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Plasmodium falciparum induces trained innate immunity

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Abstract

Malarial infection in naïve individuals induces a robust innate immune response. In the recently described model of innate immune memory, an initial stimulus primes the innate immune system to either hyperrespond (termed "training") or hyporespond ("tolerance") to subsequent immune challenge. Previous work in both mice and humans demonstrated that infection with malaria can both serve as a priming stimulus and promote tolerance to subsequent infection. In this study, we demonstrate that initial stimulation with *Plasmodium falciparum* infected red blood cells (iRBCs) or the malaria crystal hemozoin (Hz) induced human adherent PBMCs to hyperrespond to subsequent ligation of TLR2. This hyperresponsiveness correlated with increased H3K4me3 at important immunometabolic promoters, and these epigenetic modifications were also seen in Kenyan children naturally infected with malaria. However, the use of epigenetic and metabolic inhibitors indicated that the induction of trained immunity by malaria and its ligands may occur via previously unrecognized mechanism(s).

Introduction

A hallmark of malaria is robust proinflammatory cytokine production induced by widespread innate immune activation. Multiple innate immune receptors are involved in the recognition of *Plasmodium*-infected red blood cells (iRBCs) and other malarial ligands [reviewed in (1)]. For example, the malaria crystal hemozoin (Hz), which becomes coated with *Plasmodium*-derived pathogen-associated molecular patterns (PAMPs) including genomic DNA, activates TLR9 in the phagolysosome (2). Hz crystals can also induce

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phagolysosomal rupture resulting in the activation of the nucleotide-binding domain, leucine-rich repeat–containing, pyrin domain containing 3 (NLRP3) inflammasome as well as deliver *Plasmodium* DNA to the cytosol, where it is recognized by cytosolic DNA receptors including those that sense AT-rich stem-loop structures in *Plasmodium* genomes (3, 4). This innate immune response, although beneficial through limiting parasitemia and assisting in the activation of adaptive immunity, induces the systemic symptoms of fever, nausea, and malaise. Proinflammatory cytokinemia has been implicated in the development of cerebral malaria (5).

Multiple studies have demonstrated memory phenotypes in innate immune cells (6–8). In the prevailing model of innate immune memory, an initial stimulus primes the innate immune system, which induces epigenetic and metabolic changes that result in an increased or decreased response—termed training or tolerance, respectively—to a subsequent challenge occurring days to months later (9). Malarial infection serves as a robust priming stimulus, as whole blood samples from experimentally infected individuals and PBMCs from patients with acute febrile disease are hyperresponsive to *ex vivo* TLR ligand stimulation—a phenotype that can be recapitulated *in vitro* (10, 11).

Malaria can induce tolerance to subsequent infection or other immune challenge [reviewed in (12)]. The pyrogenic threshold, i.e., the level of parasitemia required to provoke fever, was higher for individuals after reinfection compared to initial infection (13). In an area of Mali with seasonal malaria transmission, nearly 50% of healthy individuals had detectable parasitemia at the end of the dry season in the absence of symptoms (14). Individuals infected with malaria as fever therapy for neurosyphilis and then challenged 2-3 days post final defervescence with heat-killed *Salmonella* exhibited depressed febrile responses (15). Tolerance and training appear to be two ends of the same spectrum, as LPS and other ligands induce tolerance at higher concentrations but produce training at much lower concentrations (16). We hypothesized that malarial stimulation would also induce trained immunity and set about to evaluate this possibility directly using human PBMCs.

Materials and Methods

Malaria cultures and iRBC/hemozoin isolation

Plasmodium falciparum clone 3D7 iRBCs were passed through a magnetic field resulting in enrichment consistently ≥90% iRBCs. Hz was isolated by passing malaria culture supernatants through a magnetic field as described previously (2).

