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The prenatally acquired T cell response to *Plasmodium falciparum* malaria and the role of regulatory T cells

Dissertation

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Human Cord Blood $CD4^+CD25^{hi}$ Regulatory T Cells Suppress Prenatally Acquired T Cell Responses to *Plasmodium falciparum* Antigens

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In malaria endemic regions, a fetus is often exposed in utero to *Plasmodium falciparum* blood-stage Ags. In some newborns, this can result in the induction of immune suppression. We have previously shown these modulated immune responses to persist postnatally, with a subsequent increase in a child's susceptibility to infection. To test the hypothesis that this immune suppression is partially mediated by malaria-specific regulatory T cells (T_{regs}) in utero, cord blood mononuclear cells (CBMC) were obtained from 44 Kenyan newborns of women with and without malaria at delivery. $CD4^+CD25^{lo}$ T cells and $CD4^+CD25^{hi}$ $FOXP3^+$ cells (T_{regs}) were enriched from CBMC. T_{reg} frequency and HLA-DR expression on T_{regs} were significantly greater for Kenyan as compared with North American CBMC ($p < 0.01$). CBMC/$CD4^+$ T cells cultured with *P. falciparum* blood-stage Ags induced production of IFN-γ, IL-13, IL-10, and/or IL-5 in 50% of samples. Partial depletion of $CD25^{hi}$ cells augmented the Ag-driven IFN-γ production in 69% of subjects with malaria-specific responses and revealed additional Ag-reactive lymphocytes in previously unresponsive individuals ($n = 3$). Addition of T_{regs} to $CD4^+CD25^{lo}$ cells suppressed spontaneous and malaria Ag-driven production of IFN-γ in a dose-dependent fashion, until production was completely inhibited in most subjects. In contrast, T_{regs} only partially suppressed malaria-induced Th2 cytokines. IL-10 or TGF-β did not mediate this suppression. Thus, prenatal exposure to malaria blood-stage Ags induces T_{regs} that primarily suppress Th1-type recall responses to *P. falciparum* blood-stage Ags. Persistence of these T_{regs} postnatally could modify a child's susceptibility to malaria infection and disease. ***The Journal of Immunology*****, 2011, 186: 2780–2791.**

Malaria infection during pregnancy constitutes a major public health problem in malaria-endemic regions of the world. Pregnant women, particularly those in their first pregnancy, are highly susceptible to malaria (1, 2). Malaria during pregnancy can lead to the sequestration of *Plasmodium falciparum*-infected erythrocytes in the placenta through adhesion to molecules such as chondroitin sulfate A (3–5) and is associated with increased risk of maternal anemia, low birth weight, growth retardation, and premature birth (6, 7). The accumulation of infected erythrocytes in the placenta may result in transplacental transport of infected erythrocytes or their soluble components, thereby exposing and sensitizing the fetal immune system to *P. falciparum* Ags (8–12). The reported frequency of malaria blood-stage–specific T and B cell responses in cord blood mononuclear cells (CBMC) ranges from ~5% to >70% (13–18). The consequences of this prenatal exposure of the infant to *P. falciparum* remain poorly understood.

Several observations indicate that some newborns may become immune tolerant to malaria blood-stage Ags in utero. Epidemiological studies show that offspring of women with placental malaria are more susceptible to *P. falciparum* infection and demonstrate higher parasitemia compared with offspring of women without placental malaria (19–21). Recently, we found that a subset of newborns of women infected with malaria during pregnancy acquired an immune tolerant phenotype, which persisted into childhood, characterized by increased IL-10 production, T cell anergy, and failure of CBMC to produce primarily IFN-γ and IL-2 in response to malaria blood-stage Ags (22). Importantly, these same children had increased risk for malaria infection compared with children who did not acquire this tolerant phenotype. Similar observations have been made for other human parasitic diseases such as lymphatic filariasis and onchocerciasis (23–25).

The mechanistic basis for this tolerant phenotype acquired in utero remains unclear. Possible explanations include clonal deletion of or anergy in malaria-specific T cells (26, 27) due to impaired APC function in cord blood (28–32). Alternatively, in utero exposure to malaria blood-stage Ags may trigger activation and expansion of regulatory T cells (T_{regs}) and/or increased production of immunomodulatory cytokines such as IL-10 or TGF-β (33–35). Recently, several studies have identified expanded populations of $CD4^+$ T cells capable of producing IL-10 in cord blood from offspring of women with placental malaria when compared with those without placental malaria (36, 37). In some of these studies, specifically $CD4^+CD25^{hi}$ cells have been shown to be an important source of IL-10 (36, 38). Depletion of $CD4^+C25^{hi}$ T cells from cord blood augmented the IFN-γ production of

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Abbreviations used in this article: CBMC, cord blood mononuclear cell; cRPMI, complete RPMI; FMO, fluorescence minus one; MSP, merozoite surface protein; PfP0, *Plasmodium falciparum* phosphoriboprotein P0; T_{eff}, effector T cell; T_{reg}, regulatory T cell.

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CBMC cultures stimulated with either malaria blood-stage Ags or mitogens, suggesting an immunoregulatory function of these cells (36–38). Further characterization indicated an expanded population of $CD4^+CD25^+FOXP3^+$ T cells after in vitro culture of CBMC with merozoites or staphylococcal enterotoxin B among offspring of mothers with chronic or past placental malaria (37). These studies, however, failed to isolate and fully characterize these T_{regs}. Because activated nonregulatory $CD4^+$ T cells can also express high levels of CD25, and FOXP3 expression can be induced in effector T cells (T_{eff}) upon activation in vitro (39), it remains unclear whether these cells are activated or directly suppressive.

In the current study, we focus on the potential role of T_{regs} in the fetal immune response to *P. falciparum* Ags and whether these regulatory cells suppress malaria Ag-driven responses by $CD4^+CD25^{lo}$ T cells. Newborns who have been exposed and/or sensitized to malaria blood-stage Ags in utero provide a unique opportunity to isolate and further characterize malaria-specific T_{regs} because of the large number of lymphocytes often available in cord blood. Isolation of T_{regs} from *P. falciparum* malaria-infected/exposed newborns, children, or adults has not been previously reported. In this study, we enriched for $CD4^+CD25^{hi}$ cells, the majority of which express the T_{reg} maker FOXP3, and evaluated their ability to actively suppress both spontaneous and *P. falciparum* blood-stage Ag-specific T_{eff} responses in vitro. We further evaluated the frequency, phenotype, and activation of $CD4^+$ T cell subsets among *P. falciparum*-sensitized versus not sensitized Kenyan neonates (born to women with and without malaria at delivery) relative to the frequency and phenotype of those cells in naive North American controls.

Materials and Methods

Study population

Mothers participating in the study delivered their children at Msambweni District Hospital (Kwale District, Coast Province, Kenya), where perennial *P. falciparum* transmission occurs. Umbilical cord blood was collected from full-term newborns immediately after parturition and was anticoagulated with heparin. Additionally, maternal peripheral blood and placental intervillous blood were obtained for malaria diagnosis as described (15). Full-thickness placental biopsies of ~1 cm square were obtained and stored in 10% buffered formalin. Subsequently, the sections were embedded in paraffin, sectioned, stained with H&E and Giemsa stain, and examined for the presence of malaria parasites in the placenta and/or hemozoin deposits. Control cord blood was obtained from healthy North American newborns delivered at University Hospitals, Cleveland, OH. Ethical approval was obtained from the Human Investigations Institutional Review Boards of University Hospitals (Case Western Reserve University, Cleveland, OH) and the Kenya Medical Research Institute in Nairobi.

Determination of malaria infection status

Plasmodium infections were identified via two methods: 1) blood smear; and 2) a post-PCR oligonucleotide ligation assay. Thick and thin blood smears were stained with 4% Giemsa for 20 min and examined under oil immersion (original magnification ×100). DNA was extracted from 200 μl erythrocyte pellet obtained from fetal cord blood and 200 μl whole maternal intervillous placental blood using Qiagen DNA extraction kits (DNeasy Kit; Qiagen). The post-PCR oligonucleotide ligation assay based on amplification of the small subunit rRNA gene was performed as previously described (40).

Ags and mitogens

Cytokine responses to two *P. falciparum* blood-stage Ags, merozoite surface protein (MSP)-1_{42} and *P. falciparum* phosphoriboprotein P0 (PfP0), were examined. rMSP-1_{42} [3D7 allele, the most common allele in the study population (I. Malhotra and C.L. King, unpublished observations)] was provided by Drs. C. Long, S. Singh, and D. Narum (Malaria Vaccine Development Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Three peptides corresponding to N- and C-terminal regions of PfP0 were synthesized and purified to 70–80% (Chiron, Clayton, Victoria, Australia). The peptides were designated N1 (DNVGSNQMASVRKSLR; codons 33–48), N2 (SVRKSLRGKATILMGKNT; codons 42–59), and C1 (AKADEPKKEEAKKVE; codons 285–299) and correspond to T cell epitopes identified by lymphocyte proliferation responses of immunized mice (41). PHA (Sigma-Aldrich) or anti-CD3/28–coated T cell expander beads (Dynal) were used as positive controls.

Isolation of mononuclear cells

CBMC were isolated within 2 h of collection by standard density gradient centrifugation on Ficoll-Paque (Amersham Biosciences). The overall scheme for cell preparation is shown in Fig. 1. Only freshly isolated CBMC were used in immunomagnetic cell separation steps and cell-culture experiments including suppression experiments. Cord blood samples from which $>1.2 \times 10^8$ CBMC were obtained ($n = 44$; shown in Table I) underwent immunomagnetic cell separation to isolate $CD4^+$ T cells, monocytes, and $CD4^+CD25^{hi}$ cells (Fig. 1). If $>1.8 \times 10^8$ CBMC were isolated, the excess CBMC were immediately cryopreserved ($n = 5$). If $<1.2 \times 10^8$ CBMC were obtained, they were not used for $CD4^+CD25^{hi}$ enrichment, and an aliquot of CBMC were cryopreserved. Irrespective of whether cells were used for selection of $CD4^+CD25^{hi}$, a small subset of freshly isolated CBMC were resuspended at a density of 10^6/ml in RPMI 1640 supplemented with 10% pooled human AB serum (Sigma-Aldrich), 4 mM L-glutamine, 25 mM HEPES, and 80 μg/ml gentamicin (complete RPMI [cRPMI]; BioWhittaker, Gathersburg, MD) and cultured with malarial Ags to detect cytokine production and lymphocyte proliferation.

For immunomagnetic cell separation, the remaining CBMC (if $>1.2 \times 10^8$) were washed and resuspended in MACS buffer (PBS, 2 mmol/l EDTA, and 0.5% BSA).

$CD25^{hi}$ cells were isolated by immunomagnetic positive selection using microbeads directly conjugated to anti-CD25 Abs (Miltenyi Biotec) at 2 μl/10^7 CBMC as previously described (42). This amount of anti-CD25 is 5-fold lower than the recommended 10 μl by the manufacturer. We did this to ensure selection of only $CD25^{hi}$ cells, those with high expression of CD25. This protocol reduced the recovery of $CD25^{hi}$ cells, but increased enrichment of $CD25^{hi}FOXP3^+$ cells.

After the first round of positive selection, the selected $CD25^{hi}$ cells were subjected to a second round of immunomagnetic separation that produced two populations of $CD25^{hi}$ cells: the double positively selected cells, which we designate $CD25^{hi++}$, and the cells remaining from the first round of positive selection (i.e., those not positively selected in the second round), which we designate $CD25^{hi+}$ (Fig. 1). $CD4^+CD25^{lo}$ T cells and $CD14^+$ monocytes were then isolated from $CD25^{hi}$ diminished CBMC by negative selection using the Isolation Kit II and the $CD4^+$ T Cell Isolation Kit II (both Miltenyi Biotec) following the manufacturer's instruction (Fig. 1).

After separation, cell populations were washed and resuspended in cRPMI and immediately used for cell cultures and add-back suppression assays. All samples that underwent the above described immunomagnetic cell separation and were used for suppression assays are listed in Table I.

