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Supplementary Materials for

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

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at www.sciencetranslationalmedicine.org/cgi/content/full/10/463/eaat6176/DC1)

Materials and Methods

Cord blood collection and processing

At the time of delivery, whole cord blood was collected using umbilical cord blood collection kits (Pall Medical). CBMCs were isolated by density gradient centrifugation (Ficoll-Histopaque; GE Life Sciences), cryopreserved in liquid nitrogen, and shipped to UCSF for immunologic evaluation. Analysis of post-thaw CBMC viability using a Millipore cell counter was consistently >80%.

Flow cytometry and intracellular cytokine staining

Surface and intracellular staining of thawed CBMCs was performed with standard protocols and using the antibodies listed in **Supplemental Table 3**. All samples were stained with LIVE/DEAD aqua amine (Invitrogen) to discriminate live from dead cells and included a "dump" channel with CD14, CD19, and γδTCR for exclusion gating (**Fig. S8**). For CD4+ T helper subsets, chemokine receptor gating strategy was used to identify the following populations, based on previously published studies*(30, 71, 72)*: Tregs (CD25+CD127-FoxP3+), T_{CTLS} (Eomes+ CX3CR1+), cTfh (CXCR5+CXCR3-), Th1 (CXCR3+CXCR5-CCR4+/-CCR6-CCR10-), Th2 (CXCR3-CXCR5-CCR4+CCR6-CCR10-), Th1/17 (CXCR3+CXCR5-CCR4+CCR6+CCR10-), Th17 (CXCR3-CXCR5-CCR4+CCR6+CCR10-), Th22 (CXCR3-CXCR5-CCR4+CCR6+CCR10+). For intracellular cytokine staining, thawed CBMCs ($1x10^6$ cells/condition) were re-stimulated with phorbol 12-myristate 13-acetate (0.1 ug/mL) and ionomycin (1.0 ug/mL) or media alone (R10; 10% fetal bovine serum) for 5h at 37°C. Brefeldin A and monensin (BD Pharmingen) were added after 1h of incubation at a final concentration of 10 mg/mL. After 5 hours, cells were washed, fixed and permeabilized, as per the manufacturer's instructions (FoxP3 Fix/Perm kit; eBiosciences). Data were collected on an LSR II (BD) and analyzed with FlowJo software (Treestar).

Malaria antigens

P. falciparum blood-stage 3D7 parasites were grown by standard methods and harvested at 5-10% parasitemia. Red blood cells infected with mature asexual stages (iRBCs) were magnetically purified, washed, and cryopreserved in glycerolyte, as previously described*(46)*. Uninfected red blood cells (uRBCs) were used as controls. *P. falciparum* schizont extract (*Pf*SE) was prepared by three freeze-thaw cycles of iRBCs in liquid nitrogen and a 37°C water bath. *Pf*SE was then resuspended in R10 media and stored at -20°C. Merozoite surface protein-1 (MSP1) peptide pools were previously synthesized and pooled *(35)*, and consist of overlapping peptides (18mers overlapping by 11 amino acids) spanning the 3D7 sequence of the blood-stage *P. falciparum* protein.

CFSE proliferation assays

For subjects with sufficient CBMCs (n=98), thawed CBMCs were rested for 1 hr at 37°C, washed in 10% Human AB (HAB) medium, and 3-4 x 10^6 cells were labeled with 1 µM CFSE (Molecular Probes) for 7 mins. CFSE-labeled CBMCs were plated in 96 deep-well culture plates (Nunc) (1x10^6 cells/condition) and incubated with *Pf*SE, MSP1 peptide pools (10ug/mL)*(35)*, uRBCs, DMSO, or media alone. After 6 days, cells were washed twice in culture medium and counted. CBMCs were then plated in 96 well round-bottom plates and re-stimulated with PMA/ionomycin or media alone for 5 hrs, as described above. CBMCs were washed and stained with surface and intracellular antibodies, as listed in **Supplemental Table 3**. For MHC-I and –II blockade experiments, purified monoclonal antibodies were added to CFSE-labeled CBMCs (5 mg/mL) and incubated for 30 mins prior to addition of *Pf*SE or uRBC and added again on d3 of culture, as previously described *(73)*. The following antibodies were used: anti-HLA-A, B, C (clone W6/32, Biolegend), anti-HLA-DR, DP, DQ (BD Biosciences), IgG2a isotype control (Biolegend).

