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Pregnancy and Malaria Exposure Are Associated with Changes in the B Cell Pool and in Plasma Eotaxin Levels

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Pregnancy triggers immunological changes aimed to tolerate the fetus, but its impact on B lymphocytes is poorly understood. In addition, exposure to the *Plasmodium* parasite is associated with altered distribution of peripheral memory B cell (MBC) subsets. To study the combined impact of high malaria exposure and pregnancy in B cell subpopulations, we analyzed PBMCs from pregnant and nonpregnant individuals from a malaria-nonendemic country (Spain) and from a high malaria-endemic country (Papua New Guinea). In the malaria-naive cohorts, pregnancy was associated with a significant expansion of all switched (IgD^-) MBC and a decrease of naive B cells. Malaria-exposed women had more atypical MBC and fewer marginal zone–like MBC, and their levels correlated with both *Plasmodium vivax*– and *Plasmodium falciparum*–specific plasma IgG levels. Classical but not atypical MBC were increased in *P. falciparum* infections. Moreover, active atypical MBC positively correlated with proinflammatory cytokine plasma concentrations and had lower surface IgG levels than the average. Decreased plasma eotaxin (CCL11) levels were associated with pregnancy and malaria exposure and also correlated with B cell subset frequencies. Additionally, active atypical and active classical MBC expressed higher levels of eotaxin receptor CCR3 than the other B cell subsets, suggesting a chemotactic effect of eotaxin on these B cell subsets. These findings are important to understand immunity to infections like malaria that result in negative outcomes for both the mother and the newborn and may have important implications on vaccine development. ***The Journal of Immunology*****, 2014, 193: 000–000.**

Malaria is caused by protozoan parasites of the genus *Plasmodium*. *Plasmodium falciparum* and *Plasmodium vivax* are the most widely distributed species in humans, causing >200 million episodes and 655,000 known deaths in 2010 (1). Although sterile immunity against *Plasmodium* infection is never acquired, immunity to malaria disease is developed with age and repeated infections. Therefore, in endemic areas, adults are usually asymptomatic but can be chronically infected with low parasitemias (2). However, pregnant women are at a higher risk of malaria infection compared with nonpregnant adults. Malaria infection in pregnancy is associated with maternal and infant morbidity and mortality through maternal anemia, clinical malaria, low birth weight, and prematurity (3–8). Increased infection risk and disease morbidity during pregnancy or postpartum have been described in other infectious diseases such as influenza or tuberculosis (9, 10).

Ab responses to placental *P. falciparum* isolates have been related to protection against malaria and to exposure to *P. falciparum* infections during pregnancy (11, 12), and the levels of IgG correlate with parity in endemic areas (13, 14). Although multiple studies have analyzed Ab responses to malaria parasites during pregnancy, only two recent studies have analyzed B cells in malaria during pregnancy. The first one reported an increase in B cells and activated B cells in this condition (15); the second one showed altered frequencies of B cell subsets (16). However, the phenotypic resolution of these studies was very limited.

Flow cytometric immunophenotyping has been used to classify human B cells into distinct subsets according to their state of maturation and differentiation. For instance, CD10 is expressed in immature B cells (17), CD38 and IgD are markers of Ag encounter, and CD27 is used to distinguish human memory B cells (MBC) (18). However, the delineation of human MBC by expression of CD27 has lately been challenged by the characterization

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Abbreviations used in this article: CI, confidence interval; ENP, malaria-exposed nonpregnant; EP, malaria-exposed pregnant; FcRL, Fc receptor-like protein; Hb, hemoglobin; MBC, memory B cell; MFI, geometric mean fluorescence intensity; MZ, marginal zone; NNP, malaria-naive nonpregnant; NP, malaria-naive pregnant; PNG, Papua New Guinea; VBC, mature viable B cell.

of $CD27^-$ MBC (19). A population of hyporesponsive MBC characterized as $CD21^{low}CD27^-$, called exhausted MBC, was reported to be expanded in HIV-infected individuals with high viral loads (20). A phenotypically similar population was found to be significantly expanded in Malian individuals with persistent *P. falciparum* infections (21). Because the functionality of this population was not confirmed in the malaria cases, the population was called atypical MBC. Later, several studies showed further associations between malaria exposure and expansion of atypical MBC (22, 23); however, in these studies, concomitant factors such as other infections, nutrition, or genetic differences could not be ruled out. Only a very recent study has demonstrated that *P. falciparum* infection indeed results in an expansion of atypical MBC, analyzing this population in malaria-naive adults before and after a controlled human malaria infection (24).

Nevertheless, the role of atypical MBC is not yet clear in malaria. Two different studies have established that, unlike HIV, the malaria-driven expanded atypical MBC population produces regular amounts of functional Abs (25, 26). Moreover, it seems that Ab and especially MBC responses to malaria Ags can be stably maintained over time in the absence of reinfection (27, 28). Therefore, further studies are needed to understand the significance of the malaria-induced expansion of atypical MBC and the mechanisms driving this expansion.

Other B cell populations have been shown to be affected by malaria exposure. Infants from a malaria-endemic area in Kenya had reduced peripheral levels of unswitched $CD19^+IgD^+CD27^+$ B cells compared with infants living in an area with unstable malaria transmission (29). This subset resembles the splenic marginal zone (MZ) MBC cells ($CD19^+IgM^+IgD^+CD27^+$), which are very important for protection against encapsulated bacteria (30).

In pregnancy, B cells and especially Abs have been associated with pregnancy well-being as well as pregnancy-associated pathologies (31); however, MBC and their different subsets have received little attention in the context of pregnancy. One very recent study (16) reported pregnancy-associated differences in atypical and classical MBC proportions in a malaria-exposed cohort, suggesting that pregnancy itself drives changes in the distribution of B cells. However, due to limitations in the flow cytometry panel, these authors could not distinguish between naive and resting atypical MBC, or classical and MZ-like MBC, resulting in partial understanding of the effect of pregnancy in those B cell subsets. In addition, a malaria-naive pregnant (NP) cohort was not included for comparison.

In summary, the association of malaria exposure with alterations in the distribution of B cells is now largely accepted, but there is limited information on the distribution of B cells during pregnancy and during malaria in pregnancy, as well as their association with *P. vivax* exposure. Therefore, we set out to investigate the independent effect of pregnancy and malaria exposure on the relative frequencies of B cell subsets by conducting an in-depth characterization of the B cell phenotypes in PBMCs of pregnant and nonpregnant women from Spain (malaria-free area) and Papua New Guinea (PNG; high malaria transmission area). We describe the association of distinct B lymphocyte subsets with various pregnancy parameters, rates of malaria infection, and *Plasmodium*-specific IgG responses, and provide some insights into the immune mediators associated with the frequencies of B cell subsets.

Materials and Methods

Study site and population

This study was performed in the context of the PregVax project (FP7-HEALTH-201588), aimed at describing the burden of *P. vivax* malaria in pregnancy in five endemic countries (www.pregvax.net). The present analysis was conducted in pregnant women from PNG enrolled at their first antenatal clinic visit and followed up until delivery at several health centers in the Madang Province, on the north coast of mainland PNG, and at the Modilon Provincial Hospital, between 2008 and 2010. The region is characterized by year-round, high-level malaria transmission. In 2005–2006, the reported prevalence of women with detectable peripheral parasitemia (all species) at first antenatal care visit was up to 34 and 14% at delivery (D.I. Stanisic, K. Moore, F. Baiwog, C. Clapham, C. King, P.M. Siba, J.G. Beeson, I. Mueller, F.J. Fowkes, and S.J. Rogerson, unpublished observations) (32). *P. vivax* and *P. falciparum* parasitemias (by microscopy and real-time PCR) and hemoglobin (Hb) were assessed at enrollment and delivery, and birth weight was recorded. For this study, infection was defined as a positive smear and/or PCR. Women with clinical symptoms who had a positive rapid diagnostic test and/or smear were treated according to the national guidelines.

Study design

This is a not-paired cohort study aimed to analyze the individual and combined impacts of high malaria exposure and pregnancy in B cell subpopulation distributions. For this purpose, 90 pregnant women randomly selected at their first antenatal visit (enrollment) or at delivery were included in the study. As enrollment rates in the first trimester of pregnancy were low, 12 additional women recruited in this trimester were included in the study, for a total of 102. One of the main objectives of the study was to compare B cell frequencies in pregnant versus nonpregnant women, but postpartum samples were not available. Therefore, to increase the sample size in the pregnant cohort in relation to the nonpregnant cohort, samples in the pregnant cohort were not paired (enrollment and delivery). A venous maternal blood sample of 10 ml was collected in heparin vacutainers at recruitment and delivery. In addition, peripheral blood samples were collected from 38 nonpregnant women from the same area in PNG during 2012. Blood samples from malaria-naive nonpregnant (NNP; $n = 21$, 7 women and 14 men) and NP ($n = 24$) donors, who were residents in Spain and had never traveled to malaria-endemic areas, were also collected at the blood bank and at the antenatal care of the Hospital Clinic (Barcelona, Spain). Participants were grouped as follows: 1) NNP; 2) NP; 3) malaria-exposed nonpregnant (ENP); and 4) malaria-exposed pregnant (EP).

Ethical approval

Written informed consent was obtained from all study participants. This study was approved by the Medical Research Advisory Committee in PNG (MRAC 08.02), and by the Hospital Clinic of Barcelona Ethics Review Committee, Spain (Comitè Ètic d'Investigació Clínica).