Human subject use

Human subject use was approved by the UMMS IRB (H-10368), University Hospitals Cleveland Medical Center IRB (06-11-22), and the KEMRI Ethical Review Committee (SSC No: 2207). Samples from Kenyan children aged 1-10 years with febrile malaria were obtained from Chulaimbo Sub-County Hospital Kisumu. Venous blood was obtained at presentation and 6 weeks after curative treatment. Individuals with submicroscopic infections detected at recovery visits by PCR (17) were excluded from further analysis. PBMCs from Kenyan children and healthy adult North American controls (3 male, 3 female,

aged 33-68 years) were cryopreserved (18). Monocytes were negatively selected from thawed PBMCs using a Pan Monocyte Isolation Kit (Miltenyi Biotec).

Human adherent PBMC isolation and stimulation

PBMCs from healthy donors were plated at 5×10^5 cells/well (in 96-well round-bottom plates) or 10×10^6 cells/well (in 6- or 12-well flat-bottom plates) and incubated at 37°C for ≥1 hr. Non-adherent cells were removed by washing 3× with PBS. Adherent PBMCs were then incubated in RPMI supplemented with 10% human serum (RPMI+) and stimulated for 24 hr. Stimulation with iRBCs or Hz did not decrease cell number or viability (data not shown). Cells were washed with PBS and allowed to rest in RPMI+ for 3 days. Cells were then harvested for ChIP analysis or stimulated with Pam3CSK4 (Invivogen) for 4-24 hr. Stimulated cells were harvested for mRNA analysis or supernatants were frozen at −20°C for subsequent cytokine measurement.

Cytokine measurement

TNFα and IL-6 ELISA kits were from R&D. A MTS tetrazolium dye assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (Promega) was used to determine relative cell number post stimulation. Cytokine values were normalized to cell number.

mRNA expression

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA synthesis and qPCR were performed using an iScript cDNA synthesis kit and iQ SYBR Green supermix (both Bio-Rad). The following primer pairs were used: *ACTB*, FW 5′-TGAAGTCTGACGTGGACATC-3′, RV 5′-ACTCGTCATACTCCTGCTTG-3′; *GAPDH*, FW 5′-GAGTCAACGGATTTGGTCGT-3′, RV 5′-TTGATTTTGGAGGGATCTCG-3′; *IL6*, FW 5′-ACTCACCTCTTCAGAACGAATTG-3′, RV 5′-CCATCTTTGGAAGGTTCAGGTTG-3′; *TNF*, FW 5′-TGCTTGTTCCTCAGCCTCTT-3′, RV 5′- GGTTTGCTACAACATGGGCT-3′. Ct values were normalized to *ACTB* and *GAPDH* expression and analyzed using Bio-Rad software. NanoString analysis was performed using the nCounter Human Inflammation v2 Codeset, which simultaneously measures mRNA expression levels of 249 inflammation-related genes, according to the manufacturer's instructions (NanoString Technologies).

Neutralizing antibodies and inhibitors

Mouse anti-human IFNγ IgG1 and IgG1 isotype control were from Biolegend. 5′-Deoxy-5′-(methylthio)adenosine (MTA) and rapamycin were from Sigma.

ChIP analysis

Briefly, cells were fixed in 1% formaldehyde for 10 min and quenched with glycine. Chromatin was sonicated from these cells using a Bioruptor UCD-300 (Diagenode) for four cycles of 10× (30 sec ON, 30 sec OFF) on the HIGH setting. Chromatin precipitation was performed using rabbit anti-human H3K4me3 IgG antibody (Diagenode) as described previously (7). DNA was then quantified using qPCR with the following primer pairs:

GAPDH, FW 5′-ATCCAAGCGTGTAAGGGTCC-3′, RV 5′-GACTGAGATTGGCCCGATGG-3′; *IL6*, FW 5′-AGCTCTATCTCCCCTCCAGG-3′, RV 5′-ACACCCCTCCCTCACACAG-3′; *MTOR*, FW 5′-ATAAAGAGCGCTAGCCCGAA-3′, RV 5′-GACCCCTCCCGGTGTAATTC-3′; *TNF*, FW 5′-CAGGCAGGTTCTCTTCCTCT-3′, RV 5′-GCTTTCAGTGCTCATGGTGT-3′. For all ChIP experiments, qPCR values were normalized as percent recovery of the input DNA.

