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BRIEF REPORT

Sex Disparity in Cord Blood FoxP3$^+$ CD4 T Regulatory Cells in Infants Exposed to Malaria In Utero

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Sex differences in the immune response and in infectious disease susceptibility have been well described, although the mechanisms underlying these differences remain incompletely understood. We evaluated the frequency of cord blood CD4 T cell subsets in a highly malaria-exposed birth cohort of mother-infant pairs in Uganda by sex. We found that frequencies of cord blood regulatory T cell ([T_{reg}] CD4$^+$CD25$^+$FoxP3$^+$CD127$^{lo/-}$) differed by infant sex, with significantly lower frequencies of T_{regs} in female than in male neonates ($P = .006$). When stratified by in utero malaria exposure status, this difference was observed in the exposed, but not in the unexposed infants.

Keywords. immunity; malaria; sex; T regulatory cells; vaccines.

In both adults and children, there is an abundant precedent for sex disparities in the immune response to infections [1, 2] and to vaccinations [3]. In general, females exhibit more robust innate and adaptive immunity than males. Females develop higher postvaccination antibody titers, clear antigen more quickly, and exhibit lower levels of viremia during chronic viral infections, but they are more susceptible to autoimmune and inflammatory disease and have higher rates of postvaccination adverse effects including local and systemic reactions [3]. Several mechanisms have been shown to contribute to these differences, including the effects of sex steroid hormones, as well as intrinsic genetic differences arising from X chromosome-encoded genes [3]. Disparities in the immune response and manifestations of infection are evident in prepubertal children [2], suggesting that sexual differentiation in the immune response may begin during infancy or gestation.

It is noteworthy to mention that, in a recent phase III trial of the RTS,S/AS01 malaria vaccine, RTS,S/AS01 vaccination was associated with higher all-cause mortality in female but not male children compared with the control arm [4]. This difference was highly statistically significant and was observed in both age groups in which the vaccine trials were conducted. Furthermore, there was a tendency towards an increased risk of fatal malaria in females but not males [4]. As the authors noted, there is precedent for increased vaccine-associated mortality in females: after introduction of the high-dose measles vaccine, a doubling of all-cause mortality among girls (but not boys) led to eventual withdrawal of the vaccine from the market [5]. The potential immunologic basis for sex differences in postvaccination mortality after RTS,S/AS01 was not explored.

Malaria is known to trigger numerous immunoregulatory pathways, including induction of regulatory T cell (T_{reg}) populations [6]. Clinical manifestations of malaria infection are likely to result from an imbalance of inflammatory and regulatory aspects of the immune response. For instance, malaria infection in a naive host results in peripheral induction and expansion of suppressive FoxP3$^+$ T_{regs} [6], and these T_{regs} have been shown to impact subsequent T-cell effector responses. After acute malaria, T_{reg} induction may limit the magnitude of subsequent Th1 response. Lower levels of FoxP3 messenger ribonucleic acid during acute malaria are associated with higher malaria-specific interferon (IFN)-γ responses to repeated malaria antigen exposure during follow-up [7]. Although sex differences in the prevalence, severity, and pathophysiology of numerous infectious diseases have been described [1, 2], including malaria-related mortality [8], there are few published studies examining potential sex differences in effector and regulatory immune responses to malaria, which is a leading cause of childhood deaths in Africa.

We sought to investigate sex differences in T-cell differentiation among infants in a highly malaria-endemic setting in Eastern Uganda, where the majority of pregnancies are complicated by placental malaria. Using cord blood samples from a birth cohort of pregnant mothers and their infants, we evaluated the frequency of total, regulatory, and activated CD4 cells in cord blood by sex.

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METHODS

Study Populations

Cord blood samples were collected from infants born to mothers enrolled in a clinical trial of prenatal malaria chemoprevention conducted in Tororo, Uganda, an area of high malaria endemicity. Clinical trial outcomes are described in a prior publication [9]. In brief, between June 2014 and October 2014, 300 human immunodeficiency virus negative mother-infant pairs were enrolled between 12 and 20 weeks of gestation. Informed consent was obtained from the parent or guardian of all study participants. The study protocol was approved by the institutional review boards of the Uganda National Council of Science and Technology, the University of California, San Francisco, and Makerere University. We collected cord blood from 166 participants from which sufficient cord blood mononuclear cells (CBMCs) were available. Participants received routine medical care at the study clinic, and mothers were evaluated monthly throughout pregnancy for *Plasmodium* parasitemia by peripheral blood via loop-mediated isothermal amplification (LAMP), which detects *Plasmodium* deoxyribonucleic acid (DNA). During febrile episodes, mothers were evaluated with blood microscopy and, if positive, treated per local guidelines for clinical malaria, as previously described [9]. At the time of delivery, maternal peripheral blood, placental blood, and cord blood were tested for parasitemia by both LAMP and microscopy. Placental tissue was processed for histopathologic evidence of malaria infection, determined by standardized placental malaria histopathology criteria as previously described [9].

