) were compared among children of different age groups ( $<2$  years,  $n = 11$ ; 2–2.9 years,  $n = 16$  or 3–4 years,  $n = 11$ ) and adults ( $\geq 15$  years,  $n = 29$ ) and associated  $p$  values obtained as outlined in the Materials and Methods. The horizontal dashed line indicates the cutoff for significance ( $p \leq 0.05$ ) and insignificant ( $p > 0.05$ ) VAR proteins for the children  $<2$  years comparison to adults are indicated by the horizontal brackets. Supplemental Table S2 contains the list of the VAR proteins from left to right.

cumulative acquisition of antibodies to the range of PfEMP1 variants within the parasite population, we would expect the magnitude, seroprevalence and the breadth of response to increase with age. Figs. 1C–1E show the association of these response outcomes with age group, which were defined on the basis of epidemiological patterns of naturally acquired immunity (children:  $<2$  years (years),  $n = 11$ ; 2–2.9 years,  $n = 16$ ; 3–4 years,  $n = 11$ ; adults:  $\geq 15$  years,  $n = 29$ ) (53). For all parameters, the USA adults had a negligible antibody response in comparison to the *P. falciparum*-exposed children and adults ( $p < 0.0001$ , Figs. 1C–1D). For the PNG children, there was no difference in the median magnitude (MFI, Fig. 1C), median seroprevalence (Fig. 1D) or median breadth (Fig. 1E) between the  $<2$  and 2–2.9-year-old children ( $p = 0.353$ ,  $p = 0.731$  and  $p = 0.941$  respectively), but this was followed by a sharp increase in the 3–4-year-old children. The increase was significant for the magnitude and seroprevalence (Figs. 1C, 1D;  $p < 0.0001$  for 3–4 year compared with  $<2$  year and 2–2.9 years) but not breadth (Fig. 1E;  $p = 0.177$  and 0.134 respectively) as a result of the smaller sample numbers (*i.e.* one measurement per sample *versus* 123 measurements per serum sample for the magnitude and seroprevalence). The PNG adults had significantly lower median magnitude and median seroprevalence than the 3–4-year-old children ( $p = 0.007$  and  $p = 0.012$  respectively), but these response outcomes remained significantly higher than that of the two youngest age groups ( $p < 0.0001$ ; Figs. 1C, 1D). There was some evidence that the median breadth of response by the adults (median = 13) was higher than the youngest age groups (Fig. 1E;  $<2$  years, median = 5;  $p = 0.07$ ; median = 6, 2–2.9 years,  $p = 0.07$ ) but it was only slightly and insignifi-

cantly higher than that of 3–4-year-old children (median = 12;  $p = 0.67$ ). However, adults had a narrower distribution of breadth values compared with the 3–4-year-old children (Fig. 1E: inspect IQR and error bars) and the proportion seropositive for at least 20 variants increased with age (Fig. 1F) suggesting that responses to a broad range of PfEMP1 stabilize with age.

Variant-specific antibody signatures were then defined by comparing the MFI per VAR protein between age groups. This analysis demonstrated that adults had significantly higher levels of antibody than  $<2$ -year-old children and 2–2.9-year-old children for 49 (Fig. 2A) and 48 (Fig. 2B) VAR proteins respectively and similar levels of antibody for the remaining VAR proteins. In addition, the antibody signatures of the two youngest age groups overlapped substantially with significantly higher MFI than adults for the same 41 VAR proteins (84% of “significant VAR proteins,” Fig. 2) and no difference in MFI for the same 70 VAR proteins (94.6% of the “insignificant VAR proteins”). This is consistent with the two youngest age groups being exposed to the same restricted subset of PfEMP1 variants. The 3–4-year-old PNG children showed equivalent responses to that of adults for all VAR proteins (Fig. 2) indicating that by this age, the antibody repertoire had expanded to the same extent as that of an adult. Significant VAR proteins included four encoded by group A *var* genes (*var1607*, 1749, 70, 22), one encoded by a *var1csa*-like gene (*var1671*) and two encoded by high frequency *var* genes (*var6*, 33%; *var22*, 27%; supplemental Table S1).