Isolation of plasma and PBMCs

Blood was collected in heparin vacutainers. Plasma was separated from the cellular fraction within 16 h of collection by centrifuging at $600 \times g$ for 10 min at room temperature, aliquoted, and stored at $-80^{\circ}C$. Cells were further fractioned in a density gradient medium (Histopaque-1077; Sigma-Aldrich). PBMCs were frozen in FCS supplemented with 10% DMSO and stored in liquid nitrogen. PNG samples were shipped to and analyzed at Barcelona Centre for International Health Research (Barcelona, Spain).

Immunophenotyping and gating strategy

PMBCs were slowly thawed, and their viability was measured on a Guava cytometer using Guava ViaCount reagent (Millipore, Madrid, Spain). Only samples with a viability >70% were used for the assays. All Abs and reagents came from BD Biosciences (Madrid, Spain), unless otherwise indicated. For compensation controls, BD Comp Beads were used. Cells and beads were acquired on a BD LSR Fortessa cytometer.

One million PBMCs per sample were used for B cell staining. Cell suspensions were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Madrid, Spain), washed, and blocked with 10 μg/ml mouse IgG (Jackson ImmunoResearch Laboratories, Suffolk, U.K.). After washing, cells were stained with the following Abs: anti-CD3 Horizon v500, anti-CD14 Horizon v500, anti-CD16 Horizon v500, anti-IgD PE-Cy7, anti-IgG PE, anti-IgM PE, anti-CD10 PE-Texas Red (electron-coupled dye) (Beckman Coulter), anti-CD19 Alexa Fluor 700, anti-CD21 eFluor 450 (eBiosciences, Hatfield, U.K.), anti-CD27 allophycocyanin, and anti-CD38 FITC. Fluorescence minus one controls were used for a better demarcation between CD10, CD21, and CD27 negative and positive events. Briefly, for each marker, one sample was stained containing all the Abs of the panel except that of the aforementioned marker. These samples were used to unequivocally determine the negative population for each staining experiment. Fig. 1 illustrates the flow cytometry gating strategy. Lymphocytes were gated using forward and side light scatter, and live B cells were

displayed according to $CD19^+$ expression and a dump channel containing a viability marker, CD3, CD14, and CD16. Mature viable B cells (VBC) were gated through a boolean gate containing live B cells and not $CD10^+$ cells. Displaying IgD versus CD38, VBC were further divided in IgD switched or unswitched populations, and plasma cells (IgD^-CD38^{high}) were excluded from further analysis. Switched and unswitched populations were displayed according to their expression of CD21 and CD27 to identify naive B cells ($IgD^+CD27^-CD21^+$), MZ-like MBC ($IgD^+CD27^+CD21^+$), active ($IgD^-CD27^+CD21^-$) and resting ($IgD^-CD27^+CD21^+$) classical MBC, and active ($IgD^-CD27^-CD21^-$) and resting ($IgD^-CD27^-CD21^+$) atypical MBC. Surface IgG or IgM expression was also analyzed. Similarly to a recent publication (24), and in contrast with previously published B cell panels identifying atypical MBC, our panel included IgD as a marker of isotype switching to distinguish naive B cells from the MBC population lacking CD27 (33). We have noted significant variations in subset phenotypes when including IgD and CD21 markers in the panel (J.J. Campo, I. Ubillos, P. Requena, D. Barrios, A. Jimenez, and C. Dobaño, unpublished observations). To facilitate understanding of data, we applied the classical MBC classifications of active ($CD21^-$) and resting ($CD21^+$) to the atypical MBC subsets. Every B cell subset was expressed as percentage of total VBC.

Additionally, PBMC samples from 45 EP women (PregVax) and 8 NNP donors were tested to determine the levels of transferrin receptor (CD71) and eotaxin chemokine receptor (CCR3) within the B cell subsets. For this analysis, PregVax samples taken only during recruitment were randomly selected to contain women with low to high plasma malaria IgG responses (see below). From half of a million to one million PBMCs per sample were used. Cell suspensions were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit. After washing, cells were stained with the following Abs: anti-IgD allophycocyanin/H7, anti-CD10 BV421 (BioLegend), anti-CD19 PE/CF594, anti-CD21 FITC (Beckman Coulter), anti-CD27 allophycocyanin, anti-CD38 PerCP, anti-CD71 PE/Cy7 (eBioscience), and anti-CCR3 PE. The IgD^+, $CD19^+$, $CD10^+$, $CD21^+$, $CD27^+$, $CD71^+$, and $CCR3^+$ populations were determined by the same fluorescence minus one criteria, and the same gating strategy was used to define the different B cell subsets (Fig. 1). Percentage and geometric mean fluorescence intensity (MFI) of CD71 and CCR3 were calculated for each B cell population. Data were analyzed using FlowJo software (Tree Star).

Quantification of Abs and cytokines

Plasmodium Ags included in this study were as follows: $PfMSP\text{-}1_{19}$ (34), PfAMA-1 (35), PfEBA175 (PfF2) (36), DBL3X, DBL5ε, DBL6ε (37), Pv200L ($PvMSP1_{121–416}$) (38), $PvMSP\text{-}1_{19}$ (39), PvCSP-N, PvCSP-C, PvCSP-R (40), full-length PvCSP, full-length PvMSP-5 (41), PvDBP (RII) (42), PvLP1, and PvLP2 (43). In addition, three *P. vivax* vir genes were expressed using the cell-free Wheat Germ system (41) (P. Requena, E. Rui, N. Padilla, F.E. Martínez-Espinosa, M.E. Castellanos, C.G. Bôtto-Menezes, A. Malheiro, M. Arévalo-Herrera, S. Kochar, S.K. Kochar, et al. manuscript in preparation; C. Fernandez-Becerra, M. Bernabeu, E. Rui, B. Correia, A. Castellanos, M. Ramirez, M. Ferrer, R. Thomson, F. Hentzchel, M. Lopez, et al., manuscript in preparation). Due to Ag-coupled beads limitation, the measurement of IgG Ab levels was done in the first 105 samples analyzed, corresponding to the NNP, NP, and EP groups, using Luminex technology with a panel developed in-house (P. Requena et al., manuscript in preparation). Briefly, microspheres with unique fluorescent spectral signatures using xMAP technology were covalently coupled with *Plasmodium* Ags, and ~1000 beads per analyte were incubated with each plasma sample (dilution 1:100) in duplicates, and subsequently with anti-human IgG-biotin (Sigma-Aldrich), followed by streptavidin-conjugated R-PE (Fluka, Madrid, Spain). Beads were analyzed on the BioPlex100 system (Bio-Rad, Hercules, CA), and results were expressed as median fluorescence intensity. Value against GST alone was subtracted for Ags bearing a GST tag.

Total plasma IgM and IgG concentrations were assessed using ELISA kits (eBioscience). Plasma cytokine concentration was quantified in a selection of samples using the Cytokine Magnetic 30-Plex Panel (Life Technologies) multiplex suspension detection system, which allows the detection of IFN-α, IFN-γ, TNF, IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, G-CSF, GM-CSF, eotaxin, IFN-γ–inducible protein-10, MCP-1, monokine induced by IFN-γ, MIP-1α, MIP-1β, RANTES, epidermal growth factor, basic fibroblast growth factor, hepatocyte growth factor, and vascular endothelial growth factor. A total of 50 μL plasma was used, and assays were conducted according to manufacturer's instructions. Pro–TGF-β was measured using an ELISA kit (R&D Systems, Abingdon, U.K.).

Plasmodium spp. *detection by PCR*

Plasmodium species molecular detection by PCR methods was performed at the Istituto Superiore di Sanità (Rome, Italy) or at the Institute of Medical Research (Madang, PNG). The protocol followed at Istituto Superiore di Sanità has been previously described (44). Briefly, DNA was extracted from dried blood spots following manufacturer's instructions (PureLink Genomic DNA Kits; Invitrogen) and eluted in 150 μl elution buffer. *P. vivax* and *P. falciparum* parasites were detected using species-specific primers and probes and LightCycler 480 Instrument. The PCR was composed of 1 cycle at 95°C 10 min; 50 cycles at 95°C 10 s, 50°C 20 s, and 72°C 5 s; and 1 cycle at 40°C 1 min. The threshold for positivity for each species was established as cycle threshold < 45, according to negative controls. At PNG-Institute of Medical Research, PCRs were done as previously described (45). Briefly, DNA was extracted from erythrocyte pellets using QIAamp96 DNA Blood Mini Kit (Qiagen, Valencia, CA) and eluted in 200 μl dH_2O. Amplification and detection of the template DNA were performed in an iQcycling system using iQSupermix (Bio-Rad). The thermal profile used was 2 min at 50°C, followed by 10 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 58°C. The threshold for positivity for each species was established as cycle threshold < 40, according to negative controls.

Statistical analysis

Quantitative variables for cell population frequencies, cellular marker MFI values, Ab levels (median fluorescence intensity), cytokine concentrations (pg/ml), and age (years) were summarized using the arithmetic or geometric means and their SD or 95% confidence interval (CI), or with the medians and interquartile ranges, and were compared between groups using ANOVA or Kruskal-Wallis test. Qualitative characteristics for *Plasmodium* infection rates (positive/negative), gestational age (≤12 wk, 13–24 wk, ≥25 wk), and gravidity (number of previous gestations: 0, 1–3, ≥4) were described with absolute frequency and percentage and compared between groups by means with the χ^2 or Fisher's exact tests. B cell variables were log transformed in those cases in which a more Gaussian distribution of residuals was required.

