

Kinetics of B Cell Responses to *Plasmodium falciparum* Erythrocyte Membrane Protein 1 in Ghanaian Women Naturally Exposed to Malaria Parasites

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Naturally acquired protective immunity to *Plasmodium falciparum* malaria takes years to develop. It relies mainly on Abs, particularly IgG specific for *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins on the infected erythrocyte surface. It is only partially understood why acquisition of clinical protection takes years to develop, but it probably involves a range of immune-evasive parasite features, not least of which are PfEMP1 polymorphism and clonal variation. Parasite-induced subversion of immunological memory and expansion of “atypical” memory B cells may also contribute. In this first, to our knowledge, longitudinal study of its kind, we measured B cell subset composition, as well as PfEMP1-specific Ab levels and memory B cell frequencies, in Ghanaian women followed from early pregnancy up to 1 y after delivery. Cell phenotypes and Ag-specific B cell function were assessed three times during and after pregnancy. Levels of IgG specific for pregnancy-restricted, VAR2CSA-type PfEMP1 increased markedly during pregnancy and declined after delivery, whereas IgG levels specific for two PfEMP1 proteins not restricted to pregnancy did not. Changes in VAR2CSA-specific memory B cell frequencies showed typical primary memory induction among primigravidae and recall expansion among multigravidae, followed by contraction postpartum in all. No systematic changes in the frequencies of memory B cells specific for the two other PfEMP1 proteins were identified. The B cell subset analysis confirmed earlier reports of high atypical memory B cell frequencies among residents of *P. falciparum*-endemic areas, and indicated an additional effect of pregnancy. Our study provides new knowledge regarding immunity to *P. falciparum* malaria and underpins efforts to develop PfEMP1-based vaccines against this disease. *The Journal of Immunology*, 2014, 192: 5236–5244.

Protective immunity to *Plasmodium falciparum* malaria acquired after natural exposure is mediated to a large extent by IgG Abs targeting the asexual blood stages of the parasites (1, 2). The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of high-m.w. proteins mediates adhesion of erythrocytes by mature *P. falciparum* parasites to a range of vascular host receptors (3–6). This sequestration of infected erythrocytes (IEs) is a major virulence factor (reviewed in Ref. 7), and the PfEMP1 family therefore constitutes a major target of acquired protective immunity to *P. falciparum* malaria (reviewed in ref. 8).

However, the PfEMP1 Ags display an extensive clonal diversity, which probably goes a long way toward explaining the sluggish rate at which immunological protection is acquired (reviewed in Ref. 8). Other immune-evasive mechanisms may also contribute to delaying acquisition of protection, including parasite interference with formation and maintenance of immunological memory (reviewed in Ref. 9). It has been proposed that parasite-driven accumulation of atypical memory B cells (phenotypically similar to the functionally “exhausted” B cells described in HIV-infected individuals) (10) among residents in *P. falciparum*-endemic areas may be one such mechanism (11, 12). Nevertheless, the association between *P. falciparum* exposure, atypical memory B cells, and slow acquisition of protective immunity (12–14) remains circumstantial, in contrast with studies of individuals infected by HIV where dysfunction (“exhaustion”) of pathogen-specific memory B cells has been demonstrated (10). Furthermore, these previous studies compared *P. falciparum*-exposed individuals with separate nonexposed individuals. Their cross-sectional design, and the lack of information on the Ag specificity of the B cells examined, ignores the contribution of factors not controlled for (such as coinfections, physiological and genetic differences, etc.) to the observed phenotypic differences that were attributed to contrasting *P. falciparum* exposure alone. Finally, several recent studies have documented that *P. falciparum*-specific B cell memory can be efficiently acquired and maintained for extended periods in the absence of exposure (15–17), and that “atypical” *P. falciparum*-specific memory B cells are not dysfunctional (18).

On this basis, this study was designed to provide further and more direct evidence regarding the relationship among exposure to *P. falciparum* Ags, Ab levels, and immunological memory. We recruited a longitudinal cohort of pregnant women attending antenatal clinics in an area of southern Ghana with stable *P. falciparum* transmission. The participants were followed for up to 1 y postdelivery,

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Abbreviations used in this article: CSA, chondroitin sulfate A; IE, infected erythrocyte; FV2, full-length recombinant IT4VAR04 (VAR2CSA); FV6, full-length recombinant HB3VAR06; FV60, full-length recombinant IT4VAR60; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; TT, tetanus toxoid.

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and peripheral venous blood samples were obtained at recruitment, near delivery, and at the end of individual follow-up. We used the samples to measure IgG levels and memory B cell frequencies specific for a pregnancy-restricted, VAR2CSA-type PfEMP1 protein and two other PfEMP1 proteins not restricted to pregnancy. In addition, we measured the relative frequencies of phenotypically defined subsets of CD19⁺ B cells in these samples. The results were analyzed in terms of time relative to delivery, parity, and parasitemia. We provide the first direct evidence from a longitudinal study regarding induction, boosting, and contraction of B cell memory to the clinically important PfEMP1 Ags during pregnancy. We also provide evidence that pregnancy has an effect on the composition of particular B cell subsets. Our findings have important implications for the understanding of immunity to *P. falciparum* malaria and for efforts to develop PfEMP1-based vaccines against this disease, in particular, vaccines aimed at protecting against placental malaria, a major cause of maternal suffering and perinatal morbidity and mortality.

Materials and Methods

Study site and study participants

The study was conducted in Assin Foso, a rainforest area ~80 km North of Cape Coast, the capital of Central Region, Ghana. Transmission of *P. falciparum* parasites remains high in Ghana (19), and historically our study area is characterized by intense transmission of *P. falciparum* parasites with limited seasonal variation (20, 21). Although transmission appears to have declined in recent years (22), reliable and generally accessible area-specific information is not available.