Cord Blood Mononuclear Cells Collection

At the time of delivery, whole cord blood was collected in umbilical cord blood collection kits (Pall Medical). Cord blood mononuclear cells were isolated by Ficoll-histopaque density centrifugation (GE Life Sciences) and cryopreserved in liquid nitrogen.

Flow Cytometry Immunophenotyping

Cord blood mononuclear cells were thawed, aliquoted at 1 × 10^6 cells, and surface stained using standard protocols with the following antibodies: APC/Cy7-conjugated CD3, PerCP-conjugated CD4, BV421-conjugated CD25, BV650-conjugated CD127 (BioLegend). Cells were then fixed and permeabilized with FoxP3 transcription factor staining buffer set (eBioscience). After washing, cells were then intracellularly stained with PE-conjugated FoxP3 (eBioscience). BV510-conjugated CD8, CD14, CD19 (BioLegend), and LIVE/DEAD aqua amine (Invitrogen) were used as exclusion markers to minimize nonspecific binding. Flow cytometry data were collected on an LSRII 4-laser flow cytometer with FACSDiva software. Color compensations were performed using compensation beads. An isotype control was used to define negative and positive populations for FoxP3 and CD25. Cellular profiles were gated on live $CD3^+CD4^+$ lymphocytes. Fresh cord blood CD4 T cells were enumerated from 50 µL of whole cord blood stained with antibodies in BD TruCount tubes or with 20 µL of CountBright counting beads (ThermoFisher Scientific) on 152 available cord blood samples. Cells were incubated for 20 minutes, and 900 µL of BD FACS lysis solution was added for 15 minutes. CD4 T cell staining was performed using PerCP-conjugated CD3, APC-conjugated CD4 antibodies. Cells were immediately analyzed on an Accuri C6 cytometer. Absolute CD4 counts were recorded per microliter of whole cord blood. To normalize the frequency of T_{regs} and activated CD4 T cells from cryopreserved CBMCs to absolute rates of CD4 per microliter of fresh whole cord blood, we calculated absolute CD4 subset counts (absolute subset count = (subset frequency) × (absolute CD4 count per µL of whole cord blood)).

Statistical Analysis

Cell frequencies were compared between sexes using Wilcoxon rank-sum testing. Associations between sex and malaria exposure outcomes were compared using χ^2 testing. Statistical analyses were completed using PRISM 7.0 (GraphPad). Two-sided P values were calculated for all test statistics, and $P < .05$ was considered significant.

RESULTS

We evaluated the frequency of total, regulatory (T_{regs}; $CD25^+$ $FoxP3^+$ $CD127^{lo/-}$), and activated ($CD25^+FoxP3^-CD127^{hi}$) CD4 T cells (Figure 1A) in the cord blood of 166 infants born to mothers enrolled in a randomized clinical trial of prenatal malaria chemoprevention. Pregnancy-associated malaria was common, with 79% of infants exhibiting evidence of in utero malaria exposure by either maternal, cord, or placental microscopy or *P falciparum* DNA testing, and/or positive placental histopathology for malaria infection. Rates of in utero malaria exposure did not differ between male and female infants (75.8% vs 82.7%, $P = .282$) (Table 1). However, we found that frequencies of T_{regs} in cord blood differed significantly by infant sex, with female neonates having lower frequencies of T_{regs} than males (1.82% vs 2.61%, $P = .006$) (Figure 1B; Table 2). In addition, absolute T_{reg} counts, calculated by normalizing subset frequency to CD4 counts per volume of whole blood, were also lower in females than in males (24.4 vs 35.8 cells/µL whole cord blood, $P = .012$) (Table 2).

To explore whether exposure to malaria antigens during fetal life may have contributed to differential expansion of T_{regs} in males and females, we examined the relationship of infant sex to T_{regs} following stratification for in utero malaria exposure status (positive maternal, cord, or placental microscopy or *P falciparum* DNA testing, and/or positive placental histopathology). A sex disparity was observed in the exposed infants with females having lower T_{reg} frequencies than males ($P = .012$; $n = 131$), but not in the unexposed infants ($P = .353$, $n = 35$) (Figure 1C). However, the small number of infants who lacked evidence of