Treg and Vδ2 T cell depletion

Thawed CBMCs were sort-purified to deplete Tregs (CD3+CD4+CD25+CD127-) or Vδ2 T cells (CD3+ Vδ2+) to >95% purity with a FACSAria II (BD). Treg-depleted, Vδ2 T cell-depleted, or mock-sorted CBMCs were washed twice with culture medium, CFSE-labeled and stimulated with *Pf*SE or uRBCs, as described above. After 6 days, CBMCs were stained with surface antibodies, as described above.

qRT-PCR

Thawed CBMCs were sorted into non-naïve CD4+ and CD8+ T cells based on the expression of CCR7 and CD45RA. For $CFSE^{low}$ cells, CFSE-labeled CBMCs were stimulated with *Pf*SE for 6 days and CD4+ or CD8+ T cells were sorted based on dilution of CFSE expression. All populations of interest were sorted to >95% purity. Total RNA was isolated using the RNeasy Plus Micro kit (Qiagen) and cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific), according to manufacturer's instructions. Real-time quantitative PCR was performed in triplicate for each sample on a StepOnePlus Real-Time PCR System (Life Technologies) to quantify transcripts of interest using pre-designed, commercially-available TaqMan Gene Expression Assays and reagents, as per the manufacturer's instructions. Probes with the following Applied Biosystems assay identification numbers were used: *EOMES* (Hs00172872_m1), *TBX21* (Hs00894392_m1), *BATF* (Hs00232390_m1), *GATA3* (Hs00231122_m1), *PRDM1* (Hs00153357_m1), *RORC* (Hs01076112_m1). Transcripts were normalized to the amount of beta-actin mRNA in each sample and expressed as arbitrary units.

FISH staining

Non-naïve CD4+ or CD8+ T cells were sorted from thawed CBMCs based on the expression of CCR7 and CD45RA from male infants. For $CFSE^{low}$ cells, CFSE-labeled CBMCs were stimulated with *Pf*SE for 6 days and CD4+ or CD8+ T cells were sorted based on dilution of CFSE. All populations of interest were sorted to >95% purity. FISH staining was performed by the UCSF Cytogenics Laboratory for X (green) and Y (red) chromosomes together with DAPI for nuclear staining. Nuclei were counted (approximately 200/sample) and quantified as the number associated with XX or XY for each cell.