Statistical analysis

Statistical comparisons were made between the indicated condition and control-trained cells (RPMI+). Multiple analyses were carried out for experiments with multiple replicates for an individual donor performed on different days; for the experiments represented in Fig. 1*B–C*: a mixed model regression analysis, a Wilcoxon signed-rank test with clustering (analysis at the replicate level accounting for correlation of data within donor), a Wilcoxon signed-rank test using one averaged value per donor (mean of all replicates), and a paired *t*-test using one averaged value per donor. All four tests provided nearly identical hypothesis decisions with an α=0.05, so paired *t*-tests using one value per donor was used for all other experiments. When ratios to controls were compared, one sample t-tests (H_0 log(ratio)=0) were performed on log-transformed ratio values.

Results

Training with malaria parasites or ligands induces increased proinflammatory and decreased anti-inflammatory cytokine production in response to secondary TLR stimulation

Adherent PBMCs from healthy donors were stimulated with RPMI+ alone, *P. falciparum* iRBCs, uninfected red blood cells (uRBCs), or Hz for 24 hr. The cells were washed and allowed to rest in RPMI+ for three days. At this point, the cells were either harvested for ChIP analysis or stimulated with the TLR2 ligand Pam3CSK4 for 4-24 hr to measure cytokine production (Fig. 1A). Training with either 1×10^5 or 1×10^6 iRBCs—but not uRBCs—induced increased production of proinflammatory cytokines TNFα and IL-6 but decreased production of the anti-inflammatory cytokine IL-10 24 hr after second stimulation with Pam3CSK4 as compared to controls (Fig. 1B and Supplemental Fig. 1). Similarly, primary stimulation with Hz induced a similar pattern of training following Pam3CSK4 stimulation (Fig. 1C).

Malarial training has wide-ranging effects on the inflammatory transcriptome post-TLR second stimulus

The prevailing model of trained immunity is that increased responsiveness to secondary stimuli results from epigenetic remodeling that increases chromatin accessibility and that increased proinflammatory gene transcription follows secondary stimulation. In our *in vitro* model, stimulation of iRBC- or Hz-trained adherent PBMCs with Pam3CSK4 increased transcription at the *TNF* locus as compared to cells trained with media alone (Fig. 2A). Surprisingly, malarial training did not lead to increased *IL6* transcription after 4 hr Pam3CSK4 second stimulation, although there was a trend towards increased *IL6* transcription in iRBC- and Hz-trained cells after 12 hr Pam3CSK4 stimulation (Fig. 2B). To

determine if malarial training has a more global effect on inflammatory gene expression, we performed a NanoString analysis on RNA harvested from trained cells given Pam3CSK4 as a secondary stimulus. For each gene, we normalized the iRBC-, uRBC-, or Hz-trained transcript count to the control-trained transcript count at each secondary stimulus time point. These normalized ratios are displayed as a heatmap (Fig. 2C), demonstrating that while uRBC training had little effect on the inflammatory transcriptome, both iRBC and Hz training had wide-ranging and similar effects. The majority of transcripts could be classified into the following cohorts: globally upregulated (C1), upregulated after 4 hr Pam3CSK4 stimulation (C2), upregulated after 12 hr Pam3CSK4 stimulation (C3), downregulated after 12 hr Pam3CSK4 stimulation (C4), downregulated after 4 hr Pam3CSK4 stimulation (C5), or globally downregulated (C6). A subset of genes previously implicated in malarial pathogenesis and/or trained immunity are listed for each cohort (19–23), and a complete list of transcripts in each cohort can be found in Supplementary Table I.

Malarial training does not depend on IFNγ signaling

IFNγ was one of the cytokines highly upregulated by malarial training. NK cells produce IFNγ starting ~6 hr post-iRBC stimulation (24, 25). In a murine malaria model, malarial priming was IFNγ-dependent (11), and IFNγ was required for *C. albicans*-induced trained immunity (26). We used a neutralizing antibody against IFNγ to test the role of this cytokine in malaria-induced training. Treatment of adherent PBMCs with anti-IFNγ IgG during the 24 hr first stimulation did not inhibit iRBC- or Hz-induced training (Fig. 3). We conclude that IFNγ signaling is dispensable for malarial training.