Differences on B cell frequencies and surface IgG MFI levels between the four study groups (NNP, NP, ENP, and EP) were analyzed using linear regression models adjusted by age (as the median age differed between groups). To investigate which pregnancy parameters influenced B cell percentages and IgG levels in the EP group, we estimated simple and multiple regression models with the following independent variables: age, time point (enrollment and delivery), gestational age, number of previous gestations, and *P. vivax* and *P. falciparum* infections (both defined as a positive slide and/or PCR). To analyze the association of B cell frequencies and IgG levels with delivery outcomes (Hb concentration and birth weight), simple and multiple linear regression models were fit adjusting for age, gestational age, number of previous gestations, *P. vivax* infection, *P. falciparum* infection, and Hb at recruitment when analyzing Hb at delivery. Differences in surface IgG and CD71 MFI levels between the VBC and active atypical MBC populations were assessed with the Wilcoxon signed-rank test.

Correlations between B cell frequencies and Abs or cytokines were measured with the Spearman's rank correlation coefficient. The p values were corrected for multiple comparisons (133 comparison B cells–Abs; 180 comparisons B cells–cytokines) using the Benjamini-Hochberg method. Plasma eotaxin and IL-8 concentrations were further studied due to a significant and relatively strong correlation (rho $> |0.35|$) with key B cell subsets and/or biological interest/novelty. Differences in eotaxin and IL-8 concentrations across the four study groups were assessed using linear regression models adjusted by age. As eotaxin concentrations were affected by malaria exposure, the correlations between eotaxin and malaria IgGs were also assessed and p values were adjusted together with the p values of the correlation of B cells with malaria Abs (133 comparisons). Differences in the $CCR3^+$ percentage and MFI levels between the different B cell subsets were assessed with the Friedman test, with Dunn post hoc test to assess comparisons between active atypical MBC and the other subsets. Analyses and figures were performed using Stata (StataCorp, 2013, Stata: Release 13; Statistical Software, College Station, TX: StataCorp LP) or GraphPad Prism (La Jolla, CA).

Results

Study population

PBMCs from 21 nonpregnant and 24 pregnant individuals from Spain (malaria-free country) and from 38 nonpregnant and 102 pregnant women from PNG (high malaria transmission area) were

analyzed. Participants were grouped as follows: 1) NNP; 2) NP; 3) ENP; and 4) EP. Five samples in the NP group and two samples in the EP group were discarded because of low viability (<70%). In addition, seven samples from the EP group were not included in the analysis as the age of the donors could not be established. Final study sample size was as follows: NNP, $n = 21$; NP, $n = 19$; ENP, $n = 38$; EP, $n = 93$. The median age differed between groups: the NNP group was the oldest (median [interquartile ranges] = 36 [14] y), followed by the NP (31 [7]), ENP (28 [19]), and EP (26 [6]) groups (Kruskal-Wallis test, $p < 0.001$). Thus, the comparison between the four groups was always adjusted by age. Both NP and EP groups included samples obtained at recruitment and delivery (never paired), and no significant differences in gestational age were found between the two groups (data not shown). *P. falciparum* infection rates did not differ between ENP (8%) and EP (6%) groups (Fisher's exact test, $p = 0.687$), but *P. vivax* prevalence was higher in the EP (13%) group compared with ENP (0%) ($p = 0.033$). Low sample size may have resulted in lack of power to detect differences on *P. falciparum* infection rates between ENP and EP groups. Fig. 1 displays the gating strategy for each B cell subset.

Plasmodium *exposure and pregnancy effects on B cell subpopulations*

To analyze the individual and combined impacts of high malaria exposure and pregnancy in B cell subpopulation distributions, the proportion of different B cell subsets was assessed in the four study groups described above (Fig. 2A). It should be noted that the different B cell subset percentages were calculated as a proportion of total VBC; thus, changes in one population will be perceived as altered percentages in other subsets, without the latter demonstrating an absolute change. We observed a higher percentage of active atypical MBC ($IgD^-CD27^-CD21^-$) in malaria-exposed compared with nonexposed donors, in both nonpregnant (ENP vs NNP) and pregnant (EP vs NP) groups (Fig. 2B). Also, an expansion of the resting atypical subset ($IgD^-CD27^-CD21^+$) was observed in malaria-exposed women and a slight increase of active classical MBC ($IgD^-CD27^+CD21^-$) in the ENP compared with the NNP group (Fig. 2B). This was accompanied by a decrease in MZ-like MBC ($IgD^+CD27^+CD21^+$) (Fig. 2B) in malaria-exposed women. IgM was not included in the cytometry panel, but IgM expression within the MZ-like MBC gate was analyzed in a separate experiment of 12 samples; as expected, in this gate 80% of the cells expressed IgM compared with 2% in the classical MBC subset.

To assess the effect of pregnancy, the differences in NNP versus NP and ENP versus EP were studied. Interestingly, when NP were compared with NNP, a significant expansion of all subsets of IgD^- MBC (Fig. 2B) and of VBC (data not shown), and a borderline significant decrease of naive B cells (overall age-adjusted difference between the four study groups, $p = 0.053$; NNP vs NP age-adjusted effect = 1.32, 95% CI = 1.04–1.69, $p < 0.05$) was observed (Fig. 2B). In the malaria-exposed cohort (ENP vs EP), the differences were only maintained in VBC (data not shown), active classical MBC, and active atypical MBC (Fig. 2B), although the latter did not reach statistical significance (ENP vs EP age-adjusted effect = 0.76, 95% CI = 0.56–1.04, $p > 0.05$).

These findings suggest that pregnancy, independently of malaria exposure, promotes maturation of B cells together with an expansion of IgD^- MBC and/or a decrease of naive B cells, whereas malaria exposure results in a further expansion of atypical MBC and a decrease of MZ-like MBC.

Association between B cell levels and pregnancy variables and outcomes

To identify associations between B cell subsets and parity, age, gestational age, and *P. vivax* infection, and to assess associations with *P. falciparum* infection, we analyzed the association between B cell subsets and pregnancy variables in the EP group (Table I displays the characteristics of this cohort). Univariate analysis showed a significant expansion of naive B cells and decrease of resting MBC and MZ-like MBC at delivery compared with recruitment (Fig. 3A). In addition, women with a *P. falciparum* infection at the bleeding time had lower levels of resting classical MBC (Fig. 3B). Age, gestational age, and *P. vivax* infection were not associated with any of the B cell subset percentages, whereas gravidity had a significant, but weak positive effect on the levels of VBC and resting atypical MBC (data not shown). Adjusted analysis provided the same associations (data not shown).

Next, we asked whether the changes in B cells could have an impact on poor delivery outcomes, such as anemia or low birth weight. Thus, we evaluated the association between B cell levels

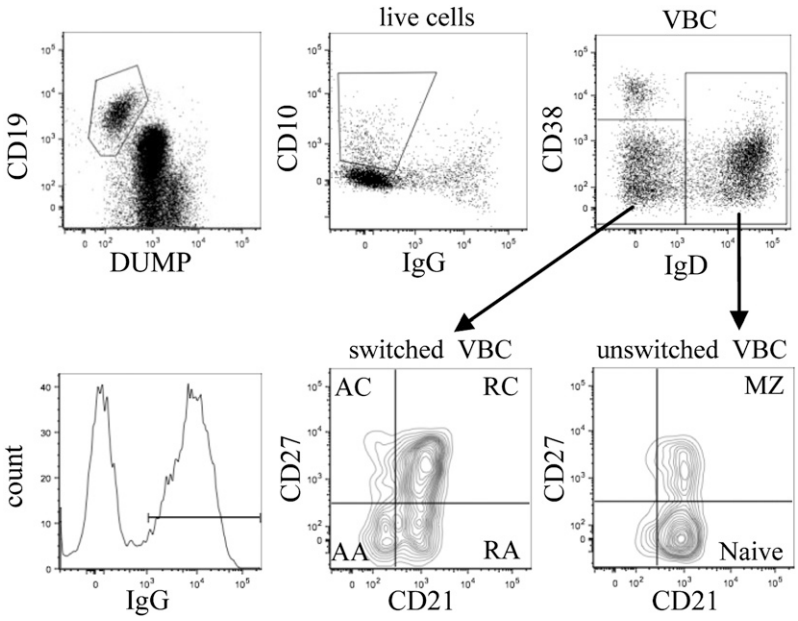

FIGURE 1. Gating strategy. After exclusion of debris and doublets, lymphocytes were displayed according to CD19 expression and a dump channel containing a viability marker, CD3, CD14, and CD16. Live $CD19^+$ B cells were displayed according to CD10 and IgG and VBC were gated through a boolean gate containing live and not $CD10^+$ cells. VBC were further divided in IgD switched or unswitched populations, and plasma cells ($CD38^{high}$) were excluded from further analysis. Switched and unswitched populations were displayed according to their expression of CD21 and CD27 to identify naive B cells (Naive), MZ-like MBC (MZ), active classical MBC (AC), resting classical MBC (RC), active atypical MBC (AA), and resting atypical MBC (RA). Surface IgG expression was analyzed.