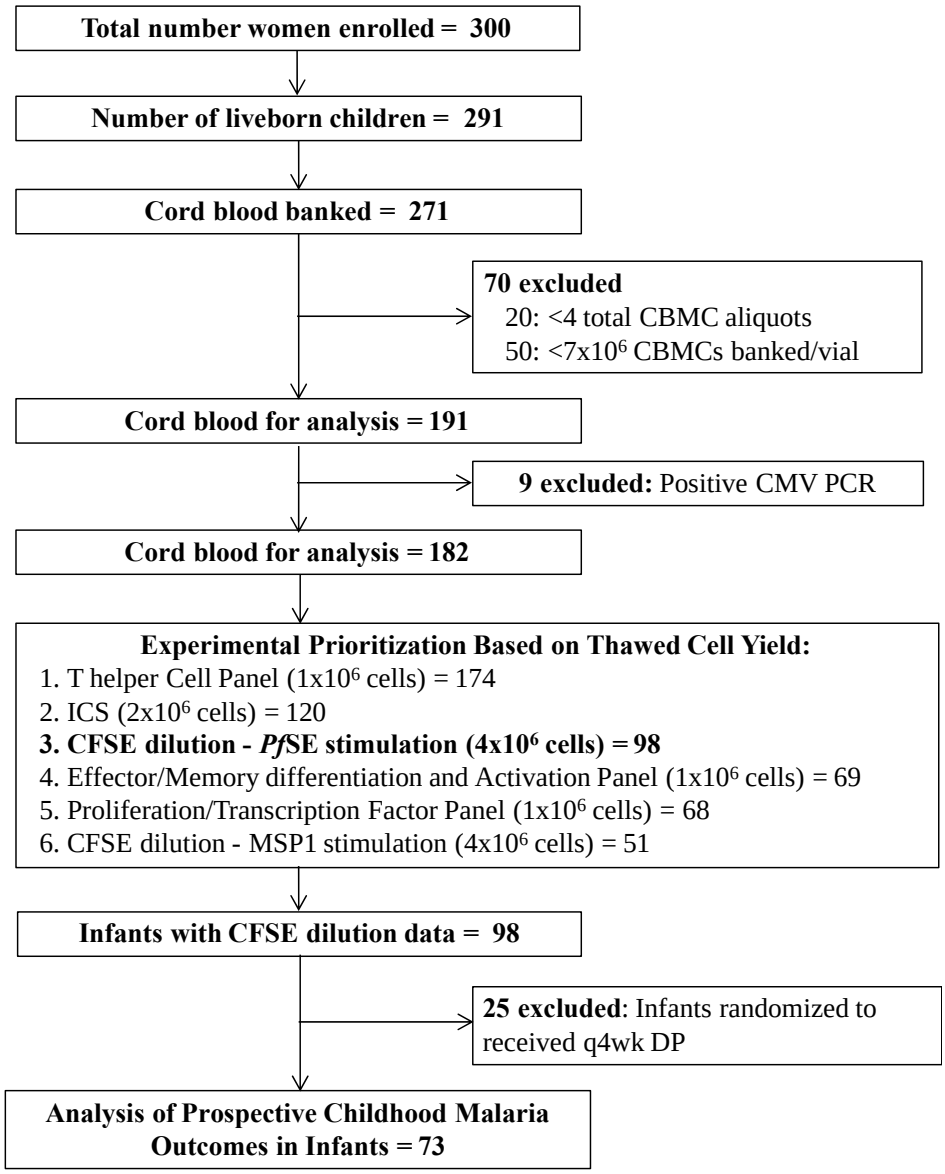


Fig. S1. Outline of inclusion criteria and experimental prioritization of cord blood samples.

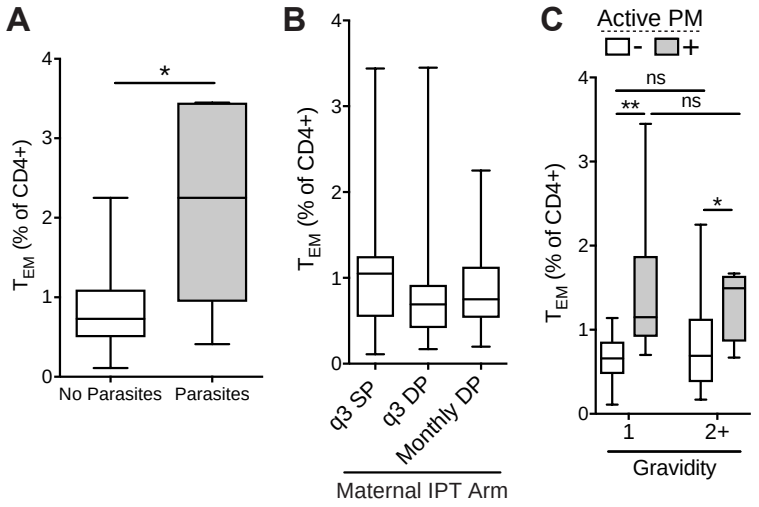


Fig. S2. Fetal effector memory CD4$^+$ T cell frequencies are altered by parasites in the placenta but not by maternal gravidity or IPT arm. (A) Frequency of CD4+T_{EM} cells in infants born to mothers with (grey bar; n=65) or without (white bar; n=5) parasites in the placenta as determined by histopathology. **(B)** Frequency of CD4+ T_{EM} cells in infants born to mothers randomized to receive SP every 3 months (n=26), DP every 3 months (n=30), or monthly DP (n=13). **(C)** Frequency of CD4+ T_{EM} cells in infants born to mothers with (grey bar: primigravidas n=6, multigravidas n=4) or without (white bar: : primigravidas n=14, multigravidas n=37) active PM, stratified by maternal gravidity. *p<0.05, **p<0.01, ***p<0.001, Kruskal Wallis and/or Wilcoxin Rank-Sum Test.

