HHS Public Access

Author manuscript

Sci Transl Med. Author manuscript; available in PMC 2019 January 06.

Published in final edited form as:

Sci Transl Med. 2018 October 17; 10(463): . doi:10.1126/scitranslmed.aat6176.

In utero priming of highly functional effector T cell responses to human malaria

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Abstract

Malaria remains a significant cause of morbidity and mortality worldwide, particularly in infants and children. Some studies have reported that exposure to malaria antigens in utero results in the development of tolerance, which could contribute to poor immunity to malaria in early life. However, the effector T cell response to pathogen-derived antigens encountered in utero, including malaria, has not been well characterized. Here, we assessed the frequency, phenotype, and function of cord blood T cells from Ugandan infants born to mothers with and without placental malaria. We found that infants born to mothers with active placental malaria had elevated frequencies of proliferating effector memory fetal $CD4^+$ T cells and higher frequencies of $CD4^+$ and $CD8^+$ T cells that produced inflammatory cytokines. Fetal $CD4^+$ and $CD8^+$ T cells from placental malaria-exposed infants exhibited greater in vitro proliferation to malaria antigens. Malaria-specific $CD4^+$ T cell proliferation correlated with prospective protection from malaria during childhood. These data demonstrate that placental malaria is associated with the generation of proinflammatory malaria-responsive fetal T cells. These findings add to our current understanding of fetal immunity and indicate that a functional and protective pathogen-specific T cell response can be generated in utero.

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Author contributions: P.M.O. designed, performed, and analyzed all experiments and drafted the manuscript. P.J., T.I.M., R.B., and T.D.B. helped design experiments and analyze data. P.J., T.I.M., R.B., M.P., F.N., M.N., E.S., K.M., and K.N. were involved in data collection. A.A. and M.P. designed protocols for cord blood collection. PJ. and G.D. performed analysis for correlations with prospective malaria (Table 1), and PJ. provided statistical guidance for all figures. A.K., M.R.K., and G.D. led the parent study clinical trial. M.E.F. designed and coordinated the project, evaluated the data, provided funding for all experiments, and helped draft the manuscript. All authors reviewed and approved the manuscript.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data associated with this study are present in the paper or in the Supplementary Materials.

INTRODUCTION

Pregnancy-associated malaria, including placental malaria (PM), remains a significant global health threat, resulting in low birth weight, preterm birth, and other complications that contribute to an estimated 100,000 deaths per year (1, 2). PM is characterized by the accumulation of parasite-infected erythrocytes in the placenta, accompanied by pathological changes, including the presence of hemozoin, infiltration of monocytes and macrophages, and deposition of perivillous fibrin (3). Although true congenital malaria infection is rare, malaria antigens are known to cross the placental barrier and enter fetal circulation (4, 5). Some studies have reported that the cord blood of infants born to women with PM contains increased frequencies of regulatory T cells (T_{regs}) that could suppress malaria-specific T cell responses (6–11), whereas other groups have reported no association of T_{regs} with PM (12, 13). Thus, it remains unclear precisely how the fetal immune system responds to in utero malaria exposure and whether this exposure has consequences for antimalarial immunity during childhood.

The fetus is predisposed toward the induction of tolerance upon encounter with foreign antigens (14). At birth, cord blood fetal T cells are primarily naïve in phenotype ($CD45RA^+$) and fetal $CD4^+$ T cells generally exhibit a differentiation bias away from T helper 1 (T_H1) cytokine production and toward T_H2 and T_{reg} functions (14–18). However, recent work has demonstrated that even during healthy pregnancy, in the absence of known exposure to an intrauterine pathogen, human cord blood contains effector memory $CD4^+$ T cells (19). Although in utero exposure to some viruses, including hepatitis B virus (20), hepatitis C virus (21), HIV (22–24), and cytomegalovirus (CMV) (25, 26), has been reported to result in priming of T cells before birth, there has been limited characterization of their phenotype, function, and antigen specificity. Moreover, protection against infection during infancy by T cells primed in utero has not been demonstrated.

To investigate the impact of in utero malaria exposure on fetal T cell immunity, we assessed $CD4^+$ and $CD8^+$ T cell frequency, phenotype, and function in the cord blood of a cohort of182 infants born to mothers with and without PM, acquired as part of a study of pregnant women and infants in a highly malaria-endemic region of Uganda (27). Our findings indicate that the human fetus is capable of mounting functional effector T cell responses in utero and suggest that these responses may afford protection against pathogen exposure in early childhood.

RESULTS

Fetal effector memory T cells in cord blood of PM-exposed infants

To better understand how PM alters the fetal T cell compartment, we used cord blood samples from a subset of infants enrolled in a clinical trial of prenatal and early childhood malaria intermittent preventative treatment (IPT) in malaria-endemic Uganda. In this study, HIV-negative pregnant women were enrolled between 12 and 20 weeks of gestation and randomized to receive standard versus enhanced malaria IPT during pregnancy (27). Women were evaluated monthly for malaria parasitemia from enrollment until delivery, and women

presenting with fever or other symptoms of malaria were evaluated by blood smear. At birth, cord blood and placental tissue were assessed for the presence of malaria parasite DNA by loop-mediated isothermal amplification (LAMP) and for histopathologic evidence of PM, as previously described (27–29). All infants in the clinical trial were included in this analysis if they had cord blood mononuclear cells (CBMCs) collected in sufficient quantity and were CMV negative at birth (fig. S1 and table S1).

We compared the frequency and phenotype of effector and memory $CD4^+$ and $CD8^+$ T cells in the cord blood of infants born to mothers with and without active PM, as defined by the presence or absence of malaria parasite DNA in the placenta. As flow cytometric staining controls, North American healthy adult PBMCs (peripheral blood mononuclear cells) and CBMCs were assessed in parallel. On the basis of the expression of CCR7 and CD45RA, we identified populations of central memory (T_{CM}), effector memory (T_{EM}), and effector memory RA^+ (T_{EMRA}) $CD4^+$ and $CD8^+$ T cells in cord blood (Fig. 1A). We observed a significantly higher frequency of $CD4^+$ T_{EM} cells in infants born to mothers with active PM compared to those born to mothers without active PM ($P = 0.0001$; Fig. 1B). The subset of these infants whose placentas had parasites visualized by histo-pathology also had significantly higher frequencies of $CD4^+$ T_{EM} cells than those without parasites ($P = 0.018$; fig. S2A). We further stratified mothers without active PM into those with no evidence of malaria infection during pregnancy and those with past malaria infection (based on detection of malaria parasite DNA in maternal peripheral blood during pregnancy or positive placental histopathology, but no malaria parasite DNA in the placenta). The frequency of $CD4^+$ T_{EM} cells among infants born to mothers with past malaria was higher than in those with no evidence of infection but was lower than in those with active PM (Fig. 1C). $CD4^+$ T_{EM} frequencies did not differ by maternal gravidity or IPT randomization arm (fig. S2, B and C). Effector memory differentiation was also observed among cord blood $CD8^+$ T cells; however, the frequency of $CD8^+$ T_{CM}, T_{EM}, and T_{EMRA} cells did not differ between exposed and unexposed infants (fig. S3, A and B). To confirm that the activated T cell populations seen in PM-exposed infants were fetally derived, as opposed to cells of maternal origin (30, 31), we performed fluorescence in situ hybridization to detect X and Y chromosomes in sorted non-naïve $CD4^+$ T cells from male infant cord blood. We detected exclusively fetal T cells (that is, XY) in these samples (fig. S4, A and B), confirming their fetal origin.