Differential epigenetic and metabolic regulation of malaria-induced trained immunity

Trained immunity is believed to be the result of intracellular metabolic shifts leading to changes in epigenetic regulation of immune-related genetic loci (9). To determine the role of naturally-acquired malaria on epigenetic remodeling, we performed H3K4me3 ChIP on monocytes isolated from Kenyan children with acute febrile malaria, the same children 6 weeks after curative antimalarial treatment, and malaria naïve adult North American controls (Fig. 4A). The Pan Monocyte Isolation Kit (Miltenyi) utilized for this monocyte isolation enriches for both $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{++}$ monocytes. Increased H3K4me3 was seen at the *TNF*, *IL6*, *MTOR*, and *GAPDH* promoters during acute malaria compared to controls. Increased H3K4me3 levels were unchanged 6 weeks after antimalarial treatment. We performed a similar experiment using our *in vitro* model on adherent PBMCs given a 24 hr first stimulation and then rested for three days. Increased H3K4me3 was seen at the *TNF*, *PTGS2*, and *IL6* promoters after iRBC (but not Hz) training compared to RPMI+ training (Fig. 4B).

We utilized the methyltransferase inhibitor MTA as well as the mechanistic target of rapamycin (mTOR) inhibitor rapamycin, which have been demonstrated to inhibit BCG-induced training (7) and *C. albicans* β-glucan-induced training (27), respectively, to investigate the importance of these mechanisms in malaria-induced training. Treatment with 1 mM MTA for the 24 hr first stimulation had no effect on iRBC-induced training (Fig. 4C). MTA treatment appeared to inhibit Hz-induced training, although this did not achieve statistical significance due to the relatively small sample size. Treatment with 10 nM

rapamycin during the 24 hr first stimulation blocked iRBC-induced training as measured by IL-6 protein production but had no effect on malarial training as measured by TNFα protein levels (Fig. 4D).

Discussion

Malaria is an extraordinarily complicated disease. Individuals from endemic regions of the world have a clinical syndrome that is influenced by age and previous exposure. Children with little or no immunity to malaria are at highest risk for acute febrile disease that may be complicated by severe sequelae such as cerebral disease (28). This corresponds with our findings that Kenyan children have increased H3K4me3 at promoters relevant to inflammation and immunometabolism both during acute malaria and after curative therapy as compared to healthy North American adult controls. This is especially striking as baseline monocyte H3K4me3 levels are higher in otherwise healthy adults than neonates at both proinflammatory and metabolic promoters (29). Monocytes were chosen for ChIP analysis because enriching for the population of cells that produce the cytokines we measured in our *in vitro* studies seemed logical. It is worth noting that the $t_{1/2}$ of monocytes is 1-3 days, suggesting that training almost certainly occurred at the level of myeloid progenitor cells (9). We recognize that this early data using patient specimens was necessary to show, but not conclusive. Long term approaches to definitively identifying training would require following individuals over the course of years (as has been done for BCG vaccine). These data support our hypothesis that malarial infection serves as a robust priming and training stimulus. Indeed, we predict that malaria-infected children are at risk to hyperrespond to superinfection in ways that might be predicted based on the *in vitro* responses we observed.

Older children and adults from endemic areas are likely to have experienced multiple episodes of malaria. These individuals will often have detectable parasitemia without symptoms (14). While the level of parasitemia in these asymptomatic malaria cases are often quite low, some individuals have levels of parasites in their blood that would be incapacitating in the immunologically naïve. It is our hypothesis that the differences in outcome in disease between young individuals or even immunologically mature adults who become infected for the first time later in life (*e.g.*, travelers) compared to multiply infected asymptomatic individuals, are due to epigenetic changes at proinflammatory and immunometabolic promoters.