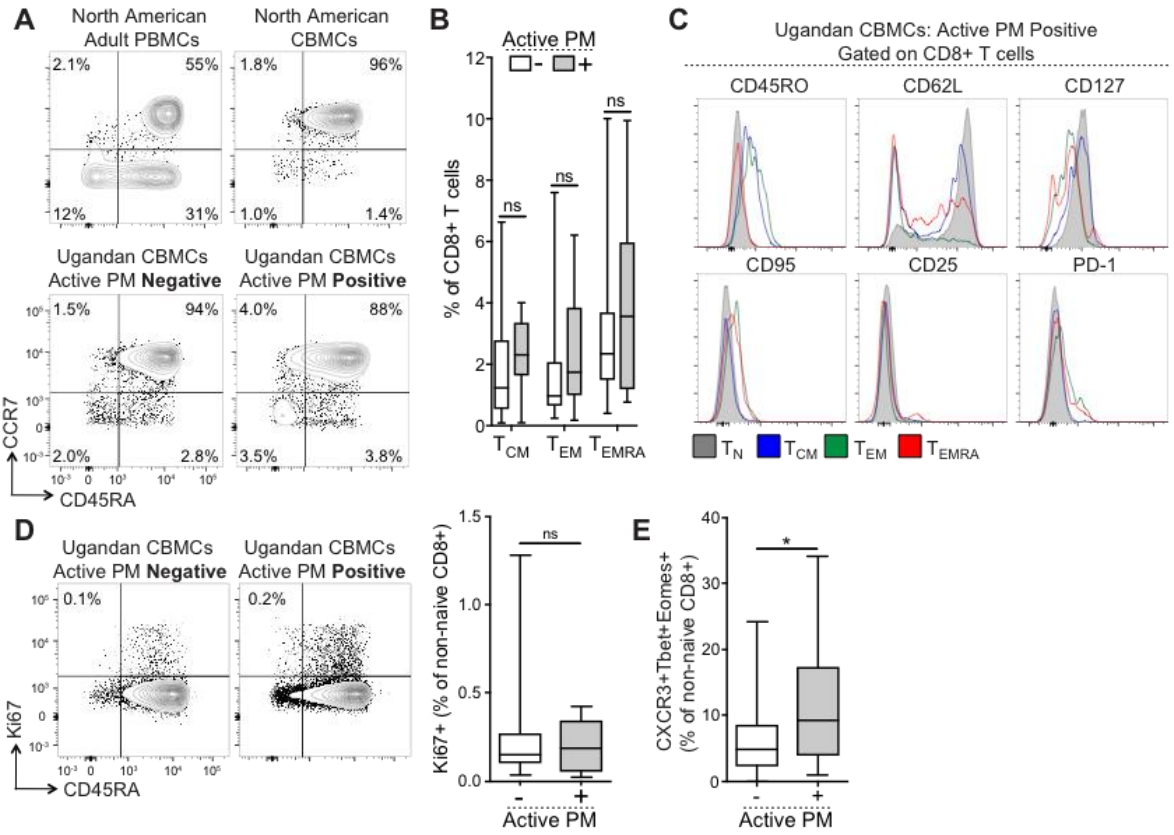
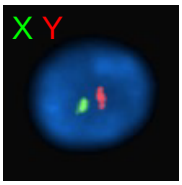


Fig. S3. Fetal effector and memory CD8⁺ T cell subsets do not differ with PM exposure. (A) Representative flow plots of CD8+ T cell effector-memory subsets. T_{CM}: CD45RA-CCR7+, T_{EM}: CD45RA-CCR7-, and T_{EMRA}: CD45RA+CCR7-. Gated on live singlets CD14-CD19-γδTCR-CD3+CD4-CD8+ T cells. Values indicate the frequency of each population as % of CD8+ T cells. **(B)** Quantification of T cell subsets in infants born to mothers with (grey bar, n=14) or without active PM (white bar, n=55). (**C**) Histograms of CD8+ T cell subset phenotype from a representative PM-exposed infant. (**D**) Representative flow plots of Ki67 and CD45RA expression. Values indicate % Ki67+ cells of non-naïve CD8+ T cells (CD45RA-). Quantification of Ki67+ T cells in active PM-exposed (grey bar; n=11) versus unexposed (white bar; n=57) infants to the left. (**E**) Quantification of CXCR3+Tbet+Eomes+ CD8+ T cells in active PM-exposed (grey bar; n=20) versus unexposed (white

bar; n=154) infants to the left. All representative flow plots were gated on live CD14-CD19-γδTCR-CD3+CD8-CD8+ T cells. *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis and Wilcoxin Rank-Sum Test.