The anti-VSA response (*i.e.* the antibody signal to the whole surface of blood stage parasites) is considered a measure of exposure to *P. falciparum* (58). Anti-VSA antibody responses



**FIG. 3. Association of antibodies to 123 distinct PfEMP1 variants with age and infection status.** Antibody responses for each of 123 VAR proteins were analyzed by infection status, as defined by light microscopy, and age group. Children aged <3 years were combined and compared with adults  $\geq 15$  years. **A**, Boxplots showing the distribution of mean signal intensity (MFI) for 123 VAR proteins for each age and infection subgroup. Boxes show the interquartile range, midline the median, whiskers the 95% confidence interval and open circles the outliers (95–99%). The median MFI's were compared and  $p$  values obtained by Mann Whitney test and are presented in the main text. Results for individual VAR proteins for **(B)** *P. falciparum* (Pf) negative samples and **(C)** Pf positive samples are shown as heatmaps (*left*) and plots of MFI (*right*) per age group and corresponding  $p$  values determined as outlined in the Materials and Methods. The horizontal dashed line indicates the cutoff for significance ( $p \leq 0.05$ ). Significant ( $p \leq 0.05$ ) and insignificant ( $p > 0.05$ ) VAR proteins for the comparison between children and adults are indicated by the vertical brackets. [Supplemental Table S2](#) contains a list of VAR proteins from top to bottom.

were previously defined by flow cytometry for 550 Amele residents participating in the same survey as those included in the current study (58). This anti-VSA data was reanalyzed in the same age categories as above in addition to age groups between 4 and 15 years. PNG children aged 1–2, 3–4, and 5–6 years have uniformly low anti-VSA signals ( $p > 0.11$ ); however, high anti-VSA signals were noted for a small number of 3–4 and 5–6-year-old children ([supplemental Fig. S4A](#)). From 5–6 to 7–9-year-old, the anti-VSA signal increased significantly with age into adulthood ( $p < 0.05$ , [supplemental Fig. S4A](#)). Taken together, these data suggest that the anti-PfEMP1 response is similar to the anti-VSA response in early childhood, but that there is a decrease in the anti-PfEMP1 response in adults despite an increase in anti-VSA responses and presumably, exposure to *P. falciparum*.

**Anti-PfEMP1 Antibodies Are Associated with *P. falciparum* Infection**—To determine whether active parasitemia influences the age-dependence of the anti-PfEMP1 antibody response, the *P. falciparum* infection status of samples, as assessed by light microscopy, was included as a variable in the analysis. Because of the small sample sizes in each of the previously defined age groups, only two age groups could be meaningfully compared: children <3 years (*P. falciparum* (Pf) negative,  $n = 18$ ; Pf positive,  $n = 9$ ) and adults  $\geq 15$  years (Pf negative,  $n = 20$ ; Pf positive,  $n = 9$ ). For uninfected individuals, the overall magnitude of the anti-PfEMP1 response (median MFI) was strongly associated with age ( $p < 0.0001$ ). In infected individuals, the median MFI was associated with age, although to a lesser extent than in uninfected individuals ( $p =$

0.003, Fig. 3A). There was also a highly significant increase in median MFI in association with *P. falciparum* infection in children ( $p < 0.0001$ ) but it decreased by a minor and probably biologically insignificant extent with infection in adults ( $p = 0.03$ , Fig. 3A). The variant-specific antibody signature was also influenced by active parasitemia with striking differences between uninfected and infected individuals (Figs. 3B, 3C). For uninfected individuals, MFI was significantly associated with age for 57 VAR proteins (46.3%) and there was a similar trend for the majority of nonsignificant VAR (Fig. 3B). For infected individuals, MFI was significantly associated with age for only 6 VAR proteins (4.9%) and MFI was similar between age groups for the majority of nonsignificant VAR (Fig. 3C). Therefore, the age-dependence of the antibody response is more pronounced for uninfected than infected hosts.