We studied 192 adult women (58 primigravidae, 33 secundigravidae, 101 multigravidae) who consented in writing to participate after receiving explanation of the study design and purpose at their first visit to antenatal clinics. All the recruited women were pregnant (217 to 13 d before delivery), and were followed for up to 14 mo after delivery (see Fig. 1). Apart from pregnancy and first antenatal visit, no special inclusion or exclusion criteria were applied. Anamnestic information (age, number of previous pregnancies, time since last pregnancy, malaria prophylaxis while pregnant, use of insecticide-impregnated bed nets; Table I) and a venous blood sample were obtained from all participants at recruitment. From each participant, a second blood sample was collected near (either before or after) delivery and a third sample several months after delivery. Three samples were collected from all but 17 women, who were lost to follow-up (only two samples collected from each; see Fig. 1). The women lost to follow-up did not differ significantly from the rest for any of the study parameters. Samples from 74 nonpregnant women from the same study area and from 13 nonpregnant Danish women without exposure to *P. falciparum* malaria collected as part of a previous study (23) were included as *P. falciparum*-exposed and non-exposed controls for the B cell phenotyping (see later).

The study was approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research, University of Ghana (study no. 038/10-11), and by the Regional Research Ethics Committees, Capital Region of Denmark (protocol H-4-2013-083).

Blood sample collection and preparation

PBMCs and plasma were separated from venous blood samples (8 ml) collected in heparinized tubes and cryopreserved as described previously (23), using a controlled temperature gradient device that preserves lymphocyte function and phenotype (24). *P. falciparum* asexual blood-stage parasitemia at the time of each blood sampling was determined by microscopic examination of Giemsa-stained blood smears.

Antigens

Recombinant proteins representing the entire ectodomains of three PfEMP1 proteins were produced in *Baculovirus*-infected insect cells as described previously (23). These full-length proteins represented the VAR2CSA-type PfEMP1 protein IT4VAR04 (FV2) and two PfEMP1 proteins involved in rosetting, HB3VAR06 (FV6) and IT4VAR60 (FV60). Tetanus toxoid (TT) from Statens Seruminstitut (<http://www.ssi.dk>) was used as a nonmalaria control Ag.

Plasma IgG Ab measurements by ELISA

Plasma levels of IgG with specificity for FV2, FV6, and FV60 were measured by ELISA as described (23). In brief, 96-well, flat-bottom

microtiter plates were coated with Ag, blocked with BSA, and incubated with prediluted plasma samples for 1 h. Wells were washed and bound Ab was detected with HRP-conjugated rabbit anti-human IgG and o-phenylene diamine. The enzymatic reaction was stopped by addition of H₂SO₄, optical densities read at 492 nm, and the specific Ab levels expressed in arbitrary units: AU = (OD_{SAMPLE} - OD_{BLANK})/(OD_{POSCTRL} - OD_{BLANK}).

Memory B cell frequency determination by ELISPOT

Frequencies of memory B cells with specificity for FV2, FV6, and FV60 were measured by ELISPOT as described previously (23). In brief, cryopreserved PMBCs were thawed, washed, adjusted to 2.5×10^6 /ml, and memory B cells were induced to start Ab secretion by stimulating them with IL-2 and the polyclonal activator R-848 for 72 h. The Ags FV2, FV6, FV60, and TT were added to separate wells of prewetted ELISPOT plates, and the plates were incubated overnight (4°C). After washing and blocking of the plates, stimulated PBMCs were added (duplicate wells) and the plates were incubated (37°C, 5% CO₂, 24 h). After washing, evidence of Ab-secreting cells was detected by HRP-conjugated rabbit anti-human IgG followed by 3,3',5,5'-tetramethylbenzidine. The plates were finally washed under running tap water and dried in the dark. The number of spots was determined with an automated ImmunoSpot reader.

B cell phenotyping

Phenotyping of B cell subpopulations was done essentially as described elsewhere (12). In brief, PBMC aliquots (1×10^6 cells, 100 μ l) were labeled (20 min, room temperature) with saturating amounts of PE.CF594-CD10 (catalog no. 562292; BD Biosciences, Albertslund, Denmark), PerCP-CD19 (catalog no. 561295; BD Biosciences), FITC-CD20 (catalog no. 555742; BD Biosciences), PE-CD21 (catalog no. 555749; BD Biosciences), and PE.Cy5-CD27 (catalog no. 560609; BD Biosciences). After removal of unbound Ab by washing, forward scatter/side scatter-gated events were collected on a Beckman Coulter FC500 instrument. List-mode data files were subsequently analyzed in Kaluza software (Beckman Coulter, Albertslund, Denmark) to determine the frequencies of B cell subsets using a predefined gating strategy (see Fig. 4).

Statistical analysis

The impact of time of sampling (recruitment, near-delivery, follow-up) and parity (primigravid, multigravid) on IgG levels, memory B cell frequencies, and B cell phenotypes were evaluated by two-way ANOVA. Cohort running means (and the associated 95% confidence intervals) of Ab levels and memory B cell frequencies were determined from interpolated values for individual donors. For this, the Ab level and memory B cell frequency were assumed to change linearly from the values obtained at the first to the values obtained at the second, or from the second to the third, time points. Calculated values for individual donors at time points before the first or after the last were not included, and cohort means were not calculated where <10 individual estimates were available. One-way ANOVA was used for intergroup comparisons (unexposed control donors versus longitudinal or cross-sectional cohort donors and cross-sectional cohort donors versus pregnant or nonpregnant longitudinal cohort donors). Confidence intervals were calculated as described previously (25).

Results

Plasma levels of PfEMP1-specific IgG reflect recent Ag exposure

We collected peripheral blood samples from the study participants at recruitment, near delivery, and several months postpartum (Fig. 1). A postpartum sample was not collected from a minority of women ($n = 17$), who were lost to follow-up. Levels of IgG specific for the three recombinant PfEMP1 constructs FV2, FV6, and FV60, and for the control Ag TT were measured in plasma isolated from each of these samples. Individual responses to the PfEMP1 constructs varied among donors and over time, but were consistently above negative cutoff in the large majority of the women at most time points (Fig. 2A–C). The high variability among donors and over time is not surprising and is in line with previous data (26). It almost certainly reflects heterogeneity in age, parity, bed net use, and so on (Table I), not least because of the highly likely marked heterogeneity in parasite Ag exposure (whether and when infection occurred during pregnancy). The variability of TT-specific responses was much lower, as a sharp increase in TT-specific