In this work, we report that the malaria parasite and its crystal Hz can induce trained immunity as measured by inflammatory gene expression after a secondary TLR ligand challenge. Although these two stimuli have similar effects on the inflammatory transcriptome post-Pam3CSK4 challenge, the differential regulation of iRBC- and Hz-induced training by known trained immunity inhibitors and differing levels of H3K4me3 at immunometabolic promoters suggest divergent training mechanisms for the two stimuli. One potential explanation for this divergence is that the parasite contains multiple additional ligands with innate immune stimulatory capacity. Although these TLRs utilize the same signaling cascades and transcription factors as the receptors stimulated by Hz, the balance in signaling between the cascades may be sufficiently different to explain these differences.

Rapamycin treatment only inhibited training as measured by IL-6 and not TNFα, which differs from previous studies on other training stimuli (27, 30). This discrepancy in rapamycin inhibition is not completely surprising given that the *TNF* and *IL6* loci have different initiation kinetics and epigenetic requirements for transcription (31). However, along with the apparent dispensability of IFNγ signaling for malaria training, this provides further evidence that both malaria parasites and Hz induce innate immune training by mechanisms that differ from other infectious stimuli. Any hope for immunomodulation of malaria, either for the purposes of improving outcome in the critically ill or enhancing acquired immunity, requires a more thorough understanding of the basic pathogenesis of the disease. The methods for acquiring this understanding are at hand.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Footnotes

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Abbreviations in this article

iRBC	*Plasmodium falciparum*-infected red blood cell
uRBC	uninfected red blood cell
Hz	hemozoin
PAMP	pathogen-associated molecular pattern
NLRP3	nucleotide-binding domain, leucine-rich repeat–containing, pyrin domain containing 3
AIM2	absent in melanoma 2
mTOR	mechanistic target of rapamycin
BCG	bacille Calmette-Guérin

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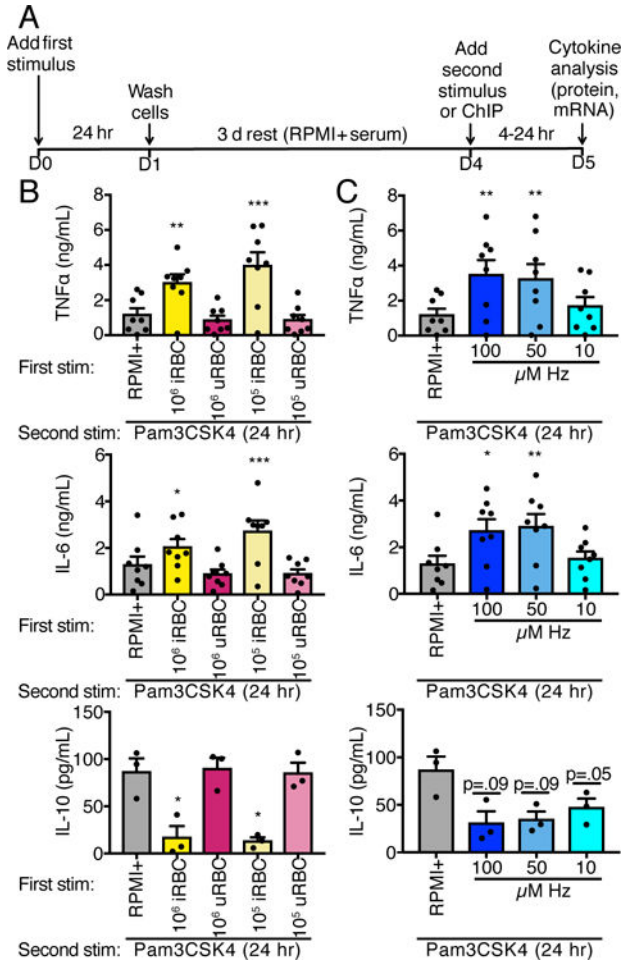

Figure 1.

P. falciparum iRBCs and Hz induce trained immunity. (**A**) Schematic of trained immunity assay. Adherent PBMCs were treated with media (RPMI+), iRBCs, uRBCs, or Hz for 24 hr and then washed. After 3 d rest, cells were harvested for ChIP or challenged with Pam3CSK4 (10 μg/mL) for 4-24 hr. (**B** and **C**). TNFα, IL-6, and IL-10 ELISA measurements of supernatants from adherent PBMCs trained with iRBCs (B) or Hz (C), rested for 3 d, then challenged with Pam3CSK4 for 24 hr. Bars represent mean ± SEM for three (IL-10) or eight (TNFα, IL-6) donors (all comparisons to RPMI+ trained, *p<0.05, **p<0.01, ***p<0.001, paired *t*-test).

