Genome-Wide Expression Analysis of Placental Malaria Reveals Features of Lymphoid Neogenesis during Chronic Infection[1]

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Chronic inflammation during placental malaria (PM) is most frequent in first time mothers and is associated with poor maternal and fetal outcomes. In the first genome-wide analysis of the local human response to sequestered malaria parasites, we identified genes associated with chronic PM and then localized the corresponding proteins and immune cell subsets in placental cryosections. B cell-related genes were among the most highly up-regulated transcripts in inflamed tissue. The B cell chemoattractant CXCL13 was up-regulated >1,000-fold, and B cell-activating factor was also detected. Both proteins were expressed by intervillous macrophages. Ig L and H chain transcription increased significantly, and heavy depositions of IgG3 and IgM were observed in intervillous spaces. The B cell phenotype was heterogenous, including naive (CD27-negative), mature (CD138-positive), and cycling (Ki-67-positive) cells. B cells expressed T-bet but not Bcl-6, suggesting T cell-independent activation without germinal center formation. Genes for the Fc binding proteins FcγRIa, FcγRIIIa, and C1q were highly up-regulated, and the proteins localized to intervillous macrophages. Birth weight was inversely correlated with transcript levels of CXCL13, IgG H chain, and IgM H chain. The iron regulatory peptide hepcidin was also expressed but was not associated with maternal anemia. The results suggest that B cells and macrophages contribute to chronic PM in a process resembling lymphoid neogenesis. We propose a model where the production of Ig during chronic malaria may enhance inflammation by attracting and activating macrophages that, in turn, recruit B cells to further produce Ig in the intervillous spaces. ***The Journal of Immunology,*** **2007, 179: 557–565.**

Placental malaria (PM)[3] due to *Plasmodium falciparum* is a major cause of death for mothers and their offspring, with the heaviest burden of disease occurring in first pregnancies. PM is caused by infected erythrocyte (IE) forms that bind to chondroitin sulfate A and sequester in the placenta (1).

Active PM episodes are defined by the presence of IE in the intervillous space. PM may present acutely with minimal inflammation or chronically with extensive inflammation and malarial pigment deposition (2). Chronic PM is most closely associated with maternal and fetal morbidity (3). Past PM episodes are defined by the persistence of malarial pigment (or hemozoin) in the absence of IE.

First time mothers commonly suffer chronic PM characterized by inflammatory infiltrates in the intervillous spaces and increased levels of type I cytokines (4) and β chemokines (5, 6). The intervillous infiltrate consists primarily of macrophages with a smaller number of T cells, B cells, and granulocytes (7). This infiltrate can sometimes become so extensive that it appears to occlude the maternal circulation, a condition called massive chronic intervillositis (8).

Women become resistant to PM over successive pregnancies as they acquire Abs that inhibit the binding of placental parasites to chondroitin sulfate A (9). These Abs are associated with a decreased risk of PM and increased birthweight and gestational age of the newborn (10). Abs that label laboratory parasites selected to bind chondroitin sulfate A also increase over successive pregnancies (11, 12), are elevated during infection (11), and have been associated with protection against anemia in first time mothers with chronic PM (13). First time mothers with active PM have elevated levels of total IgG and IgM (14), but these do not inhibit the binding of placental parasites (9) and this latter observation may explain the slow clearance of parasites from these women (15). In women without specific immunity, phagocytic cells may play a more prominent role in clearing parasites (16).

Abs can damage tissue. During type III hypersensitivity reactions as defined by Coombs and Gell (17), immune complexes deposit in tissue and activate phagocytic cells and complement. Immune complexes play a role in chronic inflammatory diseases such as rheumatoid arthritis (18) and Lyme arthritis (19), and the macrophage Fc receptor FcγRIIIa (CD16) is necessary in spontaneous murine autoimmune arthritis (20). Malaria has been associated with circulating immune complexes (21), and Ig and parasite

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[3] Abbreviations used in this paper: PM, placental malaria; BAFF, B cell-activating factor; C_T, threshold cycle; IE, infected erythrocyte.

Table I. *Primers used in this study*

Target	Primer Type	Primers	Product Size (bp)
IGGH (all isotypes)	Forward	CAAGTGCAAGGTCTCCAACA	138
	Reverse	AGGCTGACCTGGTTCTTGGT	
IGMH	Forward	ACCAGCACACTGACCATCAA	186
	Reverse	GGTGGACTTGGTGAGGAAGA	
IFNG	Forward	TGACCAGAGCATCCAAAAGA	147
	Reverse	TGTATTGCTTTGCGTTGGAC	
IL1B	Forward	CTGTCCTGCGTGTTGAAAGA	178
	Reverse	CTGCTTGAGAGCTGCTGATG	
IL18	Forward	TGCATCAACTTTGTGGCAAT	220
	Reverse	ATATGGTCCGGGGTGCATTA	
TNF	Forward	CACGCTCTTCTGCCTGCT	161
	Reverse	CAGCTTGAGGGTTTGCTACA	
BAFF	Forward	CGTTCAGGGTCCAGAAGAAA	115
	Reverse	AAAGCTGAGAAGCCATGGAA	
Hepcidin	Forward	GACCAGTGGCTCTGTTTTCC	193
	Reverse	CTACGTCTTGCAGCACATCC	
CXCL10	Forward	CCACGTGTTGAGATCATTGC	180
	Reverse	CCTCTGTGTGGTCCATCCTT	
CXCL9	Forward	GAAGCAGCCAAGTCGGTTAG	75
	Reverse	TGGAAGGAGGTTTCCACATC	
CXCL13	Forward	GGGAATGGTTGTCCAAGAAA	213
	Reverse	CAGAGCAGGGATAAGGGAAG	
CXCL16	Forward	GCCCTTTCCTATGTGCTGTG	121
	Reverse	AGCTTCCATTCTTGGCTCAG	
CCL5	Forward	CGCTGTCATCCTCATTGCTA	196
	Reverse	ACACACTTGGCGGTTCTTTC	
CCL4	Forward	CTTCCTCGCAACTTTGTGGT	88
	Reverse	GCTTGCTTCTTTTGGTTTGG	
CCL18	Forward	CCTGGCAGATTCCACAAAAG	126
	Reverse	CCCACTTCTTATTGGGGTCA	
KRT7	Forward	GGCTGAGATCGACAACATCA	103
	Reverse	CTTGGCACGAGCATCCTT	