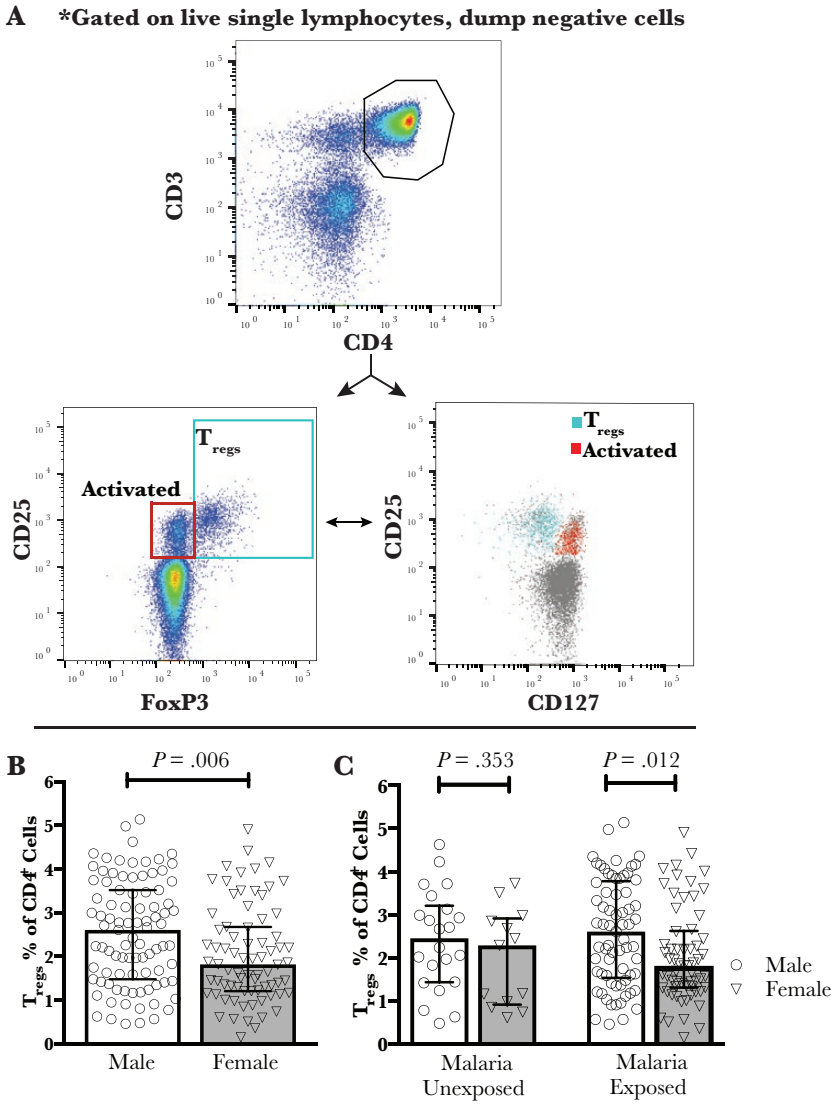

Figure 1. Lower frequency of cord blood T regulatory cells (T_{regs}) in females vs males in in utero malaria-exposed infants. (A) Cord blood cells were gated on live, single lymphocytes, dump-negative T cells. Gating of $CD4^+CD25^+$ cord blood T cells revealed 2 distinct subsets, T_{regs} ($CD25^+FoxP3^+CD127^{lo/-}$) and activated CD4 T cells ($CD25^+FoxP3^-CD127^{hi}$). (B) The frequency of cord blood T_{regs} ($CD25^+FoxP3^+CD127^{lo/-}$) from all infants evaluated, regardless of in utero malaria exposure, differs by sex ($P = .006$; Wilcoxon rank-sum testing; error bars indicate median with interquartile range; $n = 166$). (C) The frequency of T_{regs} differs by sex in the in utero malaria-exposed infants ($P = .012$), but not the malaria-unexposed infants ($P = .353$; Wilcoxon rank-sum testing; error bars indicate median with interquartile range).

any malaria exposure limited the power of this comparison in the unexposed subgroup.

We also compared the frequency of activated CD4 T cells ($CD127^{hi}$ $CD25^+$ but lacking FoxP3 expression), because we have previously shown this population to be expanded in infants who were exposed to malaria in utero [10]. There was no sex difference in the frequency of these activated CD4 cells ($P = .884$) (Table 2) in the cohort as a whole or in the exposed and unexposed subgroups.

Several studies have documented that female newborns have higher CD4 percentages and that this trend persists through childhood [2, 3]. In our cohort, there was no difference in the frequency ($P = .993$) or absolute counts of total CD4 T cells ($P = .669$) by infant sex. Thus, only the $FoxP3^+$ regulatory CD4 T-cell compartment, and not total or activated CD4 T cells, differed between male and female neonates (Table 2).