In a subset of samples ($n = 8$), freshly isolated cells were directly stained for flow cytometric analysis to verify the purity of isolated cell populations (Fig. 2). CBMC contained 2–3% $FOXP3^+$ cells. After one round of magnetic selection, this was reduced by 40–81% in $CD25^{hi}$ diminished CBMC. $CD25^{hi+}$ showed an average enrichment of 62% for FOXP3 positivity ($CD25^{hi+}$, range 59–70%; $n = 8$). The twice positively selected $CD25^{hi++}$ were further enriched to an average of 76% for FOXP3 positivity ($CD25^{hi++}$, range 73–80%; $n = 8$).

Cell culture and suppression assay

Cell cultures were performed in round-bottom 96-well microtiter plates on freshly isolated cells. CBMC and CBMC diminished in $CD25^{hi}$ cells were cultured at 1×10^6/ml, and 10^5 $CD4^+$ T cells were cultured with 5×10^4 monocytes per well. $CD25hi^{++}$ or $CD25hi^+$ cells were added to $CD4^+$/monocyte cultures at different ratios (ratio $CD4^+/CD25^{hi}$ at 1:0, 1:1, and 1:0.5) to assess suppressive activity of isolated $CD25^{hi}$ populations. Additional medium was added to wells, so that the final volume was 200 μl. Lymphocytes were stimulated in separate cultures under the following conditions: 1) with highly purified MSP-1_{42} (5 μg/ml, kindly provided by Carole Long and David Narum at Malaria Vaccine Development Unit, National Institutes of Health); 2) with PfP0 N1, N2, and C1 peptides (10 μg/ml); 3) with either anti-CD3/28 beads (one bead/five $CD4^+$ T cells) or PHA (10 μg/ml) as a positive control; and 4) with medium alone (negative control). Optimal concentrations had been determined in previous studies and pilot experiments (14, 15). Neutralizing anti–IL-10 and/or anti–TGF-β were added to a subset of samples to assess the role of immunosuppressive cytokines ($n = 12$; samples were selected based on available number of cells). Culture wells containing $CD4^+$ T cell/APCs with and without

$CD25^{hi++}$ or $CD25^{hi+}$ cells were supplemented with 1 μg/ml anti–IL-10 (JES3-9D7; BD Biosciences) and/or 1 μg/ml anti-TGF-β (clone MAB 1835; R&D Systems) based on the manufacturer's recommendation. Cultures were set up in triplicate where sufficient cell numbers were available. Due to limitation on samples and number of isolated $CD25^{hi}$ cells, not all tests could be carried out on all samples.

Quantification of cytokines and lymphocyte proliferation

Quantification of the cytokines IFN-γ, IL-13, IL-5, IL-2, IL-6, and IL-10 was performed on culture supernatants collected at 120 h. IFN-γ was measured by ELISA. The Ab pair for cytokine capture and detection (biotinylated) was as follows: M-700A and M-701B (Endogen, Cambridge, MA). IL-5, IL-10, IL-2, IL-6, and IL-13 were measured using a bead-based multicytokine immunoassay (Upstate Luminex kit) following the manufacturer's instruction. The lower limit of detection for the various cytokines that were evaluated ranged from 5–10 pg/ml depending on the cytokine (5 pg/ml for IL-5 and 10 pg/ml for IL-10, IL-2, IL-6, and IL-13). A positive response was scored when the following criterion was fulfilled: for both CBMC and $CD4^+$ T cell cultures, an Ag-driven cytokine production that was at least 2-fold greater than that of parallel cultures containing medium alone. If cytokine production was not detectable in the negative control cultures, ≥20 pg/ml for the Ag-specific cytokine production was considered to be a positive response. No *P. falciparum* Ag-driven cytokine response was detected in test cultures of CBMC from 16 healthy North American newborns.

Lymphocyte proliferation was performed as previously described (14). Samples were performed in triplicate. A positive response was a stimulation index (cpm in test sample/cpm in cultures with medium alone) >2.

Flow cytometric analysis

To evaluate the purity of selected cells, isolated cell populations (CBMC, $CD25^{hi}$ diminished CBMC, $CD25^{hi++}$, $CD25^{hi+,}$ $CD4^+$ T cells, and monocytes) from eight donors were washed in FACS buffer (PBS, 2% FBS, and 0.09% sodium azide) directly after cell separation and surface-stained with anti-CD25 (clone MA-251) and anti-CD4 (clone SK3) (both from BD Biosciences). For FOXP3 expression analysis, intranuclear staining was conducted using the anti-human FOXP3 staining kit according to manufacturer's instruction (clone PCH101; eBioscience). Monocyte populations were stained with CD14 (clone M5E2; BD Biosciences). Stained cells were refrigerated at 4°C and read within 12 h on a four-color flow cytometer (FACS scan with additional second diode to allow detection of allophycocyanin staining) at Coast Province General Hospital in Mombasa, Kenya. Assessment of purity could not be undertaken on all the samples due to limited cell numbers, in particular of the $CD25^{hi}$ populations, and very limited access to a flow cytometer in Kenya.

For a broader characterization of $CD4^+$ T cells and T_{reg} surface molecules and FOXP3 expression by flow cytometry, liquid nitrogen-stored Kenyan CBMC were transferred to the United States. Based on cytokine responses in cell culture assays and availability of sufficient frozen Kenyan CBMC, 17 samples were selected and grouped as sensitized ($n = 9$) and not sensitized ($n = 8$). Of the selected Kenyan CBMC, 5 samples represented the same individuals used for $CD25^{hi}$ enrichment experiments and suppression assays (numbers 31, 37, 38, 42, and 44, Table I, Supplemental Table I), and 12 samples were chosen from additional frozen CBMC samples from the same population collected in the same time period (Supplemental Table I, samples F1–F12). In addition, nine frozen North American CBMC samples were included in experiments.

Cryopreserved cells were quick-thawed, washed with PBS, and incubated in cRPMI at 37°C for 2 h. Cells were then washed in FACS buffer and surface stained with the following Abs: CD4 (SK3), CTLA-4 (BNI3) (both from BD Biosciences), CD3 (UCHT1), CD45RO (UCHL1), CD25 (BC96), CD127 (eBioRDR5), HLA-DR (LN3), CD62L (DREG56) (all from eBioescience) and intracellularly stained for FOXP3 (clone PCH101; eBioscience). Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Unstained cells and single stained cells/beads were included in all experiments. Cells were first gated based on forward and side scatter to exclude dead cells and cell debris. For characterization of $CD4^+$ T cells and T_{regs}, lymphocytes were first gated for $CD3^+CD4^+$ cells (Fig. 3). The gate in the *right panel* of Fig. 3 shows designation for $CD25^+CD127^{lo}$ cells. $CD4^+$ T cells and $CD4^+CD25^+CD127^{lo}$ cells were further analyzed for expression of CD45RO, CTLA-4, HLA-DR, and CD62L. Fluorescence minus one (FMO) controls were used to define the gates. It is a staining control that employs all reagents used in a flow cytometry assay except for one fluorochrome of interest (termed FMO) to control for the contribution of spectral overlap to the background when using multiple fluorochromes (43, 44).

Statistical analysis

The significance of differences among three groups was assessed using Kruskal-Wallis testing with Dunn's posttesting (GraphPad Software v4.0, GraphPad). For paired comparisons between two groups, Student *t* test was performed on log-transformed data. Correlation analysis was conducted using Pearson's rank-correlation test.

Results

Study population

The criterion for including individual samples from the study population for functional assays was the ability to obtain sufficient number of lymphocytes ($>1.2 \times 10^8$ CBMC) for $CD25^{hi}$ purification ($n = 44$; Table I; see Figs. 1 and 2 for $CD25^{hi}$ purification). Mothers of these offspring were primarily primigravid (22 out of 42, 52%) and secundigravid (10 out of 42, 24%) women. Of 36 mother/infant pairs tested, 8 (22%) women and/or their newborns showed evidence of active or prior malaria infection during pregnancy as determined by blood smear, PCR, and/or placental microscopy at delivery. Twenty-three of 44 (52%) CBMC samples showed recall responses to one or both malaria blood-stage Ags based on lymphocyte proliferation and/or cytokine production (IFN-γ, IL-13, IL-5, IL-6, IL-2, and/or IL-10; Table I, section 1). There was a mixture of Th1- and Th2-type cytokine production by cord blood lymphocytes in response to malaria blood-stage Ags. Sixteen out of 42 samples tested for IFN-γ (38%) produced IFN-γ in response to *P. falciparum* Ags, and 15 of 32 (46%) showed an IL-13 response (Table I, section 1). Four CBMC samples produced IL-5 (numbers 18, 34, 38, and 40). Cytokine concentrations measured for IL-5 were low, between 15 and 44 pg/ml. Of note, we were unable to detect malaria Ag-induced IL-2 at 120 h due to its consumption in 5-d cultures. Only one sample (number 38) produced IL-6. Kenyan newborns whose samples showed ≥1 positive cytokine recall response and/or lymphocyte proliferation to *P. falciparum* Ags were subsequently classified as sensitized (Table I, section 1). The majority of sensitized samples (16 out of 23) produced either multiple cytokines to *P. falciparum* Ag(s) or produced one cytokine in response to both Ags (i.e., samples 21, 39, and 40, Table I, section 1).

Of note, 6 of 8 (75%) CBMC samples from malaria-infected women demonstrated recall responses to malaria blood-stage Ags, whereas CBMC from 13 of 36 (36%) malaria not-infected women also had fetal priming to malaria Ags indicative of prior malaria exposure.

Higher numbers of T_{regs} are observed in cord blood of offspring sensitized or exposed to malaria Ags in utero

To determine whether CBMC from Kenyan newborns sensitized to malarial Ags in utero have increased frequency of memory T cells or lymphocytes with a T_{reg} phenotype, we did the following: first, we classified newborns as sensitized or not sensitized based on cell culture secretion of cytokines in response to *P. falciparum* Ags. A sample was classified as sensitized if at least one positive cytokine response (IFN-γ, IL-13, IL-5, and/or IL-10) was measured. Newborns were classified as not sensitized if none of the cytokine measurements were positive in response to MSP-1 and PfP0 (Supplemental Table I). We then examined expression of T_{reg}, memory, and activation markers by flow cytometry for: 1) Kenyan newborns sensitized to malaria ($n = 9$); 2) Kenyan newborns not sensitized to malaria ($n = 8$); and 3) healthy North American newborns ($n = 9$).

As expected the overall frequency of $CD4^+$ T cells that expressed the memory cell marker, $CD4^+CD45RO^+$ was low in cord blood, 10–12%, and was similar among the three groups (Supplemental Table II). The majority of memory T cells possessed a central

Table I. Study subjects of cell separation and suppression experiments

Sample Identification No.[a]	Parity	Presence of Malaria Infection			Lymphocyte Sensitization to *P. falciparum* Blood-Stage Ags (net pg/ml)				
		IVPB	CB	Placental Biopsy	IFN-γ	IL-13	IL-10	LP (SI)	Ag Response
Samples that underwent magnetic bead separation and showed cytokine response to *P. falciparum* Ags (sensitized samples)									
2	Unknown	ND	ND	ND	ND	206	–[b]	2	MSP-1
3	3	ND	ND	ND	296	–	15	2	MSP-1
4	0	ND	ND	ND	–	–	–	2.8	MSP-1
6	1	ND	–	ND	–	66	–	–	MSP-1
9	4	–	–	Chronic	430	–	–	–	PfP0
10	0	–	–	–	132	–	–	–	PfP0
11	3	–	–	–	15	–	35	–	MSP-1
18	0	–	–	Chronic	97	280	128	–	MSP-1/PfP0
21	0	–	–	–	–	168	295	–	PfP0
27	0	–	–	–	–	107	–	–	MSP-1
28	1	–	–	–	39	165	–	ND	MSP-1/PfP0
30	1	–	–	–	46	239	–	ND	PfP0
32	2	–	–	–	–	200	–	ND	PfP0
33	2	–	+	Acute/chronic	25	53	–	ND	MSP-1/PfP0
34	0	–	–	–	118	–	–	ND	PfP0
35	0	–	–	–	244	–	181	ND	MSP-1/PfP0
37[c]	0	–	–	–	20	20	–	ND	PfP0
38[c]	0	+	–	–	–	266	–	ND	MSP-1
39	0	–	–	–	72	–	–	ND	MSP-1/PfP0
40	1	+	–	Acute	154	442	186	ND	MSP-1/PfP0
41	0	–	–	ND	445	539	–	ND	MSP-1
43	0	–	–	–	895	420	–	ND	MSP-1/PfP0
44[c]	0	–	–	Acute/chronic	278	187	–	ND	MSP-1
Remaining samples that underwent magnetic bead separation									
1	Unknown	ND	ND	ND	–	–	–	–	–
5	0	ND	–	ND	–	–	–	–	–
7	0	ND	–	ND	–	ND	ND	–	–
8	0	ND	–	ND	–	ND	ND	–	–
12	0	–	–	–	–	ND	ND	–	–
13	2	–	–	–	–	–	–	–	–
14	2	–	–	–	–	ND	ND	–	–
15	2	–	–	–	–	ND	ND	–	–
16	1	–	–	–	–	ND	ND	–	–
17	0	+	–	–	–	–	–	–	–
19	1	–	–	–	–	ND	ND	–	–
20	1	–	–	–	ND	ND	ND	–	–
22	1	+	–	Acute	–	–	–	–	–
23	0	–	–	–	–	ND	ND	–	–
24	0	–	–	–	–	ND	ND	–	–
25	1	–	–	–	–	ND	ND	–	–
26	0	–	–	–	–	ND	ND	–	–
29	3	–	–	–	–	–	–	ND	–
31[c]	0	–	–	–	–	–	–	ND	–
36	1	–	–	–	–	–	–	ND	–
42[c]	4	–	–	–	–	–	–	ND	–

Maternal parity, presence of malaria infection, and cytokine production by CBMC to *P. falciparum* blood-stage Ag from all study subjects undergoing magnetic bead separation and suppression experiments.