Ag depositions have been observed in the basement membrane of endothelial cells during cerebral malaria (22) and in that of trophoblast during PM (23). Serum Ig is elevated during PM (14), yet the relationship between Ig and inflammation during PM is not known, nor has Fc receptor expression been examined in the abundant macrophages that accumulate in the placenta.

Lymphoid neogenesis is the process that results in the ectopic accumulation of lymphoid cells in chronically inflamed tissues. These cellular accumulations, known as tertiary lymphoid organs, have been observed in inflamed tissue resulting from autoimmunity, allograft rejection, and some microbial infections (24). Inflammatory cytokines, lymphoid chemokines, and various developmental stages of B cells are features of tertiary lymphoid organs during rheumatoid arthritis (25), Sjogren's syndrome (26), and Lyme borreliosis (27). The lymphoid chemokine CXCL13 is chemotactic for B cells expressing the Burkitt's lymphoma receptor CXCR5 and is essential for lymph node development in mice (28). CXCL13 expression has been associated with lymphoid neogenesis in autoimmune diseases (26, 27, 29) and during *Helicobacter pylori* and *Bartonella henselae* infection (30, 31). Although B cells contribute to the placental infiltrate during PM, no reports have characterized the B cells nor has CXCL13 been examined in malaria-infected individuals.

Genome-wide expression analysis of the host response during malaria infection has been examined in animal models of malaria infection (32, 33) and in human peripheral blood (34, 35). P.C.C. Garnham regarded the changes observed in the peripheral blood to be "merely a mild reflection of the real mechanism occurring in the internal organs" during *P. falciparum* infections (16). In this study we report the first genome-wide analysis of malaria-positive tissue in humans that reveals features of lymphoid neogenesis during chronic PM. The data suggest that macrophage CXCL13 expression, B cell recruitment, local Ab production, and Ab-mediated activation of phagocytes contribute to the pathogenesis of chronic placental malaria.

Table II. *Antibodies used in this study*

Host	Antigen	Dilution	Manufacturer
Mouse	FcγRIII (CD16)	1/500	Chemicon
Mouse	FcγRI (CD64)	1/500	Chemicon
Mouse	CD138	1/1,000	Chemicon
Mouse	CXCL13	1/50	R&D Systems
Mouse	BAFF	1/500	ID Labs
Mouse	IgG3-HRP	1/500	Zymed Laboratories
Goat	IgM-HRP	1/1,000	Chemicon
Rabbit	C1q	1/20,000	DakoCytomation
Rabbit	Hepcidin (hepc12A)	1/200	Alpha Diagnostics
Rabbit	CD79a	1/100	NeoMarkers
Mouse	CD27	1/50	Chemicon
Mouse	Ki-67	1/200	DakoCytomation
Mouse	T-bet	1/200	Santa Cruz Biotechnology
Mouse	Bcl-6	1/50	Chemicon
Mouse	Isotype (IgG1)	1/50	eBiosciences

Table III. *Clinical characteristics of women who donated samples examined for global gene expression*

	PM-Negative	PM-Positive	*p* Value
Age (years)	22.5 (3.0)	19.6 (1.8)	0.018
Infant weight (kg)	3.22 (0.31)	2.80 (0.26)	0.006
Female infants (*n*)	4/10	6/10	0.498
Placental parasite density (%)[a]	0	1.5 (0.6–63)	NA[b]
Pigment-positive (*n*)	5/10	10/10	NA[b]
Inflammation-positive (*n*)	1/10	7/10	0.01

[a] Percentage of IE.
[b] Not applicable.

Materials and Methods

Human subjects

Placental samples and clinical information were provided by Tanzanian women aged 18 to 45 years delivering at the Muheza Designated District

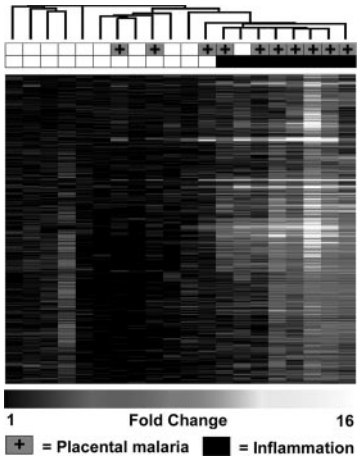

FIGURE 1. Hierarchical clustering of 728 probes associated with PM and hierarchical clustering of samples. Data are presented as fold change over the mean of PM-negative samples.