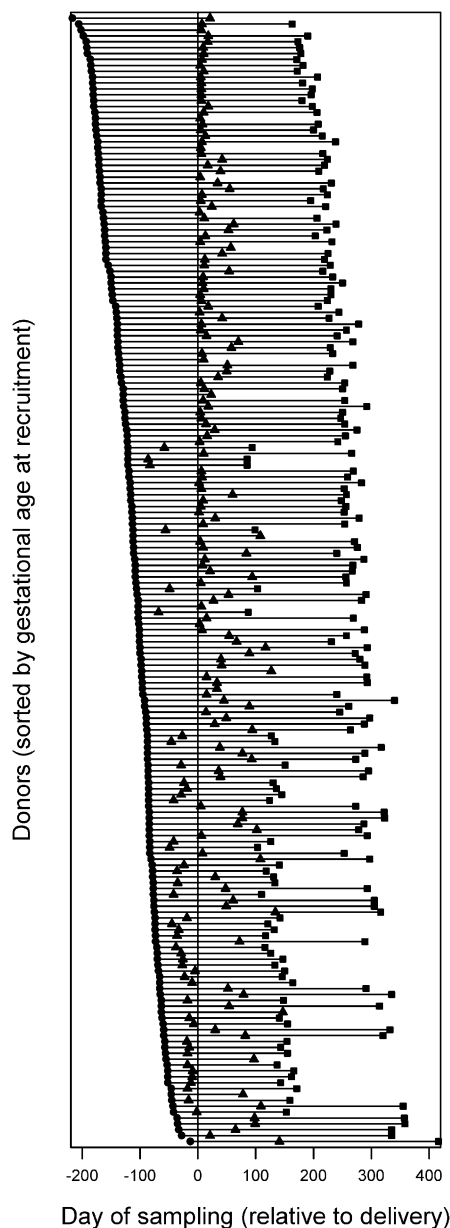


FIGURE 1. Schematic diagram of timing of sample collection from longitudinal cohort women. Blood samples were collected at recruitment (●), near delivery (▲), and 3–14 mo postdelivery (■). Seventeen women were lost to postdelivery follow-up. Collection times are shown relative to time of delivery (day 0).

IgG from recruitment to delivery, followed by steady levels postpartum was seen for most participants (Fig. 2D). Again, these more uniform responses are not surprising, because TT vaccination of all pregnant women is a standard part of antenatal care in Ghana. The pattern we observed is thus evidence of an overall good adherence to this practice, and it is likely that the minority of women who did not show the typical response were women who did not receive the scheduled TT vaccination.

To reveal population-level patterns in Ag-specific IgG reactivity, we plotted the changes in the overall mean IgG level and associated 95% confidence intervals (see *Materials and Methods* for details on calculations) over time (Fig. 2E–H). Despite the individual variability, this revealed clear differences between responses to the VAR2CSA-type PfEMP1 Ag FV2 (Fig. 2E) and the other two PfEMP1 constructs (FV6, FV60; Fig. 2F and 2G).

FV2-specific IgG levels increased from the earliest time points included (~210 d before delivery, corresponding to about week 10 of gestation) until delivery, followed by a steady decline until ~250 d postpartum (Fig. 2E). The average FV2-specific IgG response was consistently lower among primigravidae than among multigravidae at all but the earliest time points. Furthermore, FV2-specific IgG levels started to increase earlier, increased at a higher rate, and peaked higher among multigravidae than among primigravidae (Fig. 2E). The rate of postpartum decline, which was lower than what would be expected from simple catabolic decay (the overall catabolic half-life of IgG is ~26 d) was similar regardless of parity. Average levels of FV2-specific IgG started increasing again ~250 d postpartum, possibly reflecting a new pregnancy, and thereby likely (re)exposure to VAR2CSA-expressing *P. falciparum*, in some donors, although this was not assessed at time of follow-up. The observed pattern was supported by two-way ANOVA, which revealed highly significant effects on FV2-specific IgG responses of time of sampling (recruitment, near-delivery, or follow-up; $p = 0.005$) and parity (primigravid or multigravid; $p < 0.001$).

In marked contrast with the FV2-specific IgG responses, average levels of FV6- (Fig. 2F) and FV60-specific IgG (Fig. 2G) did not vary systematically with time ($p = 0.78$ and $p = 0.20$ for FV6 and FV60, respectively). As for FV2, levels of FV6- and FV60-specific IgG depended significantly on parity ($p = 0.01$ and $p = 0.001$, respectively); but in contrast with FV2, levels of FV6- and FV60-specific IgG were higher among primigravidae than among multigravidae. Finally, a strong effect of time, but not parity, on TT-specific IgG responses was apparent (Fig. 2H), and this was confirmed by statistical analysis ($p < 0.001$ and $p = 0.97$, respectively). No significant interaction between time and parity was detected for any of the Ab specificities ($p = 0.96, 0.48, 0.41$, and 0.26 for FV2, FV6, FV60, and TT, respectively).