[a]Sample identification numbers correspond to the chronology of sample acquisition.

[b]– indicates values considered to be background or zero values.

[c]Samples 31, 37, 38, 42, and 44 were also included in flow cytometry experiments to further characterize T cell phenotype (see Supplemental Table I).

CB, cord blood; IVPB, intervillous placental blood; LP, lymphocyte proliferation; ND, not done; SI, stimulation index.

memory phenotype ($CD45RO^+CD62L^{hi}$). There was also no difference in HLA-DR and CTLA-4 expression on $CD4^+$ T cells among the three groups (Supplemental Table II).

To evaluate the relative proportion of T_{regs} among the three groups, gated $CD4^+$ T cells were further gated for a subpopulation associated with a T_{reg} phenotype, $CD25^+CD127^{lo}$ (gating schema shown in Fig. 3) (45–48).

Although FOXP3 is a more robust marker of T_{regs}, we found intranuclear staining of FOXP3 technically difficult to perform simultaneously with some of our chosen surface markers, such as CTLA-4, CD45RO, and HLA-DR. Therefore, to validate the association of $CD25^+CD127^{lo}$ with $FOXP3^+$ cells, lymphocytes from nine cord blood samples were examined with a reduced panel that included CD4, CD25, CD127, and FOXP3.

$FOXP3^+$ cells were mainly found within the $CD25^+CD127^{lo}$ population. Whereas 69–80% ($n = 9$) of $CD4^+$ T cells staining for $CD25^+CD127^{lo}$ were $FOXP3^+$, only 1 to 2% of $CD4^+CD25^-$ cells and 4–6% of $CD4^+CD25^+CD127^+$ cells expressed FOXP3 (see Supplemental Fig. 1 for one representative sample). Conversely, 72–83% of $CD4^+$ $FOXP3^+$ T cells were $CD25^+CD127^{lo}$ (data not shown). The percentages of FOXP3 and $CD25^+CD127^{lo}$ staining $CD4^+$ cells were therefore highly correlated ($r^2 = 0.84$, $p < 0.0001$; Supplemental Fig. 1) in CBMC. Together, this suggests that cell populations expressing the markers FOXP3 and $CD25^+$ $CD127^{lo}$ are very similar and representative of T_{regs}.

Kenyan newborns sensitized in utero to malaria blood-stage Ags had an average of 4.7% of their $CD4^+$ cells expressing $CD25^+$ $CD127^{lo}$ compared with an average of 3.7% and 3.1% of cord

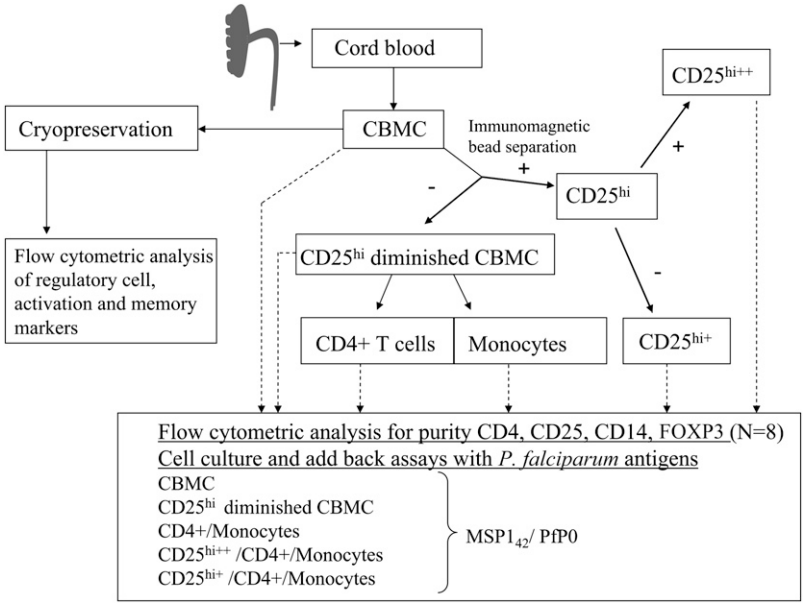

FIGURE 1. Experimental overview and cell separation schema. The flow chart presents an overview of the experimental setup as well as the overall scheme for CBMC cell separation and enrichment of $CD25^{hi}$ cells. Freshly isolated CBMC were used for immunomagnetic bead separation. The first round of enrichment produced $CD25^{hi}$ cells that underwent a second round of separation, generating two different populations: the twice positively selected $CD25^{hi++}$ cells and the remaining cells, referred to as $CD25^{hi+}$. $CD4^+$ T cells and monocytes were further enriched from $CD25^{hi}$ diminished CBMC as described in the *Materials and Methods* section. Flow cytometry was performed on a subset of these isolated cells to demonstrate purity and FOXP3 positivity (n = 8). CBMC and isolated lymphocyte fractions were set up for cell culture and add-back experiments. Cryopreserved CBMC were later used for flow cytometric analysis of regulatory cells, memory, and activation marker expression.

blood $CD4^+$ cells from not sensitized newborns or unexposed North American newborns, respectively (Fig. 4*A*). The frequency of $CD25^+CD127^{lo}$ cells in the malaria-sensitized group was significantly greater compared with North Americans ($p < 0.01$; Fig. 4*A*). Significantly more $CD25^+CD127^{lo}$ cells from Kenyan newborns expressed HLA-DR^+ (0.95%) compared with North American controls (0.32%, $p < 0.01$; Fig. 4*B*), indicating greater activation or expansion of these T_{regs} in Kenyan newborns (49).

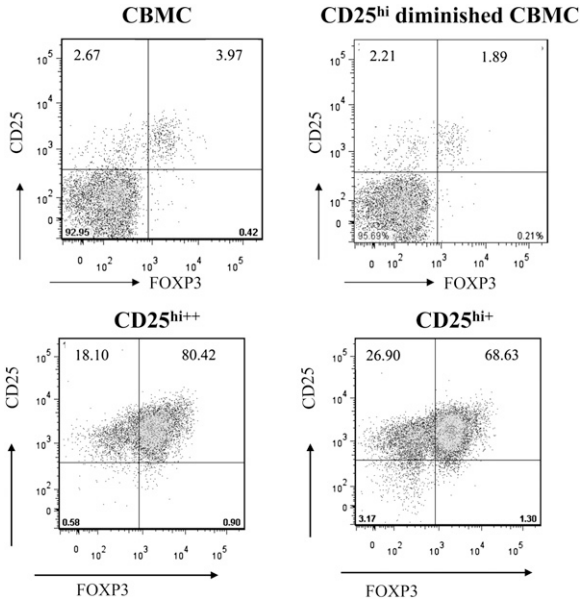

FIGURE 2. Cell separation and T_{reg} isolation. The flow cytometry dot plots show $FOXP3^+$ and $CD25^{hi}$ expression on one representative sample of CBMC, CBMC diminished in $CD25^{hi}$, $CD25^{hi++}$, and $CD25^{hi+}$ cells after gating for $CD4^+$ T cells. Cells were first gated based on forward and side scatter to exclude dead cells and cell debris and then gated for $CD4^+$ T cells based on side scatter and expression of CD4. Gates for FOXP3 and CD25 were based on FMO controls. Total of 4.4% of $CD4^+$ cells in CBMC expressed FOXP3, which was reduced by ~50% in $CD25^{hi}$ diminished CBMC after one round of CD25 selection. Total of 80% of the $CD4^+$ cells in the $CD25^{hi++}$ population were $FOXP3^+$ compared with 69% in the $CD25^{hi+}$ population.

By contrast, there was no difference in CTLA-4 expression among the three groups (mean percentage was 0.24, 0.23, and 0.2% for Kenyan-sensitized, not sensitized, and North Americans, respectively) nor for the memory effector cell phenotype $CD45RO^+$ $CD62L^{lo}$ (mean percentage was 16, 16.5, and 14.5%) or for the central memory phenotype $CD45RO^+CD62L^{hi}$ (mean percentage was 7.2, 7.7, and 9.6%).

Effect of $CD25^{hi}$ depletion on recall responses to malaria blood-stage Ags by CBMC

To assess whether $CD4^+CD25^{hi}$ T cells suppress malaria Ag-induced cytokine production by CBMC, we partially depleted $CD25^{hi}$ T cells from CBMC using a single round of immunomagnetic selection in subjects shown in Table I. Examination of $FOXP3^+CD25^{hi}$ in CBMC before and after depletion (CBMC versus $CD25^{hi}$ diminished CBMC) showed an average reduction of 64% (range 40–81%, n = 8; Fig. 2). Partial depletion of $CD4^+$ $CD25^{hi}$ augmented the net malaria blood-stage Ag-driven IFN-γ in 11 of 16 malaria-sensitized subjects (previously shown malaria Ag-specific IFN-γ recall response), whereas in the remaining 5 subjects, there was a decrease or no change in IFN-γ release (Fig. 5*A*). Among the 26 subjects that were not identified as sensitized, partial depletion of $CD25^{hi}$ cells resulted in detection of Ag-driven IFN-γ production in three subjects (Fig. 5*B*), indicating that the failure to observe Ag-driven cytokine production in some cord blood cells may result from active suppression through T_{regs}. In contrast to IFN-γ, partial depletion of $CD4^+CD25^{hi}$ resulted in a significant decrease in IL-13 production for seven subjects, no change in four, and an increase in four subjects (data not shown). Comparatively fewer subjects showed malaria Ag-driven IL-5 and IL-10 (Table I). $CD4^+CD25^{hi}$ cell depletion produced no consistent effect on increased or decreased IL-5 or IL-10 production (data not shown). Thus, $CD25^{hi}$ cells have a variable effect on modulating Ag-driven cytokine production in CBMC, with a generally suppressive effect on malaria Ag-driven Th1-like cytokine production, but not on Th2-type cytokine production (i.e., IL-13 and IL-5).