A similar effect was observed when anti-VSA data was stratified by infection status ([supplemental Fig. S4B](#)). For uninfected individuals, anti-VSA antibodies were highly significantly associated with age (3–4 years compared with 15–30 years,  $p < 0.0001$ ). For infected individuals, a significant association with age was also observed but it was much weaker ( $p = 0.009$ ). The anti-VSA response was significantly associated with infection in children aged from 1–2 years to 10–11 years ( $p < 0.05$ ) but was not in early adolescents (12–14-year-old,  $p = 0.832$ ) and adults (15–30-year-old,  $p = 0.739$ ).

**Age-specific Seroprevalence Patterns of 123 PfEMP1 Variants**—Because age-specific patterns of recognition of individual VAR proteins might reveal insights into hierarchical pat-

## DISCUSSION

terms of PfEMP1 exposure, we investigated the VAR-specific seroprevalence with age. Small sample sizes prevented the inclusion of *P. falciparum* infection as a variable in this analysis. Of the 123 variants evaluated, var1886 (supplemental Table S1) was the most seroprevalent from a young age and into adulthood, being recognized by 45.5% (<2 years), 37.5% (2–2.9 years), 73% (3–4 years) and 45% ( $\geq 15$  years) of individuals. The majority of VAR were recognized more frequently by the 3–4-year-old children than adults (Fig. 4). Cluster analysis revealed three distinct patterns with age including (1) 25 VAR (20.3%) that were seroprevalent from <3-year-old and maintained into adulthood (early); (2) 29 VAR (23.5%) that were seroprevalent only in 3–4-year-old and/or  $\geq 15$ -year-old (late) and (3) 69 VAR (56.1%) rarely recognized by all age groups (rare, Fig. 4). Of the “early” variants, several were highly seroprevalent (>36%) in the youngest children (e.g. var51, 77, 28, 1886, 1747, 26). Of the “late” variants, some remained seroprevalent until adulthood (e.g. 48, 74, 6, 1742, 134) whereas others became less seroprevalent by adulthood (e.g. var203, 1999, 1616, 1735, 218) (Fig. 4). There was no difference in the distribution of six DBL $\alpha$  subgroups (see below) in the early and late clusters ( $p = 0.95$ ) nor did inspection of the sequence alignments reveal any motifs or characteristics that differed between the primary amino acid sequences of these clusters (supplemental Fig. S5).