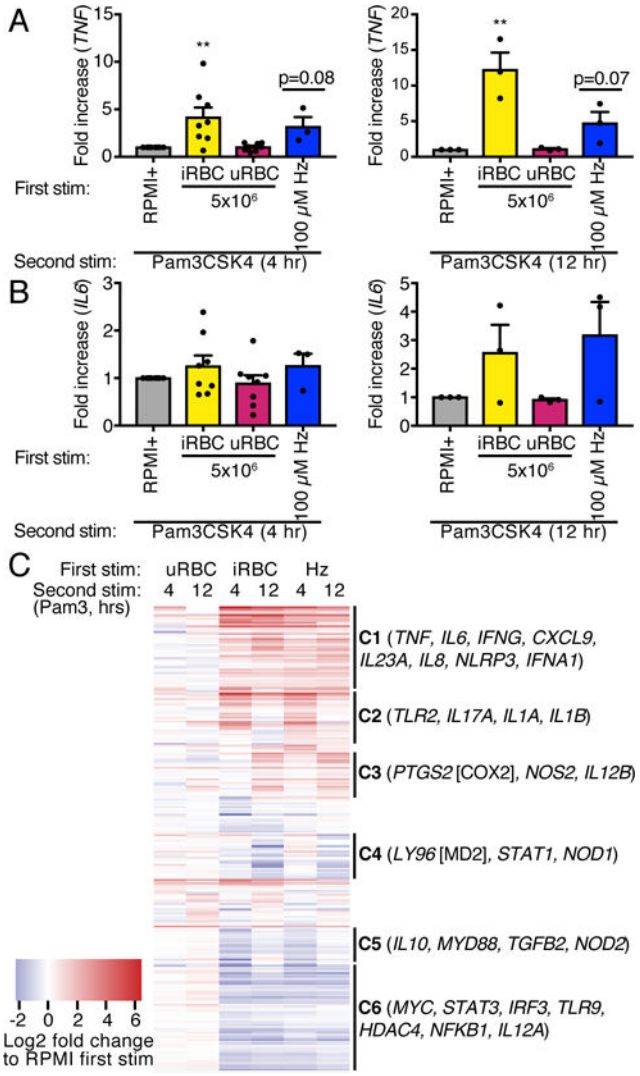

Figure 2.

Malaria-induced training has wide-range effects on the transcriptional response to subsequent challenge. (**A** and **B**) mRNA was harvested from adherent PBMCs trained with iRBCs or Hz, rested for 3 d, and challenged with Pam3CSK4 for 4 or 12 hr. Levels of *TNF* (A) and *IL6* (B) relative to RPMI+ trained cells are shown. Bars represent mean ± SEM for eight (4 hr Pam3CSK4) or three (12 hr Pam3CSK4) donors (**p<0.01, paired *t*-test). (**C**) Gene expression from cells treated as in (A) and (B) was quantified using NanoString technology. Expression counts are represented as log2 fold change compared to control-trained cells using hierarchical clustering. Each row represents one gene. A subset of the total genes measured is listed after each cohort. Values shown are the means of two independent experiments from one of the donors in (A) and (B).

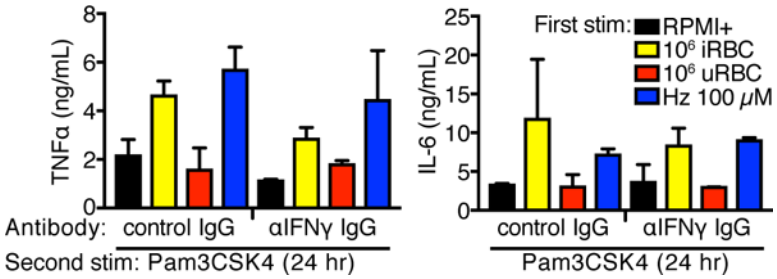

Figure 3.