Given the elevated frequencies of $CD4^+$ TEM cells in infants born to mothers with active PM, we further characterized the activation profile, differentiation status, and TH subset distribution of fetal non-naïve $CD4^+$ and $CD8^+$ T cells (T_{EM}, T_{CM}, and T_{EMRA}) directly ex vivo. Compared to naïve T cells (T_N), $CD4^+$ T_{EM} cells were $CD45RO^{high}CD62L^{low}CD127^{low}$ and expressed the proapoptotic marker CD95/Fas, the high-affinity interleukin-2 (IL-2) receptor CD25, and the inhibitory receptor programmed cell death-1 (PD-1) (Fig. 1D). $CD4^+$ T_{EM} cells from active PM-exposed infants expressed significantly more PD-1 than did those from unexposed infants ($P = 0.006$), but there were no differences in other activation markers (Fig. 1E). We also used a combination of activation marker, chemokine receptor, and transcription factor expression to identify eight distinct T_H subsets (32) within non-naïve $CD4^+$ T cells (Fig. 1F). We found this compartment to consist predominantly of T_H1-, T_H2-, and T_{reg}-phenotype $CD4^+$ T cells. The frequency of $CD4^+$ T_H subsets did not differ between infants born to mothers with or

without active PM (Fig. 1G). Heterogeneity in the non-naïve $CD4^+$ T cell compartment was also evident at the transcriptional level, with coexpression of multiple transcription factors associated with T_H1 (*Prdml*, *Tbx21*, and *BATF*), T_H2 (*Gata3*), and T_H17 (*RoRγT*) cells (fig. S5), again with no difference observed between active PM-exposed and unexposed infants. However, expression of Ki67 by non-naïve $CD4^+$ T cells was significantly higher in infants born to mothers with active PM ($P < 0.0001$), suggesting greater in vivo proliferation of $CD4^+$ T cells (Fig. 1, H and I). We found no significant difference in the activation status (fig. S3B) or Ki67 expression (fig. S3C) of non-naïve $CD8^+$ T cells. However, we did observe an increased frequency of $CXCR3^+$ $Tbet^+$ $Eomes^+$ non-naïve $CD8^+$ T cells among infants born to mothers with active PM (fig. S3D), suggesting some effector differentiation of the $CD8^+$ T cell compartment. Thus, we have identified a population of fetal effector memory $CD4^+$ T cells that is enriched in the cord blood of infants born to mothers with active PM and exhibits evidence of in vivo activation and proliferation.

Inflammatory cytokine production by cord blood $CD4^+$ and $CD8^+$ T cells from PM-exposed infants

We next evaluated the functional capabilities of cord blood $CD4^+$ and $CD8^+$ T cells in PM-exposed infants by assessing cytokine production by intracellular cytokine staining (ICS) after in vitro stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin. Infants born to mothers with active PM had significantly higher frequencies of non-naïve $CD4^+$ T cells producing the proinflammatory cytokine interferon-γ (IFN-γ) compared to infants born to mothers with past infection ($P = 0.002$) or infants born to mothers with no malaria infection during pregnancy ($P < 0.001$). Similar results were observed for production of tumor necrosis factor-α (TNF-α) and coproduction of IFN-γ and TNF-α (Fig. 2, A and B). Production of IL-2 and IL-8 by $CD4^+$ T cells was also observed but did not differ between exposure groups (fig. S6A), whereas IL-17A and IL-10 were not detected. Similarly, in the $CD8^+$ T cell compartment, active PM-exposed infants had higher frequencies of non-naïve $CD8^+$ T cells producing IFN-γ ($P = 0.05$) and significantly higher frequencies of non-naïve $CD8^+$ T cells co-producing IFN-γ and TNF-α (no malaria: $P = 0.03$/past infection: $P = 0.005$) (Fig. 2, C and D, and fig. S6B). Increased cytokine production by T cells from infants born to mothers with active PM is consistent with the higher frequency of phenotypically activated fetal TEM cells. Together, these findings indicate that in utero exposure to active PM alters both the phenotype and function of the fetal $CD4^+$ and $CD8^+$ T cell compartments by inducing the differentiation of fetal effector T cells, suggesting either an antigen-specific response to malaria or nonspecific bystander activation.

Proliferation of cord blood $CD4^+$ and $CD8^+$ T cells in response to malaria antigens

Given the increased frequency of cytokine-producing $CD4^+$ and $CD8^+$ T cells in PM-exposed infants, we next asked whether these cells were specifically responsive to malaria antigens in vitro. To test this, CBMCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated with *Plasmodium falciparum* schizont extract (PfSE) or uninfected red blood cells ($_uRBC_s$) for 6 days. Proliferating $CD4^+$ T cells ($CFSE^{low}$, having undergone at least one division) were identifiable in most cord blood samples, whereas $CFSE^{low}$ $CD8^+$ T cells were much rarer (Fig. 3A). We found that infants born to mothers with active PM had significantly higher frequencies of $CFSE^{low}$ $CD4^+$ T