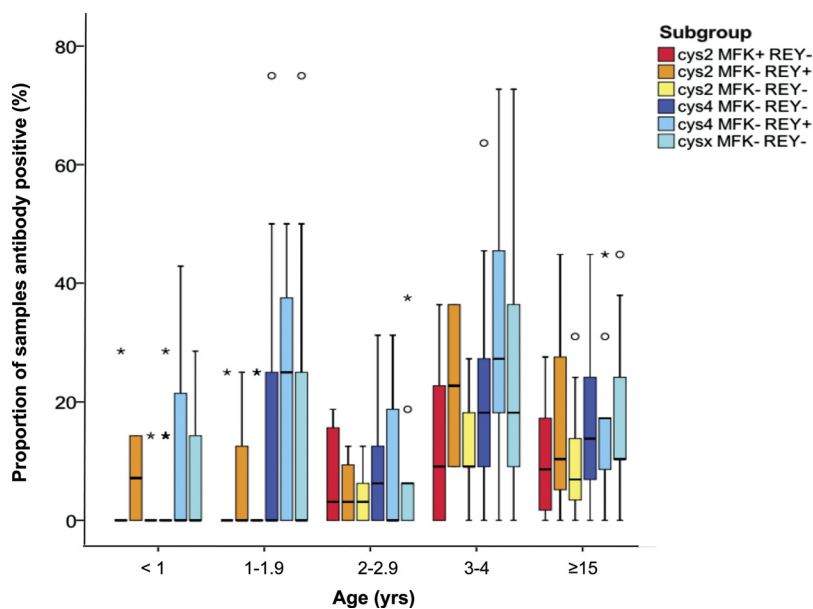
**Age-specific Seroprevalence Patterns of Six DBL $\alpha$  Subgroups**—The DBL $\alpha$  domains of group A var genes expressed by parasites infecting young children and that cause severe disease contain either cys2/MFK+REY– (group 1), cys2/MFK–REY+ (group 2) or cys2/MFK–REY– (group 3) signatures whereas group B and C var genes that are often associated with mild and asymptomatic illness tend to have DBL $\alpha$  domains with cys4/MFK–REY– (group 4), cys4/MFK–REY+ (group 5) and cysX/MFK–REY– (group 6) signatures (21, 57). To further investigate a possible hierarchy in the antibody response, we stratified the age-specific seroprevalence data by the six DBL $\alpha$  subgroups. In endemic areas, many children receive their first few infections before the age of 1-year-old, so we divided the <2-year-old into < 1-year-old ( $n = 7$ ) and 1–1.9-year-old ( $n = 4$ ) age groups. Again, small sample sizes prevented the inclusion of *P. falciparum* infection as a variable in this analysis. For the <1-year-old children, cys2/MFK–REY+ (group 2) variants were serodominant, followed by cys4/MFK–REY+ (group 5) and the default group, cysX/MFK–REY– (group 6) whereas the other three subgroups were rarely recognized (Fig. 5). During childhood the median seroprevalence for each subgroup increased with age, with frequent recognition of all subgroups achieved only after the age of 2 years. By adulthood, the median seroprevalence to all subgroups had decreased relative to the 3–4-year-old children, but remained higher than that of age groups <3 years. This suggests that antibodies to DBL $\alpha$  group 2 sequences are acquired first in a hierarchy of antibody acquisition, at least in the malaria endemic region of Amele, PNG.

PfEMP1 is a dominant target of variant-specific antibodies implicated in naturally acquired immunity to malaria (9, 59). Approaches that can measure antibodies to large numbers of well-characterized proteins are urgently needed to identify key variants among the thousands circulating in the parasite populations of malaria endemic areas (60). To date, the ability to do large-scale immuno-epidemiological surveys has been limited by the technology available. Researchers have determined antibody reactivity to PfEMP1 using whole parasites (infected erythrocytes) or purified recombinant PfEMP1 domains using a variety of approaches including agglutination assays, ELISA analysis and flow cytometry (e.g. (10, 26, 61–64)). Whole parasite approaches are confounded by the presence of other VSA molecules such as Rifin (65), Stevor (66) and Surfin (67) and all of the techniques used only measure antibodies to one variant at a time. Recent development of a high throughput BioPlex assay to 100 well-characterized recombinant proteins per experiment has increased throughput but the use of variants from an established laboratory clone (3D7 (37, 68)) may not recall antibody response patterns as well as variants from the infecting parasite population. Here we have combined high throughput sampling of var gene sequences from *P. falciparum* field isolates with a protein microarray approach to measure antibody responses to PfEMP1. Included on the microarray were 123 recombinant PfEMP1-DBL $\alpha$  domains (VAR proteins) representing  $\sim 40\%$  of the variants found in the Amele region of PNG (20). We applied this approach to concurrently sampled plasma from children who are in a state of active acquisition of clinical immunity to malaria and adults who are immune to malaria. The results revealed important features of anti-PfEMP1 antibody response patterns with age.