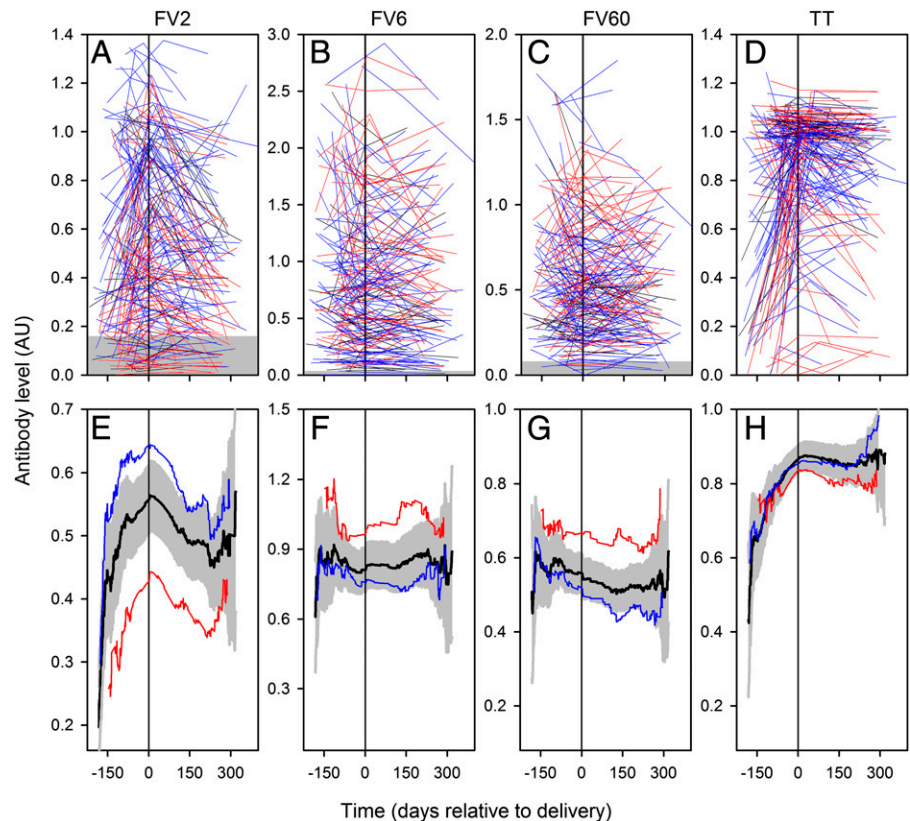
These findings confirm and markedly strengthen earlier data that PfEMP1-specific IgG levels reflect recent natural exposure to *P. falciparum* parasites (reviewed in Ref. 27). Second, they show that pregnancy is associated with a marked IgG response to VAR2CSA-type PfEMP1 (reviewed in Ref. 28). Third, the levels of these latter Abs decline fairly rapidly postdelivery, when pregnancy-restricted *P. falciparum* Ags are no longer present (23, 29, 30). This contrasts with the postdelivery levels of TT-specific IgG, which remained high throughout follow-up as expected.

Frequencies of PfEMP1-specific memory B cells expand and contract in response to Ag

Much less is known about PfEMP1-specific B cell memory. In a previous cross-sectional study, we concluded that FV2-specific B cell memory can be maintained stably for many years after exposure (23), but the study design did not allow detection and analysis of transient changes in memory B cell frequencies during and shortly after pregnancy. This deficiency was therefore addressed in this study. As was the case for IgG levels, frequencies of PfEMP1-specific memory B cells varied considerably among donors and over time (Fig. 3A–C). However, plotting changes in overall mean memory B cell frequencies (Fig. 3E–G) again revealed temporal patterns and differences among the Ags that were not easily appreciated in the plots of individual memory B cell frequency kinetics.

The temporal changes and the parity-dependent differences in the memory B cell frequencies (Fig. 3) were consistent with the corresponding IgG responses (Fig. 2). Frequencies of FV2-specific memory B cells (Fig. 3A) initially increased earlier and faster, and also peaked earlier and higher, among multigravidae than primigravidae. This was followed by a decline that started near delivery

FIGURE 2. Temporal changes in plasma IgG levels. Levels of IgG specific for FV2 (**A**), FV6 (**B**), FV60 (**C**), and TT (**D**) in individual primigravid (red lines) and multigravid (blue lines) donors at different time points before and after delivery are shown. Negative cutoffs (means + 2 SD of levels in negative control donors) are indicated by gray shading (**A–C**). Overall mean levels (heavy black lines) and 95% confidence intervals (gray shading) of IgG specific for FV2 (**E**), FV6 (**F**), FV60 (**G**), and TT (**H**) at different time points before and after delivery are also shown. Finally, mean levels among primigravidae only (red lines) or multigravidae only (blue lines) are shown (**E–H**). Only mean levels based on data from at least 10 individual donors are shown (**E–H**). Time of delivery is marked by a vertical black line in all panels.



in the multigravidae and several months later among the primigravidae. As for the FV2-specific IgG responses, a secondary increase in FV2-specific memory B cells was apparent from ~250 d postpartum (Fig. 3A). This supports the earlier mentioned hypothesis of re-exposure to parasites expressing VAR2CSA-type PfEMP1 after becoming pregnant again, indicated by the coincident increase in FV2-specific IgG levels. Results of the two-way ANOVA were consistent with the observed pattern. Thus, both parity and time had an effect on FV2-specific memory B cell frequencies. However, the levels of significance ($p = 0.12$ and 0.09 , respectively) were considerably lower because of interaction between time and parity ($p = 0.08$), reflecting the substantially slower response kinetics among primigravidae than multigravidae.

Frequencies of FV6- and FV60-specific memory B cells (Fig. 3F and 3G) were much more stable over time ($p = 0.87$ and 0.29 , respectively), and with less parity-dependent differences ($p = 0.27$ and 0.73 , respectively). Again, nontrivial interaction between time and parity was evident for both Ags ($p = 0.05$ and 0.17 , respectively). This appeared mainly because of a modest and transient postpartum increase in the average FV6- and FV60-specific memory B cell frequencies among primigravidae (Fig. 3F and 3G). Frequencies of TT-specific memory B cells (Fig. 3H) depended strongly on time ($p > 0.001$) as expected because of the TT

booster vaccination received as part of antenatal care, but they were not affected by parity ($p = 0.97$). There was no significant interaction between the effect of time and parity on frequencies of TT-specific memory B cells ($p = 0.26$).

Taken together, these results strongly indicate that memory B cell response to PfEMP1 follows the general pattern of induction, expansion, and contraction, and also shows the faster and stronger response upon recall compared with primary exposure.

B cell subset composition is affected by pregnancy and is associated with residence in an area of stable P. falciparum transmission

Several studies have reported that frequencies of atypical B cells are increased in *P. falciparum*-exposed individuals, and that this may be related to an assumed dysfunction in the acquisition of protective immunity to this infection (reviewed in Ref. 9). To investigate this in more detail, we determined the relative proportions of various phenotypically defined CD19⁺ B cell subsets (Fig. 4) in all samples where sufficient cells were available ($n = 173$, $n = 180$, and $n = 161$ at recruitment, near-delivery, and follow-up, respectively), and compared the results with data obtained from a separate cohort of 74 nonpregnant women living in the same area and 13 nonpregnant Danish women without *P. falciparum* exposure (Fig. 5).