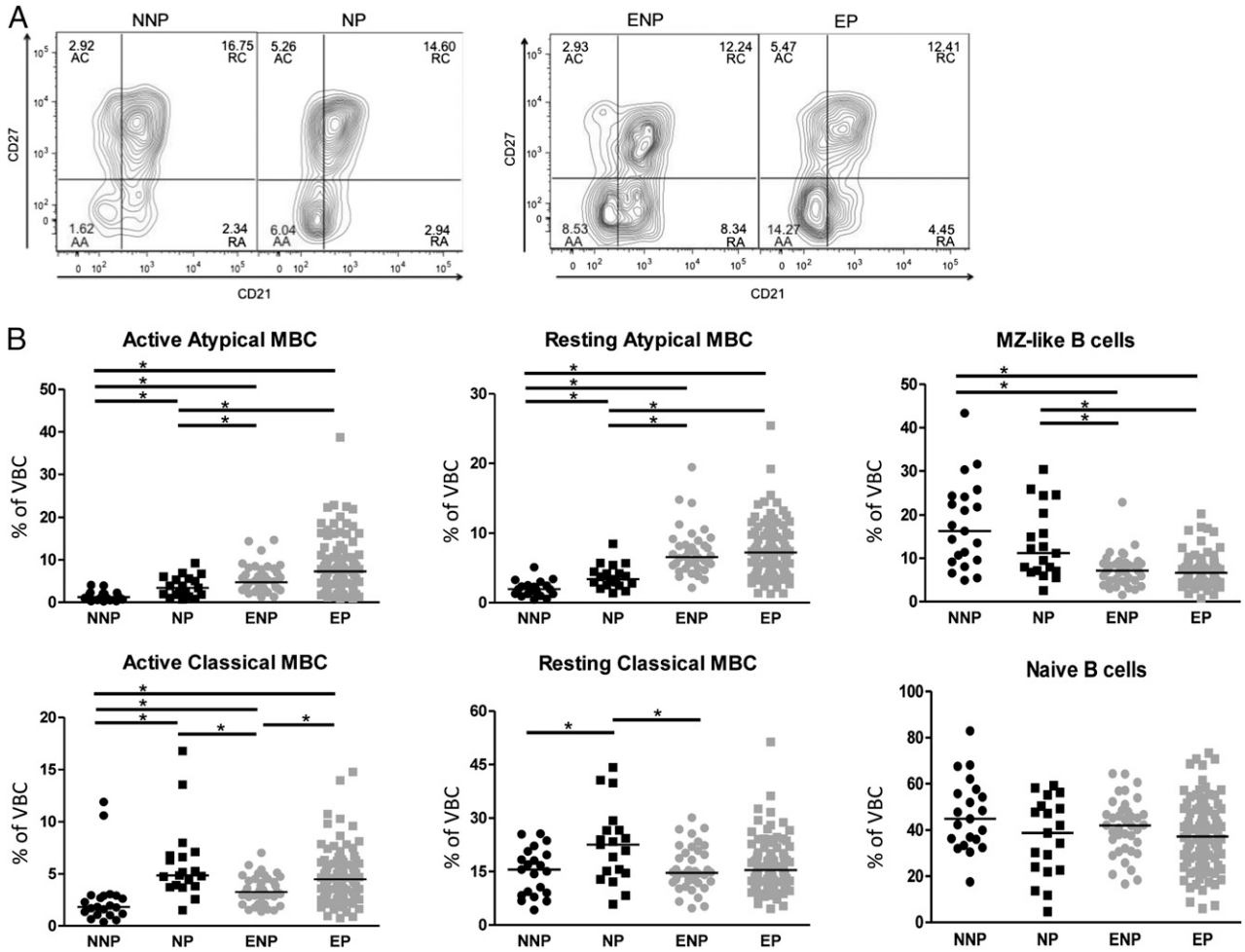

FIGURE 2. Malaria exposure and pregnancy are associated with changes in the distribution of different B cell subsets. Peripheral blood B cells were characterized by flow cytometry in the different groups, as follows: NNP (n = 21), NP (n = 19), ENP (n = 38), and EP (n = 93). (**A**) Representative contour plots of the IgD^- MBC subset distribution in the four groups are shown. Numbers indicate the percentage (%) of cells within each gate. (**B**) Dot plots show the median percentage of different B cell subsets in the four groups (*$p < 0.05$, age-adjusted log-normal regression estimation). AA, active atypical MBC; AC, active classical MBC; RA, resting atypical MBC; RC, resting classical MBC.

measured at recruitment or at delivery with delivery outcomes (Hb levels and birth weight) in the EP group. MZ-like MBC levels measured at recruitment were positively associated with Hb levels at delivery (unadjusted effect estimate on Hb levels [g/dL] per 2-fold increase in MZ-like MBC: 1.2 U [g/dL], 95% CI: 0.3; 2.1, p = 0.009, n = 34). This observation was maintained when the analysis was adjusted by age, gestational age, parity, Hb levels at recruitment, and *P. vivax* and *P. falciparum* infection. In addition, VBC measured at delivery had a negative association with Hb levels (unadjusted effect per 2-fold increase in VBC: −12.1 U (g/dL), 95% CI: −20.5; −3.6, p = 0.006, n = 46); however, adjusted effect was not statistically significant.

In summary, we show changes in the proportion of different B cell subsets at delivery, but not necessarily along pregnancy (no association with gestational age), and a decrease of resting classical MBC with *P. falciparum* infection. Only MZ-like MBC levels were associated with delivery outcomes.

Analysis of surface IgG levels

To better characterize atypical MBC functionality, the IgG surface expression in the different MBC subsets (Fig. 4A) and among the four study groups (Fig. 4B) was compared. When we analyzed the amount of IgG expression in the IgG^+ population (MFI), NP women presented the lowest values in all the MBC subsets, whereas IgG MFI levels in the EP group were as high as ENP and NNP groups, with the exception of active atypical MBC (Fig. 4C). Compared with NNP, malaria-exposed women had lower IgG expression in the active atypical MBC (Fig. 4B, 4C). Therefore, the IgG MFI levels on this subset were compared with the average IgG levels in the VBC population. Interestingly, in the malaria-

Table I. Characteristics of the EP cohort (n = 93)

Variable		Summary Statistics
Age[a]		25.74 (5.53)
n° previous gestations[b]	Primigravida	37 (40%)
	(1–3)	38 (41%)
	(4 or more)	18 (19%)
Gestational age[b]	0–12 wk	10 (11%)
	13–24 wk	22 (24%)
	25+ wk	61 (66%)
Present *P. vivax* infection[b]	Negative	82 (88%)
	Positive	11 (12%)
Present *P. falciparum* infection[b]	Negative	88 (95%)
	Positive	5 (5%)

[a]Arithmetic mean (SD).
[b]n (column percentage); positive refers to a smear and/or PCR-positive result.

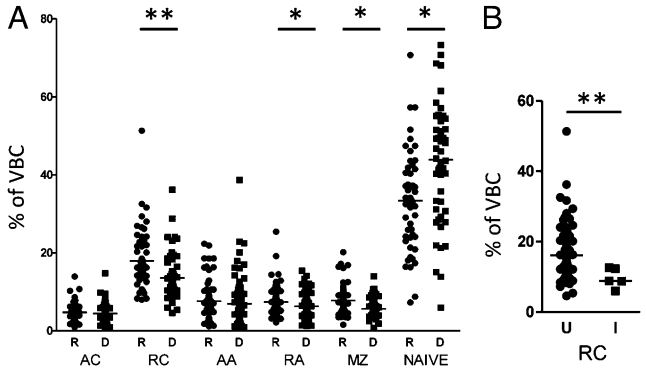

FIGURE 3. Time point of bleeding and infection is associated with changes in the levels of certain B cell subsets. (**A**) Dot plots show the median percentage of different B cell subsets at recruitment (R, $n = 46$) and at delivery (D, $n = 47$) in the EP group. (**B**) Dot plot shows the median percentage of resting classical MBC in uninfected women (U, $n = 88$) and *P. falciparum*–infected women (I, $n = 5$) in the EP group. Log-normal simple regression was estimated. $*p < 0.05$, $**p < 0.01$. AA, active atypical MBC; AC, active classical MBC; MZ, MZ-like MBC; NAIVE, naive B cells; RA, resting atypical MBC; RC, resting classical MBC.

exposed groups (and to a lower extent in the NP group), active atypical MBC presented much lower IgG levels than the B cell average (Fig. 4D). To investigate whether the lower surface IgG levels found in active atypical MBC were a consequence of IgG internalization after BCR engagement (46, 47), in another set of samples we studied the surface expression of transferring receptor (CD71), which is known to recycle constitutively between early endosomes and the cell surface (48). Conversely, higher CD71 expression was found in active atypical MBC compared with the average B cell levels (Fig. 4E).

Regression models were fit to estimate pregnancy factors influencing IgG expression, and decreased IgG MFI values in all IgD^- MBC subsets were found at delivery (Fig. 4F) and in women with a *P. falciparum* infection (Fig. 4G). Adjusted regression analysis showed the same results, except in the case of active atypical MBC in which the association with *P. falciparum* infection was not significant.

When the percentage of IgG positivity rather than MFI was analyzed, malaria exposure was associated with an increase in classical MBC IgG^+ percentages, whereas pregnancy did not have an impact (Fig. 5). In contrast, atypical MBC did not have higher IgG^+ frequencies in the exposed groups, but EP had lower proportions than ENP (Fig. 5). In the EP group, a negative association was found between the percentage of IgG^+ cells in all IgD^- MBC subsets and age (data not shown).