Table IV. *Expression data for selected genes up-regulated during inflammation (>2.5 fold change and $p < 0.01$)*

Gene Name	Gene Symbol	No. of Probes	*p* Value	Fold Change
Chemokine-related				
Chemokine (CXC motif) ligand 13	*CXCL13*	1	8.77E-08	129.3
Chemokine (CC motif) ligand 18	*CCL18*	2	2.53E-10	47.3
Chemokine (CXC motif) ligand 9	*CXCL9*	1	6.43E-09	32.0
Chemokine (CC motif) ligand 3	*CCL3*	1	6.03E-06	13.7
Chemokine (CC motif) ligand 4	*CCL4*	1	1.27E-04	13.5
Chemokine (CXC motif) ligand 10	*CXCL10*	1	1.56E-04	8.1
Chemokine (CX3C motif) receptor 1	*CX3CR1*	1	9.05E-04	5.5
Chemokine (CC motif) ligand 5	*CCL5*	3	2.25E-03	4.3
Chemokine (CXC motif) ligand 16	*CXCL16*	1	3.81E-07	4.1
Chemokine (CC motif) receptor 5	*CCR5*	1	1.72E-04	4.1
Chemokine (CC motif) receptor 1	*CCR1*	2	3.48E-03	2.8
Cytokine-related				
BAFF (TNF (ligand) superfamily, member 13b)	*TNFSF13B*	2	6.92E-09	8.7
IL-1R antagonist	*IL1RN*	1	5.44E-04	5.3
IL-10R α	*IL10RA*	1	3.79E-05	3.9
IL-1β	*IL1B*	1	2.49E-03	3.6
IL-18 (IFN-γ -inducing factor)	*IL18*	1	8.29E-05	3.4
TNF (ligand) superfamily, member 13	*TNFSF13*	1	7.91E-05	2.9
Caspase 1 (IL-1β, convertase)	*CASP1*	1	3.80E-03	2.6
Immunoglobulin				
Igλ locus	*IGL*	4	5.51E-06	33.0
Igκ locus	*IGK*	6	1.26E-05	25.0
Ig heavy locus	*IGH*	1	1.54E-04	11.8
Ig J polypeptide	*IGJ*	1	4.18E-04	8.1
Fc receptor				
Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	*FCGR3A*	1	8.98E-06	6.1
Fc fragment of IgE, high affinity I, receptor for; γ polypeptide	*FCER1G*	2	1.05E-04	4.3
Fc fragment of IgG, low affinity IIIb, receptor (CD16b)	*FCGR3B*	1	1.36E-03	3.1
Fc fragment of IgG, high affinity Ia, receptor (CD64)	*FCGR1A*	2	1.46E-03	2.9
Complement				
Complement component 1, q subcomponent, β polypeptide	*C1QB*	1	3.82E-05	3.6
Complement component 3a receptor 1	*C3AR1*	1	6.51E-05	3.2
Complement component 3	*C3*	1	3.14E-04	3.0
Complement component 5 receptor 1 (C5a)	*C5R1*	1	4.00E-03	2.9
Complement component 1, q subcomponent, γ polypeptide	*C1QG*	1	4.06E-04	2.8
Complement component 1, q subcomponent, α polypeptide	*C1QA*	1	5.90E-04	2.7
B cell expressed				
Regulator of G protein signaling 1	*RGS1*	2	1.41E-06	11.9
Protein kinase C, β 1	*PRKCB1*	1	2.48E-06	8.6
CD48 Ag (B cell membrane protein)	*CD48*	1	1.95E-07	8.5
SAM domain, SH3 domain and nuclear localization signals, 1	*SAMSN1*	2	6.23E-05	6.9
CD72 Ag	*CD72*	1	4.84E-04	4.4
CD37 Ag	*CD37*	1	1.48E-04	3.9
Bruton agammaglobulinemia tyrosine kinase	*BTK*	1	1.58E-04	2.6
T cell expressed				
SLAM family member 7	*SLAMF7*	1	3.70E-05	11.4
Granzyme A (CTLA3)	*GZMA*	1	1.51E-04	7.1
TCR α locus	*TRA*	2	2.64E-04	6.8
TCR β locus	*TRB1*	2	1.05E-04	6.0
Pleckstrin homology, Sec7 and coiled-coil domains, binding protein	*PSCDBP*	1	6.00E-07	5.4
Fibrinogen-like 2	*FGL2*	1	2.58E-05	4.6
CD2 antigen	*CD2*	1	2.87E-03	4.2
TCR γ locus	*TRG*	2	3.05E-03	4.1
Granzyme B (CTLA1)	*GZMB*	1	5.34E-03	3.6

(*Table continues*)

Table IV. (*Continued*)

