Human Cord Blood CD4⁺CD25hi Regulatory T Cells Suppress Prenatally Acquired T Cell Responses to *Plasmodium falciparum* Antigens

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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.
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 Tregs. Because activated nonregulatory CD4+ T cells can also express high levels of CD25, and FOXP3 expression can be induced in effector T cells (Teff) 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 Tregs in the fetal immune response to P. falciparum Ags and whether these regulatory cells suppress malaria Ag-driven responses by CD4+ 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 Tregs because of the large number of lymphocytes often available in cord blood. Isolation of Tregs from P. falciparum malaria-infected/exposed newborns, children, or adults has not been previously reported. In this study, we enriched for CD4+CD25hi cells, the majority of which express the Treg maker FOXP3, and evaluated their ability to actively suppress both spontaneous and P. falciparum blood-stage Ag-specific Teff 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 perinatal P. falciparum transmission occurs. Umbilical cord blood was collected from full-term newborns immediately after parturition and was anti-coagulated with heparin. Additionally, maternal peripheral blood and placental biopsies of full-term newborns immediately after parturition were procured (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 intervillosal 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)-142 and P. falciparum phosphoriboprotein P0 (PfP0), were examined. rMSP-142, 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 (DVNGSNQMASVRKSLR; codons 33–48), N2 (SV-RKSLRKATILMGKNT; codons 42–59), and C1 (AKADEPKEE-AKKVE; 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 × 108 CBMC were obtained (n = 44; shown in Table I) underwent immunomagnetic cell separation to isolate CD4+ T cells, monocytes, and CD4+CD25lo cells (Fig. 1). If >1.8 × 108 CBMC were isolated, the excess CBMC were immediately cryopreserved (n = 5). If <1.2 × 108 CBMC were obtained, they were not used for CD4+CD25hi enrichment, and an aliquot of CBMC were cryopreserved. Irrespective of whether cells were used for selection of CD4+CD25hi, a small subset of freshly isolated CBMC were reseeded at a density of 106/ml in RPMI 1640 supplemented with 10% pooled human AB serum (Sigma-Aldrich), 4.5 g/l glucose, sodium bicarbonate, 25 g/l NaHCO3, and 80 μg/ml G418 (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 × 108) were washed and resuspended in MACS buffer (PBS, 2 mMol/l EDTA, and 0.5% BSA).

CD25lo cells were isolated by immunomagnetic positive selection using microbeads directly conjugated to anti-CD25 Abs (Miltenyi Biotec) at 2 μl/107 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 CD25lo cells, those with high expression of CD25. This protocol reduced the recovery of CD25hi cells, but increased enrichment of CD25loFOXP3lo cells.

After the first round of positive selection, the selected CD25hi cells were subjected to a second round of immunomagnetic separation that produced two populations of CD25lo cells: the double positively selected cells, which we designate CD25lohi; and the cells remaining from the first round of positive selection (i.e., those not positively selected in the second round), which we designate CD25lohii (Fig. 1). CD4+CD25lohi T cells and CD4+ monocytes were then isolated from CD25lohii 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% FOXP3lo cells. After one round of magnetic selection, this was reduced by 40–81% in CD25hi diminished CBMC. CD25lohi showed an average enrichment of 62% for FOXP3pos cells (CD25lohi). The twice positively selected CD25lohi were further enriched to an average of 76% for FOXP3pos cells (CD25lohi).

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 CD25lo cells were cultured at 1 × 106/ml, and 106 CD4+ T cells were cultured with 5 × 104 monocytes per well. CD25shit* or CD25shii* cells were added to CD4+ monocyte cultures at different ratios (ratio CD4+CD25lohi at 1:0, 1:1, and 1:0.5) to assess suppressive activity of isolated CD25lo populations. Additionally, 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-142 (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/ACPs with and without...
CD25\textsuperscript{hi} or CD25\textsuperscript{hi} cells were supplemented with 1 µg/ml anti-IL-10 (JES5-9D7; 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\textsuperscript{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 multiplex cytokine 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 cytokine production 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 (IL-10 and/or IL-13) 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\textsuperscript{hi} diminished CBMC, CD25\textsuperscript{hi}, CD25\textsuperscript{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 the manufacturer’s instruction (clone PCH101; eBioscience). Monocyte populations were stained with CD14 (clone MSE2; BD Biosciences). Stained cells were rehydrated 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\textsuperscript{hi} populations, and very limited access to a flow cytometer in Kenya.