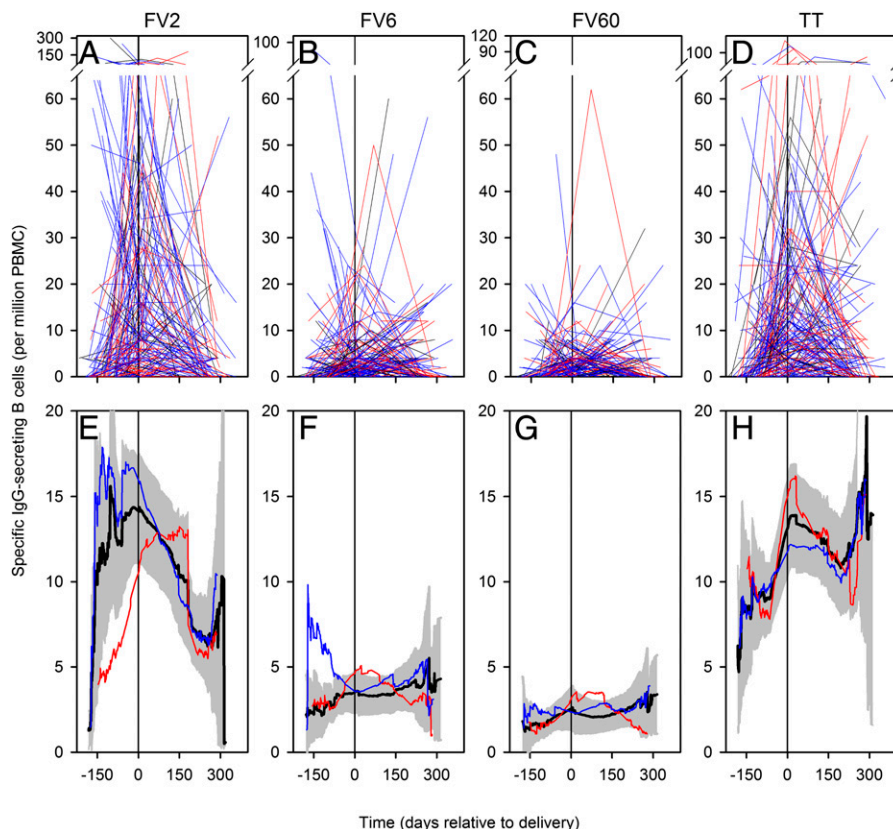
Table I. Characteristics of study participants

	Exposed, Pregnant	Exposed, Nonpregnant	Nonexposed, Nonpregnant
Donors, <i>n</i>	192	74	13
Median age at enrollment, y (range) ^a	28 (15–44)	30 (17–54)	37 (23–54)
Mean no. of pregnancies (range) ^a	2 (0–7)	2 (0–11)	2 (0–4)
Parasitemic at sampling (proportion)	0.4 ^b	0.09	—
Malaria prophylaxis during pregnancy ^a	0.62	—	—
ITN use ^a	0.47	0.61	—

^aSelf-reported.

^bAt any sampling. The prevalence of parasitemia at recruitment was much higher (0.33) than at the two subsequent samplings (0.07 each).

FIGURE 3. Temporal changes in memory B cell frequencies. Frequencies of B cells secreting IgG specific for FV2 (**A**), FV6 (**B**), FV60 (**C**), and TT (**D**) in individual primi-gravid (red lines) and multigravid (blue lines) donors at different time points before and after delivery are shown. Overall mean levels (heavy black lines) and 95% confidence intervals (gray shading) of B cells secreting IgG specific for FV2 (**E**), FV6 (**F**), FV60 (**G**), and TT (**H**) at different time points before and after delivery are also shown. Finally, mean levels among primi-gravidae only (red lines) or multigravidae only (blue lines) are shown (E–H). Only mean levels based on data from at least 10 individual donors are shown (E–H). Time of delivery is marked by a vertical black line in all panels.



Within the longitudinal cohort, the composition of CD19⁺ B cell subsets varied over time, and statistically significant variation was detectable in several subsets. Thus, the relative frequency of

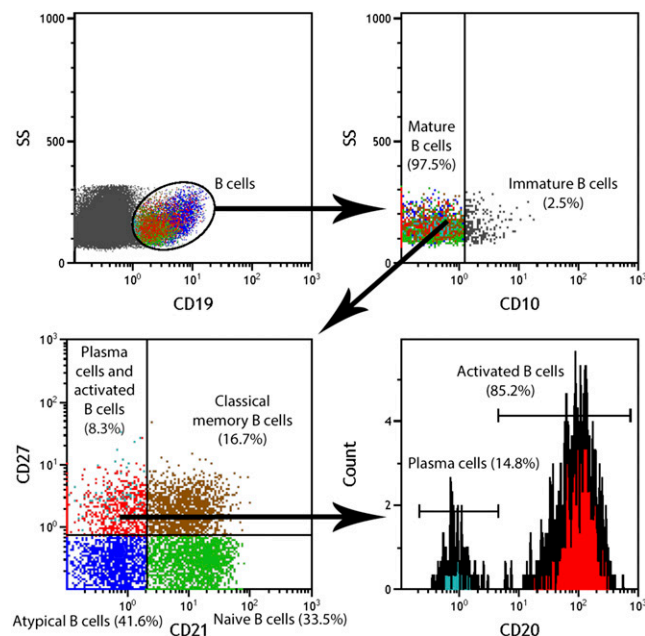
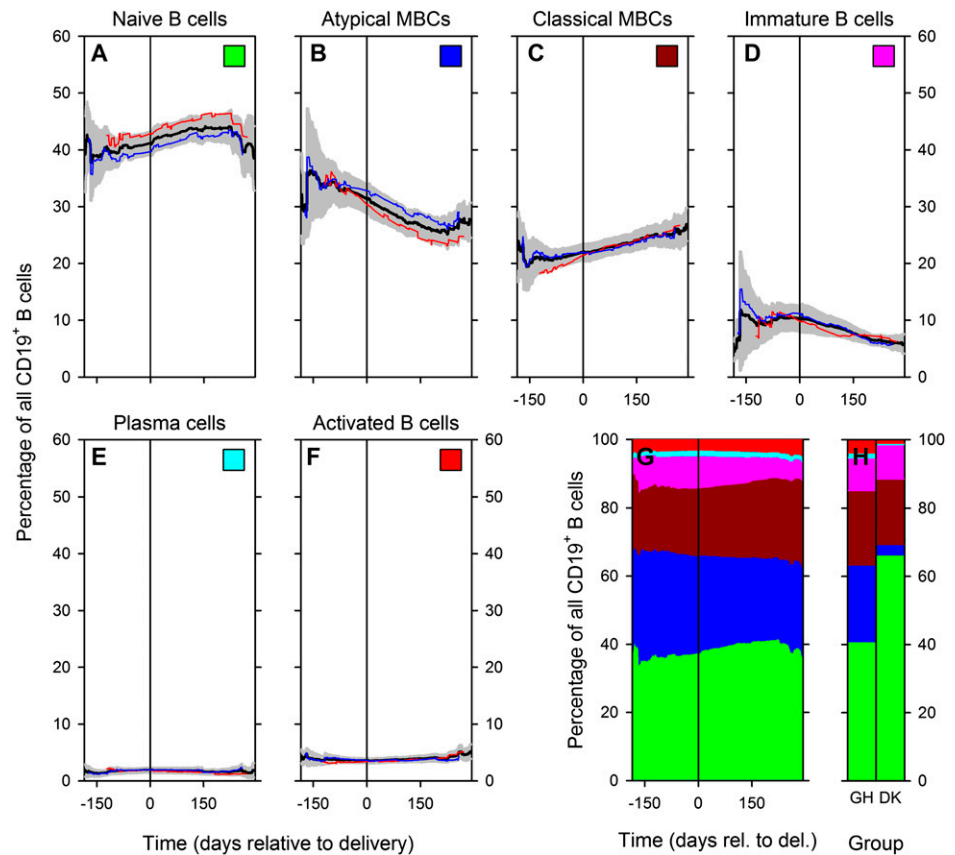