A Ugandan CBMCs: Active PM Positive
Sorted on Non-naive CD4+ T cells



B

Sorted Population	XY Frequency per 200 nuclei
Non-naive CD4+ T cells	200/200
Non-naive CD8+ T cells	200/200
$CFSE^{low}$ CD4+ T cells	200/200
$CFSE^{low}$ CD8+ T cells	200/200

Fig. S4. Cord blood $CD4^+$ T cell responses are fetal in origin. (A) Representative FISH staining of sorted non-naïve CD4+ T cells from cord blood of a male infant born to a mother with active PM. **(B)** Quantification of XX and XY chromosomes counted in 200 nuclei/individual (n=3 individuals) from sorted cord blood T cell populations.

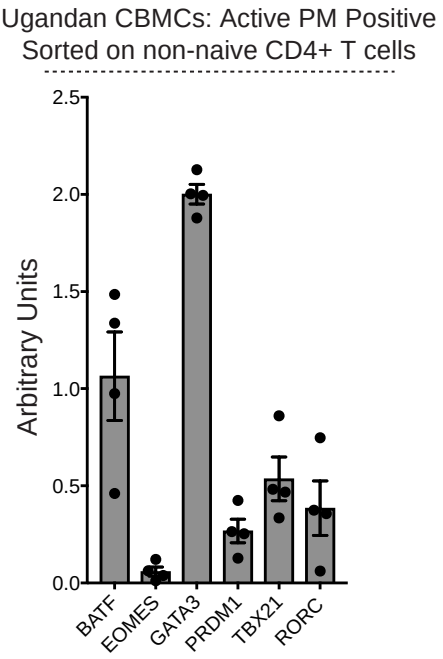


Fig. S5. Cord blood CD4$^+$ T cell responses are transcriptionally heterogeneous. Transcription factor mRNA expression in sorted non-naïve CD4+ T cells from cord blood of infants born to mothers with active PM (n=3-5). Data are representative of 4 independent experiments.

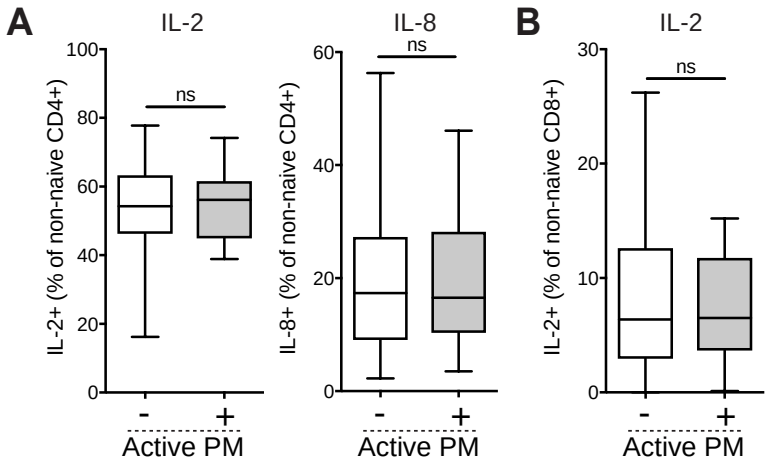


Fig. S6. Cytokine production by cord blood CD4$^+$ and CD8$^+$ T cells. (A) Quantification of cytokine producing non-naïve CD4+ cells in active PM-exposed (grey bar, n=21) and unexposed (white bar, n=99) infants. Values calculated by background subtraction of media-alone controls. **(B)** Quantification of cytokine producing non-naïve CD8+ cells in active PM-exposed (grey bar, n=21) and unexposed (white bar, n=99) infants. Values calculated by background subtraction of media-alone controls. *p<0.05, **p<0.01, ***p<0.001, Wilcoxin Rank-Sum Test.

