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Mild *Plasmodium falciparum* Malaria following an Episode of Severe Malaria Is Associated with Induction of the Interferon Pathway in Malawian Children

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Infection with *Plasmodium falciparum* can lead to a range of severe to minimal symptoms, occasionally resulting in death in young children or nonimmune adults. In areas of high transmission, older children and adults generally suffer only mild or asymptomatic malaria infections and rarely develop severe disease. The immune features underlying this apparent immunity to severe disease remain elusive. To gain insight into host responses associated with severe and mild malaria, we conducted a longitudinal study of five children who first presented with severe malaria and, 1 month later, with mild malaria. Employing peripheral blood whole-genome profiling, we identified 68 genes that were associated with mild malaria compared to their expression in the severe malaria episode (paired Students *t* test, $P < 0.05$). These genes reflect the interferon (IFN) pathway and T cell biology and include IFN-induced protein transcripts 1 to 3, oligoadenylate synthetases 1 and 3, and the T cell markers cathepsin W and perforin. Gene set enrichment analysis identified Gene Ontology (GO) pathways associated with mild malaria to include the type I interferon-mediated signaling pathway (GO 0060337), T cell activation (GO 0042110), and other GO pathways representing many aspects of immune activation. In contrast, only six genes were associated with severe malaria, including thymidine kinase 1, which was recently found to be a biomarker of cerebral malaria susceptibility in the murine model, and carbonic anhydrase, reflecting the blood's abnormal acid base environment during severe disease. These data may provide potential insights to inform pathogenesis models and the development of therapeutics to reduce severe disease outcomes due to *P. falciparum* infection.

Malaria is a leading cause of morbidity and mortality in children worldwide. Globally, over 3 billion people are at risk of acquiring malaria, and an estimated 250 million people become clinically symptomatic each year (8). Most of the 1 million deaths caused by *Plasmodium falciparum* occur in patients with a severe disease syndrome, which may include severe anemia, cerebral malaria (CM), respiratory distress, renal impairment, hypoglycemia, or hyperlactemia (42). Long-term complications can also occur after recovery from severe disease. A decreased language ability and vocabulary and increased likelihood of developing epilepsy and disruptive behavioral disorders have been documented in children who survived CM (7). The risk of severe disease and clinical symptoms declines as children in regions of endemicity grow older in a setting where they experience continuing exposure to infectious mosquito bites (2, 9). Thus, older malaria-infected residents in a region of *P. falciparum* endemicity experience only mild symptoms or are asymptomatic. The underlying mechanisms of protection from severe disease in human infections remain unclear (32). Defining the components of naturally acquired immunity to severe disease could inform vaccine development strategies and therapeutic interventions.

Blood transcriptional profiling has been used to elucidate host responses in HIV, adenovirus, bacterial, tuberculosis (TB), and other infections to build pathogenesis and predictive models (5, 24, 26, 38). Thus, we set out to delineate the differences in host transcriptional responses between episodes of severe malaria and mild malarial infection in the same individual. We compared the transcriptomes of host peripheral blood cells collected during an episode of severe malaria and, 1 month later, during a subsequent mild malaria infection in five Malawian children. Our analysis identified genes and gene sets that were significantly associated with mild malaria compared to their expression in cases of severe malaria. Our data suggest that the interferon (IFN) pathway and T cells may have important roles in the development of protective immune responses in Malawian children who had survived severe malaria. We have also identified genes associated with severe disease that could be further tested as biomarkers. These data provide insights into host peripheral blood transcriptional correlates associated with severe malaria and the subsequent development of mild clinical disease.

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MATERIALS AND METHODS

Study cohort. To identify children who sustained severe malaria and a subsequent mild malaria infection, we carried out a longitudinal study of children enrolled in the Blantyre Malaria Research Project, Blantyre, Malawi, during the transmission season from January to April 2009. This study enrolls children with severe malaria to define the clinicopathological features of CM (6, 13). Institutional review board (IRB) approvals were obtained from the Albert Einstein College of Medicine, Michigan State University, and The University of Malawi College of Medicine Research and Ethics Committee. Children between the ages of 6 months and 12 years with peripheral blood parasitemia and a Blantyre coma score of <3 were enrolled into the Blantyre malaria research ward after parental consent. At the time of admission, a blood sample was taken, and from this 3 ml was aliquoted into Tri-Reagent BD (Molecular Research Center), shaken vigorously for 15 s, and frozen at −80°C for transcriptional analyses of severe malaria. Admission data included a full physical exam, a fundoscopic exam for determination of malarial retinopathy, a complete blood count, glucose and lactate analysis, blood culture, and parasitemia count (37). Bacterial meningitis as a cause of coma was ruled out through analysis of cerebral spinal fluid bacterial culture, microscopy, and white cell count.