Gene Name	Gene Symbol	No. of Probes	*p* Value	Fold Change
Macrophage expressed				
Sialic acid binding Ig-like lectin 10	*SIGLEC10*	1	3.17E-10	21.6
ADAM-like, decysin 1	*ADAMDEC1*	1	9.50E-08	13.2
Epidermal growth factor-like module containing, mucin-like, hormone receptor-like 1	*EMR1*	1	6.15E-07	12.8
Epidermal growth factor-like module containing, mucin-like, hormone receptor-like 2	*EMR2*	1	2.35E-06	7.4
SLAM family member 8 (BLAME)	*SLAMF8*	2	5.18E-06	5.7
CD86 antigen (CD28 antigen, B7-2 antigen)	*CD86*	1	5.35E-05	5.3
C-type lectin domain family 7, member A	*CLEC7A*	1	1.38E-04	4.3
macrophage receptor with collagenous structure	*MARCO*	1	1.29E-04	3.9
CD163 Ag	*CD163*	2	3.53E-04	3.8
Myeloid cell nuclear differentiation antigen	*MNDA*	1	3.84E-04	3.2
Macrophage expressed gene 1	*MPEG1*	2	4.99E-03	3.1
Ficolin 1	*FCN1*	1	3.75E-03	3.1
Chitinase 3-like 1	*CHI3L1*	1	1.93E-03	3.0
Antigen presentation				
MHC, class II, DQ α 1	*HLA-DQA1*	3	1.42E-07	37.4
MHC class II, DP β 1	*HLA-DPB1*	1	1.45E-08	14.0
MHC class II, DP α 1	*HLA-DPA1*	2	3.62E-07	12.3
MMHC, class II, DM α	*HLA-DMA*	1	2.55E-07	11.8
MHC class II, DQ β 1	*HLA-DQB1*	6	1.08E-04	10.9
MHC, class II, DR α	*HLA-DRA*	2	2.65E-08	10.2
MHC, class II, DR β 1	*HLA-DRB1*	4	5.73E-08	10.1
Proteasome subunit, β type, 9	*PSMB9*	1	1.31E-05	6.0
Cathepsin S	*CTSS*	3	3.74E-06	7.9
MHC, class II, DR β 3	*HLA-DRB5*	1	3.66E-07	6.6
CD74 antigen	*CD74*	2	1.09E-06	5.8
MHC, class II, DM β	*HLA-DMB*	1	6.57E-06	3.3
Pattern recognition				
TLR-8	*TLR8*	2	1.10E-07	12.6
Formyl peptide receptor-like 2	*FPRL2*	2	5.24E-08	12.2
TLR-1	*TLR1*	1	5.61E-06	5.8
TLR-2	*TLR2*	1	1.58E-05	5.4
Formyl peptide receptor 1	*FPR1*	1	5.46E-04	3.3
TLR-4	*TLR4*	2	1.12E-03	2.9
Redox related				
Superoxide dismutase 2, mitochondrial	SOD2	2	3.95E-06	7.9
Cytochrome b-245, β polypeptide	*CYBB*	1	2.02E-04	4.4
Cytochrome b-245, α polypeptide	*CYBA*	1	2.66E-05	2.8
Heme oxygenase (decycling) 1	*HMOX1*	1	2.16E-04	2.6
Intercellular adhesion				
Epidermal growth factor-like module containing, mucin-like, hormone receptor-like 2	*EMR2*	1	2.35E-06	7.4
Integrin, β_2 (Ag CD18)	*ITGB2*	2	1.27E-06	6.0
Integrin, α_L (Ag CD11A)	*ITGAL*	1	1.78E-04	4.1
Other				
Serpin peptidase inhibitor, clade A1	*SERPINA1*	2	2.50E-08	11.3
Adenosine deaminase	*ADA*	2	1.53E-06	7.2
Lysozyme (renal amyloidosis)	*LYZ*	2	3.14E-04	7.1
Prostaglandin E receptor 4 (subtype EP4)	*PTGER4*	1	2.46E-06	6.2
Hepcidin antimicrobial peptide	*HAMP*	1	2.30E-05	4.0
Leukotriene A4 hydrolase	*LTA4H*	1	2.35E-05	3.3
Ferritin, heavy polypeptide 1	*FTH1*	1	8.12E-07	2.8

Hospital, Muheza, Tanga region, in an area of intense malaria transmission. These women were participating in a birth cohort study known locally as the Mother-Offspring Malaria Study. Women signed an informed consent form before joining the study, and those with chronic debilitating disease were excluded. Clinical information was collected by project nurses and assistant medical officers on standardized forms. Study procedures involving human subjects were approved by the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the U.S. National Institutes of Health, and ethical clearance was obtained from the institutional review boards of Seattle Biomedical Research Institute (Seattle, WA) and the National Institute for Medical Research in Tanzania.

Sample processing

The placenta was collected at delivery, and a full thickness biopsy from the middle third of the placental disc was made. Tissue was fresh frozen in liquid nitrogen or collected in RNAlater (Ambion) and stored at −80°C. Placental blood was extracted from placental tissue by mechanical grinding. Placental malaria was diagnosed by microscopy of Giemsa-stained thick and thin smears of placental blood.

Table V. *Validation of array data by quantitative RT-PCR*

	PM-negative (n = 22) compared with			
	PM-Positive (19)		PM-Positive with (++) Intervillositis (5)	
Gene	Fold change	p Value	Fold Change	p Value
CXCL13	46	<0.001	1242	<0.001
CCL18	11	<0.001	67	<0.001
IGGH	3	0.007	51	<0.001
HEPC	5.3	<0.001	38	<0.001
IFNG	4.2	0.001	37	<0.001
IGMH	2.2	0.008	34	<0.001
TNF	5.1	<0.001	19	<0.001
CCL4	4.5	<0.001	17	<0.001
CXCL9	2.9	0.006	14	<0.001
IL1B	4.3	<0.001	13	<0.001
CCL5	1.5	0.034	9.4	<0.001
IL18	2.1	<0.001	8.4	<0.001
CXCL10	1.8	0.062	8.2	0.003
BAFF	1.8	0.006	6.1	<0.001
CXCL16	1.9	<0.001	3.5	<0.001

Placental histopathology

For histologic analysis, 5-μm cryosections of placental tissue were fixed in methanol and Giemsa stained. Sections were assessed by examining >90 fields per section at ×60 magnification. Hemozoin deposition in fibrinoid was quantified by determining the proportion of fields with hemozoin present. Immune infiltrates within the intervillous spaces were qualitatively scored as negative (−) for none or very few inflammatory cells present, positive (+) for inflammatory cells present, and double positive (++) for having an extensive accumulation of inflammatory cells, i.e., massive chronic intervillositis.

Microarray analysis

For microarray analyses, placental villi were dissected at <0.5 mm^3, excluding large vessels, stem villi, infarcts, fetal membranes, or decidua from RNAlater-preserved placental tissues. Total RNA was extracted using RNeasy mini kits (Qiagen). RNA quality was assessed by an Agilent 2100 bioanalyzer, resulting in 28- to 18-s ratios of 1.1 to 1.5. Microarray assays were performed at the Center for Expression Arrays at the University of Washington (Seattle, WA). Biotinylated target cRNA was prepared and hybridized to Affymetrix Human Genome U133 Plus 2.0 GeneChip with minor modifications from the procedures recommended by Affymetrix. First-strand cDNA was produced by 5 μg of total RNA using a T7-linked oligo(dT) primer. In vitro transcription reaction was performed using biotinylated UTP and CTP. Fifteen micrograms of cRNA was fragmented, a hybridization mixture was assembled with the addition of spike-in controls, and chips were hybridized for 16 h. The chips were then washed and stained with streptavidin-PE using the Affymetrix GeneChip system and scanned using the GeneChip scanner. Transcription profiles were defined by GeneChip operating system (GCOS) absolute expression analysis. Data were normalized by the GeneChip robust multiarray analysis (GC-RMA) algorithm and then analyzed by t test and hierarchical clustering with Acuity 4.0 (Axon).