cells compared to infants born to mothers with past infection ($P = 0.002$) or infants born to mothers with no malaria infection during pregnancy ($P = 0.002$; Fig. 3B). Similarly, compared to infants born to mothers with no malaria infection, $CFSE^{low}$ $CD8^+$ T cell frequencies were increased in infants born to mothers with both past infection ($P = 0.004$) and active PM ($P < 0.001$; Fig. 3B). The proliferative T cell response was major histocompatibility complex (MHC) class restricted, as blockade of MHC-I or MHC-II abrogated $CD4^+$ or $CD8^+$ T cell proliferation, respectively (Fig. 3C). To assess whether these malaria-responsive T cells were capable of cytokine production, we restimulated CBMC cultures on day 6 with PMA/ionomycin and evaluated the production of proinflammatory cytokines by ICS. We found that $CFSE^{low}$ proliferating $CD4^+$ and $CD8^+$ T cells produced IFN-γ, TNF-α, and IL-2 (Fig. 3, D and E, and fig. S7A), but no substantial production of IL-17A or IL-8 was detected. Cord blood from infants exposed to active PM contained higher frequencies of $CFSE^{low}$ IFN-γ-producing $CD4^+$ ($P = 0.03$) and $CD8^+$ ($P = 0.05$) T cells (Fig. 3E), indicating that active PM induces the differentiation of malaria-responsive T cells in cord blood that are capable of proinflammatory cytokine production. The inflammatory bias of malaria-responsive $CD4^+$ and $CD8^+$ T cells is further supported by the increased gene expression of T_H1-associated transcription factors in sorted $CFSE^{low}$ T cells, including *TBX21*/Tbet, *BATF*/BATF, and *PRDM1*/Blimp-1 in $CD4^+$ T cells and TBX21/Tbet, *BATF*/BATF, *PRDM1*/Blimp-1, and EOMES/Eomes in $CD8^+$ T cells (Fig. 3F).

Next, because several previous studies have reported that in utero exposure to malaria is associated with the induction of T_{regs} that may suppress fetal T cell activation and proliferation (6–11), we investigated whether depletion of T_{regs} would influence $CD4^+$ or $CD8^+$ T cell proliferation in response to malaria antigens. We stimulated CFSE-labeled CBMCs with or without $CD25^+CD127^-$ T_{regs} by FACS (fluorescence-activated cell sorting) and assessed proliferation in response to *Pf*SE, as above. We found no significant difference in proliferation of $CD4^+$ or $CD8^+$ T cells after depletion of T_{regs} (Fig. 3G), suggesting that Treg suppression does not substantially affect proliferation of malaria-responsive T cells in this setting. Furthermore, because the Vδ2 subset of γδ T cells has been shown to have intrinsic reactivity to malaria antigens and is expanded in malaria-exposed neonates (33, 34), we similarly assessed whether Vδ2 cell depletion would affect proliferation in response to malaria schizont extract. We found no significant difference in CFSE dilution after depletion of Vδ2 cells (fig. S7B), indicating that bystander cytokine production by Vδ2 T cells is not responsible for the proliferation of fetal T cells in response to malaria antigens.

To further confirm the malaria specificity of fetal T cells expanded in response to in utero malaria exposure, we stimulated CFSE-labeled CBMCs with pooled overlapping peptides spanning merozoite surface protein 1 (MSP1), an abundant blood-stage plasmodial protein that is an immunodominant target of malaria-specific T cells (35). After a 6-day stimulation with MSP1 peptides, we observed proliferation of both $CD4^+$ and $CD8^+$ T cells, albeit at much lower frequencies than were observed with *Pf*SE stimulation (Fig. 4A). MSP1 peptide stimulation resulted in significantly higher frequencies of $CFSE^{low}$ $CD4^+$ and $CD8^+$ T cells from infants born to mothers with active PM compared to mothers with past infection ($CD4^+$, $P = 0.002$; $CD8^+$, $P = 0.001$) and mothers with no malaria infection during pregnancy ($CD4^+$, $P < 0.001$; $CD8^+$, $P = 0.0002$) (Fig. 4B), similar to what we observed

after *PfSE* stimulation. Furthermore, proliferating $CD4^+$ T cells ($CFSE^{low}$) in active PM-exposed infants produced more IFN-γ after restimulation with PMA/ionomycin ($P = 0.001$); however, this was not observed for the $CD8^+$ T cell population (Fig. 4C). Together, our results suggest that infants exposed to malaria in utero generate both $CD4^+$ and $CD8^+$ T cells that are responsive to malaria antigens, are MHC restricted, and are capable of proliferation and production of inflammatory cytokines upon reencounter with antigen.

Association between $CD4^+$ T cell proliferation in response to malaria antigens at birth and protection from malaria in infancy

In light of the evidence that malaria-responsive $CD4^+$ T cells capable of effector functions can be primed in utero, we investigated whether these responses were associated with protection against plasmodial infection and symptomatic malaria during infancy and early childhood. Children were followed prospectively until 24 months of age with monthly screening for peripheral parasitemia (by LAMP), as well as monitoring and treatment for symptomatic malaria. All children included in this analysis received IPT with dihydroartemisinin-piperaquine (DP) every 12 weeks (fig. S1). We assessed prospective protection using three separate outcome parameters (clinical malaria, time to first episode of malaria, and parasitemia prevalence). To best assess the relationship between malaria-responsive $CD4^+$ T cells and prospective protection from malaria (which we hypothesized would be nonlinear), we categorized infants into three parsimonious groups based on the percentage of proliferating $CD4^+$ T cells ($CFSE^{low}$) after in vitro stimulation with *PfSE*: "low" (<15% $CFSE^{low}$ $CD4^+$ T cells; $n = 11$), "intermediate" (15 to 30% $CFSE^{low}$ $CD4^+$ T cells; $n = 35$), and "high" (≥30% $CFSE^{low}$ $CD4^+$ T cells; $n = 27$). Infants with high proliferation had a >70% lower incidence of clinical malaria in the first 2 years of life compared to infants with low proliferation [incidence rate ratio (IRR), 0.28; $P = 0.02$; Table 1]. Furthermore, infants with intermediate and high malaria-specific $CD4^+$ T cell proliferation had a reduced time to first malaria episode after birth than children in the low group [intermediate group: hazard ratio (HR), 0.40; $P = 0.05$; high group: HR, 0.28; $P = 0.01$; Table 1]. For each 1% increase in $CFSE^{low}$ $CD4^+$ T cells, there was a 4% reduction in the hazard of malaria [HR, 0.96; 95% confidence interval (CI), 0.93 to 0.99; $P = 0.025$]. Finally, when assessing the risk of parasitemia based on active monthly surveillance from 0 to 24 months of life, infants with high malaria-specific $CD4^+$ T cell proliferation had significantly less parasitemia than children in the other strata [prevalence rate ratio (PRR), 0.33; $P = 0.03$; Table 1]. In all three of these analyses, the association between malaria-specific $CD4^+$ T cell pro-liferation and protection remained statistically significant after adjustment for maternal malaria during pregnancy (table S1). Together, these results suggest that a higher frequency of cord blood $CD4^+$ T cells that proliferate in response to malaria antigens in vitro ($CFSE^{low}$ $CD4^+$ T cells) results in protection from both parasite infection and symptomatic malaria during the first 2 years of life.