In addition, we investigated the association between surface IgG expression and delivery outcomes. A positive association between IgG MFI in all switched MBC at delivery and birth weight was observed (adjusted effect in birth weight [g] per 2-fold increase in IgG MFI, $n = 46$; active classical MBC effect: 325.78 U [g], 95% CI: 69.51; 582.05, $p = 0.014$; resting classical MBC effect: 285.89 U [g], 95% CI: 19.46; 552.33, $p = 0.036$; active atypical MBC effect: 369.86 U [g], 95% CI: 107.90; 631.82, $p = 0.007$; resting atypical MBC effect: 332.17 U [g], 95% CI: 78.39; 585.96, $p = 0.012$). Moreover, a positive association between the levels of surface IgG MFI in some MBC at delivery and Hb levels at delivery was observed (adjusted effect in Hb levels [g/dL] per 2-fold increase in IgG MFI, $n = 46$; active classical MBC effect: 1.12 U [g/dL], 95% CI: 0.15; 2.09, $p = 0.025$; active atypical MBC effect: 1.01, 95% CI: 0.00; 2.02, $p = 0.051$).

We also analyzed total IgG and IgM levels in plasma from EP women. No association between plasma Ig levels and infection was seen (data not shown). IgM plasma levels at delivery had a negative association with birth weight (data not shown), but this association was lost after adjusting for the potential cofounders (data not shown).

All together, these data indicate that atypical MBC have lower surface IgG levels than other MBC, and that IgG surface levels are regulated in all MBC subsets during pregnancy and in *P. falciparum* infections, showing positive associations with delivery outcomes such as birth weight and Hb levels.

Correlation of B cells with Plasmodium*-specific IgG Abs*

To further investigate the association between the changes observed in the malaria-exposed groups and *Plasmodium* exposure, the correlation between the levels of different B cell subsets and plasma IgG Ab responses to a total of 19 *P. vivax* and *P. falciparum* Ags was investigated. Most of the plasma IgGs analyzed are well-known markers of malaria exposure. A significant positive correlation between the levels of atypical MBC and plasma Abs to several *Plasmodium* Ags was seen, even after adjusting p values for multiple comparisons (Table II). Nonsignificant correlation with classical MBC (Table II) or naive B cells (data not shown) was observed, in agreement with the lack of (or weak) differences in the proportion of these B cell subsets between the exposed and nonexposed groups. A negative correlation between MZ-like MBC levels and Ab responses to several Ags was also observed (Table II).

Correlation between B cells and plasma cytokine and chemokine concentrations

To provide some insights into the immunological pathways involved in the altered distribution of B cells observed in pregnancy and after malaria exposure, the correlation between different B cells and cytokines, chemokines, and growth factors was assessed. In Table III, a heat map shows the Spearman's rho coefficients for each cellular subset and plasma cytokine/chemokine concentrations, with a scale of colors ranging between dark gray (Spearman's rho = 0.4) and white (rho = −0.4). As multiple comparisons were performed, p values were adjusted using the Benjamini-Hochberg method. The profiles of cytokines/chemokines that correlated with active and resting classical MBC were similar, whereas inverse profiles correlated with naive B cells. The profile of cytokines/chemokines associated with MZ-like MBC and atypical MBC did not cluster with that of naive or classical MBC, suggesting a different developmental origin (26). MZ-like MBC had a borderline significant positive correlation with RANTES (adjusted $p = 0.054$). Remarkably, active atypical MBC had a positive correlation with TNF and IL-8, but adjusted p value was only significant for IL-8 ($p = 0.002$) (Fig. 6A). As the correlation with IL-8 was moderately strong (rho = 0.38), we compared plasma IL-8 concentrations across the four study groups. Accordingly, malaria-exposed women had more plasma IL-8 then their nonexposed counterpart groups (Fig. 6B), but no effect of pregnancy was observed.

These data show patterns of cytokines associated with particular B cell subsets and highlight the association between active atypical MBC and proinflammatory cytokines.

Analysis of plasma eotaxin levels and CCR3 expression across study groups

The Spearman's test and heat map (Table III) were used as a first approach to find key cytokines/chemokines associated with different B cell subsets. In this regard, analyses showed that eotaxin (CCL11) could be involved in the changes occurring in the B cell subsets (as well as in T regulatory cells; P. Requena et al., manuscript in preparation) during pregnancy and malaria exposure, as

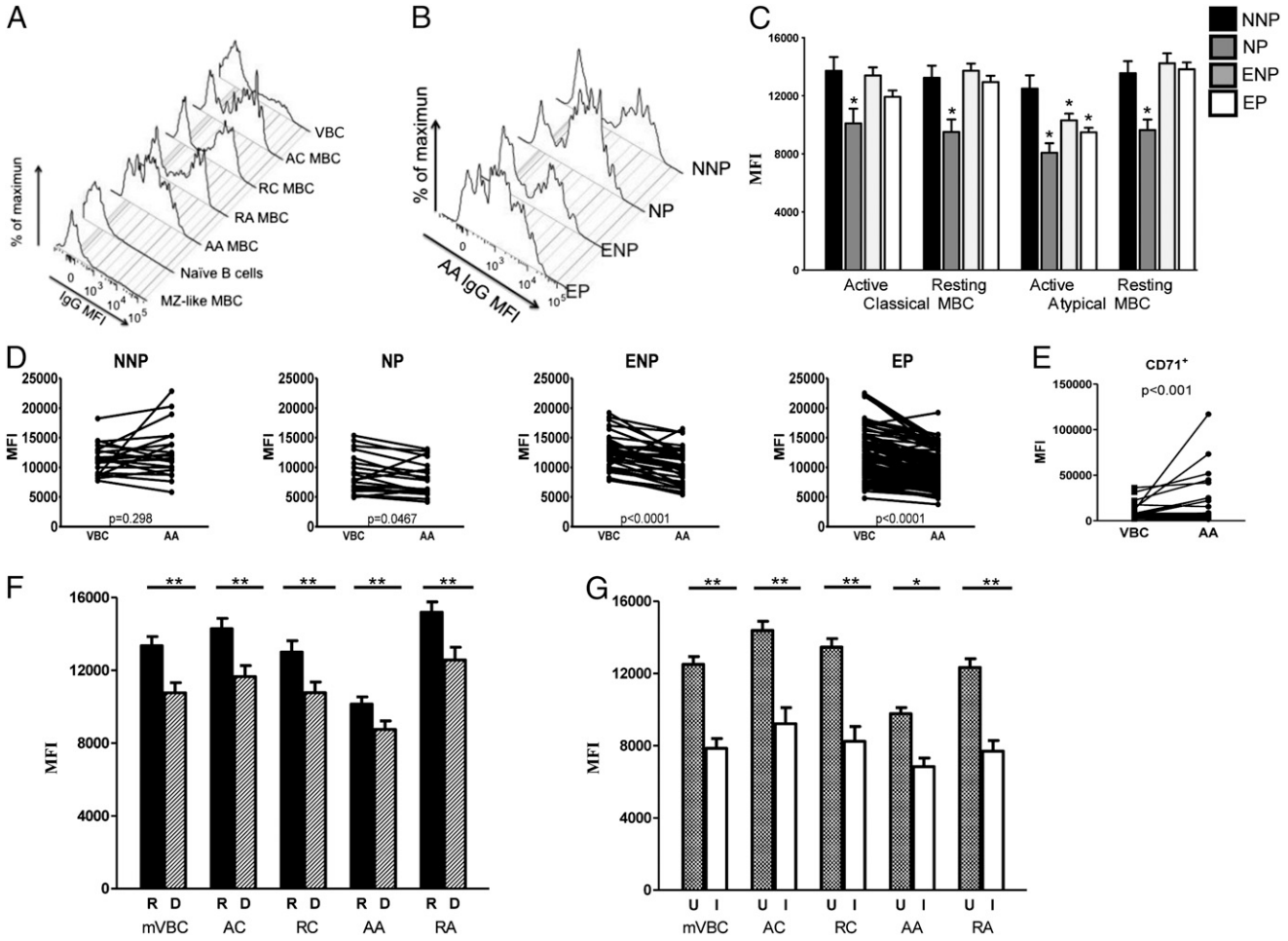

FIGURE 4. Characterization of IgG and CD71 fluorescence intensity by subset, study group, and infection status. Surface IgG MFI was analyzed in the IgG^+ fraction of peripheral blood B cells in the different groups, as follows: NNP (n = 21), NP (n = 19), ENP (n = 38), and EP (n = 93). Representative histograms of the IgG MFI values in different B cell subsets (**A**) and in the different study groups (**B**) are shown. (**C**) Bars represent mean + SEM of the surface IgG MFI. Age-adjusted log-normal regression models were estimated. $*p < 0.05$, for each MBC subset only depicted differences versus NNP. (**D**) Graphs depict IgG MFI levels for the VBC and active atypical MBC (AA) populations, within each group. P corresponds to the Wilcoxon signed-rank test. (**E**) Graph depicts CD71 MFI levels for the VBC and AA cell populations, in a different set of EP women samples (n = 45). P corresponds to the Wilcoxon signed-rank test. (**F**) Bars represent mean plus SEM of the surface IgG MFI in the EP group, stratifying by time point the following: recruitment (R, n = 46) and delivery (D, n = 47). (**G**) Bars represent mean + SEM of the surface IgG MFI in the EP group, stratifying by present *P. falciparum* infection status. U: uninfected women, n = 88; I: infected women, n = 5. In (F) and (G), p corresponds to a simple median regression model, $*p < 0.05$, $**p < 0.01$. AA, active atypical MBC; AC, active classical MBC; RA, resting atypical MBC; RC, resting classical MBC.

eotaxin plasma levels negatively correlated with resting atypical MBC (adjusted p = 0.001), active atypical MBC (adjusted p = 0.001), and active classical MBC (adjusted p = 0.018) (Fig. 7A, Table III). In addition, eotaxin presented a positive correlation with naive B cells, but, when p values were adjusted, the significance was lost. Therefore, the levels of plasma eotaxin were also compared across study groups; pregnant women had lower eotaxin plasma concentrations than nonpregnant adults in both malaria-naive and malaria-exposed cohorts (Fig. 7B). In addition, ENP had less eotaxin than NNP (Fig. 7B). Accordingly, eotaxin significantly and negatively correlated with 13 of 19 malaria IgG responses (only 4 of them remained significant after multiple comparison adjustment), including a moderate correlation with anti-$PvMSP1_{19}$ (Spearman's rho = −0.47, adjusted p = 0.002) and anti-PvMSP5 (rho = −0.44, adjusted p = 0.007).