In cultures with measurable spontaneous cytokine release, partial depletion of $CD25^{hi}$ cells increased IFN-γ levels by 2.2–13.5-fold in 6 subjects, decreased by >50% in 5 subjects, and showed no change in the remaining 31 subjects. By contrast, no

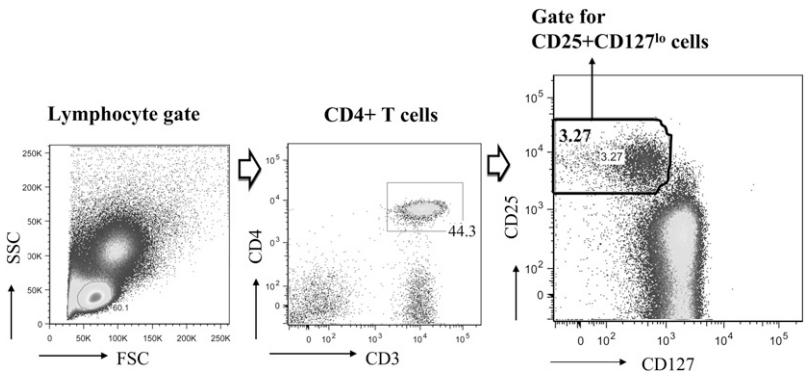

FIGURE 3. $CD25^{+}CD127^{lo}$ gating schema. Cells were first gated based on forward and side scatter to exclude dead cells and cell debris and then gated for $CD4^{+}$ T cells based on expression of CD4 and CD3. $CD4^{+}$ T cells were further gated for $CD25^{+}CD127^{lo}$ cells as shown in the *right panel*.

subject demonstrated a >2-fold change in spontaneous IL-13, IL-5, and IL-10 production following partial removal of $CD25^{hi}$ cells (data not shown).

Suppressive effects of enriched $CD4^{+}CD25^{hi}$ T_{regs} on Ag-driven cytokine production by $CD4^{+}CD25^{lo}$ T cells

To directly evaluate the ability of $CD4^{+}CD25^{hi}$ T_{regs} to suppress the production of cytokines by malaria Ag-specific T cells from cord blood, we first isolated $CD25^{hi}$ lymphocytes immunomagnetically. We adopted a strategy in which $CD25^{hi}$ cells were positively selected twice using anti-CD25–coated beads. After two rounds of immunomagnetic separation, we obtained two populations: $CD25^{hi++}$ and $CD25^{hi+}$ cells (Fig. 1). A total of 75–85% of the selected cells were $CD4^{+}$. In the $CD25^{hi++}$ population, 78–85% of the $CD4^{+}$ cells were $FOXP3^{+}$ (Fig. 2). The $CD25^{hi+}$ population (positively selected in the first round of selection, but not retained magnetically in the second round) showed lower levels of purity, with ~68–75% of $CD4^{+}$ cells expressing FOXP3.

Fig. 2 shows the CD25 and FOXP3 expression of a representative sample of $CD25^{hi++}$ and $CD25^{hi+}$ populations, both gated for $CD4^{+}$ cells. Of note, preliminary studies of enriched $CD25^{hi++}$ show low expression of CD127 (data not shown). Cell purity of selected monocytes and $CD4^{+}$ T cells was consistently >90% and >93%, respectively. Cell recovery was generally low, typically between 3 and 8×10^{5} $CD25^{hi++}$ cells from a total $>1.2 \times 10^{8}$ CBMC. Enriched $CD25^{hi++}$ cells (cultured with CD14 positively selected monocytes, ratio of 2:1 for $CD25^{hi++}$ to monocytes; $n = 5$) had a phenotype characteristic of T_{regs} (50); they failed to proliferate or produce IL-2 in response to PHA compared with strong

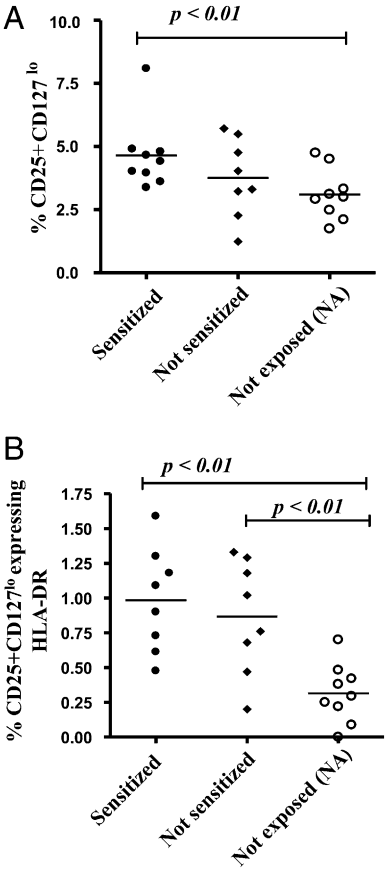

FIGURE 4. Frequency and HLA-DR expression of $CD4^{+}CD25^{+}$ $CD127^{lo}$ cells is increased in Kenyan neonates. Figures present the percentage of $CD4^{+}CD25^{+}CD127^{lo}$ cells (*A*) and $CD4^{+}CD25^{+}CD127^{lo}$ cells expressing the activation marker HLA-DR (*B*) in $CD4^{+}$ T cells from *P. falciparum*-sensitized and not sensitized Kenyan and North American newborns. Kruskal-Wallis test with Dunn's posttest comparisons were used to assess the significance of differences (shown in figure). The overall p value that includes all three groups for *A* is $p = 0.0083$ and for *B* is $p = 0.0011$.

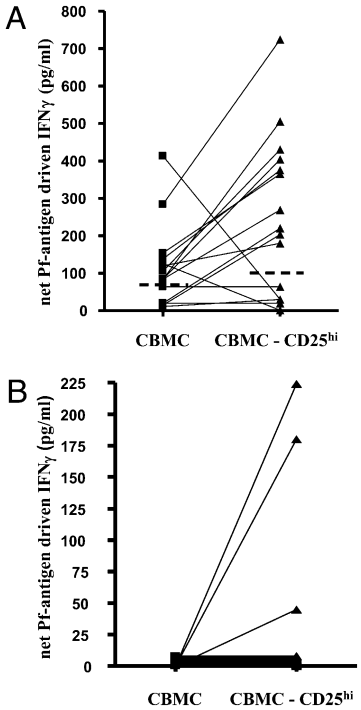

FIGURE 5. Depletion of $CD25^{hi}$ cells enhances IFN-γ responses to *P. falciparum* Ags in some individuals. The effect of partial depletion of $CD25^{hi}$ cells on net malaria blood-stage Ag-driven IFN-γ production from Kenyan CBMC sensitized (*A*) or not sensitized (*B*) as evaluated in Table I is shown. The dashed lines represent the geometric mean.

proliferation responses of similarly cultured $CD4^+CD25^{lo}$ T cells (data not shown). Enriched $CD4^+CD25^{hi++}$ lymphocytes did, however, spontaneously produce variable amounts of IL-10; four out of seven individuals tested produced from 98–627 pg/ml of IL-10, and one subject secreted 643 pg/ml IL-6. There was no spontaneous production of IL-5, IL-13, or IFN-γ.

To evaluate the suppressive capacity of enriched $CD25^{hi++}$ on $CD4^+CD25^{lo}$ T cells (subsequently referred to as $CD4^+$ T cells), $CD4^+$ T cells were cocultured with monocytes alone as APCs or with the addition of an equal number of enriched $CD25^{hi++}$ cells (e.g., a 1:1 ratio plus APCs). T_{regs} were added to cultures in the absence (spontaneous) or presence of malaria blood-stage Ag (Fig. 6). Fifteen subjects' CBMC had detectable spontaneous IFN-γ production, of which nine (60%) showed complete suppression by addition of T_{regs}, four showed partial suppression, and two showed increased IFN-γ production (Fig. 6*A*; $p = 0.01$). We next examined the effect of adding T_{regs} to cultures in the presence of malaria blood-stage Ags (Fig. 6*B*, 6*C*). Fig. 6*B* illustrates an experiment from one CBMC sample. Of note, because there were insufficient numbers of highly enriched cells to add at a lower ratio, less highly enriched $CD25^{hi+}$ cells (obtained after a single round of positive selection) were added to $CD4^+$ cells at 1:1 and 0.5:1 ratio. Often the addition of $CD25^{hi++}$ resulted in complete suppression of malaria Ag-driven IFN-γ, for which levels of suppression decreased in a dose-dependent fashion with the less enriched $CD25^{hi+}$ cells added (Fig. 6*B*, 6*C*). Fig. 6*C* summarizes the results of suppressive effects of T_{regs} on all CBMC showing malaria Ag-induced IFN-γ production. Complete suppression of Ag-driven IFN-γ was observed in 12 of 16 subjects, and 3 had partial suppression. Overall, the addition of $CD25^{hi++}$ produced a mean average suppression of 85% for malaria Ag-driven IFN-γ production by $CD4^+$ cells ($p < 0.001$), and with lower numbers of T_{regs} ($CD25^{hi+}$), the suppression was 70% ($p < 0.05$).

The effect of $CD25^{hi++}$ on suppression of Ag driven IL-13 release was less pronounced than that observed for IFN-γ (Fig. 7). Addition of $CD25^{hi++}$ to $CD4^+$ cells in the absence of malaria Ag failed to suppress spontaneous IL-13 release ($CD4^+$ alone, geometric mean = 10, and $CD4^+$ with $CD25^{hi++}$ geometric mean = 24; Fig. 7*A*). Fig. 7*B* shows an experiment from one CBMC sample. In all subjects with malaria Ag-driven IL-13, 4 of 15 subjects had complete inhibition, 1 showed no inhibition, 1 individual demonstrated an increase in IL-13, and the remaining 9 individuals showed partial suppression (Fig. 7*C*). The overall mean level of suppression was 61% with highly enriched $CD25^{hi++}$ ($p < 0.05$). Addition of less enriched $CD25^{hi+}$ cells failed to induce significant IL-13 suppression ($p > 0.05$). Although not shown, similar results were observed for malaria Ag-induced IL-5. Thus, $CD25^{hi++}$ T cells showed weaker suppression of Th2-type cytokine production compared with Th1-type responses. Of note, IL-10 production by $CD4^+$ T cells was not suppressed by adding $CD25hi^{++}$ cells (data not shown); rather the addition augmented IL-10 production in 5 of 6 subjects showing an IL-10 response to malaria Ags (data not shown).

Suppression of $CD4^+$ T cells by T_{regs} is not dependent on IL-10 or TGF-β production

Because some isolated $CD25^{hi++}$ cells from Kenyan newborns produced IL-10, and IL-10 and TGF-β are known to mediate suppression of T cells responses (36, 51–53), we evaluated whether these cytokines from T_{regs} or other IL-10/TGF-β–producing $CD4^+$ cells may contribute to the observed immune suppression. The addition of neutralizing anti–IL-10 and/or anti–TGF-β to cultures failed to significantly augment spontaneous IFN-γ (Fig. 8*A*; $n = 12$, $p = 0.14$) or IL-13 production (data not shown). Addition of $CD25^{hi++}$ T_{regs} suppressed spontaneous IFN-γ production by $CD4^+$ cells and continued to suppress $CD4^+$ cells' spontaneous IFN-γ production after addition of anti–IL-10/TGF-β (Fig. 8*A*; $p = 0.03$). With respect to Ag-driven cytokine production, neutralizing Abs to IL-10– and/or TGF-β–augmented malaria Ag-driven IFN-γ in some individuals (Fig. 8*B*; representative of 2 out of 12 studied) but not others (Fig. 8*C*; representative of 2 out of 12; the remaining 8 out of 12 did not show any Ag-

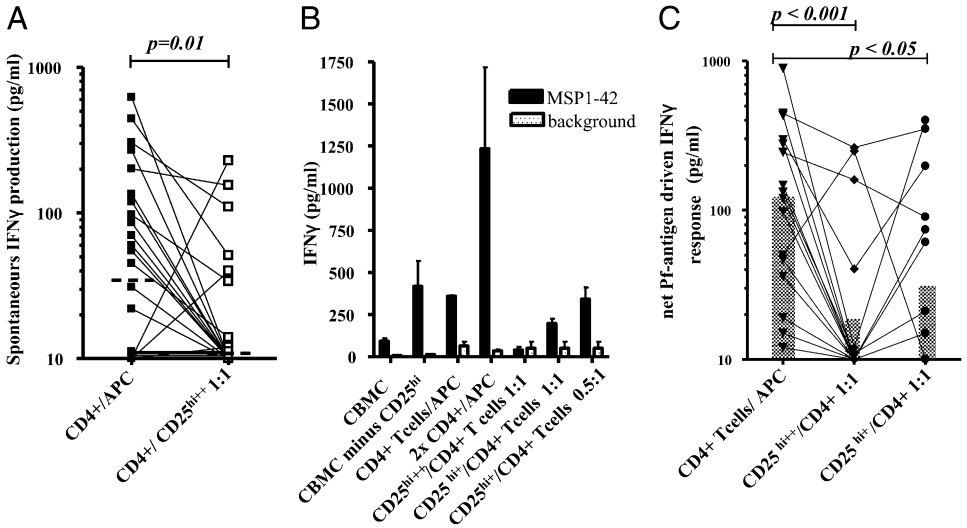

FIGURE 6. Cord blood $CD25^{hi}$ T_{regs} suppress spontaneous and *P. falciparum*-Ag–specific IFN-γ response. The figures illustrate the effect of enriched $CD25^{hi}$ cells on suppression of spontaneous (*A*) and malaria Ag-driven IFN-γ (*B*, *C*). *B* shows an experiment with CBMC from newborn number 3 (Table I) in which $CD25^{hi++}$ and $CD25^{hi+}$ cells are enriched and added back to purified $CD4^+$ T cells with APCs. This subject was shown because sufficient $CD25^{hi+}$ cells were available for add back at several ratios. Cultures were performed in triplicate unless there were insufficient cells, and values represent mean + SE. *C* summarizes the results of subjects for which there was detectable net malaria Ag-driven IFN-γ. Each point represents mean net (Ag-induced minus spontaneous) cytokine production for responders. Lines connect CBMC from the same individual for the three culture conditions indicated. The condition 2× $CD4^+$/APC was included as an additional control condition. Subsequent add-back experiments are to 1× CD4 T cells. Dashed lines (*A*) and bars (*C*) in the figures show the geometric mean cytokine production for all individuals. Kruskal-Wallis test with Dunn's posttest comparisons were used to assess the significance of differences (shown in figure). The overall p values that include all three groups for *C* is $p = 0.0002$.