DISCUSSION

Upon encounter with non-self antigens, naïve fetal $CD4^+$ T cells have been reported to preferentially differentiate into T_{regs} (14), presumably due to the need for reciprocal tolerance between the mother and fetus. T cell-intrinsic mechanisms (18, 36) [for example,

hypermethylation of *IFNG* locus (17)] and T cell-extrinsic mechanisms [for example, differing antigen presenting cell function (37–42)] are both believed to contribute to a bias away from T_H1 differentiation and inflammatory cytokine production in utero. As a result, young infants have heightened vulnerability to numerous infections, particularly those requiring cell-mediated immunity for control, and exhibit delayed development of adaptive immunity to many pathogens (43). It was recently reported that $CD4^+$ T cells with an effector memory phenotype develop during fetal life, even in the absence of any known pathogen exposure, and that these cells perform a variety of inflammatory effector functions (19, 44). However, to date, relatively few studies have performed detailed characterization of effector T cells in the setting of human fetal pathogen exposure. Here, we add to our current understanding of how the fetus is capable of responding to pathogens by demonstrating effector memory differentiation, inflammatory cytokine production, and robust antigen-specific T cell proliferation after in utero exposure to malaria.

Previous studies of pregnancy-associated malaria suggested a role for suppressive T_{regs} in dampening fetal effector T cell responses to in utero malaria exposure, but the published data are inconsistent. Some studies have reported higher frequencies of T_{regs} among infants born to mothers with PM and increased production of IFN-γ in response to malaria antigen stimulation after depletion of T_{regs} (6–11), whereas other studies, including the current study, have found no association between PM and T_{reg} frequency (12,13). Several factors could account for these discordant results, including variability in the definition of PM, as well as differences in the cellular markers and immunologic assays used to identify effector T cells and T_{regs} (6–13, 45). In addition, the timing, duration, and degree of malaria antigen exposure during pregnancy may influence the fetal T cell response, as may the degree of associated placental inflammation (13, 45). Here, we found that the most profound effector T cell differentiation occurred in infants born to mothers with active PM infection at the time of birth. This may reflect later timing of malaria infection during pregnancy (and hence greater immune maturation of the fetus) or pathogen persistence, as it is not possible to know with confidence when the placenta became infected. Infants born to mothers with evidence of past malaria infection had intermediate frequencies of effector or malaria-responsive T cells. Thus, active PM late during pregnancy or persisting throughout pregnancy may induce the most robust fetal effector T cell responses, whereas malaria infections that were treated or resolved during pregnancy may induce some combination of T_{regs} and effector T cells (13).

Our study demonstrates a relationship between in utero pathogen exposure, human fetal T cell responses, and protection from infection after birth. Our finding that malaria-specific cord blood $CD4^+$ T cell proliferation correlates with protection from malaria in the first 2 years of life is somewhat surprising, as a protective role for $CD4^+$ T cells in childhood malaria has been difficult to establish (35, 46–50). The relationship between the malaria-specific T cell response and risk of infection is complex, as children residing in highly endemic regions sustain repeated and often prolonged infections, resulting in near-constant exposure to parasites and high-level anti-genemia. Immunoregulatory mechanisms induced by this chronic malaria antigen exposure (including production of the suppressive cytokine IL-10) have been shown to interfere with the generation of fully functional and durable T cell response in children (46). To date, the best proof of principle that sterilizing immunity to

malaria in humans is achievable comes from experimental settings in which antigen exposure is limited, either by parasite attenuation or by drug treatment. Such strategies include vaccination with irradiated (or genetically attenuated) sporozoites (51–54), infection under “cover” of blood-stage chemoprevention (55–60), and low-dose blood-stage parasite vaccination (61, 62). These approaches all result in limited exposure to blood-stage parasite antigens and have been associated with the priming of $CD4^+$ T cell responses and induction of sterilizing immunity. It is likely that transplacental exposure of the fetus, as occurs with PM, similarly results in limited exposure to blood-stage antigens and may therefore lead to priming of protective memory $CD4^+$ T cells that can respond vigorously after re-exposure to malaria during infancy.

Our study is limited by the fact that all enrolled women received at least the standard-of-care three-dose IPT for malaria (2, 27), providing some protection from malaria during pregnancy. Although it could be that IPT cleared maternal parasites and altered the fetal T cell response, no differences in fetal T cells were noted between infants born to mothers receiving the standard-of-care versus enhanced IPT. However, it may be that fetal T cell responses induced after highly inflammatory or severe PM in settings of untreated pregnancy-associated malaria would differ from those found in this study. Despite intensive monitoring, it is possible that some maternal infections were missed, resulting in misclassification bias. Some pregnant women and children may have sustained greater malaria exposure than others due to heterogeneous environmental exposure to infected mosquitoes. However, this would likely bias our analysis away from observing a protective response during infancy. Although our work supports an association between in utero malaria exposure, fetal T cell responses, and prospective protection from malaria during childhood, the strength of this finding is limited by a relatively small number of infants (n = 73) included in the protection analyses. Furthermore, we cannot conclude that this relationship is causal, as it is possible that other fetal immune effector mechanisms contribute to this relationship (including B cells and antibodies). It has been shown that malaria infection during pregnancy is associated with the generation of fetal malaria-specific antibodies, indicating that fetal B cells can also be primed in utero (63–65).

Maternal malaria IPT has been shown to decrease infant morbidity (2), and enhanced regimens, recently shown to have excellent efficacy in preventing PM (27, 66), are now being considered for widespread implementation (67). Our data suggest that the immunologic consequences of preventing in utero exposure to malaria require further detailed study. It will be important to determine whether fetal T cell responses to malaria persist and provide durable protection against repeated malaria exposure in childhood. It is possible that malaria antigenemia from breakthrough infections may drive the differentiation of protective $CD4^+$ T cell responses into regulatory IL-10-producing T_H1 cells, the predominant $CD4^+$ T cell phenotype observed in highly exposed children (46). Nonetheless, our findings raise the intriguing possibility that vaccination during pregnancy could benefit infants through the induction of fetal effector memory T cell responses. Influenza-specific fetal effector memory $CD4^+$ T cell responses have been identified in cord blood after vaccination during pregnancy (68), providing evidence that transplacental exposure to vaccine antigens, in the absence of active fetal infection, can induce the differentiation of fetal T cells.