For a broader characterization of CD4+ T cells and Treg surface molecule expression by flow cytometry, liquid nitrogen-preserved 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\textsuperscript{hi} enrichment experiments and suppression assays (numbers 31, 37, 38, and 42, 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 rPfRM at 37 °C for 2 h. Cells were then washed in FACS buffer and stained with the following Abs: CD4 (SK3), CTLA-4 (BNI3), CD127 (eBioRDR5), HLA-DR (LN3), CD62L (DREG56) (all from eBioscience) 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\textsuperscript{+} T cells and Treg lymphocytes were firstly gated for CD3/CD4\textsuperscript{+} cells (Fig. 3). The gate in the right panel of Fig. 3 shows designation for CD25\textsuperscript{+} CD127\textsuperscript{+} cells. CD4\textsuperscript{+} T cells and CD4\textsuperscript{+}CD25\textsuperscript{+}CD127\textsuperscript{+} cells were further analyzed for expression of CD45R0, 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 the one fluorochrome of interest (termd 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 × 10\textsuperscript{6} CBMC) for CD25\textsuperscript{hi} purification (n = 44; Table I; see Figs. 1 and 2 for CD25\textsuperscript{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\textsubscript{eff} 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 Treg 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 Treg 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\textsuperscript{+} T cells that expressed the memory cell marker, CD4\textsuperscript{+}CD45RO\textsuperscript{*} 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...
memory phenotype (CD45RO⁺CD62Lhi). 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 Tregs among the three groups, gated CD4⁺ T cells were further gated for a subpopulation associated with a Treg phenotype, CD25⁺CD127lo (gating schema shown in Fig. 3) (45–48). Although FOXP3 is a more robust marker of Tregs, 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⁺CD127lo 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⁺CD127lo population. Whereas 69–80% (n = 9) of CD4⁺ T cells staining for CD25⁺CD127lo were FOXP3⁺, only 1 to 2% of CD4⁺CD25⁺ cells and 4–6% of CD4⁺CD25⁺CD127lo cells expressed FOXP3 (see Supplemental Fig. 1 for one representative sample). Conversely, 72–83% of CD4⁺ FOXP3⁺ T cells were CD25⁺CD127lo (data not shown). The percentages of FOXP3 and CD25⁺CD127lo 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⁺CD127lo are very similar and representative of Tregs.

Kenyan newborns sensitized in utero to malaria blood-stage Ags had an average of 4.7% of their CD4⁺ cells expressing CD25⁺CD127lo compared with an average of 3.7% and 3.1% of cord
blood CD4+ cells from not sensitized newborns or unexposed North American newborns, respectively (Fig. 4A). The frequency of CD25+CD127lo cells in the malaria-sensitized group was significantly greater compared with North Americans (p < 0.01; Fig. 4A). Significantly more CD25+CD127lo cells from Kenyan newborns expressed HLA-DR+ (0.95%) compared with North American controls (0.32%, p < 0.01; Fig. 4B), indicating greater activation or expansion of these Tregs in Kenyan newborns (49).

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+CD62Lhi (mean percentage was 16, 16.5, and 14.5%) or for the central memory phenotype CD45RO+CD62Llo (mean percentage was 7.2, 7.7, and 9.6%).

Effect of CD25hi depletion on recall responses to malaria blood-stage Ags by CBMC

To assess whether CD4+CD25hi T cells suppress malaria Ag-induced cytokine production by CBMC, we partially depleted CD25hi T cells from CBMC using a single round of immunomagnetic selection in subjects shown in Table I. Examination of FOXP3+CD25hi in CBMC before and after depletion (CBMC versus CD25hi diminished CBMC) showed an average reduction of 64% (range 40–81%, n = 8; Fig. 2). Partial depletion of CD4+ CD25hi 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. 5A). Among the 26 subjects that were not identified as sensitized, partial depletion of CD25hi cells resulted in detection of Ag-driven IFN-γ production in three subjects (Fig. 5B), indicating that the failure to observe Ag-driven cytokine production in some cord blood cells may result from active suppression through Tregs. In contrast to IFN-γ, partial depletion of CD4+CD25hi 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+CD25hi cell depletion produced no consistent effect on increased or decreased IL-5 or IL-10 production (data not shown). Thus, CD25hi 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 CD25hi 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
subject demonstrated a >2-fold change in spontaneous IL-13, IL-5, and IL-10 production following partial removal of CD25hi cells (data not shown).