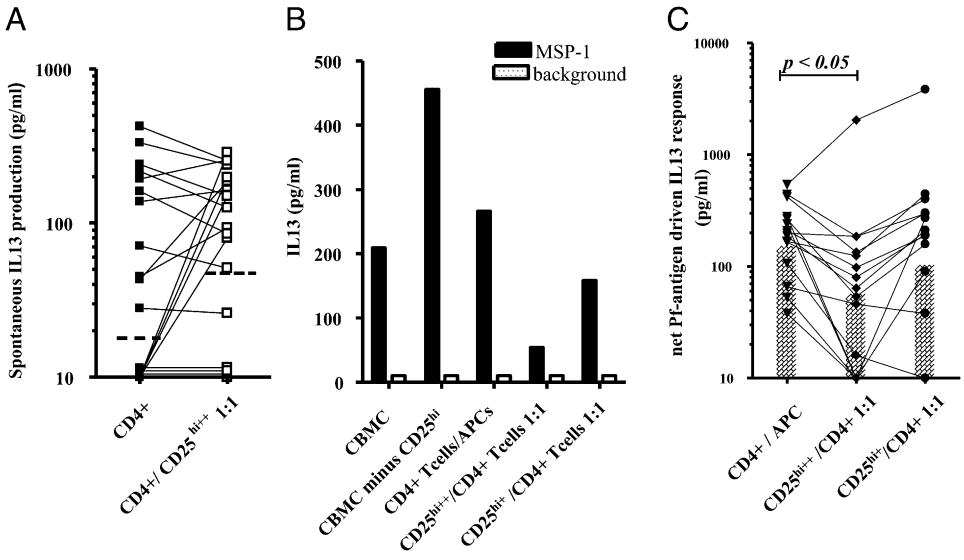

FIGURE 7. Cord blood $CD25^{hi}$ T_{regs} suppress *P. falciparum*-Ag–specific, but not spontaneous IL-13 response. Enriched $CD25^{hi}$ cells fail to suppress spontaneous IL-13 release from $CD4^+CD25lo$ T cells (*A*) but $CD25^{hi++}$ do suppress malaria Ag-driven IL-13 production (*B*, one individual) and for all malaria-Ag–reactive individuals that produced IL-13 (*C*). Panels are identical to those described in legend of Fig. 6 with the exception that *B* shows results from subject 38 in Table I. The overall *p* value (for the three groups) for *C* is $p = 0.023$, and comparison for degree of suppression of $CD4^+C25^{hi++}$ on $CD4^+CD25^{lo}$ cells is partial and significant at $p < 0.05$ level using the same analysis described in the legend of Fig. 6.

driven cytokine production above background). Importantly, the blocking of endogenous IL-10 and/or TGF-β failed to reverse the suppressive effect of $CD4^+CD25^{hi++}$ cells on malaria Ag-driven IFN-γ (Fig. 8*B*, 8*C*) or IL-13 (data not shown).

Discussion

In humans and other primates a unique maternal–fetal interface develops where the placenta of fetal origin is in direct contact with maternal blood (i.e., hemochorial placenta development). This allows for efficient gas and nutrient exchange and the transplacental transfer of Abs from maternal to fetal circulation. However, this physiology requires development of immunologic mechanisms whereby the semiallogeneic fetus escapes recognition by the maternal immune system. This requirement provides for the expression on the placenta of nonclassical MHC class I HLA-G, which stimulates inhibitory receptors on cells of lymphoid and myelomonocytic origin (54, 55). It also selects for the production of immune inhibitory cytokines, PGs, and immunoregulatory T cells (56, 57). The increasing study of $CD4^+CD25^+FOXP3$ T_{regs} in cord blood has shown they are typically immature and not activated compared with those from adult blood, yet they appear to be more suppressive (42, 58–61). When fetal T_{regs} are exposed to nonself-Ags in utero, however, they become critical in the regulation of maternal cell interaction in the fetus, and these cells persist well into childhood (62). Thus, the introduction of exogenous Ags, such as malarial products, into this potent fetal regulatory milieu may heighten stimulation of Ag-specific T_{regs}. In this study, we show the presence of malaria blood-stage Ag-specific

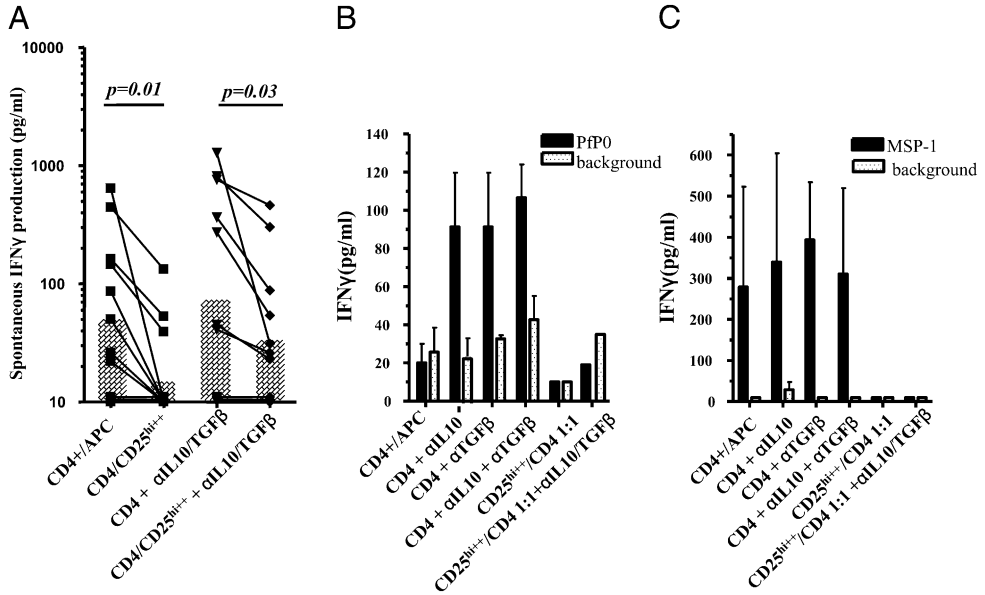

FIGURE 8. Anti–IL-10/anti–TGF-β do not abrogate suppression by $CD25^{hi}$ T_{regs}. Suppressive effects of $CD25^{hi++}$ T_{regs} on spontaneous cytokine release and malarial Ag-induced cytokine production were examined with and without neutralizing anti–IL-10 and anti–TGF-β. *A* shows the effects of neutralizing anti–IL-10/anti–TGF-β on spontaneous IFN-γ production and suppression by $CD25^{hi++}$ T_{regs} ($n = 12$). Each symbol point represents spontaneous cytokine production for one individual. Bars in *A* show the geometric mean of spontaneous IFN-γ production for all study samples in this experiment ($n = 12$). The *p* values were determined using paired *t* test of log-transformed data. *B* and *C*, Graphs present IFN-γ production by T cells plus or minus T_{regs} and/or neutralizing anti–IL-10/anti–TGF-β as indicated on the *x*-axis. *B* shows results from cultures induced with PfP0 from individual 32 and *C* from cultures induced with MSP-1 using CBMC from newborn 44 in Table I. Cultures were performed in triplicate unless there were insufficient cells, and values represent mean + SE.

$CD4^+CD25^{hi}FOXP3$ T_{regs} in cord blood from newborns of malaria-infected or malaria-exposed women. These cells, when purified, are directly suppressive of $CD4^+CD25^{lo}$ T cells in a dose-dependent fashion. This suppression is much more potent for Th1 than for Th2-type cytokine production by cord blood cells, consistent with generally Th2 cytokine bias of cord blood lymphocytes (18, 63, 64). IL-10 or TGF-β do not mediate this T_{reg} suppression, consistent with some but not all prior studies (36, 38).

This study differed from prior studies examining T_{regs} in malaria-infected individuals or in cord blood from mothers exposed to malaria in that we directly purified $CD4^+CD25^{hi}$ regulatory cells. The large amount of lymphocytes collected in some of our cord blood samples allowed such studies, which cannot be routinely performed on peripheral blood samples, particularly in young children. We undertook two rounds of positive selection using a lower concentration of anti-CD25 beads than recommended by the manufacturer, with the aim of preferentially enriching for $CD4^+$ T cells that expressed very high levels of CD25. These cells are most strongly associated with a T_{reg} phenotype as determined by the presence of FOXP3. As a consequence of this stringent selection, the number of $CD4^+CD25^{hi}$ cells recovered was low. This and practical obstacles in the field led to the limitation that not all of the enriched samples were checked for the purity of $FOXP3^+$ cells (those checked did show limited variability, however, with 78–85% of $CD4^+$ cells being $FOXP3^+$). As a consequence, the observed differences in level of suppression with add back among samples may have resulted from differences in $FOXP3^+$ enrichment. Another limitation of this purification is that not all T_{regs} have been depleted from whole CBMC. This may account for the failure to augment Ag-driven responses in some subjects following partial depletion. Only a subset of CBMC diminished in $CD25^{hi}$ was measured for the amount of $CD4^+CD25^{hi}$ depletion, which was highly variable due to the lower number of beads used to remove $CD4^+CD25^{hi}$. Therefore, we were unable to accurately associate depletion of $CD4^+CD25^{hi}$ with the magnitude of Ag-driven IFN-γ.

The enriched $CD4^+CD25^{hi}$ cells had functional characteristics of T_{regs}. They failed to proliferate or to generate IL-2 with mitogen stimulation and produced variable amounts of IL-10 (47). Of note, retention of functional suppression following enrichment of $CD25^{hi}$ T cells required the use of fresh CBMC and a larger number of available CBMC. This requirement, along with the limited number of enriched cells, precluded detailed phenotypic analysis. Therefore, we did not correlate functional activity with phenotypic expression, a limitation of the study. Enriched T_{regs} suppressed both spontaneous and malaria blood-stage Ag-driven IFN-γ production in a dose-dependent fashion. The observation that enriched T_{regs} suppressed spontaneous IFN-γ in the absence of additional exogenous Ag suggests preactivation to exogenous Ag in utero or by the process of positive selection. However, some enriched T_{regs} that showed only partial suppression in spontaneous cultures completely suppressed Ag-induced IFN-γ production (Fig. 6), suggesting that a subset of T_{regs} is malaria Ag-specific. Furthermore, not all enriched T_{regs} suppressed spontaneous or Ag-induced IFN-γ, supporting the notion of in utero activation in some infants and not others.