To test the hypothesis that eotaxin could regulate the distribution of B cells, we analyzed the expression of the eotaxin receptor CCR3 in all B cell subsets in the NNP and EP groups. As there were no remaining PBMCs from the original samples, a different set of samples was used. The NNP group was older than the EP group (data not shown). As expected, the percentage of $CCR3^+$ cells in the VBC population was low, especially in the NNP group (Fig. 7C–E). In fact, very few $CCR3^+$ events were found in naive B cells, MZ-like MBC, and resting MBC. However, the percentage of CCR3 expression in active atypical MBC was higher than in the rest of B cell subsets, except active classical MBC, which had very similar levels (Fig. 7C–E). This finding was observed in both NNP and EP groups. Similarly, when we compared the amount of CCR3 expression (MFI), we found higher values in active atypical MBC compared with the rest of subsets in the EP group (Fig. 7C, 7D, 7F). Although mean differences were similar in the NNP group, they did not reach statistical significance in some subsets. No differences in the percentage or MFI of $CCR3^+$ cells were found between NNP and EP in any cell subsets (data not shown). Lack of power due to limited sample numbers could have prevented us from finding statistical differences.

Discussion

We characterized the effect of malaria exposure/infection and pregnancy in the levels of B cell subsets. Our results add to the

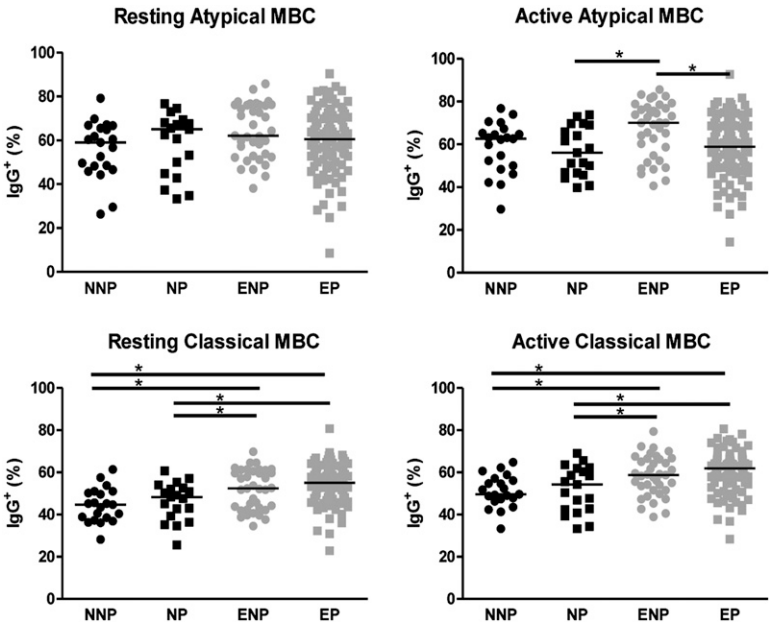

FIGURE 5. Malaria exposure and pregnancy are associated with changes in the percentages of IgG^+ of different B cell subsets. Dot plots show the median percentage of IgG^+ cells within different MBC subsets in the four groups (*$p < 0.05$, age-adjusted log-normal regression estimation), as follows: NNP ($n = 21$), NP ($n = 19$), ENP ($n = 38$), and EP ($n = 93$).

evidence of malaria-driven expansion of atypical MBC (21–24), as shown by the consistent correlation with malaria-specific IgGs in plasma. Interestingly, in this study, to our knowledge, we show for the first time that exposure to *P. vivax* may drive an expansion of atypical MBC similar to *P. falciparum.* As recently reported (16), we show that expansion of atypical MBC in high malaria trans-

Table II. Correlation between MBC and Ab responses to *Plasmodium* Ags

	Active Atypical MBC		Resting Atypical MBC		Active Classical MBC		Resting Classical MBC		MZ-Like MBC	
Ag	rho	*p*	rho	*p*	rho	*p*	rho	*p*	rho	*p*
PvCSP	**0.51**	**<0.001**	**0.40**	**0.003**	0.33	0.053	0.13	0.937	-0.28	0.363
PvCSP-C	0.18	0.937	0.03	0.937	0.15	0.937	-0.04	0.937	-0.28	0.363
PvCSP-N	**0.39**	**0.004**	0.20	0.937	0.18	0.937	-0.09	0.937	-0.30	0.172
PvCSP-R	-0.04	0.937	-0.25	0.851	0.08	0.937	-0.06	0.937	-0.14	0.937
PvDBP	**0.36**	**0.018**	0.28	0.350	0.27	0.483	-0.03	0.937	**-0.43**	**0.001**
PvMSP1-19	**0.37**	**0.018**	**0.39**	**0.009**	0.24	0.929	-0.02	0.937	**-0.45**	**<0.001**
Pv200L	**0.45**	**<0.001**	**0.48**	**<0.001**	0.21	0.937	0.07	0.937	-0.21	0.937
PvMSP5	**0.41**	**0.002**	**0.44**	**<0.001**	0.29	0.261	0.09	0.937	-0.28	0.331
vir25-related	0.11	0.937	0.03	0.937	0.09	0.937	-0.06	0.937	-0.11	0.937
Vir14-related	0.22	0.937	0.27	0.490	0.05	0.937	-0.06	0.937	-0.19	0.937
vir2/15-like	0.09	0.937	0.24	0.937	-0.10	0.937	-0.09	0.937	-0.14	0.937
LP1	0.28	0.320	0.20	0.937	0.11	0.937	-0.06	0.937	-0.18	0.937
LP2	**0.40**	**0.004**	**0.43**	**0.001**	0.11	0.937	0.01	0.937	-0.23	0.937
PfAMA-1	0.19	0.937	0.33	0.070	-0.02	0.937	-0.03	0.937	-0.13	0.937
PfDBL3x	**0.49**	**<0.001**	0.27	0.468	0.29	0.261	-0.12	0.937	-0.23	0.937
PfDBL5ε	**0.47**	**<0.001**	0.32	0.077	0.22	0.937	-0.11	0.937	-0.21	0.937
PfDBL6ε	**0.53**	**<0.001**	**0.38**	**0.009**	0.31	0.118	-0.08	0.937	**-0.36**	**0.018**
PfEBA-175	**0.38**	**0.009**	**0.42**	**0.001**	0.19	0.937	0.08	0.937	-0.13	0.937
PfMSP1-19	**0.58**	**<0.001**	**0.58**	**<0.001**	0.25	0.780	0.07	0.937	-0.29	0.251

Spearman's correlation test. rho: Spearman's coefficient ranges between 0 and |1|. The *p* values were adjusted using the Benjamin-Hochberg method. Samples included all groups except malaria-exposed nonpregnant women. Number of observations = 102. Bold indicates $p < 0.05$.

	0-|0.19| "very weak"
	|0.20|-|0.39| "weak"
	|0.40|-|0.59| "moderate"

Table III. Correlation between B cells and cytokines/chemokines

	Naive[a]	RC	AC	MZ-like[b]	RA	AA
EOTAXIN	0.22	-0.20	-0.35	0.19	-0.39	-0.40
G-CSF	0.22	-0.26	-0.09	-0.24	-0.06	0.01
IL-2R	0.21	-0.27	-0.17	-0.22	-0.09	-0.05
IL-12	0.21	-0.30	-0.27	-0.17	-0.07	-0.08
MCP-1	0.16	-0.23	-0.12	0.03	-0.35	-0.18
IL-13	0.11	-0.18	-0.16	-0.06	0.02	-0.01
IP-10	0.10	-0.26	-0.05	-0.15	-0.03	0.04
IL-17	0.09	0.07	-0.09	0.10	-0.07	-0.17
IFN-α	0.08	-0.21	-0.05	-0.20	0.16	0.12
IFN-γ	0.06	-0.09	-0.18	0.04	0.07	-0.03
IL-5	0.05	-0.05	-0.10	0.02	0.11	0.06
MIG	0.04	-0.20	0.00	-0.23	0.17	0.15
IL-4	0.04	-0.19	0.01	-0.24	0.19	0.16
IL-1RA	0.03	0.02	0.05	0.04	-0.05	0.02
IL-15	0.02	-0.21	0.02	-0.16	-0.08	0.10
IL-7	0.01	-0.04	-0.07	-0.08	0.03	0.03
HGF	-0.01	-0.18	0.15	-0.12	0.04	0.21
VEGF	-0.01	-0.17	0.08	-0.31	0.21	0.25
TNF	-0.02	-0.15	0.13	-0.21	0.11	0.27
IL-6	-0.03	-0.07	0.18	-0.19	-0.08	0.10
GM-CSF	-0.03	-0.06	0.03	0.02	0.00	-0.10
IL-1β	-0.04	-0.18	-0.02	-0.08	-0.02	0.06
FGF	-0.05	-0.01	0.07	-0.01	0.05	0.01
RANTES	-0.07	0.16	-0.09	0.32	-0.25	-0.24
MIP-1α	-0.07	-0.06	-0.01	0.05	0.04	0.06
IL-10	-0.09	-0.07	0.22	-0.09	0.05	0.18
IL-2	-0.12	-0.04	0.20	-0.08	0.02	0.16
EGF	-0.16	-0.01	0.12	0.02	0.02	0.12
TGF-β	-0.21	0.14	0.23	0.18	0.06	0.21
IL-8	-0.21	0.05	0.25	-0.04	0.21	0.38
MIP-1β	-0.23	0.09	0.13	0.11	0.04	0.16