In summary, we have identified a population of fetal malaria-responsive T cells in infants born to mothers with active PM that have phenotypic features of effector memory cells and a variety of inflammatory effector functions. These findings advance our understanding of how the fetus can respond to in utero malaria exposure, indicative of a more inflammatory T cell response than previously appreciated. We also show that fetal T cell responses at birth correlate with a reduced risk of malaria in childhood, suggesting in utero priming of a protective T cell response. These results have important implications for understanding the fetal response to pathogens and for strategies that seek to optimize infant immunity.

MATERIALS AND METHODS

Study design

The objective of this study was to evaluate fetal T cell responses in cord blood of infants born to mothers with or without PM. Of the 300 subjects enrolled in the parent clinical trial, 291 were followed until delivery and 271 had cord blood samples collected and banked. Of these available samples, the subset studied here ($n = 182$) was chosen on the basis of (i) collection and successful banking of at least four vials of CBMCs for an individual, (ii) a recorded concentration of at least 7×10^6 CBMCs per vial, and (iii) a negative result for CMV DNA in a dried blood spot polymerase chain reaction assay (because CMV is another common intrauterine infection that could prime fetal T cells) (69). Variability in the number of cells obtained after thawing resulted in the inability to perform all immunological assays in all individuals (fig. S1). The number of individuals included in each assay is indicated in corresponding figure legends and Materials and methods. Clinical details of the subjects included in this study are outlined in table S2. Primary data for all experiments, where $n < 20$, are located in table S4.

Ethical approval

Informed consent was obtained from the mother of all study participants. The study protocol was approved by the Uganda National Council of Science and Technology and the institutional review boards of the University of California, San Francisco, Makerere University, and the Centers for Disease Control and Prevention.

Study site and participants

Samples for this study were obtained from a subset of infants enrolled in a clinical trial of prenatal and early childhood malaria IPT in Tororo, Uganda, a rural district in southeastern Uganda with high malaria endemicity. Details of the larger study, including eligibility criteria for enrollment and clinical outcomes, have been previously described in detail (PROMOTE-BC1; NCT02163447) (27). Briefly, 300 HIV-negative pregnant women were enrolled between 12 and 20 weeks of gestation and were randomized to receive standard malaria IPT during pregnancy [three-dose sulfadoxine-pyrimethamine (SP)] versus enhanced IPT (three-dose or monthly DP). Participants randomized to receive standard IPT were administered SP at 20, 28, and 36 weeks of gestation. Similarly, participants randomized to receive three-dose DP were administered drug at 20, 28, and 36 weeks of gestation, whereas those in the monthly DP arm received drug every 4 weeks beginning at 16 or 20 weeks based on gestational age at enrollment. At enrollment, participants received

an insecticide-treated bed net, underwent standardized examination, and had blood samples collected. All mothers also received one dose of mebendazole in the second trimester per Ugandan Ministry of Health guidelines. Participants received all of their medical care at the study clinic and had scheduled visits every 4 weeks for routine laboratory testing. Women who presented with fever had a blood smear performed, and if positive, they were treated for malaria (detailed below). Participants were encouraged to deliver at the hospital adjacent to the study clinic.

Screening for maternal parasitemia during pregnancy

Maternal malaria parasitemia was assessed every 4 weeks throughout pregnancy, beginning at enrollment. The presence of malaria parasites in maternal peripheral blood was evaluated using LAMP kits (Eiken Chemical), as previously described (27, 28). LAMP assays were batch analyzed at the end of the trial and were not used to inform treatment (asymptomatic parasitemia is not treated per current Ugandan standard of care). Placental tissue was processed for histopathologic evidence of PM, as previously described (27, 29), and placental blood and cord blood were tested for the presence of malaria parasites by both LAMP and microscopy.

Childhood malaria outcomes

After birth, infants born to enrolled women were randomized to receive DP every 4 or 12 weeks and were followed until 24 months of age. Routine visits were also conducted in children every 4 weeks beginning at birth and included the assessment of peripheral parasitemia by LAMP. Children were encouraged to be brought to the clinic any time they were ill. Those who presented with a documented fever (tympanic temperature > 38.0°C) or history of fever in the previous 24 hours had blood collected for a thick blood smear, and if the smear was positive, the patient was diagnosed with clinical malaria. Episodes of uncomplicated malaria in children <4 months of age or weighing <5 kg, as well as episodes of complicated malaria and treatment failures within 14 days, were treated with a 7-day course of quinine (oral or parenteral).

Infants randomized to receive DP every 4 weeks were excluded from the analysis of prospective protection, as piperaquine has a very long half-life that results in a prophylactic period of ~4 weeks after DP administration (70). All infants randomized to receive DP every 12 weeks during infancy, and for whom sufficient CBMCs were available for performance of the CFSE proliferation assays, were included in the analyses of prospective protection from malaria in childhood *(n* = 73). This included infants born to mothers with no malaria infection during pregnancy (*n* = 8), past malaria infection during pregnancy (*n* = 51), and active PM during pregnancy (*n* = 13).

Statistical analysis

All statistical analyses were performed using STATA version 14.2 (College Station) and Prism 7.0 (GraphPad). Frequencies of cytokine-producing T cells (alone or in combination) were reported after background subtraction of the frequency of the identically gated population of cells from the same sample stimulated with control. In CFSE dilution assays, the percentage of divided T cells after *Pf*SE stimulation was calculated and reported after

subtraction of the percentage of divided T cells after uRBC stimulation. Negative values were graphed as zero. Comparisons of cellular frequencies and proportions between groups were performed using the Kruskal-Wallis test, Wilcoxon rank sum test, or Wilcoxon matched-pairs signed-rank test. The cumulative risk of developing malaria after birth was estimated using the Kaplan-Meier product limit formula. Associations between malaria-responsive $CD4^+$ T cells and the hazard of developing malaria were made using Cox proportional hazards models. The Schoenfeld test was used to test the proportionality of hazards. Negative binomial regression was used to estimate associations between cellular responses at birth and the incidence of malaria (IRRs) and parasite prevalence (PRRs) in the first 2 years of life. Multivariate models were adjusted for maternal malaria exposure (no malaria exposure, past malaria infection, or active PM) (table S1). In all analyses, a two-tailed P value of <0.05 was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We are grateful to all the study participants and their families for their consent and cooperation in this study. We thank all of the study team members for their tireless dedication and excellent work. We also thank B. Greenhouse, I. Roderiguez-Barraquer, and J. M. McCune for valuable discussions.

Funding: Funding was provided by the NIH/NIAID (5P01HD059454-07 to M.R.K. and G.D., 2R01AI093615-06 and 5K24AI113002-03 to M.E.F., and K23 AI100949 to P.J.).