Enriched cord blood T_{regs} were much more effective in suppressing Th1-type as compared with Th2-type cytokine production. Enriched T_{regs} consistently failed to inhibit spontaneous IL-13 release, whereas in the same cultures, IFN-γ production was completely suppressed. Similarly, enriched T_{regs} partially suppressed Ag-induced IL-13 production, but to a lesser extent than that of IFN-γ in the same cultures and only at a ratio of $CD4^+CD25^{hi++}$ to $CD4^+CD25^{lo}$ of 1:1. Although much data supports the observation that T_{regs} can suppress Th2-type responses (65, 66), this varies depending on the culture conditions used. Prior studies indicate that enriched $CD25^+$ T_{regs} show defective suppression of Th2-type cytokines to birch pollen, but only during the birch pollen season (67). Similarly, Th2 clones were less susceptible to suppression by human thymocyte-derived $CD25^+$ T_{regs} compared with Th1 clones (68). The addition of IL-4 or IL-9 could further reduce the suppressive capacity on Th2 cells, but not Th1 lines, supporting the interpretation that Th1 clones respond to primarily IL-2, whereas Th2 cells can respond to other growth factors such as IL-4 and IL-9. This is consistent with one possible mechanism of T_{regs} suppression, in which high expression of CD25, or IL-2R, depletes cultures of IL-2 necessary for cell activation and growth, especially for Th1-type cells, whereas Th2-type cells can respond to other growth factors. This may occur under conditions that produce greater amounts of these additional growth factors (e.g., IL-4 and IL-9), such as during allergy season or in the fetal environment (66, 69, 70). This observation is consistent with our prior observations that newborns who develop a tolerant phenotype in utero show persistent suppression of malaria-specific Th1, but little suppression of malaria-specific Th2-type responses in childhood (22).

Substantial amounts of IL-10 are produced by CBMC in malaria-exposed fetuses (14, 15, 17, 38), which are thought to be important for immunoregulation (36, 38, 71). This can occur both spontaneously and in response to malaria blood-stage Ags, suggesting that expansion of IL-10–producing T cells may be important for modulating malaria Ag-specific immune responses. In malaria-exposed neonates, $CD4^+CD25^{hi}$ cells appear to be a source of IL-10 (36, 38), and IL-10 has been identified as a key mediator of T_{reg} function (along with TGF-β and IL-35); the extent to which these cytokines mediate suppression by T_{regs} appears to vary greatly in different pathogenic/hemostatic settings (72). The present studies suggest that IL-10 or TGF-β are not important mediators of $CD4^+CD25^{hi}FOXP3^+$ T_{reg}-induced suppression by cord blood following malaria exposure, suggesting that other mechanisms may be involved, such as metabolic disruption of T_{eff} by consumption of locally produced IL-2 as mentioned above or by targeting dendritic cells for suppression. Our observations do show, however, an immunoregulatory role of IL-10 whereby adding anti–IL-10 augmented IFN-γ production in previously unresponsive individuals, which is consistent with previous studies (71). This phenomenon is likely mediated through other T_{reg} subsets.

Two recent studies in adults have shown that $FOXP3^+$ T_{regs} are expanded in infected versus noninfected adults as well as exposed versus nonexposed adults (73, 74). Similar to these recent studies in adults, previous studies with cord blood have examined whether T_{regs} are more likely to be obtained from mothers who have evidence of current or prior malaria. The history of prenatal exposure to malaria has been typically surmised by the presence of malaria in the placenta at the time of delivery, either by direct detection of parasites (PCR, blood smear) or histologically. The detection of hemozoin (malaria pigment) in the placenta indicates prior infection, resulting in classification of placental malaria as acute, chronic, or past infection (no evidence of active infection at delivery). Whether this histological classification accurately reflects prenatal exposure to malaria is unknown. Using this criterion, however, one study demonstrated $CD4^+CD25^{hi}$ T_{regs} are more prevalent in cord blood from offspring of women with placental malaria (36). A second study showed an expansion of $CD4^+CD25^+FOXP3^+$ cells only after in vitro stimulation with merozoites or staphylococcal enterotoxin B in offspring of women with chronic or past but not active malaria (37). A third study found no association with placental malaria and T_{regs} in cord

blood (38), similar to the lack of association with malaria infection in women for the current study. Because lack of evidence of malaria in the placenta or peripheral blood at delivery does not exclude prior malaria exposure, a better negative control is cord blood cells from newborns living in an area not endemic for malaria. Using this control, we found the proportion of $CD4^+$ cells expressing a T_{reg} phenotype was 30–40% higher in cord blood from Kenyans (many of whom have been exposed to malaria), as compared with cord blood from North Americans. More striking is the finding that T_{regs} from Kenyan CBMC were 3-fold more likely to express the activation marker HLA-DR compared with T_{regs} from North American newborns. By contrast, we found no difference in markers on T_{regs} from Kenyan and North American newborns with respect to the memory marker CD45RO, the immunoregulatory marker CTLA-4, or markers suggestive of effector memory ($CD45RO^+CD62L^-$) or central memory ($CD45RO^+CD62L^+$) cells. Together, these observations suggest activation and expansion of T_{regs} in Kenyan newborns exposed to malaria and other parasite Ags in utero. It is likely that only a small subset of T_{regs} are strongly activated at any point, especially to malaria, and they may be difficult to detect by flow cytometry.

In contrast to other studies, we also classified newborns with malaria exposure in utero based on whether they developed recall responses to malaria blood-stage Ags at birth. Such responses could have developed at any time during pregnancy, even if the mother was found to be negative for malaria at delivery. Using this classification, there was a trend toward greater numbers and frequency of activated T_{regs} in cord blood from malaria-sensitized versus nonsensitized children; however, the numbers were too small to show significant differences. This is not surprising for three reasons. First, the nonsensitized offspring may include a putatively tolerant group (i.e., prior malaria exposure) that may have expanded T_{regs}, but lack a conventional Ag recall response (20). Second, using only two purified Ags, MSP-1, and PfP0 in our experiments, it is likely that not all potentially sensitized offspring were detected. Third, T_{regs} may also have been expanded and activated in response to other Ags in utero. Pregnant women living in malaria endemic areas are often coinfected with various helminth, bacterial, and viral infections that can stimulate immune responses in the fetus.

In our study, suppressive T_{regs} could be isolated from most, but not all newborns, and the suppressive capacity varied among individuals. This observed variation may be related more to differences in purification than real functional differences. Additionally, the proportion of T_{regs} that were malaria specific was difficult to assess. Once T_{regs} are activated either to malaria or other Ags, however, they can suppress nonspecifically (75, 76). It unlikely that T_{reg} differences in cord blood could have arisen from maternal contamination because we have previously shown in our population significant admixture occurs infrequently (10).

What is clear from this and other studies is that T_{regs} are expanded and activated in cord blood from newborns living in malaria endemic areas, either as a consequence of in utero exposure to malaria or to Ags of chronic blood-borne infections found in pregnant women in these areas. A better understanding of T_{regs} function in utero will require the use of more accurate biomarkers for their presence and function, as well as a better way to correlate timing of malaria infection during pregnancy.

Recent studies show that T_{regs} can modify susceptibility to disease (77, 78) and contribute to whether the host immune responses are protective or pathological in response to infection with *P. falciparum* parasites (73, 79–82). The nature of the initial exposure to malaria Ags likely affects the potentially diverse roles assumed by T_{regs} in malaria infection. For some individuals, this first experience appears to occur in utero (10, 12, 14, 17, 83, 84). This may have an important impact on the subsequent development of an individual's immune response to malaria and potentially to other Ags. How this prenatal exposure shapes the subsequent immune response is only now beginning to be studied. The current and several previous studies indicate that the generation of T_{regs} is an important component of the response; however, further study of factors that determine how T_{regs} are generated in utero, how they function, and whether they persist into infancy and childhood as a reservoir of preactivated regulatory cells is needed. Such studies are important, as increasing efforts are made to intensively control malaria during pregnancy, such as with prophylactic drugs and through immunization programs once an effective malaria vaccine emerges. The subsequent lack of exposure to malarial Ags in utero will surely affect malarial morbidity and mortality in childhood, but in ways that we are only beginning to understand.

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Supplemental table I: Study subjects for flow cytometric analysis of CD4+Tcell phenotype and activation status [a]

Sample ID	Parity	Presence of malaria infection			Lymphocyte sensitization to Pf blood stage antigens (net pg/ml)				
		IVPB [b]	CB[c]	Placental Biopsy	IFNγ	IL13	IL5	IL10	antigen response (MSP-1 and/or PfP0)
1) **Kenyan newborns sensitized to Pf antigens**									
F1	4	-[d]	-	chronic	-	-	-	5000	MSP-1
F2	4	-	-	-	-	55	-	90	MSP-1/PfP0
F3	0	+	-	acute/chronic	260	360	30	460	MSP-1
F4	2	-	-	-	-	150	-	350	MSP-1/PfP0
F5	3	-	-	-	159	-	-	8000	MSP-1
F6	2	+	-	-	38	140	-	7000	MSP-1
37*	0	-	-	-	20	20	-	-	PfP0
38*	0	+	-	-	-	266	44	-	MSP-1
44*	0	-	-	acute/chronic	278	187	-	-	MSP-1
2) **Kenyan newborns not sensitized to Pf antigens**									
F7	1	-	-	-	-	-	-	-	-
F8	0	+	+	-	-	-	-	-	-
F9	1	-	-	-	-	-	-	-	-
F10	0	-	-	-	-	-	-	-	-
F11	1	-	-	-	-	-	-	-	-
F12	0	-	-	not done	-	-	-	-	-
31*	0	-	-	-	-	-	-	-	-
42*	4	-	-	-	-	-	-	-	-

[a] Maternal parity, presence of malaria infection and cytokine production by CBMC to Plasmodium falciparum blood stage antigens from all Kenyan study subjects included in flow cytometry experiments to characterize phenotype and activation status of $CD4^+$ Tcells

[b]IVPB=intervillous placental blood, [c]CB = cord blood, [d] the "-" indicates values considered to be background or zero values

* samples were also included in magnetic bead separation and suppression experiments, samples numbers are identical with sample numbers in table I

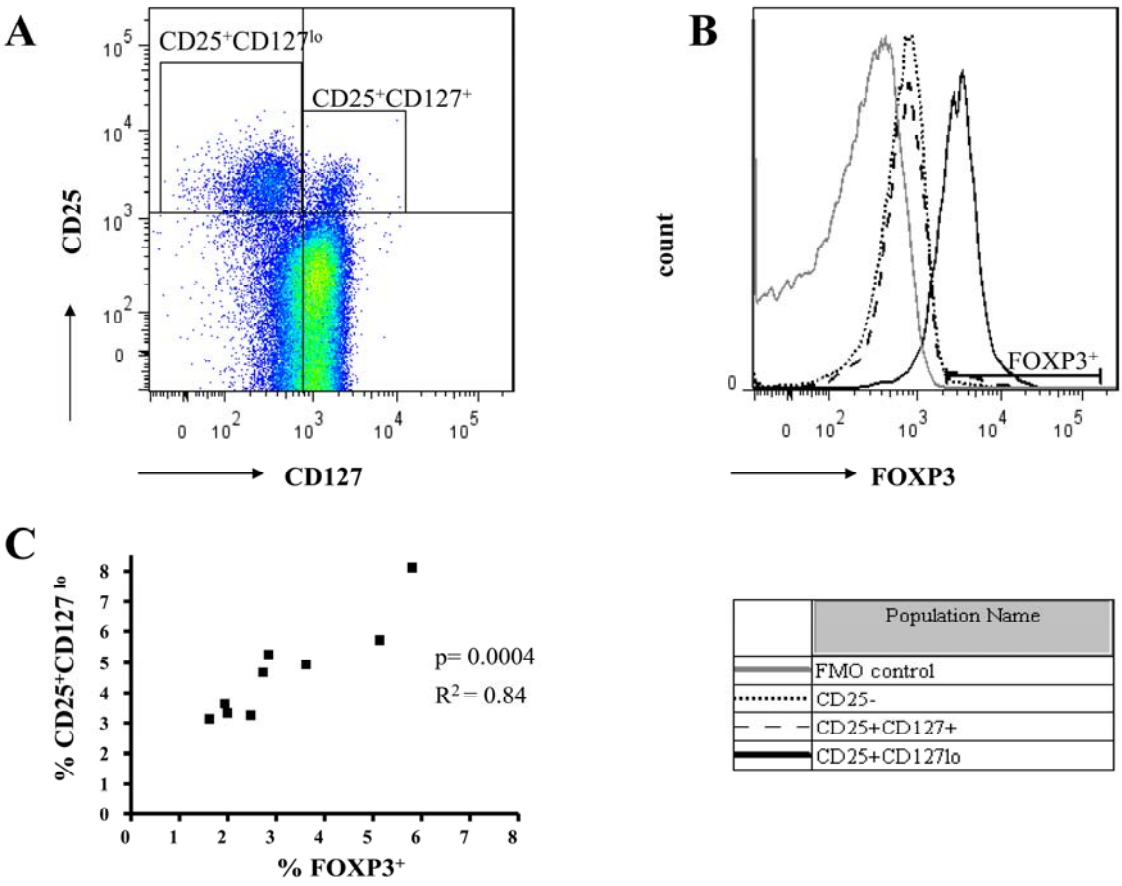
Supplemental Table II: Phenotypic characterization of Kenyan cord blood $CD4^+$ T cells[a]