Spearman's correlation coefficient is displayed in the cells. The color scale ranges between the dark gray (Spearman's rho = 0.4) and white (rho = −0.4). Samples included belonged to the four groups (malaria-naive pregnant and nonpregnant, and malaria-exposed pregnant and nonpregnant). $n = 125$.

[a]Naive B cells.

[b]MZ-like MBC.

AA, active atypical MBC; AC, active classical MBC; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IP-10, IFN-γ–inducible protein-10; MIG, monokine induced by IFN-γ; RA, resting atypical MBC; RC, resting classical MBC; VEGF, vascular endothelial growth factor.

mission areas is also observed during pregnancy, a period of clear immunological changes. In addition, we report in this work two distinct populations of atypical MBC, as follows: active ($CD21^-$) and resting ($CD21^+$) atypical MBC, following the nomenclature of classical MBC. Both subsets were highly expanded in malaria-exposed donors. The profiles of cytokines correlating with both subsets were very similar, and, likewise, the correlation profiles with malaria IgG responses, suggesting that they comprise a singular subpopulation. The fact that active atypical MBC presented a higher correlation with proinflammatory cytokines, and expressed more CCR3 and less IgG on the surface than their resting counterparts, suggests that they indeed have a more activated phenotype. However, functional analyses and finer phenotyping are necessary to demonstrate this hypothesis. Although the approach of correlating *Plasmodium*-specific IgGs as markers of malaria exposure with cellular frequencies is an adequate strategy, we cannot rule out other causative factors. However, HIV can be discarded due to its low prevalence in PNG, as follows: 0.8% in 2010 (49). Helminth infection rates are known to be high in the area (50), but acute infections do not seem to affect the levels of atypical MBC (21). In addition, a very recent manuscript demonstrated that malaria infection is followed up by an expansion of this B cell subset (24).

Nevertheless, as shown before (16, 21), we did not find any association between the expansion of atypical MBC and malaria infections. We also found no associations between atypical MBC and poor pregnancy outcomes. This is in agreement with the finding that atypical MBC can produce regular amounts of functional IgG (25, 26). However, the increased levels of atypical MBC after malaria exposure might have an impact on other diseases beyond malaria due to a potential role in innate immunity. Malaria exposure induces atypical MBC to express the inhibitory receptor Fc receptor-like protein (FcRL) 4 (21, 26). FcRL4 dampens BCR activation, but enhances TLR9 signaling, favoring a switch from adaptive to innate B cell signaling (51). Moreover, a recent study has shown that tissue-$FcRL4^+$ B cells produce proinflammatory cytokines like IL-6, TNF, and receptor activator for NF-κB ligand in rheumatoid arthritis (52). We have shown a good correlation of active typical MBC with proinflammatory IL-8 and TNF, higher IL-8 plasma levels in exposed versus unexposed donors, and higher TNF levels in EP versus NP (data not shown). All these data suggest that active atypical MBC, which are greatly expanded in highly malaria-exposed individuals, might produce proinflammatory cytokines. Although this hypothesis must yet be proven, the implications in a high proinflammatory disease as malaria will be important. In contrast, the good correlation with IL-8 and TNF might signify that inflammation occurring in malaria and expansion of active atypical MBC are processes running somehow together.

A potential role of active atypical MBC as a special class of short-lived plasma cells has also been proposed (25, 26). Consistent with this lineage, in which surface Ig expression is downregulated in favor of transition to secretory Ig (53), we did observe a lower amount of surface IgG (MFI levels) in active atypical MBC compared with the mean IgG levels in B cells in the NP, ENP, and EP groups. Nevertheless, decreased IgG levels may also result from Ig (BCR) internalization after B cell activation (46, 47). Thus, active atypical MBC may recognize and internalize Ags at a higher rate than other B cells, at least in conditions of persistent B cell priming. However, we observed higher levels of CD71 in active atypical MBC. This receptor has been used as a marker of B cell endocytosis after BCR engagement by others (48). Thus, a transcriptomic regulation of IgG in active atypical MBC seems more likely. Interestingly, malaria-naive pregnant women presented lower IgG surface levels in all MBC subsets than the other groups, although the levels in the active atypical subset were the lowest. This finding reported in this work for the first time, to our knowledge, deserves further attention in the context of pregnancy. It is currently accepted that fetal Ags are actively recognized by maternal cells (54); therefore, lower IgG levels might be a result of BCR internalization after persistent B cell priming.

Surface IgG levels in all MBC subsets and plasma IgM were associated with *P. falciparum* infection, although these data should be interpreted cautiously due to the low number of *P. falciparum*–infected women in our study. Moreover, in some MBC subsets, IgG MFI showed an association with Hb levels at delivery and birth weight. During malaria infections, naive B cells produce IgM (55) and soluble Ags engage the BCR, resulting in IgG internalization (46, 47), explaining the association observed with *P. falciparum* infection and, consequently, poor delivery outcomes. This finding must be confirmed with a longitudinal study with more malaria cases, including analysis of IgG expression at the protein

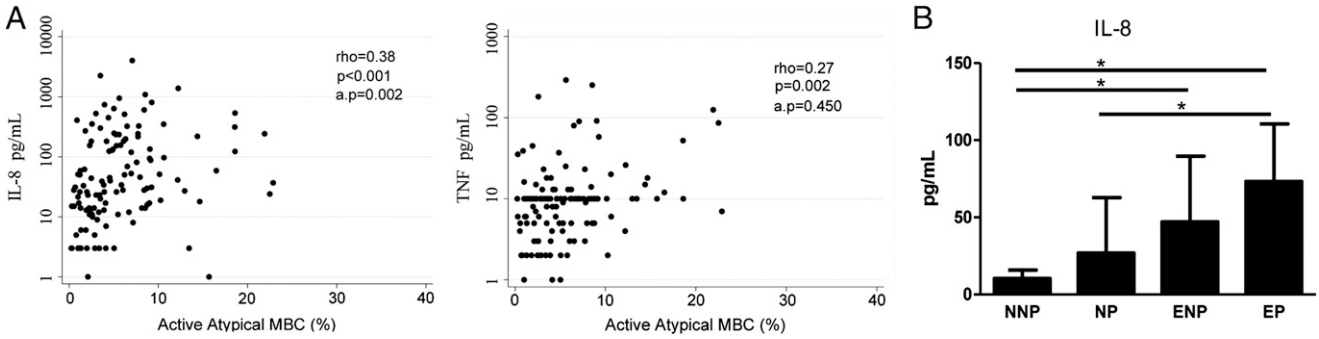

FIGURE 6. Proinflammatory cytokines correlate with active atypical MBC and are increased in malaria-exposed women. (**A**) Scatter plots show the distribution of values for active atypical memory B cells (%) and proinflammatory cytokine plasma concentration (IL-8 and TNF) in the four groups (n = 125). rho: Spearman's coefficient; p corresponds to Spearman's correlation test; a.p corresponds to the adjusted p value after correcting for multiple comparisons using the Benjamini-Hochberg method. (**B**) Bars represent geometric mean + 95% CI of plasma IL-8 in the four study groups, as follows: NNP (n = 23), NP (n = 13), ENP (n = 38), and EP (n = 69). Age-adjusted median regression models were estimated, and effects were assessed comparing the four groups (*$p < 0.05$).

and mRNA levels. However, an association between *P. vivax* infection and levels of surface IgG was not observed. Parasite rates, different cell hosts, and accumulation of *P. falciparum* in the placenta may influence the way Ags of both species are recognized by MBC in pregnancy.

A decrease in MZ-like MBC percentages was also associated with malaria exposure in pregnant and nonpregnant women, as previously described in Kenyan children (29). Some studies have shown a strong correlation between the loss of IgM^+ MBC and reduced immune responses to pneumococcal polysaccharides, which might increase the risk of invasive pneumococcal diseases (30, 56, 57). Thus, reduced levels of MZ-like MBC might explain the well-established impaired Ab responses to heterologous polysaccharide Ags associated with malaria, at least in children (58). In adults and pregnant women, the malaria-driven reduction of MZ-like MBC might increase the risk of invasive encapsulated bacterial infections, although this association must yet be proven. In this regard, we found a positive association between the levels of MZ-like MBC at recruitment and Hb levels at delivery, which suggests a protective role of this subset from poor delivery outcomes. However, we could not prove whether this association was a consequence of a protection against pneumococcal infectious disease. In addition, resting classical MBC were reduced in *P. falciparum*–infected pregnant women, in accordance with a migration of this subset to lymph nodes.