FIGURE 4. Gating strategy for phenotyping of B cell subsets. B cells were identified by gating on CD19⁺ cells. Subsets of CD19⁺ cells were subsequently designated as immature B cells (CD10⁺), naive B cells (CD10[−]CD21⁺CD27[−]), classical memory B cells (CD10[−]CD21⁺CD27⁺), atypical memory B cells (CD10[−]CD21[−]CD27[−]), plasma cells (CD10[−]CD20[−]CD21[−]CD27⁺), and activated B cells (CD10[−]CD20⁺CD21[−]CD27⁺), respectively. Plots and percentages shown are representative examples. Color coding corresponds to that used in Fig. 5G and 5H.

CD10[−]CD21⁺CD27[−] (naive, $p = 0.02$) cells among all CD19⁺ B cells (Fig. 5A) increased from recruitment until ~250 d postpartum, followed by a decrease. A similar picture was seen for CD10[−]CD21⁺CD27⁺ (classical memory, $p = 0.01$) cells (Fig. 5C), except that the decrease after 250 d postpartum was not evident. In contrast, the average relative frequency of triple-negative (CD10[−]CD21[−]CD27[−], $p < 0.001$) atypical memory B cells (Fig. 5B) decreased initially, followed by an increase toward the end of the follow-up period. This was also the case ($p = 0.04$) for CD10⁺ immature B cells (Fig. 5D) except for the late increase, which was not discernable in this subset. The CD10[−]CD20[−]CD21[−]CD27⁺ (plasma cells, $p = 0.33$) and CD10[−]CD20⁺CD21[−]CD27⁺ (activated B cells) subsets did not vary significantly over time (Fig. 5E and 5F). Parity did not significantly affect the relative frequency of any of the subsets ($p > 0.36$ in each case). Significant interaction between time and parity was only detected for atypical memory B cells ($p = 0.02$; $p > 0.23$ for all other subsets), where the rate of the postpartum decline appeared to be higher among primigravidae than among multigravidae. Subset relative frequencies did not differ according to whether the donor was parasitemic at the time of sampling ($p > 0.29$ for all subsets), whereas the relative frequencies of naive B cells and atypical memory B cells ($p = 0.06$ and $p = 0.004$, respectively) in particular, and to a lesser extent classical memory B cells and immature B cells (both $p = 0.10$), depended on pregnancy status.

The B cell subset composition among nonpregnant Danish control women without malaria exposure (Fig. 5H) was markedly different from that of *P. falciparum*-exposed Ghanaian women (Fig. 5G and 5H). Thus, the relative frequency of naive B cells was substantially higher among the Danish than among the Ghanaian women, whether samples from the longitudinal study (Fig. 5G) or the separate cross-sectional study (Fig. 5H) were used ($p < 0.001$ in both cases). In contrast, the relative frequencies of atypical memory B cells and activated B cells were substantially

FIGURE 5. Temporal changes in peripheral blood B cell subsets. Frequencies of phenotypically defined naive B cells ($CD19^+CD10^-CD21^+CD27^-$) (A), atypical memory B cells ($CD19^+CD10^-CD21^-CD27^-$) (B), classical memory B cells ($CD19^+CD10^-CD21^+CD27^+$) (C), immature B cells ($CD19^+CD10^+CD21^-CD27^-$) (D), plasma cells ($CD19^+CD10^-CD20^+CD21^-CD27^+$) (E), and activated B cells ($CD19^+CD10^-CD20^+CD21^+CD27^+$) (F) at different time points before and after delivery are shown. Graph layout and color coding of (A)–(F) corresponds to that used in Figs. 2 and 4. Relative percentages of circulating $CD19^+$ B cell subsets at different time points before and after delivery (G), and in a separate cohort of sympatric nonpregnant women (GH) and in Danish nonpregnant women without *P. falciparum* exposure (DK) (H). Color coding of (G) and (H) correspond to the little square boxes shown in the upper right corner of (A)–(F).



lower among the Danish than among the Ghanaian women, whether samples from the longitudinal study or the separate cross-sectional study were used ($p < 0.001$ in all cases). The frequencies of classical memory B cells, immature B cells, and plasma cells among the Danish donors were not significantly different from the corresponding frequencies among the Ghanaian donors ($p > 0.3$ in all cases).