Quantitative RT-PCR

For quantitative PCR, total RNA was extracted from frozen cryosections using RNeasy mini kits (Qiagen). The RNA quality of representative

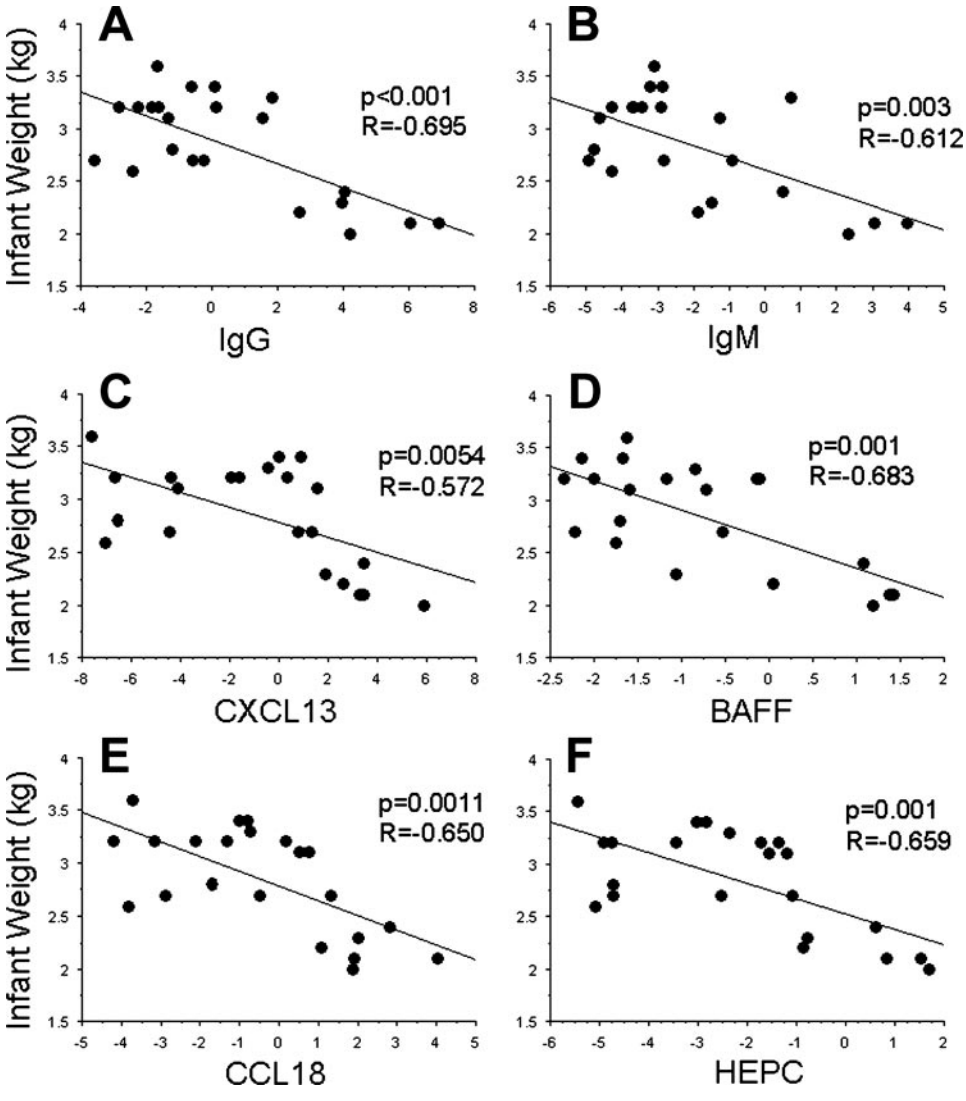

FIGURE 2. Correlation of gene expression by quantitative RT-PCR with birth weights for infected women. Gene expression for IgG (*A*), IgM (*B*), CXCL13 (*C*), BAFF (*D*), CCL18 (*E*), and hepcidin (HEPC) (*F*) is presented as $\log_2$-fold expression over KRT7. Simple regression analysis was used to calculate R and p values.