Suppressive effects of enriched CD4+CD25hi Tregs on Ag-driven cytokine production by CD4+CD25lo T cells

To directly evaluate the ability of CD4+CD25hi Tregs to suppress the production of cytokines by malaria Ag-specific T cells from cord blood, we first isolated CD25hi lymphocytes immunomagnetically. We adopted a strategy in which CD25hi cells were positively selected twice using anti-CD25-coated beads. After two rounds of immunomagnetic separation, we obtained two populations: CD25hi++ and CD25hi+ cells (Fig. 1). A total of 75–85% of the selected cells were CD4+. In the CD25hi++ population, 78–85% of the CD4+ cells were FOXP3+ (Fig. 2). The CD25hi+ population (positively selected in the first round of selection, but not retained magnetically in the second round) showed lower levels of purity, with ∼66–75% of CD4+ cells expressing FOXP3.

Fig. 2 shows the CD25 and FOXP3 expression of a representative sample of CD25hi++ and CD25hi+ populations, both gated for CD4+ cells. Of note, preliminary studies of enriched CD25hi++ 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 $\times$ 105 CD25hi++ cells from a total 1.2 $\times$ 108 CBMC. Enriched CD25hi++ cells (cultured with CD14 positively selected monocytes, ratio of 2:1 for CD25hi++ to monocytes; n = 5) had a phenotype characteristic of Tregs (50); they failed to proliferate or produce IL-2 in response to PHA compared with strong

![Figure 3](image-url)  
**FIGURE 3.** CD25+CD127lo 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+CD127lo cells as shown in the right panel.

![Figure 4](image-url)  
**FIGURE 4.** Frequency and HLA-DR expression of CD4+CD25+ CD127lo cells is increased in Kenyan neonates. Figures present the percentage of CD4+CD25+CD127lo cells (A) and CD4+CD25+CD127lo 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](image-url)  
**FIGURE 5.** Depletion of CD25hi cells enhances IFN-γ responses to *P. falciparum* Ags in some individuals. The effect of partial depletion of CD25hi 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⁺CD25lo T cells (data not shown). Enriched CD4⁺CD25hi++ 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 CD25hi++ on CD4⁺CD25lo 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 CD25hi++ cells (e.g., a 1:1 ratio plus APCs). Tregs 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 Tregs, four showed partial suppression, and two showed increased IFN-γ production (Fig. 6A; p = 0.01). We next examined the effect of adding Tregs to cultures in the presence of malaria blood-stage Ags (Fig. 6B, 6C). Fig. 6B 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 CD25hi+ 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 CD25hi++ 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 CD25hi++ cells added (Fig. 6B, 6C). Fig. 6C summarizes the results of suppressive effects of Tregs 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 CD25hi++ 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 Tregs (CD25hi+), the suppression was 70% (p < 0.05).

The effect of CD25hi++ on suppression of Ag driven IL-13 release was less pronounced than that observed for IFN-γ (Fig. 7). Addition of CD25hi++ to CD4⁺ cells in the absence of malaria Ag failed to suppress spontaneous IL-13 release (CD4⁺ alone, geometric mean = 10, and CD4⁺ with CD25hi++ geometric mean = 24; Fig. 7A). Fig. 7B shows an experiment from one CBMC sample. In all subjects with malaria Ag-driven IL-13, 4 of 15 subjects had complete suppression, 1 showed no inhibition, 1 individual demonstrated an increase in IL-13, and the remaining 9 individuals showed partial suppression (Fig. 7C). The overall mean level of suppression was 61% with highly enriched CD25hi++ (p < 0.05). Addition of less enriched CD25hi+ 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, CD25hi++ 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 Tregs is not dependent on IL-10 or TGF-β production**

Because some isolated CD25hi++ 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 Tregs 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. 8A; n = 12, p = 0.14) or IL-13 production (data not shown). Addition of CD25hi++ Tregs 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. 8A; 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. 8B; representative of 2 out of 12 studied) but not others (Fig. 8C; representative of 2 out of 12; the remaining 8 out of 12 did not show any Ag-specific response).
driven cytokine production above background). Importantly, the blocking of endogenous IL-10 and/or TGF-β failed to reverse the suppressive effect of CD4+CD25hi++ cells on malaria Ag-driven IFN-γ (Fig. 8B, 8C) 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 Tregs 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 Tregs 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 Tregs. In this study, we show the presence of malaria blood-stage Ag-specific

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Cord blood CD25hi Treg suppress P. falciparum-Ag-specific, but not spontaneous IL-13 response. Enriched CD25hi cells fail to suppress spontaneous IL-13 release from CD4+CD25lo T cells (A) but CD25hi++ 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+CD25hi++ on CD4+CD25lo cells is partial and significant at p < 0.05 level using the same analysis described in the legend of Fig. 6.