Malaria-induced training is not inhibited by co-treatment with anti-IFNγ neutralizing mAb. TNFα and IL-6 ELISA measurements of supernatants from adherent PBMCs trained with iRBCs or Hz for 24 hr, rested for 3 d, and challenged with Pam3CSK4 for 24 hr. Cells were treated with control IgG or neutralizing antibody during the 24 hr training. Bars represent mean ± SEM for two donors (all comparisons to control IgG conditions were not significant, paired *t*-test).

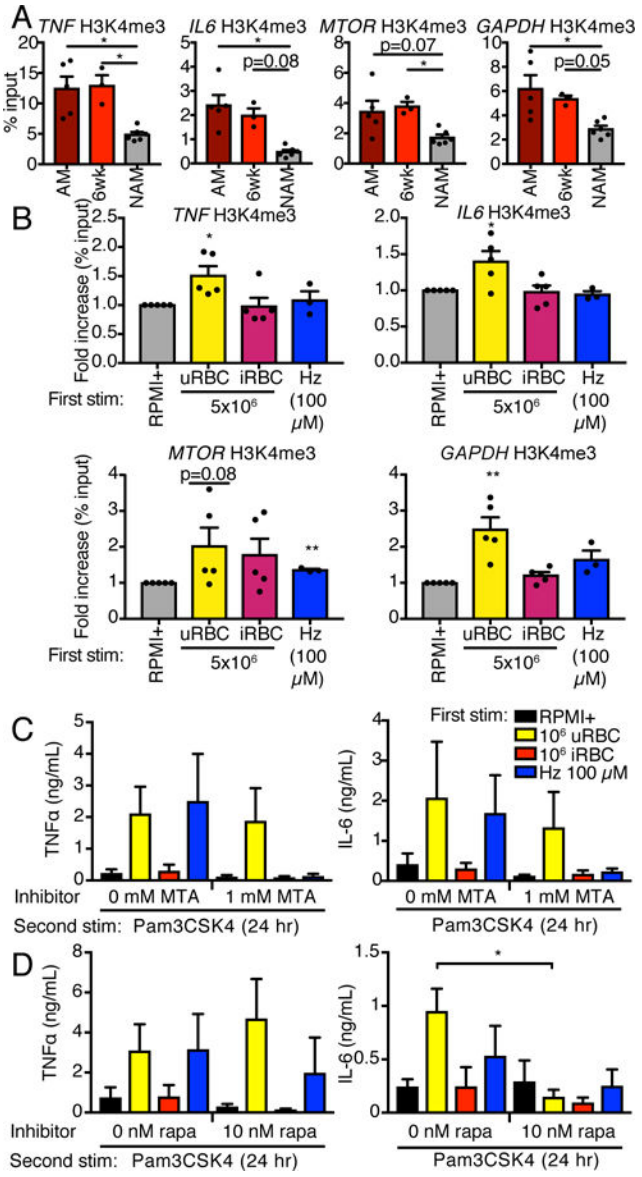

Figure 4.

Epigenetic and metabolic regulation of malaria-induced training. (**A**) H3K4me3 ChIP was performed on monocytes from Kenyan children with acute malaria (AM), the same children 6 weeks after therapy (6wk), or adult North American controls (NAM). Bars represent mean ± SEM for five (AM), three (6wk), or six (NAM) donors (*p<0.05, Kruskal-Wallis test). (**B**) H3K4me3 ChIP was performed on adherent PBMCs trained for 24 hr and rested for 3 d. Bars represent mean ± SEM for five (RPMI+, iRBC, uRBC) or three (Hz) donors (all comparisons to RPMI+ trained, *p<0.05, **p<0.01, paired *t*-test). (**C** and **D**) Adherent PBMCs were co-treated with MTA (C), rapamycin (D) or relevant vehicle controls during the 24 hr training stimulation. Cells were rested for 3 d and challenged with Pam3CSK4 for 24 hr. Cytokine concentrations in supernatants were assessed by ELISA. Bars represent

mean ± SEM for three or four donors (all comparisons to no inhibitor conditions and were not significant unless indicated, paired *t*-test).