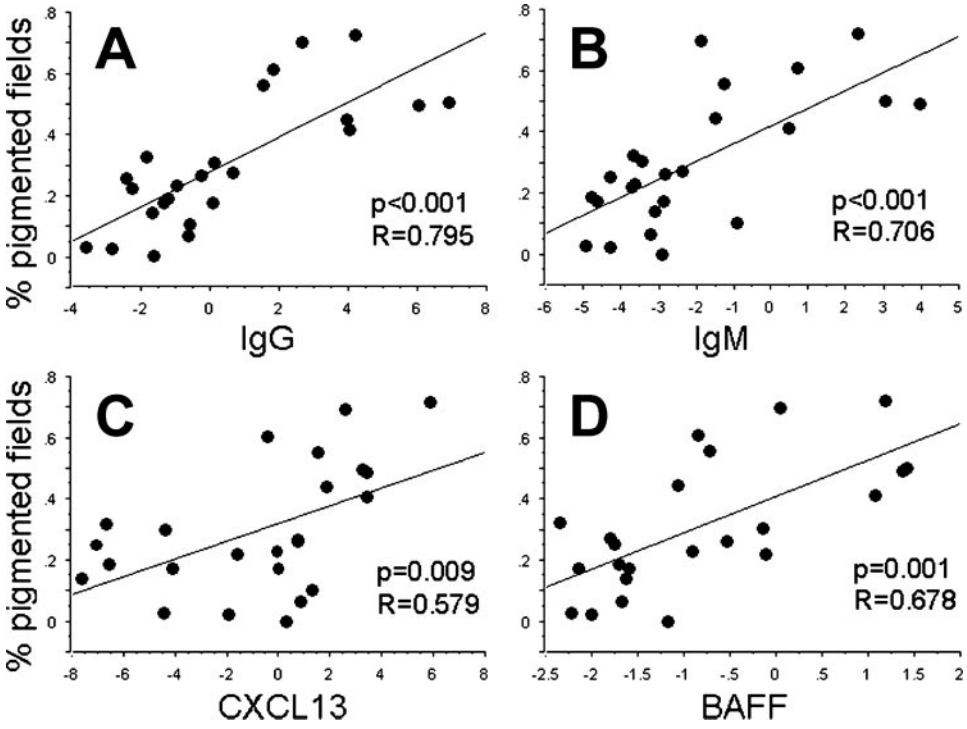

FIGURE 3. Correlation of gene expression by quantitative RT-PCR to placental malarial pigment deposition (proportion positive fields) by microscopy. Only infected women were analyzed. Gene expression for IgG (*A*), IgM (*B*), CXCL13 (*C*), and BAFF (*D*) is presented as 2-fold expression over KRT7. Simple regression analysis was used to calculate *R* and *p* values.

samples was assessed by an Agilent 2100 bioanalyzer, resulting in 28- to 18-s ratios of 1.1 to 1.5. cDNA was synthesized using a SuperScript III enzyme (Invitrogen Life Technologies) and anchored oligo$(dT)_{20}$ primers. Real-time PCR was performed in duplicate using SYBR Green Master Mix and an ABI Prism 7000 or 7500 system (Applied Biosystems). The annealing temperature was 60°C. Intron-spanning primers (except those for CXCL9, which comprise a single exon) were designed using Primer3 (Massachusetts Institute of Technology, Cambridge, MA). Primers for TNF were a gift from A. Collie (University of Washington, Seattle, WA). Primers are listed in Table I. All primers yielded single products, and amplification was linear on serial dilutions of cDNA samples. Threshold cycles (C_T) were calculated and normalized to the C_T of *KRT7* (a gene expressed by the trophoblast, not inflammatory cells) and *t* tests were performed on normalized C_T values. Data is presented as the fold difference from the control gene, calculated by $2^{(\text{control } C_T - \text{ gene } C_T)}$.

Immunohistochemistry

For immunohistochemistry, 5-μm cryosections were fixed for 10 min in 4% paraformaldehyde (for CXCL13, IgG3, IgM, C1q, and hepcidin) or acetone (for CD16, CD64, CD138, and B cell activating factor (BAFF)). Abs and dilutions are listed in Table II. Indirect staining was performed using an anti-mouse or anti-rabbit diaminobenzidine (DAB) EnVision+ kit (DakoCytomation) according to manufacturer's directions. Direct staining was performed for IgG isotypes and IgM on sections blocked with species-concordant serum. For immunofluorescence studies, tissue was fixed for 10 min in 4% paraformaldehyde and then Alexa Fluor 488 chicken anti-mouse (Molecular Probes) or tetramethylrhodamine isothiocyanate goat anti-rabbit (Sigma-Aldrich) were used as secondary Abs. The sections were stained with 4′,6′-diamidino-2-phenylindole (Sigma-Aldrich) to define nuclei, mounted in 80% glycerol, and visualized using a fluorescent microscope.

Results

For global gene expression analysis, placental samples from 20 first time mothers were selected based on PM status and RNA quality. Ten had active PM episodes, and of the ten PM-negative women five had evidence of a past PM episode. Clinical characteristics of the women are summarized in Table III.

A normalized dataset was generated. Hierarchical clustering was performed to identify coregulated genes. We detected a group of 752 probes (correlation coefficient, 0.870) representing 528 coexpressed genes (Fig. 1) that were related to PM status. These included genes previously identified to be involved in PM such as *IFNG*, *TNF*, *CCL3*, *CCL4*, and *CCL5*. In analysis limited to these 752 probes, hierarchical clustering separated the samples from women with placental inflammation from those without placental inflammation (Fig. 1). Samples from three women with active PM but minimal inflammation clustered with PM-negative samples. In addition, one sample from a woman with a past PM episode possessed inflammatory cells and clustered with the active PM inflamed samples. The placental blood smear from this subject was re-examined and confirmed to be negative for IE. Samples from the other four women with past PM episodes clustered with the other PM-negative samples. No coexpressed genes were identified that correlated with percentage of IE or with the levels of malaria pigment deposition. These data suggest that the transcriptional changes observed during PM are more closely related to placental inflammation than to parasitemia or pigment deposition.

Statistical testing for differences in gene expression between seven PM-positive women with inflammation vs nine PM-negative women without inflammation revealed 314 probes representing 234 genes that were at least 2.5-fold elevated with $p < 0.01$ (see supplemental table).[4] For each of these probes, mean intensity was above the 25th percentile of all probe intensities. Probe data for IFN-γ and TNF did not meet these criteria. The most up-regulated gene was *CXCL13* at 130-fold, followed by *CCL18* at 47-fold, *HLADQA1* at 37-fold, *Ig*λ at 33-fold, *CXCL9* at 32-fold, and *Ig*κ at 25-fold. Genes associated with the immune response, particularly with B cell, T cell and macrophage function were identified. Selected genes are listed in Table IV.