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Anti–IL-10/anti–TGF-β do not abrogate suppression by CD25hi Treg. Suppressive effects of CD25hi++ Treg 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 CD25hi++ Treg (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 Treg 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⁺CD25hi⁺FOXP3⁺ Tregs in cord blood from newborns of malaria-infected or malaria-exposed women. These cells, when purified, are directly suppressive of CD4⁺CD25⁰ 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 Tregs suppression, consistent with some but not all prior studies (36, 38).

This study differed from prior studies examining Tregs in malaria-infected individuals or in cord blood from mothers exposed to malaria in that we directly purified CD4⁺CD25⁰ 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 Tregs phenotype as determined by the presence of FOXP3. As a consequence of this stringent selection, the number of CD4⁺CD25hi 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 Tregs 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 CD25hi was measured for the amount of CD4⁺CD25⁰ depletion, which was highly variable due to the lower number of beads used to remove CD4⁺CD25hi. Therefore, we were unable to accurately associate depletion of CD4⁺ CD25hi with the magnitude of Ag-driven IFN-γ.

The enriched CD4⁺CD25⁰ cells had functional characteristics of Tregs. 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 CD25⁰ 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 Tregs suppressed both spontaneous and malaria blood-stage Ag-driven IFN-γ production in a dose-dependent fashion. The observation that enriched Tregs 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 Tregs that showed only partial suppression in spontaneous cultures completely suppressed Ag-induced IFN-γ production (Fig. 6), suggesting that a subset of Tregs is malaria Ag-specific. Furthermore, not all enriched Tregs suppressed spontaneous or Ag-induced IFN-γ, supporting the notion of in utero activation in some infants and not others.

Enriched cord blood Tregs were much more effective in suppressing Th1-type as compared with Th2-type cytokine production. Enriched Tregs consistently failed to inhibit spontaneous IL-13 release, whereas in the same cultures, IFN-γ production was completely suppressed. Similarly, enriched Tregs 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⁺ CD25hi⁺:CD4⁺CD25⁰ of 1:1. Although much data supports the observation that Tregs can suppress Th2-type responses (65, 66), this varies depending on the culture conditions used. Prior studies indicate that enriched CD25⁺ Tregs 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⁺ Tregs 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 Tregs 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 previous 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⁺CD25⁰ cells appear to be a source of IL-10 (36, 38), and IL-10 has been identified as a key mediator of Treg function (along with TGF-β and IL-35); the extent to which these cytokines mediate suppression by Tregs 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⁺⁺FOXP3⁺ Treg-induced suppression by cord blood following malaria exposure, suggesting that other mechanisms may be involved, such as metabolic disruption of Treg 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 cells, which is consistent with previous studies (71). This phenomenon is likely mediated through other Tregs subsets.

Two recent studies in adults have shown that FOXP3⁺ Tregs 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 Tregs 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⁺ Tregs are more prevalent in cord blood from offspring of women with placental malaria (36). A second study showed an expansion of CD4⁺ CD25⁺⁺FOX3⁺ 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 Tregs 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 Treg 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 Tregs from Kenyan CBMC were 3-fold more likely to express the activation marker HLA-DR compared with Tregs from North American newborns. By contrast, we found no difference in markers on Tregs 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 Tregs in Kenyan newborns exposed to malaria and other parasite Ags in utero. It is likely that only a small subset of Tregs 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 Tregs 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 Tregs, but lack a conventional Ag recall response (20). Second, using only two purified Ags, MSP-1, and PIP0 in our experiments, it is likely that not all potentially sensitized offspring were detected. Third, Tregs 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 Tregs 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 Tregs that were malaria specific was difficult to assess. Once Tregs are activated either to malaria or other Ags, however, they can suppress nonspecifically (75, 76). It is unlikely that Treg 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 Tregs 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 Tregs 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 Tregs can modify susceptibility to disease (77, 78) and contribute to whether the host immune responses are protective or pathological in response to infection with Plasmodium falciparum parasites (73, 79–82). The nature of the initial exposure to malaria Ags likely affects the potentially diverse roles assumed by Tregs 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 Tregs is an important component of the response; however, further study of factors that determine how Tregs 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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References