Overall, our B cell phenotype data confirm previous reports that the relative frequencies of different B cell subsets in individuals living in areas of stable *P. falciparum* transmission differ from those in individuals from nonendemic areas. The cause(s) of this difference is unclear, however. Although our data clearly suggest that pregnancy does have an impact on subset composition, presence or absence of *P. falciparum* parasites at the time of sampling appears to be irrelevant. Most importantly, however, our study does not resolve whether overall B cell phenotypes are affected by long-term *P. falciparum* exposure, as suggested by previous indirect evidence, or whether other differences between the Ghanaian and Danish donors (race, living conditions, other infections, etc.) are equally or more important determinants.

Discussion

Placental malaria is caused by the selective accumulation of *P. falciparum*-IEs (reviewed in Ref. 31). The sequestration of IEs on the maternal side of the placenta is mediated by parasite-encoded VAR2CSA-type PfEMP1 proteins that are expressed on the IE surface and have high affinity for low-sulfated chondroitin sulfates in the intervillous space (32, 33). Placental malaria is a well-recognized cause of maternal morbidity such as severe anemia and of substantial perinatal morbidity and mortality (34). Expression of VAR2CSA-type PfEMP1 proteins appears restricted to pregnancy, which explains why primigravidae living in areas of stable transmission of *P. falciparum* parasites are fully susceptible

to placental malaria despite enjoying substantial protection from *P. falciparum* malaria in general, acquired during childhood and adolescence (reviewed in Refs. 28, 35). In such areas, susceptibility to placental malaria declines in subsequent pregnancies, reflecting acquisition of specific immunity against the placenta-sequestering parasites. It is thus well established that levels of VAR2CSA-type, PfEMP1-specific IgG at the time of delivery depend on parity among women living in areas with stable transmission of *P. falciparum* parasites, and that these IgG levels are associated with clinical protection from placental malaria (32). The causal relation between levels of VAR2CSA-type, PfEMP1-specific IgG and clinical protection is underpinned by several lines of investigation, not least is the ability of specific IgG to inhibit and reverse adhesion of IEs to chondroitin sulfate A (CSA) (32, 36–38). A vaccine to protect pregnant women and their offspring from placental malaria thus appears feasible and is currently in development (28). All this notwithstanding, little is known about the kinetics of VAR2CSA-specific Ab responses during pregnancy, and only a single cross-sectional study has reported on their levels in nonpregnant, *P. falciparum*-exposed women (23). No longitudinal studies have compared Ab responses to VAR2CSA-type PfEMP1 with responses specific for other pregnancy-unrestricted PfEMP1 Ags in the same donors, let alone compared changes in IgG levels with the kinetics of the accompanying memory B cell response or studied constructs representing the full ectodomains of these clinically important Ags. This study was designed to overcome some of these deficiencies.

We found that levels of IgG with specificity for a recombinant protein (FV2), representing the full ectodomain of the VAR2CSA-type PfEMP1 IT4VAR04 (39), varied considerably among donors and over time (Fig. 2A), in good agreement with an earlier similar study of Abs to the immunodominant DBL_{EPAM}_D5 domain of VAR2CSA (26). Nevertheless, mean levels of FV2-specific IgG

increased over time from ~150 d before delivery (the earliest time point with sufficient data available) until delivery (Fig. 2E). The rate of increase was faster and the peak level higher, among multigravidae compared with primigravidae (Fig. 2E). After giving birth, levels started to decrease steadily until ~250 d postpartum, where averages started to increase once again. Apart from the very earliest time points studied, levels of FV2-specific IgG were above negative cutoff in the large majority of samples at all time points studied. This data set on the kinetics of IgG responses to VAR2CSA-type PfEMP1, which is arguably the most comprehensive and definitive of its kind so far, is in general in good agreement with previous reports. Thus, O'Neil-Dunne et al. (40) showed that CSA adhesion-inhibitory plasma Abs were generally absent early in pregnancy but increased steadily toward delivery, and this increase occurred earlier and faster in multigravidae than in primigravidae. Other studies have yielded similar results (30, 41, 42). However, it should be emphasized that the overall trend obviously masks transient responses where levels increase, then decline before delivery in some (mainly multigravid) women (43) or show more complex dynamics (26). Less data are available regarding changes in VAR2CSA-Ab levels postpartum. Staals et al. (30) reported that delivery levels of IgG specific for CSA-adhering IEs declined substantially over the following 6 mo, in agreement with our recent report that most previously pregnant women from an area of stable *P. falciparum* transmission had little or no FV2-specific IgG (23). Although other authors reported the half-life of VAR2CSA-specific IgG to be very long (several to many decades), their conclusions were based on extrapolations from measurements in pregnant women only, which probably leads to gross overestimation (26). Overall, the evidence suggest that levels of IgG to VAR2CSA-type PfEMP1 are not maintained between successive pregnancies and start declining as soon as the parasites expressing this Ag type disappear, that is, at delivery (29, 30). This would be in line with the general consensus that PfEMP1-specific IgG responses tend to be transient and that levels of these Abs thus reflect recent exposure (44, 45). With respect to the late postpartum secondary increase in average FV2-specific IgG levels that we observed (Fig. 2E), it is tempting to speculate that it indicates secondary pregnancies in some cohort members during follow-up. Unfortunately, this was not investigated, but the hypothesis is supported by the similar changes in Ab levels over the course of two pregnancies observed in an earlier study (30) and by our data on TT-specific IgG (Fig. 2H) and FV2- and TT-specific memory B cell kinetics (Fig. 3E and 3H).

IgG levels specific for the two other full-length recombinant PfEMP1 proteins studied, HB3VAR06 (FV6) and IT4VAR60 (FV60), did not vary systematically with time (Fig. 2B, 2C, 2F, and 2G). The FV2-specific response discussed earlier is thus in all likelihood elicited by exposure to infecting parasites expressing VAR2CSA-type PfEMP1 rather than a nonspecific or cross-reactive response to parasites expressing other PfEMP1 types. It has previously been shown that parasitemia among pregnant women in areas of stable *P. falciparum* transmission is dominated by parasites expressing VAR2CSA-type PfEMP1 proteins indicative of ongoing placental infection (46, 47). Nevertheless, it appears plausible that the higher average levels of FV6- and FV60-specific IgG observed among primigravidae than multigravidae (Fig. 2F and 2G), in marked contrast with the FV2-specific responses (Fig. 2E), reflect boosting of pre-existing immunity specific for these Ags caused by placenta-sequestering parasites (which are more prevalent in primigravidae) switching to expression of FV6- and FV60-like PfEMP1 proteins. With the exception of a minority of primigravidae, levels of IgG specific for the unrelated Ag TT increased sharply from recruitment to delivery in essentially all study

participants and were maintained at high levels throughout the follow-up period (Fig. 2H). This pattern likely reflects an encouragingly high level of adherence to the policy of TT vaccination as part of the antenatal care program in Ghana.