We validated the expression of a subset of genes by quantitative RT-PCR over a larger group of samples (Table V). Primers for the IgG H chain were designed to amplify all IgG classes. The level of gene expression correlated with the level of inflammation for all genes analyzed, including *TNF* and *IFNG*. Remarkably, CXCL13

[4] The online version of this article contains supplemental material.

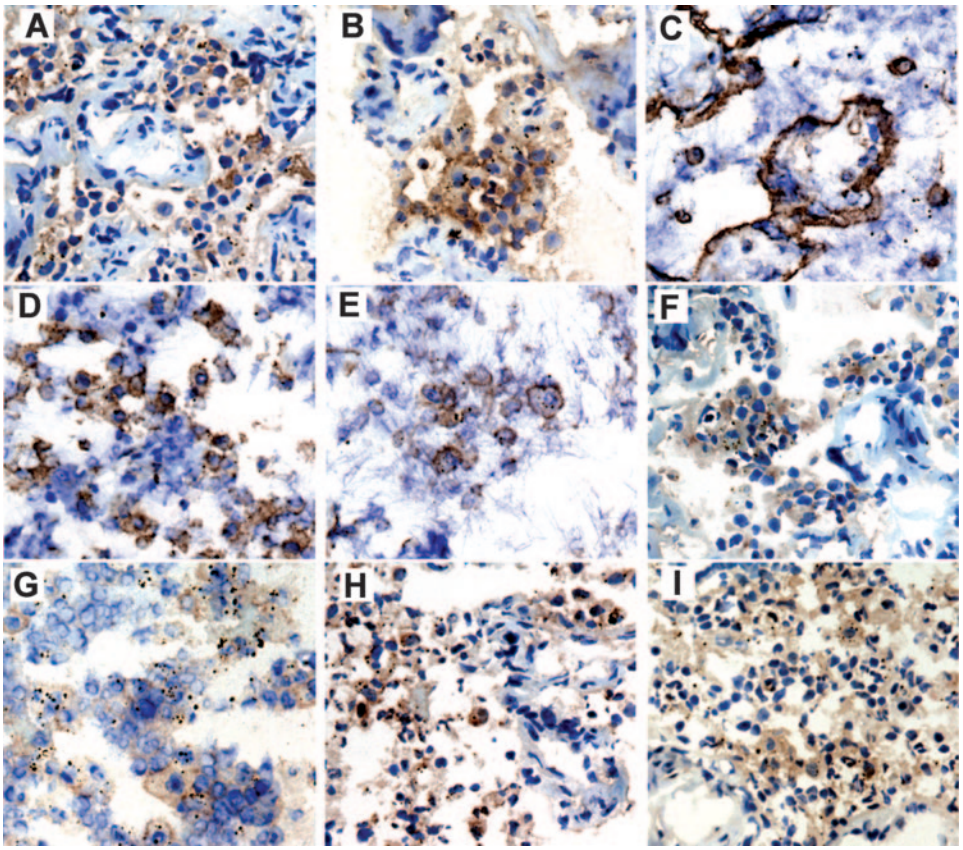

FIGURE 4. Immunohistochemistry of inflamed PM-positive tissues for IgM (*A*), IgG3 (*B*), CD138, a plasma cell marker (*C*), FcγRIII (CD16) (*D*), FcγRI (CD64) (*E*), C1q (*F*), BAFF (*G*), CXCL13 (*H*), and hepcidin (*I*). All fields are ×200 (original magnification).

was >1,000-fold up-regulated in the placentas of women with massive chronic intervillositis. Of the genes examined by quantitative RT-PCR, *CXCL13*, *IGGH*, and *IGMH* were disproportionately increased (27-, 17-, and 15-fold, respectively) in massive chronic intervillositis compared with other PM-positive samples.

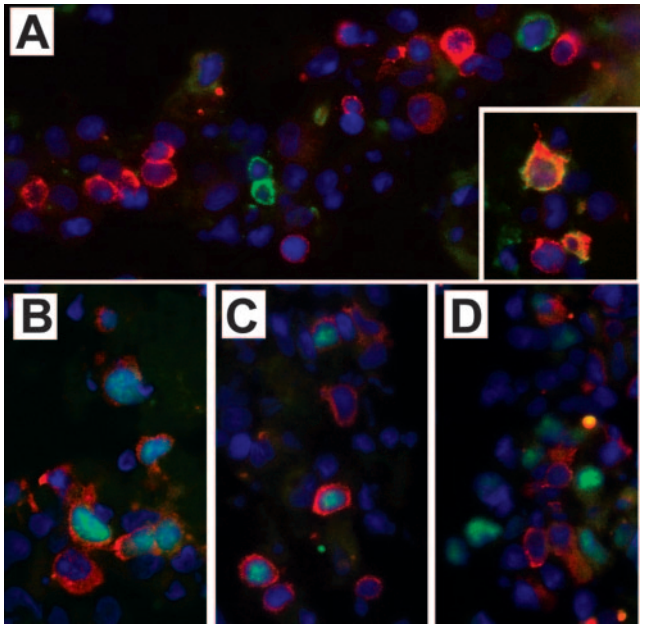

FIGURE 5. B cell phenotype analysis by double indirect immunofluorescence using CD79a to identify B cells (red), a second marker (green), and 4′,6′-diamidino-2-phenylindole to define nuclear DNA (blue). *A* and *inset*, CD27. *B*, Ki-67. *C*, T-bet. *D*, Bcl-6. All fields are ×400 (original magnification).

Several genes, including *IgG*, *IgM*, *BAFF*, *CXCL13*, *CCL18*, and hepcidin were negatively correlated with birthweight in PM-positive women (Fig. 2). Maternal hemoglobin did not correlate significantly with the placental hepcidin transcript level ($R = -0.143$, $p = 0.392$, $n = 38$). Several genes, notably *IgG*, *IgM*, *CXCL13*, and *BAFF*, were correlated with the degree of malarial pigment deposition (Fig. 3), a pathologic feature that reflects the chronicity of infection in PM-positive women.