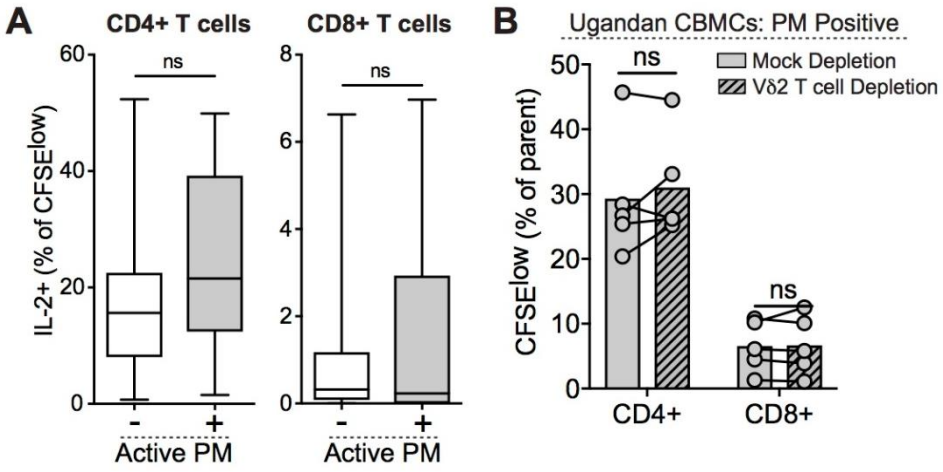


Fig. S7. Characterization of cord blood CD4$^+$ and CD8$^+$ T cell proliferation in response to malaria antigens. (A) Quantification of CFSE^{low} cytokine-producing CD4+ (left) and CD8+ (right) T cells stratified by infant malaria exposure (active PM negative, white bars, n = 79; active PM positive, grey bars, n=17). Values calculated by background subtraction of uRBC-stimulated, media-alone controls. **(B)** CFSE^{low} CD4+ T cell frequencies after 6d *Pf*SE stimulation in the presence or absence of Vδ2 T cells. Values calculated by background subtraction of uRBC-stimulated controls. Data representative of three independent experiments. Significance assessed by Wilcoxin Rank-Sum Test for all experiments except Vδ2-depletion, which was assessed by Wilcoxon Matched-Pairs Signed Rank Test.

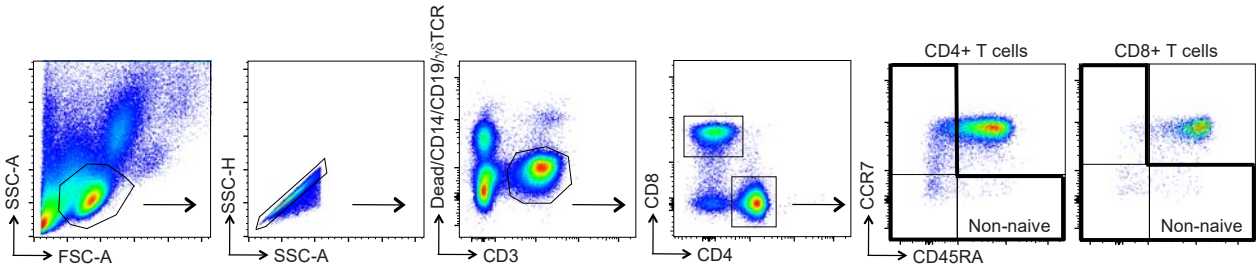


Fig. S8. Gating strategy for CD4$^+$ and CD8$^+$ T cell populations.

Table S1. CD4$^+$ T cell proliferation in response to malaria antigens is associated with protection from childhood malaria with adjustment for maternal malaria exposure.