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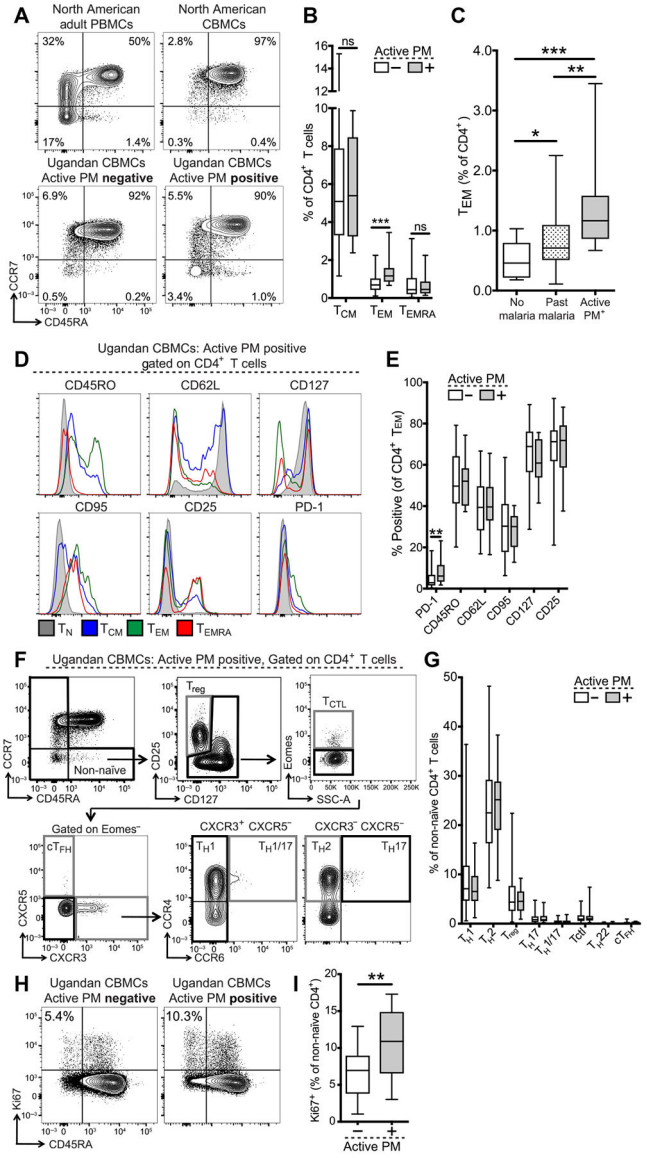

Fig. 1. Fetal effector memory CD4$^+$ T cell differentiation in cord blood of malaria-exposed infants.

(A) Representative flow plots of CD4$^+$ T cell effector memory subsets. T_{CM}, CD45RA$^-$CCR7$^+$; T_{EM}, CD45RA$^-$CCR7$^-$; and T_{EMRA}, CD45RA$^+$CCR7$^-$. Values indicate % of CD4$^+$ T cells. (B) Quantification of CD4$^+$ T cell subsets in infants born to mothers with (gray bar; $n = 14$) or without (white bar; $n = 55$) active PM. (C) Frequency of T_{EM} cells in infants born to mothers without malaria during pregnancy (white bar; $n = 10$), with past malaria infection (striped bar; $n = 45$), or with active PM (gray bar; $n = 14$). ns, not significant. (D) Representative histograms of CD4$^+$ T cell subset phenotype from a representative PM-exposed infant. T_{CTL}, cytotoxic T lymphocyte; cT_{FH}, circulating T follicular helper cell. (E) Quantification of % positive for phenotypic markers on CD4$^+$ T_{EM} cells in active PM-exposed (gray bar; $n = 14$) versus unexposed (white bar; $n = 55$) infants. (F) Gating strategy for CD4$^+$ T_H subsets; representative of an active PM-exposed infant. (G) Quantification of CD4$^+$ T_H subsets (% of non-naïve CD4$^+$ T cells) in infants born to mothers

with (gray bar; $n = 20$) versus without (white bar; $n = 154$) active PM. (H) Representative flow plots of Ki67 and CD45RA expression. Values indicate % $Ki67^+$ cells of non-naïve $CD4^+$ T cells ($CD45RA^-$). (I) Quantification of non-naïve $Ki67^+$ T cells in active PM-exposed (gray bar; $n = 11$) versus unexposed (white bar; $n = 57$) infants. All representative flow plots were gated on live $CD14^-CD19^-\gamma\delta TCR^-CD3^+CD8^-CD4^+$ T cells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Kruskal-Wallis and Wilcoxon rank sum test.