By immunohistochemistry, IgG3 and IgM stained strongly in the intervillous space of PM-positive placentas and were associated with macrophages (Fig. 4). IgG1 and IgG4 levels were not elevated and IgG2 was moderately elevated (data not shown). Infiltrating macrophages stained positively for the Fc receptors FcγRIIIa (CD16), FcγRIa (CD64), and C1q. Macrophages also stained positively for CXCL13 and BAFF. Because of the high level of Ig transcription we tested for plasma cells and identified CD138-positive cells present in the intervillous space during chronic placental malaria, but not in uninfected women. The villous trophoblast also stained positive for CD138, a marker also expressed by epithelial cells including trophoblasts (36).

We further characterized the B cell population in chronic PM using double indirect immunofluorescence (Fig. 5). B cells were identified using CD79a. The majority of B cells were CD27 negative suggesting a naive phenotype, although CD27-positive cells were present (Fig. 5*A*, *inset*). Furthermore, a population of B cells expressed the mitotic marker Ki-67. The transcription factor T-bet was also expressed by a subset of B cells. B cells did not express Bcl-6, although it was observed in other cell types during chronic PM.

Discussion

The inflammatory response to sequestered *P. falciparum* parasites is thought to play a key role in the development of severe malaria syndromes. This is the first study to examine the transcriptome of

the local response to sequestered parasites in humans. The results highlight a hitherto unappreciated role for B cells during episodes of chronic inflammatory PM that echo features of lymphoid neogenesis, including macrophage CXCL13 expression, the accumulation of naive B cells, cycling B cells, abundant plasma cells, and IgM and IgG synthesis.

Malaria infection is associated with B cell pathology, including hypergammaglobulinemia (37), autoantibody production (38), and circulating immune complexes (21, 39). Malaria also has a strong geographic overlap with Burkitt's lymphoma, a B cell neoplasm (40). Hyperreactive malarial splenomegaly is marked by elevated levels of IgM and antimalarial Abs (41), and total serum Ig levels (14) and immune complex deposition (23) increase during PM. The mechanism of B cell dysfunction during PM may involve the accumulation of CD27-negative naive B cells in the placenta and their subsequent activation to produce nonspecific Abs. Activation may involve a T cell-independent Ig class switch, as evidenced by T-bet expression (42). In our study, B cells were not aggregated in follicular structures and did not express the germinal center marker Bcl-6. Similarly, T-bet expression but not Bcl-6 expression has been observed in B cell infiltrates of *B. henselae* granulomas (31).

Our data suggest that Ig and macrophages interact to contribute to the pathology in first time mothers with chronic PM. We propose a model in which *P. falciparum* Ag-Ab complexes in the intervillous space activate monocytes through Fcγ receptors (CD16 and CD64) and complement C1q. Macrophages stained positively for IgG3 and IgM, suggesting phagocytic uptake. Macrophage expression of CXCL13 and BAFF may contribute to B cell accumulation and Ig synthesis. We speculate that further Ig synthesis activates additional monocytes, thus generating a proinflammatory feedback loop. Such a proinflammatory feedback loop, involving B cells and macrophages, may be a general phenomenon during malaria infection because hypergammaglobulinemia and circulating immune complexes are also features of malaria in nonpregnant individuals. Although our data suggest a pathologic role for the B cell infiltrate during severe malaria, they do not exclude the possibility that these B cells may eventually lead to parasite clearance.

We observed the up-regulation of several genes, in addition to *CXCL13*, that are associated with chronic inflammation. Elevated levels of the chemokines CCL18 (43), CXCL16 (44), and CXCL9 (45) have also been observed in tertiary lymphoid organs and may recruit lymphocytes to the site of inflammation. Like CXCL13, CCL18 is expressed in germinal centers and attracts naive lymphocytes (43). The chemokines CXCL16 and CXCL9 attract plasma cells (46, 47). BAFF promotes B cell survival and its overexpression in mice leads to hypergammaglobulinemia and autoantibody production (48). IL1b and IL18 are primary drivers of inflammation in mice, are elevated during chronic autoimmune diseases of humans, and are therapeutic targets in rheumatoid arthritis (49, 50).

The type I cytokines TNF and IFN-γ are involved in the immune response to PM (4). TNF regulates CXCL13 expression in some models and may be upstream of CXCL13 during PM. TNF is necessary for experimental follicle formation (51) and stimulates dendritic cell CXCL13 production (52). We also observed evidence of type I differentiation in B cells. IgG3 was the predominant IgG isotype observed, and a subset of B cells expressed T-bet, which is induced by IFN-γ and is necessary for type I differentiation (53).

The pathways identified in this study should be examined in other severe malaria syndromes, as they may be general phenomena during malaria infection. In addition, multiple soluble molecules that we identified by microarray analysis have potential use as biomarkers for diagnosing or assessing the severity of *P. falciparum* infections. Hepcidin is a cytokine-induced peptide that is a key mediator of the anemia of inflammation (54). We detected hepcidin expression in intervillous macrophages during chronic malaria infection, although in the present study hepcidin was not associated with hemoglobin concentration.

In summary, our results suggest that macrophage CXCL13 expression, B cell recruitment, Ab synthesis, and Ab-mediated activation of phagocytes contribute to the pathogenesis of chronic placental malaria, echoing features of lymphoid neogenesis. These findings may explain how malaria causes B cell dysfunction, as well as the high levels of Ig that develop during chronic infection. Future studies should assess whether the activation of immature B cells at the site of sequestered parasites may also interfere with the acquisition of protective immunity to malaria.

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Disclosures

The authors have no financial conflict of interest.

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