The data show that the anti-PfEMP1 antibody response of PNG children is limited, and directed toward specific variants until the age of 3 years after which it intensifies significantly with recognition of a broader range of variants by 3–4 years of age (>4-year-old children were not tested). By adulthood ( $\geq 15$ -year-old), although the overall anti-PfEMP1 antibody response diminishes in magnitude and seroprevalence when compared with the 3–4-year-old children it was significantly higher and more complex than that of the <3-year-old children and the magnitude of response to individual variants was similar in the 3–4-year-old children and adults. With age, there was an increase in the proportion of PNG residents who recognized at least 20 variants on the microarray. These results demonstrate that PNG children under the age of 4 years are in an active state of antibody acquisition. Each year of *P. falciparum* exposure in childhood magnifies and expands the antibody repertoire and by adulthood responses are relatively stable and complex. The data also imply that there is a peak in the anti-PfEMP1 response sometime between the ages of 4 and 15 years.



**FIG. 5. Age-specific seroprevalence patterns of six PfEMP1-DBL $\alpha$  subgroups.** The age-specific seroprevalence values for each of 123 VAR proteins were stratified by the six DBL $\alpha$  sequence subgroups, which were defined according to the classification by Bull and colleagues (21, 57) where sequences were subgrouped by the number of cysteine residues (cys2, cys4, cysx) and presence (+) or absence (-) of MFK or REY motifs. Each subgroup is represented by a different colored box and whisker plot (see key). Boxes show the interquartile range, midline the median, whiskers the 95% confidence interval, open circles the outliers (95–99%) and asterisks the extreme values (99–100%).



The pattern observed resembles that reported in other studies in PNG where the antibody response to VSA on infected erythrocytes was limited in young children, and increased with age (58) (supplemental Fig. S4A). However, all reports of the naturally acquired antibody response to recombinant PfEMP1 domains have been restricted to malaria endemic regions of Africa (36, 37, 61, 64). In the highest transmission areas of Africa (infection prevalence >90%), the overall anti-PfEMP1-DBL $\alpha$  antibody response is limited until 2–3 years, peaks at 5–7 years and decreases slightly with age after that. However, in moderate transmission villages (infection prevalence ~50%) the antibody response is limited until 5–7 years and continues to increase into adulthood (37). The transmission of *P. falciparum* in the Amele region of PNG is moderate in comparison with an infection prevalence of 37.8% (49) and the age-specific patterns of clinical immunity are similar to that of African communities (3, 52, 53); thus, in Amele, the anti-PfEMP1-DBL $\alpha$  antibody peak would be expected to occur in adulthood but we observed a decline in antibodies after the age of 15 years in comparison to the 3–4-year-old children. Several explanations for this pattern are possible. First, Amele *var* genes are less diverse than those from Africa and thus broad recognition might be achieved more rapidly (60). Second, there is evidence that the age of peak anti-PfEMP1 response increases with the length of time between the collection of parasite isolates and serum (62). In the African studies, variants isolated from the 3D7 clone, which is of unknown origin and was collected in the 1980s, were used to measure antibodies in samples collected 20 years later (37), whereas the PNG parasites and plasma were collected concurrently. Third, the prevalence of infection in 3–4 - children is higher than in adults (62, 69) and therefore antibody responses in children may be more frequently boosted to currently circulating variants whereas the adult

antibody profile may also comprise antibodies to variants experienced during childhood that may not be represented on the microarray. Finally, protection against clinical malaria is associated with antibody responses against parasite antigens exposed on the surface of merozoites or released from apical organelles at invasion (reviewed by (70)). T-cell responses are also thought to play a role in naturally acquired immunity to malaria (2). In adulthood, these other responses may become more important in the maintenance of clinical immunity than responses to a diverse repertoire of PfEMP1 variants.

The anti-PfEMP1 antibody response of children was more pronounced in the presence of *P. falciparum* parasites. This suggests that *P. falciparum* infection activates the anti-PfEMP1 antibody response in young children and that it is not maintained in the interval between infections, whereas adults have high levels of antibody irrespective of their infection status. These data confirm that the stability of the anti-PfEMP1 antibody response increases with age and this in addition to antigenic diversity, may contribute to the slow development of immunity to malaria. One caveat of our findings is that the sample size for responses to individual VAR proteins was smaller than that needed to obtain significant *p* values; nonetheless, a clear trend was seen (Fig. 3). Similar associations with parasitemia have been observed for responses to other blood-stage antigens including AMA1, MSP1–19 and MSP2 (71). Children's antibody responses are also less stable than those of adults between transmission seasons (44). To our knowledge, this is the first study to investigate the influence of concurrent *P. falciparum* infection on the anti-PfEMP1 response.