DISCUSSION

We found significantly lower frequencies of cord blood $FoxP3^+$ regulatory CD4 T cells in female than in male infants born in a region of high malaria transmission intensity, where 79% of enrolled participants had evidence of malaria infection either during pregnancy or at parturition. To our knowledge, no prior studies have reported a difference in frequencies of T_{regs} in the cord blood of male and female infants, either in uncomplicated healthy pregnancy or after in utero exposure to a pathogen. It

Table 1. In Utero Malaria Exposure by Infant Sex

Malaria Exposure Category	Female	Male	
Enrollment[a]	Positive n (%)	Positive n (%)	*P* Value
Maternal parasitemia by LAMP	41 (54.7)	48 (52.8)	.805
Monthly Screening During Pregnancy			
Maternal parasitemia by LAMP	42 (56.0)	39 (42.9)	.092
Delivery			
Maternal parasitemia			
LAMP	13 (17.3)	7 (7.7)	.058
Microscopy	2 (2.67)	2 (2.2)	.649
Placenta			
LAMP	11 (14.7)	9 (9.9)	.347
Microscopy	2 (2.67)	2 (2.2)	.845
Histopathology	30 (40.0)	33 (36.3)	.622
Cord Blood			
LAMP	1 (1.3)	2 (2.2)	.501
Any malaria during pregnancy or delivery	62 (82.7)	69 (75.8)	.282

Abbreviations: LAMP, loop-mediated isothermal amplification.

[a]12 to 20 weeks gestational age.

is not clear whether this difference reflects an intrinsic biologic difference between male and female neonates that is independent of pathogen exposure, or if alternatively, in utero exposure to malaria antigens drives T_{reg} differentiation more strongly in males than females.

Several potential mechanisms might contribute to the observed sex differences in cord blood T_{regs}. First, differences in sex steroid hormones have direct effects on numerous innate and adaptive immune cells [3]. Although infancy and childhood have historically been regarded as a time of hormonal quiescence, some data suggest that differences in the hormonal milieu of males and females emerge in utero, with testosterone first produced by male testes as early as gestational week 10 [11]. Thus, sex-based differences in the immune response may begin even before birth. In addition to hormonal influences, genetic differences between male and female individuals may drive sexual dimorphism in the immune response. Experiments comparing transgenic XY and XX mice with a common gonadal type indicate that it is the XX sex chromosome complement, rather than female steroid hormones, that predisposes females to autoimmunity [12]. Many genes critical for immune function and immunoregulation are encoded by the X chromosome (including FoxP3, TLR7, TLR9, and IRAK), and an estimated 15% of these may escape X inactivation to some degree, making them subject to gene dosage effects [13]. Together, these factors may contribute to a bias towards less T_{reg} differentiation and more vigorous inflammatory immune responses in females.

Table 2. Cord Blood T-Cell Subsets by Infant Sex

T-Cell Subset	Female	Male	
	Median [IQR]	Median [IQR]	*P*-value
Frequency (%)[a]			
Total CD4	74.4 [68.2–83.1]	76.3 [68.6–80.4]	.993
T_{regs}	1.82 [1.2–2.7]	2.61 [1.5–3.5]	**.006**
Activated ($CD25^+$ $FoxP3^-CD127^{hi}$)	2.17 [1.1–3.5]	2.39 [1.4–3.0]	.884
Absolute Counts[b]			
Total CD4	1356 [1252–1778]	1527 [1056–1872]	.669
T_{regs}	24.4 [15.6–42.2]	35.8 [18.3–57.8]	**.012**
Activated ($CD25^+$ $FoxP3^-CD127^{hi}$)	31.8 [18.5–49.0]	32.0 [18.8–49.5]	.956

Statistically significant *P*-value in bold. Abbreviations: IQR, interquartile range; T_{regs}, T regulatory cells.

[a]Frequency of parent subset.

[b]Absolute count per microliter of whole cord blood.

In light of recent reports of increased postvaccination mortality in females receiving the RTS,S/AS01 malaria subunit vaccine [4], it is clear that sex differences in the immune response to malaria require further evaluation, both in naturally exposed populations and in malaria vaccine trials. The lower frequencies of T_{regs} observed among female infants in this malaria-endemic setting could contribute to a more vigorous effector response and increased immunopathology after natural malaria exposure or vaccination. In murine vaccination models, T_{reg} depletion at the time of vaccination enhances CD4 IFN-γ and interleukin-2 production upon restimulation with antigen [14]. Additional studies are needed in both malaria-exposed and unexposed populations to validate our findings of sex differences in T_{regs} at birth. Moreover, future field studies of malaria-specific immunity, including vaccine-induced responses, should pay careful heed to potential sex differences in regulatory and effector responses, which may shed light on the mechanisms underlying the increased mortality seen in female RTS,S/AS01 malaria vaccine recipients.

CONCLUSIONS

Our findings add to the mounting evidence indicating that females and males differ in their intrinsic immune responsiveness and susceptibility to infection, and that these differences arise during early life—even in utero. Moreover, although both biological sex and societal gender roles and behaviors have been shown to contribute to differences in infectious disease outcomes, the observation that sex disparities are evident at birth highlights the biological underpinnings of sexual dimorphism in the immune response.

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