Cellular subsets[b]	Kenyan CBMC (Mean % positives, range)		North American naïve
	Sensitized (N=9)	Not Sensitized (N=8)	CBMC (N=9)
CD4+ T cells			
$CD45RO^+$	10.22 (5.04; 23.16)	11.06 (3.49; 26.6)	11.95 (2.95; 18.7)
CTLA-4^+	0.15 (0.047; 0.24)	0.12 (0.03; 0.2)	0.23 (0.07; 0.3)
HLA-DR^+	0.19 (0.04; 0.44)	0.19 (0.09; 0.36)	0.11 (0; 0.24)
$CD45RO^+CD62L^{lo}$	3.2 (1.94; 5.3)	3.94 (0.92; 8.09)	3.33 (1.69; 6.41)
$CD45RO^+CD62L^{hi}$	7.3 (2.67;17.7)	5.94 (2.72; 18.5)	8.32 (1.29; 12.1)

[a] Phenotypic characterization of Kenyan cord blood CD4+Tcells according to whether they generated recall responses (sensitized) or not (not sensitized) to malaria blood stage antigens and North American control cord blood cells

[b] $CD4^+$ subsets represent the percent of cells staining with the various markers gated on $CD4^+$ T cells.

Supplemental Figure 1



Supplemental Figure 1. The relationship of CD25, CD127 and FOXP3 positivity.

CBMC were first gated for $CD4^+$ T cells based on CD3 and CD4 expression as shown in figure 3. $CD4^+$ T cells were subsequently gated for FOXP3, CD25 and CD127 expression.

A) CD25/CD127 dot plot of one representative sample. $CD25^+CD127^{lo}$ and $CD25^+CD127^+$ populations were gated as indicated.

B) The histogram presents the FOXP3 expression of different subpopulations of $CD4^+$ T cells shown in A. Gray line: fluorescence minus one (FMO) control to define gate for FOXP3 positivity. Dotted line: $CD25^-$ population presenting the two lower quadrants of dot plot in A. 1.4% of $CD25^-$ cells are $FOXP3^+$. Dashed line: $CD25^+CD127^+$ population as gated in A. 4.6% of $CD25^+CD127^+$ cells are FOXP3. Solid black line: $CD25^+CD127^{lo}$ cells as gated in A. 71.5% of $CD25^+CD127^{lo}$ cells are $FOXP3^+$.

C) Correlation between FOXP3 and $CD25^+CD127^{lo}$

Summary

In our study we examined how regulatory T cells (T_{regs}) may affect the neonatal immune response to Plasmodium falciparum (*P. falciparum*) malaria following prenatal exposure and T cell activation to *P. falciparum* antigens. This study attempts to contribute to the understanding of the longer term consequences of fetal exposure to malaria on the immune system and the immune response of neonates and young children to malaria and the impact this may have on children´s health in endemic areas.

Every year about 125 million women begin a pregnancy in malaria endemic areas. 70% of these women live in areas with transmission of the parasite species *P. falciparum* which causes the most serious forms of malaria disease (Dellicour et al., 2010). Pregnant women have an increased susceptibility to Plasmodium infection with the result that they and their offspring are at high risk for anemia, premature birth and growth retardation (Uneke, 2007, Desai et al., 2007, Hartman et al., 2010). In addition to these direct risks, malaria infection during pregnancy can lead to exposure of the fetal immune system to malaria antigens. This can happen through the crossing of parasitized erythrocytes through breaches in the placental wall or transfer of soluble antigens or antigen-immunoglobulin complexes through the placenta (May et al., 2009, Malhotra et al., 2006a, Jakobsen et al., 1998, Parekh et al., 2010). Both of these phenomena are facilitated by specific sequestering of the *P. falciparum* parasites in the placenta, referred to as placental malaria (Fried and Duffy, 1996, Duffy and Fried, 2003, Maubert et al., 2000). Exposure to malarial antigens during fetal development is a common phenomenon. Several studies from areas with stable Plasmodium falciparum transmission in sub-saharan Africa conducted since 1980 have reported an average of 25% of the mothers (range 5-52%) having evidence of malaria infection at time of delivery based on parasite microscopy (Steketee et al., 2001, Desai et al., 2007, Guyatt and Snow, 2004). Although congenital clinical malaria disease is rare, about 6 -13% of neonates show direct evidence of malaria infection at birth, determined by microscopy, antigen test or PCR in cord blood (Malhotra et al., 2006a, Mayengue et al., 2004, Bardaji et al., 2011, Oringanje et al., 2010). But in-utero exposure to malaria antigens, which can happen at any time during pregnancy, seems to be much higher than these numbers would indicate, which only measured infection at one single time point, the birth.

Immunological studies from different African countries have demonstrated specific T- and B cell responses to *P. falciparum* blood stage antigens in cord-blood lymphocytes (Fievet et al., 1996, Malhotra et al., 2008, Malhotra et al., 2005, King et al., 2002, Brustoski et al., 2005a, Metenou et al., 2007), which is the result of previous antigen exposure. Prevalences ranged from 10-70% of cord blood samples. The immunological consequences of this in-utero exposure are still poorly understood. With such substantial numbers of neonates, in some endemic areas even the majority, having been exposed to *P. falciparum* antigens in-utero, more knowledge is urgently required to understand how this prenatal exposure can shape and influence the fetal/neonatal immune system and the immune response to *P. falciparum* malaria at birth, during infancy, early childhood and even later in life.

In addressing this question, it is important to consider that the fetal immune system during its development is in a very unique and precarious situation where it has to balance a response to foreign pathogens against the dangers of reacting either to self-antigens or generating excessive, harmful immune responses to pathogens. For decades the fetal/neonatal immune system was thought to be immature and prone to development of tolerization. A series of newer studies broke with that paradigm by showing that a mature, adult-like immune response could be achieved under certain circumstances, depending on the level of costimulation and dose of antigen (Marchant and Goldman, 2005, Forsthuber et al., 1996).

With that in mind, different outcomes of prenatal exposure to malarial antigens are imaginable. It could lead to a faster development of semi-immunity, therefore accelerating protection. It could potentially increase or decrease the risk of severe malaria which occurs in children lacking partial-immunity and results from the cytoadherence of *P. falciparum* parasites and immune mediated pathology through a strong host response. Or it could impair development of immunity by mechanism of immune tolerance. It is thinkable that different outcomes are possible individually, depending on timing and intensity of antigen exposure.

What conclusions we have so far paint a complex picture but some trends are appearing. Several studies indicate that tolerance can indeed be induced through prenatal exposure. Epidemiological observations suggest that placental malaria increases the susceptibility of infants to *P. falciparum* parasitemia (Schwarz et al., 2008, Le Hesran et al., 1997)

with this effect being influenced by the number of previous pregnancies (Mutabingwa et al., 2005). Recent studies by Malhotra et al and Dent et al found that a subset of newborns of women infected with *P. falciparum* malaria during pregnancy acquired an immune tolerant phenotype, which persisted into childhood, that was characterized by increased IL-10 production, T cell anergy, lower levels of invasion-inhibitory antibodies and failure of cord blood mononuclear cells (CBMC) to produce the pro-inflammatory cytokines IFNγ and IL-2 in response to malaria blood stage antigens (Malhotra et al., 2009, Dent et al., 2006). Importantly, these same children had increased risk for malaria infection in early childhood compared to children who did not acquire this tolerant phenotype. Similar observations have been made for other human parasitic diseases such as lymphatic filariasis and onchocerciasis (Elson et al., 1996, Malhotra et al., 2006b, Malhotra et al., 2003).

To further address the question of how prenatal *P.falciparum* exposure can shape the development of immunity during early childhood an ongoing prospective cohort study was initiated in 2005 in Msambweni in the coastal region of Kenya. Pregnant women were enrolled during antenatal visits, cord blood was collected at birth and their offspring followed up to 36 months of age with blood-drawings every 6 months to study immune responses, development of immunological memory and factors influencing the susceptibility to infection, clinical disease and severe malaria in early childhood.

The current study, presented here, was conducted as part of the cohort study to focus on potential mechanisms of immune regulation. We decided to specifically investigate the role of regulatory T cells (T_{regs}) after several studies had suggested that T_{regs} play a role in the regulation of the immune response to parasitic diseases and specifically the neonatal immune response to malaria (Walther et al., 2005, Hisaeda et al., 2004, Brustoski et al., 2006, Belkaid et al., 2002). T_{regs} are an important branch of the immune system, whose role is to suppress activated immune cells, balance pro-inflammatory responses and maintain tolerance to self-antigens. Several subsets of T_{regs} have been identified so far. Among these are the so called natural, thymus derived T_{regs} which are $CD4^+$ and are identified based on high expression of CD25, the transcription factor FOXP3 and low expression of CD127. Their suppressive mechanisms have been suggested to include metabolic disruption of T cells, targeted aggregation and inhibition of dendritic cells, cytolysis of effector cells and release of immunoregulatory cytokines

such as IL10, IL35 and TGFβ. $FOXP3^+$ can also be induced in $CD4^+$ T cells peripherally or in vitro in the presence of TGFβ but the suppressive function of these cells in human beings is in question (Miyara and Sakaguchi, 2011, Tran et al., 2007). Other CD4+ T cells with regulatory function are adaptive, peripherally induced non-foxp3-expressing T_{regs} that can produce IL10 and/or TGFβ. But with our current knowledge a distinct separation between the different regulatory T cell groups is difficult to draw and none of the mentioned markers are exclusive for T_{regs}.

Recently, several studies have identified expanded populations of $CD4^+$ T cells capable of producing IL-10 in cord blood from offspring of women with placental malaria when compared to those without placental malaria (Brustoski et al., 2006, Flanagan et al., 2009). Depletion of $CD4^+C25^{hi}$ T cells from cord blood augmented production of IFNγ by cord blood mononuclear cells (CBMC) cultures stimulated with either malaria antigens or mitogens, suggesting an immunoregulatory function of these cells (Brustoski et al., 2006, Bisseye et al., 2009). These studies, however, failed to isolate, functionally test and fully characterize these T cells.

Based on the previous findings we hypothesized that exposure to *P. falciparum* antigens in utero induces the activation of $CD4^+CD25^{hi}FOXP3^+$ T regulatory cells that recognize blood stage malaria antigens and contribute to the observed immunosuppression in prenatally exposed children.

The specific aims of our study were to:

1. Evaluate the frequency, phenotype and activation status of T_{regs} in Kenyan neonates with and without positive T cell recall response to *P. falciparum* antigens relative to naïve North American neonates.
2. Isolate cord blood $CD4^+CD25^{hi}$ $FOXP3^+$ T_{regs} and test their ability to actively suppress *P. falciparum* antigen-specific $CD4^+$ T cell response in vitro.
3. Assess the mode of the suppressive function of T_{regs} in response to malaria antigens.

In order to investigate if prenatal exposure to *P. falciparum* antigens leads to expansion and activation of T_{regs} (aim 1) we conducted flow cytometry experiments on CBMC from Kenyan neonates and from malaria-naïve neonates in Cleveland, Ohio, USA.