Other studies have suggested that young children with a limited antibody response favor the growth of parasites expressing relatively conserved and thus serodominant PfEMP1 variants whereas hosts with a broad antibody repertoire favor

the growth of parasites expressing more diverse variants (21, 34, 36, 37, 72, 73). In the current study, the antibody signatures of the two youngest age groups overlapped almost completely (84% of significant VAR proteins and 94.6% of insignificant VAR proteins) suggesting that children <3-years old are exposed to a restricted subset of PfEMP1 variants. When the variant-specific seroprevalence was measured, three distinct age-specific patterns emerged with 25 variants recognized frequently from an early age (<3 years), 29 recognized later (>3 years) and 69 recognized rarely by all age groups. Although some variants in the latter cluster may be recently introduced into the parasite population, the early *versus* late recognition may reflect a hierarchy of *var* gene expression (34) and consequent PfEMP1 exposure (36, 37). Although we did not find an association of this result with primary sequence characteristics, we cannot rule out that distinguishing motifs are present in other domains or the higher order structure of the protein. Further work is required to elucidate any functional significance associated with these particular patterns.

Greater insight into the variant-specific response was gained when we investigated the age-specific seroprevalence patterns for previously defined DBL $\alpha$  subgroups (21). The antibody response of PNG infants (<1 year) was directed predominantly at DBL $\alpha$  bearing *cys2/MFK-REY+* signatures (subgroup 2) and to a lower extent those bearing *cys4/MFK-REY+* signatures (group 5) and the default *cysX* subgroup (group 6). After 2 years of age all subgroups showed similar seroprevalence. The expression of the relatively conserved subgroup 2 has previously been associated with low host age and a limited antibody response (21, 34). Subgroups 2, 5, and 6 which were frequently recognized by PNG infants have all been linked to rosetting in symptomatic patients from Thailand (74) and there is evidence for subgroup 2 *var* gene expression in parasites isolated from symptomatic but not asymptomatic cases of malaria in Africa (21, 29, 30) and PNG (32). Our results provide further support for hierarchical expression of *var* genes and show that such patterns are conserved over large geographic and evolutionary distances. Importantly, *var* genes containing DBL $\alpha$  subgroup 2 represent only 3.5% of the total circulating *var* genes in Amele (20) and <6% in Thai and African populations (21, 29, 30, 74) thus restricting the number of potential vaccine candidates among the vast diversity of *var* genes.

In conclusion, we have presented a protein microarray platform for measuring antibodies to large numbers of PfEMP1 variants using small volumes of plasma. Data obtained using this platform confirm that the anti-PfEMP1-DBL $\alpha$  antibody response increases in magnitude and prevalence with age and further demonstrates that it increases in stability and complexity in PNG, a region of high *P. falciparum* transmission. High frequency recognition of specific PfEMP1 variants and in particular those containing DBL $\alpha$  subgroup 2 were observed from an early age whereas others were not recog-

nized until after the age of 3 years thus providing further evidence of a hierarchy of antibody responses to diverse PfEMP1 variants (36, 37). Protein microarray strategies incorporating different antigen variants have the potential to uncover the role of antibodies not only against PfEMP1 but also to other parasite antigens in the development of naturally acquired immunity to *P. falciparum* malaria. Given the availability of high throughput genomic and proteomic technology, surveys of naturally acquired immunity in the context of *P. falciparum* diversity as we have done here will provide valuable information to facilitate the development of an effective malaria vaccine.

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 This article contains [supplemental Figs. S1 to S5 and Tables S1 and S2](#).

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