Pregnancy, independently of malaria exposure, had a marked effect in the peripheral distribution of almost all the cellular subsets studied. Globally, we observed an expansion of IgD^- MBC and a nonsignificant decrease of naive B cells and MZ-like MBC in NP compared with NNP. Similarly, a recent publication reported increased atypical MBC and a borderline significant decrease of naive B cells during pregnancy in a malaria-exposed cohort (16), although the cell populations defined in that study differed in resolution from those of our study. The expansion of IgD^- MBC may be necessary to produce enough IgGs to be transferred to the fetus through the placenta. Although we show some associations between certain B cells and poor delivery outcomes, a larger study in malaria-free populations should be conducted to determine whether specific MBC subsets are associated with pregnancy pathologies.

The correlation between eotaxin, B cell, and T regulatory cell levels (P. Requena et al., manuscript in preparation) suggests an association between this chemokine and these subsets, at least during pregnancy, but, to our knowledge, this relation has not been previously established. Eotaxin recruits eosinophils to different tissues through interaction with CCR3. B cells do not express CCR3 regularly, but they do it under the influence of IL-2 and IL-4, and eotaxin induces apoptosis in B cells (59). To test the hypothesis that eotaxin exerts chemoattractive effect on B cells, we analyzed CCR3 levels in the B cell subsets. As expected, CCR3 expression was low in B cells, but active atypical MBC and, to a lesser extent, active classical MBC had higher percentages and expression levels of this chemokine receptor than the other B cell subsets, suggesting that this chemokine could be involved in these two B cell subset migrations to tissues.

Plasma eotaxin levels were noticeably decreased in pregnancy (NP vs NNP and EP vs ENP), in agreement with previous reports (60), which further suggest a role of this chemokine in pregnancy. CCR3 is present in the placenta, and interaction with eotaxin-2 (CCL24) seems to benefit a process called decidualization (61), which is essential in early pregnancy. Thus, a reduction of peripheral eotaxin might favor the interaction of eotaxin-2 with CCR3 by decreasing competition, at least in the first trimester of pregnancy. Indeed, eotaxin levels increased at delivery (data not shown). Thus, lower levels of eotaxin could prevent active MBC from localizing to specific lymphoid tissues, resulting in increased levels in periphery.

Interestingly, eotaxin may also be associated with the malaria-specific expansion of active atypical MBCs. Plasma eotaxin concentration was lower in ENP compared with NNP; no differences were observed between EP and NP, probably because the levels were already low in both groups. The lower levels found in PNG were somewhat unexpected considering the high helminth infection rate in the study area (50). In contrast, it suggests that the pressure of Th1 responses (as those induced by *Plasmodium*) is higher in this area, hence the significant negative correlation of eotaxin with four malaria-specific Abs. Of note, Scholzen et al. (24) recently reported that the expansion of atypical MBC (with the same phenotype as active atypical MBC reported in this work) after a malaria infection is more likely to depend on chemotactic redistribution of B cells rather than on B cell proliferation, with a potential role for BAFF enhancing the chemotactic effect. Whether BAFF has the potential to enhance B cell chemotaxis to eotaxin as it does for other chemokines (62) is unknown; however, our results point to a similar chemotactic mechanism. Finally, the ultimate pathway resulting in a decrease of eotaxin in pregnancy and in malaria must be investigated.

In conclusion, we demonstrate that both human pregnancy and malaria exposure trigger important changes in peripheral B cell distributions. As new characteristics of atypical MBC in

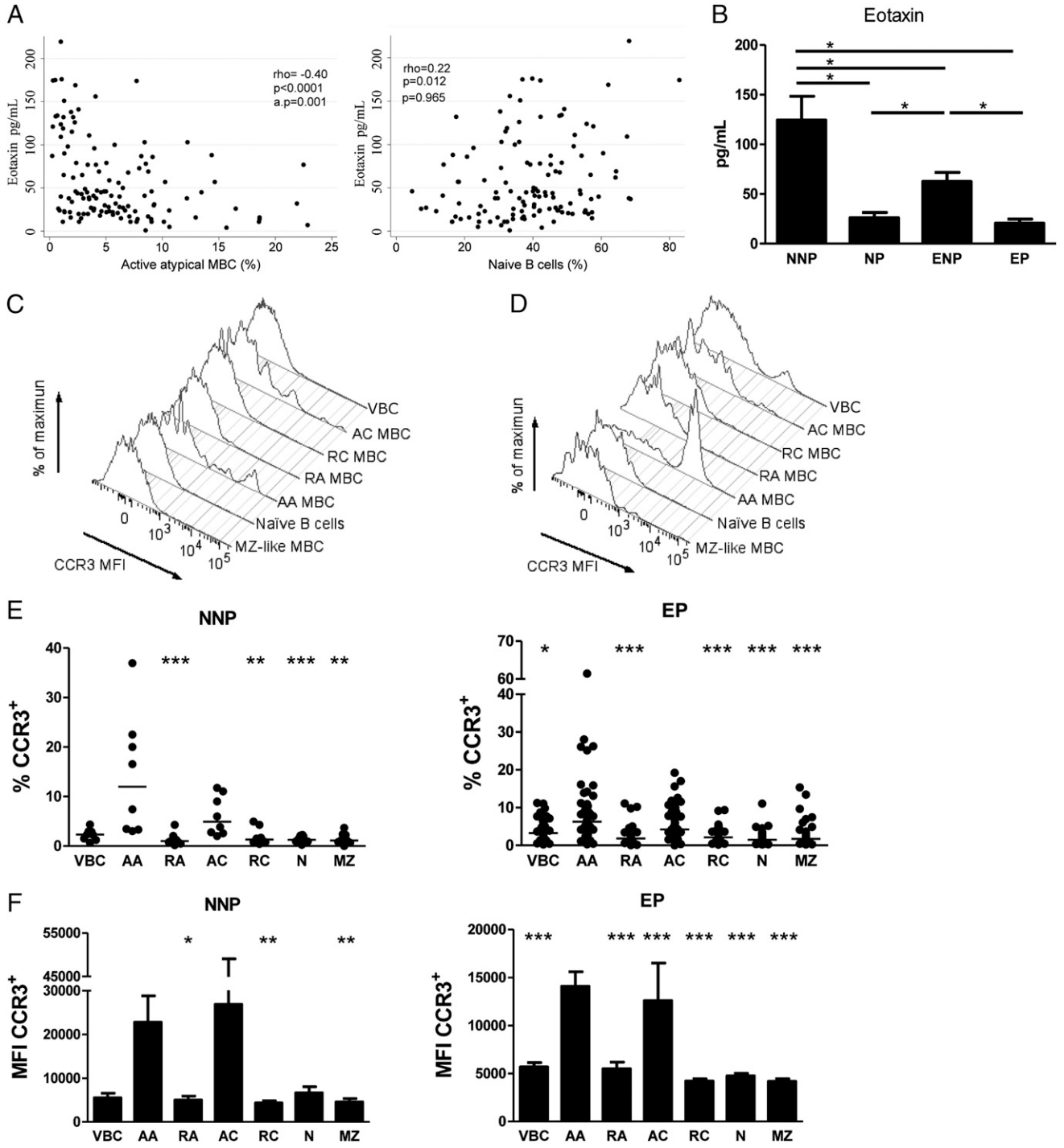

FIGURE 7. Eotaxin and its receptor CCR3 are associated with changes in B cells. (**A**) Scatter plots show the distribution of values for different B cell subsets (%) and eotaxin plasma concentration in the four groups (n = 125). rho: Spearman's coefficient; p corresponds to Spearman's correlation test; a.p correspond to the adjusted p value after correcting for multiple comparisons using the Benjamini-Hochberg method. (**B**) Bars represent geometric mean + 95% CI of eotaxin plasma concentration in the four study groups, as follows: NNP (n = 23), NP (n = 13), ENP (n = 38), and EP (n = 69). Age-adjusted median regression models were estimated, and effects were assessed comparing the four groups (*$p < 0.05$). (**C**) Histograms show the CCR3 MFI values in different B cell subsets in a NNP donor. (**D**) Histograms show the CCR3 MFI values in different B cell subsets in a EP donor. (**E**) and (**F**) show, respectively, the percentage and MFI values of $CCR3^+$ events within every B cell subset in a different set of NNP donors (n = 8) and EP women (n = 45). Differences were assessed with the Friedman test plus Dunn post hoc test, comparing active atypical (AA) MBC versus every other B cell subset. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. AA, active atypical MBC; AC, active classical MBC; MZ, MZ-like MBC; N, naive B cells; RA, resting atypical MBC; RC, resting classical MBC.

malaria, we present low levels of surface IgG, correlation with *Plasmodium*-specific IgGs and IL-8, and expression of CCR3. Moreover, we show that plasma eotaxin is decreased in both pregnancy and after malaria exposure, which might contribute to the altered distribution of B cell subsets in these two conditions, as active MBC express CCR3. Finally, we discuss how these alterations can influence the outcomes of malaria and other diseases, such as pneumococcal infections. Due to the importance of MBC

in immunity to infections and success of vaccination, these altered distributions of B cells associated with pregnancy and high malaria exposure must be taken into account when testing vaccines for high-burden infections during pregnancy, especially in tropical areas.

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Disclosures

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