The observed transience of the FV2-specific IgG response (Fig. 2E), in marked contrast with the sustained TT-specific response (Fig. 2H), might suggest an underlying defect in the memory B cell response to VAR2CSA-type PfEMP1 proteins, and by extension to PfEMP1 and other clinically relevant *P. falciparum* Ags in general. In fact, this has repeatedly been proposed as an important underlying cause of the sluggish development of protective immunity to *P. falciparum* malaria (reviewed in Ref. 9). However, we did not find evidence of such a defect in our previous cross-sectional study on this issue (23), and other reports support this conclusion (15–17, 48). As in the earlier study (23), we analyzed in this study the frequency of B cells secreting IgG specific for the study Ags after nonspecific in vitro stimulation, as a measure of Ag-specific circulating memory B cells (49). The overall frequency of FV2-specific memory B cells increased from low levels at the earliest time point studied to a peak 10–15 times higher near delivery, then declining again (Fig. 3A and 3E). However, the initial increase happened earlier, was faster, and peaked earlier among multigravidae compared with primigravidae (Fig. 3E). This parity-related difference in kinetics strongly implies that vigorous recall responses dominated among the multigravidae, in contrast with slower primary FV2-specific immune responses among the primigravidae. The increase in the frequency of FV2-specific B cells was followed by a contraction of the B cell response after disappearance of specific Ags. This would be expected to occur earlier and often well before delivery (because of the rapid immune response) among multigravidae than among primigravidae, where parasitemia often persists until delivery (reviewed in Ref. 50). As was the case for the FV2-specific IgG responses, a secondary increase in the mean frequency of FV2-specific memory B cells was observed from day 250 onward (Fig. 3E), likely reflecting new pregnancies during follow-up as mentioned earlier.

We did not observe convincing systematic temporal changes in the frequencies of memory B cells specific for the two PfEMP1 proteins not restricted to pregnancy (Fig. 3B, 3C, 3F, and 3G). However, the minor peaks in both FV6- and FV60-specific memory B cell frequencies observed among primigravidae shortly after delivery might be related to the proposed boosting effect of placenta-dwelling parasites switching from VAR2CSA-type PfEMP1 to PfEMP1 proteins resembling FV6 and FV60. Because additional supportive evidence is lacking, this is presently conjecture. The kinetics of the memory B cell response to TT (Fig. 3D and 3H) resembled the FV2-specific response (Fig. 3A and 3E), including a secondary increase beginning about day 250, which lends further support to the notion of new pregnancies in some cohort members during follow-up. The only real difference was that TT-specific memory B cell kinetics unsurprisingly did not depend on parity (Fig. 3H).

Could the similar memory B cell responses to these two Ags, combined with the markedly different (transient versus sustained) IgG kinetics, indicate the existence of fundamentally different defensive strategies against different types of infections? In the case of infections like malaria, “mobilization” (of memory B cells) might suffice for adequate control, whereas other infections (e.g., with toxin-producing bacteria like *Clostridium tetani*) can only be successfully controlled if a “standing army” (of Abs) is present at all times. If so, it might be unjustified to interpret the often low and transient Ab responses to *P. falciparum* Ags as evidence of immune dysfunction. Further studies are clearly required to confirm or refute this postulate.

The hypothesis that parasite-specific immune dysfunction can explain the slow rate at which protective immunity is acquired has received new attention following a series of studies reporting increased frequencies of so-called atypical memory B cells among residents of areas with stable transmission of *P. falciparum* parasites (reviewed in Ref. 9). These cells are phenotypically similar to the predominantly HIV Ag-specific “exhausted” memory B cells, which can be found in HIV-infected individuals and are functionally impaired relative to classical memory B cells (10). Importantly, however, it is not yet known whether the expanded atypical memory B cell subset seen in *P. falciparum*-endemic areas has a corresponding bias toward *P. falciparum* Ags. Our phenotypic B cell data support the earlier reports (11, 12, 14), as we also found much higher frequencies of atypical (CD10⁺CD21⁺CD27⁺) memory B cells in our Ghanaian donors compared with Danish control donors without *P. falciparum* exposure (Fig. 5G and 5H). In addition, we provide evidence that pregnancy may also cause a (modest) increase of this subset, and present the first detailed data on changes in various B cell subsets during and after pregnancy. As in the earlier studies, we can only speculate on the Ag specificity of the cells in these subsets. The very similar B cell phenotype data that we obtained from primigravida and multigravida donors, respectively, despite major differences in their functional B cell responses, do not lend further support for a specific role of *P. falciparum* in explaining high frequencies of atypical B cells. However, the available evidence suggests that an impact on atypical B cell frequencies requires long-term parasite exposure, whereas current parasitemia is of limited consequence (12). It is thus possible that bouts of parasitemia during pregnancy do not result in a B cell phenotypic perturbation detectable by a study of our size. In any case, the proposed links between atypical memory B cells and *P. falciparum* remain largely circumstantial (see Ref. 51 for an exhaustive review). A resolution of this conundrum must await studies of *P. falciparum* Ag-specific B cells rather than general B cell populations.

In conclusion, to our knowledge, we have reported the first detailed longitudinal study of B cell responses to pregnancy-restricted and -unrestricted PfEMP1 proteins in a cohort of Ghanaian women followed from early pregnancy up to about a year postdelivery. We found no functional or phenotypic evidence of malaria-specific immune dysfunction. Rather, our data show that exposure to VAR2CSA-type PfEMP1 during pregnancy results in a textbook-like primary and secondary immune response in primigravidae and multigravidae, respectively. Our findings contribute to the basic understanding of immunity to this important infectious disease, to the clinically important PfEMP1 Ags, and bode well for current efforts to develop PfEMP1-based malaria vaccines, in particular, to control the burden of placental malaria in areas of stable *P. falciparum* transmission.

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Disclosures

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