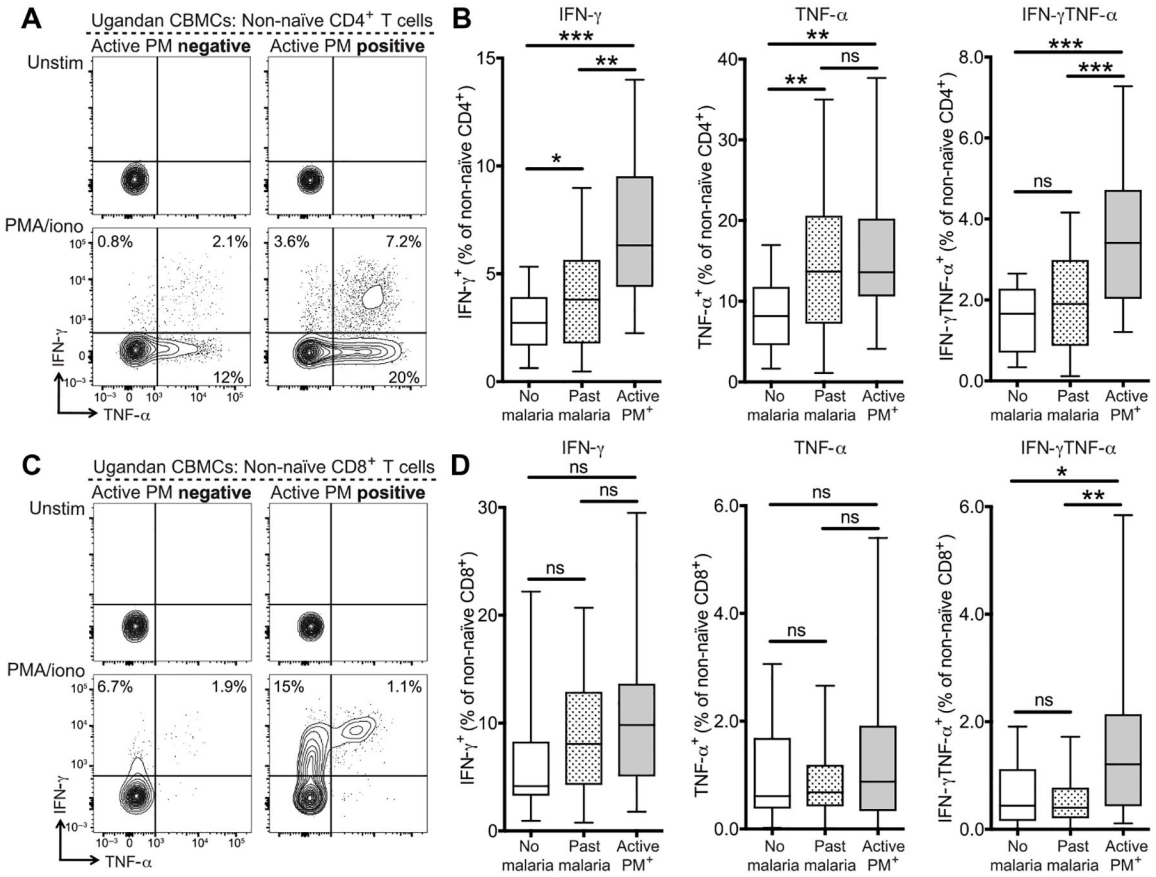

Fig. 2. Inflammatory cytokine production by cord blood CD4$^+$ and CD8$^+$ T cells from PM-exposed infants.

(A) Representative flow plots of ICS for production of IFN-γ and TNF-α by non-naïve CD4$^+$ T cells after 5-hour in vitro stimulation of CBMCs with medium alone (upper) or PMA/ionomycin (lower). Gated on live CD14$^-$CD19$^-$γδTCR$^-$CD3$^+$CD8$^-$CD4$^+$CD45RA$^-$ T cells. Values indicate the frequency of cytokine-positive cells within the non-naïve population. (B) Quantification of cytokine-producing cells in infants born to mothers without malaria during pregnancy (white bar; $n = 16$), with past malaria infection (striped bar; $n = 83$), or with active PM (gray bar; $n = 21$). (C) Representative flow plots of ICS for production of IFN-γ and TNF-a by non-naïve CD8$^+$ T cells after 5-hour in vitro stimulation of CBMCs with medium alone (upper) or PMA/ionomycin (lower). Gated on live CD14$^-$CD19$^-$γδTCR$^-$CD3$^+$CD4$^-$CD8$^+$CD45RA$^-$ T cells. Values indicate the frequency of cytokine-positive cells within the non-naïve population. (D) Quantification of cytokine-producing cells in infants born to mothers without malaria during pregnancy (white bar; $n = 16$), with past malaria infection (striped bar; $n = 83$), or with active PM (gray bar; $n = 21$). Values calculated by background subtraction of medium-alone controls. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Kruskal-Wallis and Wilcoxon rank sum test.

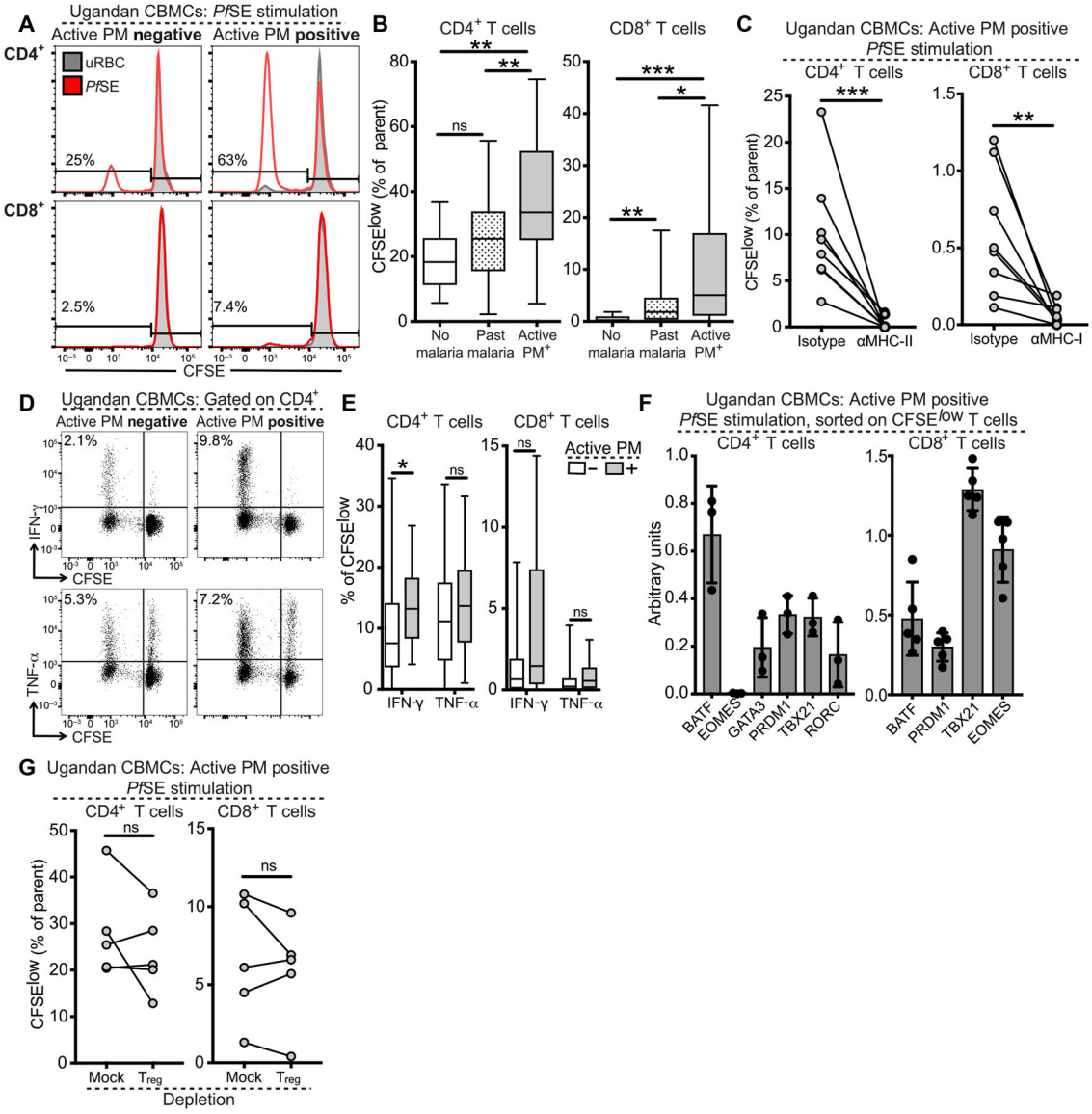

Fig. 3. Proliferation of cord blood CD4+ and CD8+ T cells in response to malaria antigens.