Based on T cell sensitization measured by cytokine response in separately conducted cell culture experiments with 2 different *P. falciparum* blood stage antigens, MSP-1 and PfP0, we identified and compared 3 groups: 1) Kenyan newborns sensitized to *P. falciparum* antigens, which included only CBMC samples that showed a positive cytokine response to one or both of the *P. falciparum* antigens, 2) Kenyan newborns not-sensitized to *P. falciparum* antigens with no positive recall response and 3) North American naïve controls.

Regulatory T cells were defined by flow cytometric assessment of surface markers as $CD3^{+}CD4^{+}CD25^{hi}CD127^{lo}$ cells. In addition, CBMC were stained for the activation marker HLA-DR, the memory marker CD45RO, the homing receptor CD62L and the inhibitory signal transducing receptor CTLA4. In our hands intranuclear staining for FOXP3, to date the most robust marker for a regulatory phenotype in unstimulated cells, was incompatible with staining for some of the surface antigens, such as HLA-DR due to the fixation and permeabilization process required for FOXP3 staining (Fazekas de St Groth et al., 2011). For this reason we decided to use $CD25^{hi}CD127^{lo}$ as T_{reg} marker (Liu et al., 2006, Seddiki et al., 2006) after establishing that percentages of $FOXP3^{+}$ and $CD25^{hi}CD127^{lo}$ were highly correlated in a subset of simultaneously stained samples.

We observed that Kenyan neonates sensitized to *P. falciparum* antigens have a significantly higher frequency of $CD4^{+}CD25^{hi}CD127^{lo}$ T_{regs} (4.7% of $CD4^{+}$ T cells) than North American controls (3.1%) with not-sensitized Kenyans showing an intermediate frequency (3.7%). In addition, both sensitized and not-sensitized Kenyans had a significantly higher percentage of activated ($HLA\text{-}DR^{+}$) T_{regs} than North American neonates (0.95 and 0.85 versus 0.32%). The percentages of cells expressing the inhibitory receptor CTLA-4 were, however, not different in the 3 groups. The majority of T_{regs} in Kenyan and North American neonates were $CD45RO^{-}$ (74 – 79%) and therefore displayed a naïve phenotype. There was no difference in expression of the memory marker CD45RO, the central memory phenotype $CD45RO^{+}CD62L^{hi}$ or the effector memory cell phenotype $CD45RO^{+}CD62L^{lo}$ in $CD4^{+}$ T cells and $CD4^{+}CD25^{hi}CD127^{lo}$ T_{regs} among the 3 groups.

These findings suggest that exposure to malaria and potentially other parasite antigens in utero leads to expansion and activation of T_{regs}. It does not, however, give us any information about their functional activity which is particularly important to assess since

none of the currently known markers for T_{regs} are exclusive and CD25 and FOXP3 can also be expressed in activated cells (Wang et al., 2007, Gavin et al., 2006). We therefore isolated putative T_{regs} to directly test their suppressive activity in vitro (aim 2). Isolation of T_{regs} from *P. falciparum* malaria exposed neonates, children or adults has not been previously reported. Cord blood provides a unique opportunity to conduct these experiments due to the high number of mononuclear cells that can be obtained from it which are required for isolation and functional testing of T_{regs}. T_{regs} were purified from fresh Kenyan CBMC with directly-conjugated anti-CD25 microbeads. We aimed at isolating only the subgroup with the highest expression of CD25 by using a very low number of microbeads. Flowcytometric assessment confirmed that the majority of the most stringently isolated cells were $CD4^+CD25^{hi}$ ($\geq$80%) and $FOXP3^+$ (>70%).

We were then able to assess the direct suppressive function by comparing the cytokine production in cell cultures of $CD4^+$ T cells/monocytes stimulated with *P. falciparum* antigens to results in co-cultures with $CD4^+$ T cells/$CD25^{hi}$ T_{regs} in ratios of 1:1 and, where possible, lower ratios. About half of the collected CBMC samples showed a positive T cell recall response to the tested *P. falciparum* antigens, MSP-1 and PfP0. IFNγ and IL-13 were the two dominant cytokines produced while IL-5 and IL-10 were less frequently detected. The addition of highly purified $CD25^{hi}$ T_{regs} to $CD4^+$ T cells led to a strong, dose-dependent suppression of *P. falciparum* antigen-specific IFNγ responses (average suppression of 85% at a ratio of 1:1). IL-13 production was also suppressed, but the overall level of suppression was lower than observed with IFNγ (average suppression of 61% at a ratio of 1:1) and a similar picture was seen with IL-5. In contrast, there was no suppression of IL-10, an immunoregulatory cytokine.

Interestingly, we also observed that isolated $CD25^{hi}$ cells of some subjects produced IL-10 both spontaneously and as response to *P. falciparum* antigens. The antigen levels were comparable to the amount produced by $CD4^+$ T cells. Since IL-10, as well as TGFβ, another immunoregulatory cytokine, are released by subsets of T_{regs} to mediate suppression, we decided to further evaluate if IL-10 and/or TGFβ contributed to the observed immunosuppression by isolated $CD25^{hi}$ cells (aim 3). Importantly, adding neutralizing anti-IL10 and anti-TGFβ to CD4+ T cells/ $CD25^{hi}$ $FOXP3^+$ T_{regs} cocultures stimulated with *P. falciparum* antigens failed to reverse the suppression of IFNγ- and IL-13 production mediated by $CD25^{hi}$ $FOXP3^+$ T_{regs}. It did, however, induce measureable

IFNγ production in $CD4^+$ T cell cultures in 2 out of 10 previously unresponsive individuals.

In summary, the results of our study indicate that isolated cord blood $CD25^{hi}$ cells from Kenyan neonates, the majority of which expressed FOXP3 and therefore displayed a regulatory phenotype, suppressed $CD4^+$ T cell cytokine responses to *P. falciparum* antigens in add-back cultures. The suppressive mechanism was independent of IL-10 and TGFβ. In addition, populations of $CD25^{hi}CD127^{lo}$ T_{regs} were expanded in *P. falciparum* sensitized Kenyan neonates compared to naïve North American controls. Furthermore, significantly more $CD25^{hi}CD127^{lo}$ T_{regs} of Kenyan compared to North American neonates expressed the activation marker HLA-DR.

Stated briefly, we observed that in addition to having T_{regs} specific for *P. falciparum* malaria, Kenyan neonates exposed to *P. falciparum* in utero have an increased number of T_{regs} in total and more highly activated T_{regs} than North American controls.

These results provide an important new insight to the current understanding of prenatal immunity to malaria. We were able to demonstrate for the first time that purified cord blood $CD4^+CD25^{hi}FOXP3^+$ T_{regs} directly suppress prenataly acquired *P. falciparum* antigen-specific T cell responses in vitro. This finding advances previous studies from Gaboon and Gambia that had shown enhanced IFNγ responses to *P. falciparum* antigens and mitogens in exposed neonates after depleting CBMC of $CD25^{hi}$ cells (Bisseye et al., 2009, Brustoski et al., 2006). Contrary to the depletion study from Gambia (32) we could also show a suppressive effect of isolated $CD25^{hi}$ T_{regs} on IL-13 and IL-5 production in response to *P. falciparum* antigens (Bisseye et al., 2009). The suppression of IFNγ, a Th1-type cytokine, was, however, much stronger than suppression of the Th2-type cytokines, IL-13 and IL-5, which is consistent with a general Th2 cytokine bias of cord blood lymphocytes. This could offer an explanation for the observation by Malhotra et al in an earlier cohort study that IFNγ but not IL-13 and IL-5 production was continuously reduced in a subgroup of children up to 3 years of age that had been born to *P. falciparum* infected mothers (Malhotra et al., 2009).

In malaria-exposed neonates, $CD4^+CD25^{hi}$ cells appear to be a source of IL-10 (Brustoski et al., 2006, Bisseye et al., 2009) and IL-10 has been identified as one

possible key mediator of T_{reg} suppression for subgroups of T_{regs}, along with TGFβ (Maynard and Weaver, 2008). In our study we could observe spontaneous and *P. falciparum* antigen-induced IL-10 production by purified $CD25^{hi}$ cells; the levels, however, did not exceed those observed in $CD4^{+}$ T cells. Importantly, suppression by purified $CD25^{hi}$ cells was not mediated by TGFβ and/or IL10 as it was not abrogated with addition of anti-IL10/-TGFβ. We could, however, confirm the important role of IL-10 in modulating the immune response to malaria by showing that addition of anti-IL10 to cell cultures could reveal IFNγ production in some previously unresponsive individuals. This is consistent with prior studies (Bisseye et al., 2009, Brustoski et al., 2005b) and likely mediated through other T_{reg} subsets.

Two recent studies in adults have shown that FOXP3+ T_{regs} are expanded in infected versus non-infected as well as exposed versus nonexposed adults (Minigo et al., 2009, Finney et al., 2009). Studies in cord blood reported mixed results using placental malaria as the criterion for *P. falciparum* exposure and $CD25^{hi}$ or $CD25^{hi}FOXP3^{+}$ as markers for T_{regs} (Brustoski et al., 2006, Flanagan et al., 2009). Since a lack of apparent infection at birth does not exclude infection earlier during pregnancy, we used naïve North American neonates as negative controls. Classifying Kenyan neonates based on the presence of positive T cell response to *P. falciparum* antigens (sensitized versus not-sensitized) we could show a statistically significant increase in T_{reg} numbers using the marker $CD25^{hi}CD127^{lo}$ in sensitized Kenyan compared to North American control samples. There was an increase compared to not-sensitized Kenyans but that difference was not significant. Both Kenyan groups expressed about 3-times more HLA-DR on $CD25^{hi}CD127^{lo}$ cells than North American neonates, indicating an increased subset of strongly activated T_{regs} at that stage (Baecher-Allan et al., 2006). Exposure to other antigens such as helminthes is likely to contribute to the observed difference between Kenyan and North American neonates.

Our current study as well as several previous studies indicate that generation and activation of T_{regs} is an important component of the immune response to *P. falciparum* malaria in neonates as well as adults (Brustoski et al., 2006, Bisseye et al., 2009, Todryk et al., 2008, Torcia et al., 2008). While earlier studies in cord blood focused on IL10-producing T_{regs} our data suggest that natural $FOXP3^{+}$ T_{regs} also contribute to a suppression of the fetal immune response to *P. falciparum* malaria via a mechanism

independent of IL-10/TGFβ. A study by Mold et al showed that natural $FOXP3^+$ T_{regs} play a major role in the fetal tolerance of maternal alloantigens and these T_{regs} can persist well into early adulthood (Mold et al., 2008). It is likely that malaria-specific T_{regs}, activated in the pro-regulatory milieu of the fetal immune system, equally persist through childhood and beyond. Persisting malaria-specific T_{regs} could have major consequences not only for susceptibility to natural disease and development of protective semi-immunity but also for the effectiveness of potential future vaccines against malaria to be administered during infancy or early childhood that are currently under trial.

Further studies are needed to document the development of the frequency, activation and memory status of T_{regs} from birth to adulthood and investigate potential correlations between susceptibility to infection, clinical disease or severe malaria and the frequency/activation and suppressive capacity of T_{regs}. The feasibility of such investigations during the ongoing cohort study in Msambweni, however, is greatly limited due to the current lack of an exclusive marker to isolate and identify T_{regs}, the necessity of substantial numbers of mononuclear cells, which cannot be obtained from infants and young children and the technical and logistic restrictions of the research site. More accurate biomarkers for the presence and function of T_{regs} would greatly facilitate further studies.

Presently, the importance of controlling malaria during pregnancy has become more recognized world-wide. This has resulted in increased efforts to that end through intermittent presumptive antimalarial treatment during pregnancy, bednet distribution and efforts to develop a vaccine against placental malaria.
Preventing malaria during pregnancy will also influence when and how children are first exposed to malaria. More detailed knowledge about the role of timing and intensity of first malaria exposure in regards to T_{reg} activation, how T_{regs} affect clinical disease and whether fetal malaria-specific T_{regs} persist during infancy and childhood is urgently required. A better understanding of the immunological processes taking place in utero will have important consequences for public health and treatment recommendations during pregnancy as well as for effective vaccination scheduling once an effective vaccine against malaria has been developed.

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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