					Adjusted for Maternal Malaria Exposure[a]	
Incidence of clinical malaria through 24 months of age						
% *Pf*SE-specific CFSE^{low} CD4+ T cells	**N**	**Episodes**	**PY[c]**	**Incidence**	**IRR (95% CI)**	**p-value**
Low < 15%	11	16	21.0	0.76	reference	
Intermediate 15<30%	35	36	62.5	0.58	0.74 (0.28-1.92)	0.53
High ≥ 30%	27	10	48.3	0.21	0.29 (0.09-0.86)	0.03
Time to first episode of malaria following birth						
	N	**Cumulative risk (95% CI)**			**HR (95% CI)**	**p-value**
Low < 15%	11	79.6% (51.8-96.8%)			reference	
Intermediate 15-<30%	35	41.1% (26.3-60.1%)			0.45 (0.18-1.10)	0.08
High ≥ 30%	27	33.8% (18.5-56.5%)			0.35 (0.13-0.95)	0.04
Detection of malaria parasites at routine monthly visits between 2-24 months of age[d]						
	n/N	**%**			**PRR (95% CI)**	**p-value**
Low < 15%	25/269	9.29%			reference	
Intermediate 15-<30%	56/797	7.03%			0.78 (0.33-1.88)	0.58
High ≥ 30%	19/620	3.06%			0.34 (0.13-0.89)	0.03

[a] Malaria in pregnancy exposure categories: No malaria exposure, any malaria exposure without placental malaria by LAMP, placental malaria by LAMP
[b] In cord blood
[c] Person years of observation
[d] By blood smear and/or LAMP

Table S2. Details of clinical cohort.

	Clinical Cohort (n=182)
Maternal Characteristics	
Age (years) – mean (IQR)	22.1 (18.9-24.9)
Gravidity – n (%)	
1	62 (34%)
2	59 (32%)
≥3	61 (34%)
Malaria IPT arm – n (%)	
3 dose SP	78 (43%)
3 dose DP	29 (16%)
Monthly DP	75 (41%)
Malaria Exposure During Pregnancy	
Malaria Exposure Categories – n (%)	
No malaria infection	29 (16%)
Past malaria infection	132 (72%)
Active placental malaria infection	21 (12%)
Infant Characteristics and Clinical Outcomes	
Gestational age (weeks) – mean (IQR)	39.3 (38.6-40.4)
Low birth weight (<2500 g) – n (%)	18 (10%)
Preterm birth (<37 weeks) – n (%)	13 (7%)
Gender (female) – n (%)	89 (49%)

Table S3. Details of flow cytometric antibodies.

Marker	Clone	Fluorophore	Manufacturer
CD3	SK7	PE-Cy5.5	eBioscience
CD4	RPA-T4	BV570	Biolegend
CD8	RPA-T8	BV711	Biolegend
CD45RA	HI100	BV650	Biolegend
CD45RO	UCHL1	BV605	Biolegend
γδTCR	B1	BV510	Biolegend
CD14	M5E2	BV510	Biolegend
CD19	HIB19	BV510	Biolegend
CCR7	G043H7	BV785, FITC	Biolegend
CXCR3	1C6	PE-Cy5	BD Bioscience
CXCR5	J252D4	BV421, PE	Biolegend
CCR10	314305	PE	R&D
CCR6	11A9	BV605	BD Bioscience
CCR4	L291H4	PE-Cy7	Biolegend
PD-1	EH12.2H7	APC-Cy7, PE	Biolegend
CD27	O323	A700	Biolegend
ICOS	C398.4A	PE-Dazzle	Biolegend
CD25	BC96	PE, AF488	Biolegend
CD95	DX2	PE-Cy7	BD Bioscience
CD62L	DREG-56	PE-Cy5	eBioscience
CD127	A019D5	BV421	Biolegend
TNFα	MAB11	PE-e610	eBioscience
IFNγ	4S.B.3	PE-Cy7	Biolegend
IL-17A	eBio64DEC17	APC-Cy7	eBioscience
IL-2	MQ1-17H12	BV421	Biolegend
IL-8	E8N1	AF488	Biolegend
IL-10	JES3-19F1	PE	BD Bioscience
Eomes	WD1928	e660	eBioscience
FoxP3	PCH101	PE	eBioscience
Ki67	B56	FITC	BD Bioscience
Granzyme B	GB11	A700	BD Bioscience
Tbet	4B10	BV421	Biolegend