(A) Representative CFSE dilution profiles for $CD4^+$ (upper) and $CD8^+$ (lower) T cells after 6-day P*f*SE (red histogram) or uRBC (gray histogram) stimulation. Values indicate frequency of $CFSE^{low}$ T cells (% of $CD4^+$ or $CD8^+$ T cells). (B) Quantification of $CFSE^{low}$ $CD4^+$ (left) or $CD8^+$ (right) T cells in infants born to mothers without malaria during pregnancy (white bar; $n = 11$), with past malaria infection (striped bar; $n = 69$), or with active PM (gray bar; $n = 18$). (C) $CFSE^{low}$ $CD4^+$ (left) and $CD8^+$ (right) T cell frequencies after 6 days of P*f*SE stimulation in the presence or absence of αMHC-II or αMHC-I ($n = 8$). Data are representative of three independent experiments. (D) ICS for cytokine production by CFSE-labeled P*f*SE-stimulated CBMCs restimulated on day 6 with PMA/ionomycin in infants born to mothers with (gray bar) or without (white bar) active PM. (E) Quantification of $CFSE^{low}$ cytokine-producing $CD4^+$ and $CD8^+$ T cells stratified by infant malaria exposure (active PM^-, white bars, $n = 79$; active PM^+, gray bars, $n = 17$). (F) Transcription

factor mRNA expression in sorted $CFSE^{low}$ $CD4^+$ (left) and $CD8^+$ T cells (right) from infants born to active PM^+ mothers (n = 3 to 5). Data are representative of three independent experiments. (G) $CFSE^{low}$ $CD4^+$ T cell frequencies in the presence or absence of T_{regs}. Data are representative of three independent experiments. All representative flow plots were gated on live $CD14^-CD19^-\gamma\delta TCR^-CD3^+$ T cells. $CFSE^{low}$ frequencies were calculated by background subtraction of uRBC-stimulated and medium-alone controls. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, significance assessed by Kruskal-Wallis and Wilcoxon rank sum test for all experiments except MHC blockade and T_{reg} depletion, which were assessed by Wilcoxon matched-pairs signed-rank test.

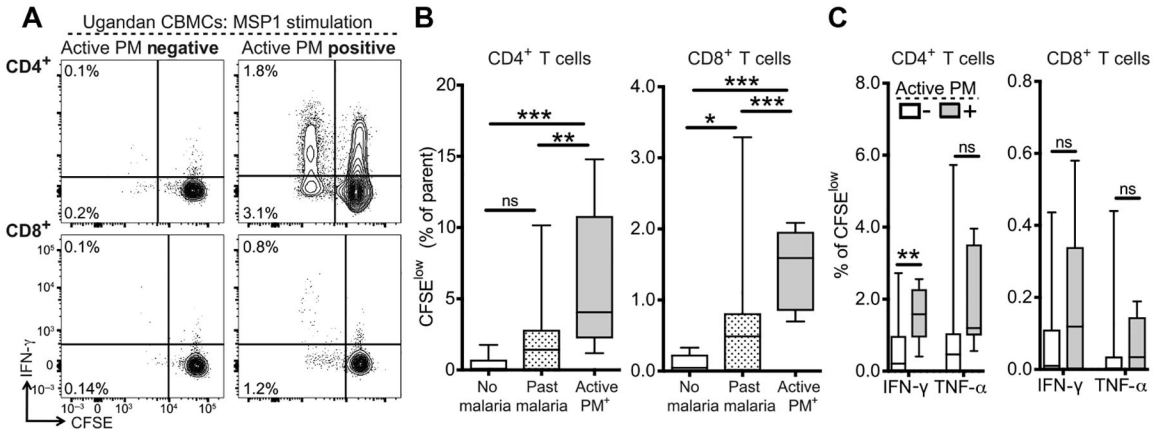

Fig. 4. Proliferation of cord blood CD4$^+$ and CD8$^+$ T cells in response to MSP1 peptide stimulation.

(A) Representative CFSE dilution profiles for CD4$^+$ (upper) and CD8$^+$ (lower) T cells stimulated with MSP1 peptide pools for 6 days and restimulated with PMA/ionomycin. Gated on live CD14$^-$CD19$^-$γδTCR$^-$CD3$^+$ T cells. Values indicate frequency of CFSE^{low} T cells as % CD4$^+$ or CD8$^+$ T cells. (B) Frequency of CFSE^{low} CD4$^+$ (left) or CD8$^+$ (right) T cells in infants born to mothers without malaria during pregnancy (white bar; $n = 4$), with past malaria infection (striped bar; $n = 38$), or with active PM (gray bar; $n = 9$). (C) Quantification of CFSE^{low} cytokine-producing CD4$^+$ and CD8$^+$ T cells stratified by infant malaria exposure (PM$^-$, white bars, $n = 41$; PM$^+$, gray bars, $n = 8$). For all experiments, values are calculated by background subtraction of dimethyl sulfoxide-stimulated and medium-alone controls. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Kruskal-Wallis and Wilcoxon rank sum test.

Table 1.

CD4$^+$ T cell proliferation in response to malaria antigens associated with protection from childhood malaria.

PY, person years of observation.

Incidence of clinical malaria through 24 months of age						
% PfSE-specific CFSE^{low} CD4+ T cells	***n***	**Episodes**	**PY**	**Incidence**	**IRR (95% CI)**	***P* value**
Low (<15%)	11	16	21.0	0.76	Reference	
Intermediate (15 to 30%)	35	36	62.5	0.58	0.79 (0.30–2.06)	0.63
High (≥30%)	27	10	48.3	0.21	0.28 (0.09–0.84)	0.02
Time to first episode of malaria after birth						
	n	**Cumulative risk (95% CI)**			**HR (95% CI)**	***P* value**
Low (<15%)	11	79.6% (51.8–96.8%)			Reference	
Intermediate (15to30%)	35	41.1% (26.3–60.1%)			0.40 (0.16–0.98)	0.05
High (≥30%)	27	33.8% (18.5–56.5%)			0.28 (0.11–0.77)	0.01
Detection of malaria parasites at routine monthly visits between 2 and 24 months of age*						
		n/N		**%**	**PRR (95% CI)**	***P* value**
Low (<15%)		25/269		9.29%	Reference	
Intermediate (15 to 30%)		56/797		7.03%	0.80 (0.33–1.93)	0.63
High (≥30%)		19/620		3.06%	0.33 (0.13–0.89)	0.03

*By blood smear and/or LAMP.