Study patients were followed up 1 month after discharge, at which time a thick blood smear was prepared, and a physical exam was performed including measurement of body temperature. Patients with a positive smear at this visit were sampled again by venipuncture to obtain 3 ml of whole blood that was processed for transcriptional analysis as above, and this sample comprised the episode of mild malaria. Peripheral parasite load was determined on a thick smear using the semiquantitative method; scoring was as follows: 2+ represents 11 to 100 parasites per 100 high-powered fields (HPF), 3+ represents 1 to 10 parasites per HPF, and 4+ represents >10 parasites per HPF.

RNA isolation and data analysis. The samples were shipped to New York in nitrogen gas and thawed at room temperature, and total RNA was isolated according to the manufacturer's protocol (Tri-Reagent BD; Molecular Research Center). High-quality RNA samples with an A_{260}/A_{280} ratio of >1.6 and with distinct ribosomal bands on ethidium bromide-stained agarose gels were subsequently hybridized to Affymetrix GeneChip Standard 1.0 ST Arrays. Gene expression values were calculated from Affymetrix microarray probe-level data using the robust multiarray average (RMA) procedure, resulting in 32,321 probe sets which were then quantile normalized (28). The data were thresholded to a minimum of 20 expression units (EU) and a maximum of 20,000 EU. A filtered gene list of 3,110 genes was generated by removing genes that varied less than 3-fold or 100 EU in at least one sample across the 10-sample data set. Differentially abundant transcripts between the severe and mild clinical states for each paired sample were identified using a paired Student *t* test of $\log_2$-transformed data. To identify gene sets that were associated with each state, a library of Gene Ontology (GO) functions containing 15 to 500 genes was constructed from the Bioconductor library (accessed 4 September 2010) using all parent and child nodes. Gene sets with all evidence codes except IC, for inferred by curator, or ND, for no evidence, were included. Differentially regulated GO gene sets were identified using gene set enrichment analysis (GSEA) against a ranked list of genes containing the paired Student *t* test statistic of $\log_2$-transformed data (35). A weighted enrichment statistic was employed.

Microarray data accession number. All microarray data were deposited in the Gene Expression Omnibus (GEO) database under accession number GSE33811.

RESULTS

Patients ($n = 119$) were admitted to the Blantyre Malaria Research Project with CM or CM/severe anemia in the 2009 transmission season. All patients received intravenous quinine and routine clinical care.

Patients were documented to become smear negative during the hospital admission. Eighteen patients died, and the rest improved clinically and were discharged. Of the 101 survivors, 81% ($n = 82$) presented for the routine 1-month follow-up study visit. At this time 10% of these patients ($n = 8$) had a positive blood smear for parasites. None of the eight infected patients exhibited evidence of severe disease. A second blood draw was collected from six patients at this second time point. Five severe/mild paired samples had sufficiently high-quality RNA for microarray and further analysis.

The exam and laboratory results of five children with severe and mild malaria are shown in Table 1. These children ranged from 8 to 45 months of age, and all presented with fever on admission. Upon admission to the study, they displayed many manifestations of severe malarial disease, with all patients manifesting WHO-defined CM; four out of five had severe anemia (hematocrit of <15%), and four of the five had hyperlactemia (>10 mmol/liter). Four of five patients had evidence of retinopathy, suggestive of parasite brain sequestration (37). White blood cell counts varied from 8,100 to 75,390 cells/μl, and platelet counts ranged from 48,000 to 299,000 cells/μl. The peripheral parasite load also varied over a large range, from 2,270 to 400,400 parasites/μl. Clearance of peripheral blood parasites was noted in all patients by 48 h. All patients who presented with coma recovered from coma by 12 h.

At the follow-up visit after 1 month, some children manifested mild malaria with symptoms of fever and vomiting. They all had a normal clinical exam. Their peripheral parasitemia ranged from 2+ to 4+ using a semiquantitative system. The hematocrit during this evaluation was nearly double the initial hematocrit level, despite the second infection.

Given that these children had experienced two bouts of *P. falciparum* malaria with markedly different disease classifications, we wanted to determine whether differences existed in the peripheral blood transcriptional profiles that were associated with mild versus severe disease presentations. Total RNA was extracted from peripheral blood cells collected at each visit and analyzed by microarray. Figure 1 reports the transcripts that had the greatest fold difference between severe and mild samples (paired Students *t* test, $P \leq 0.05$; fold change of ≥2.0). Many of the upregulated genes associated with the mild malaria episode involved the interferon pathway, including IFN-induced protein transcripts (IFIT1 to IFIT3), myxovirus resistance 1 (MX1), oligoadenylate synthetases 1 and 3 (OAS1 and OAS3, respectively), and T cells with upregulation of cathepsin W (CTSW) and perforin 1 (PRF1). The complete list of 68 gene transcripts is given in Table S1 in the supplemental material ($P \leq 0.05$). Six genes were associated with severe malaria, including carbonic anhydrase 1 (CA1), G-protein-coupled receptor 89B (GPR89B), lipocalin 2 (LCN2), thymidine kinase 1 (TK1), small nucleolar RNA, C/D box 30 (SNORD30), and TBC1 domain family member 3 (TBC1D3) ($P \leq 0.05$; fold change of ≥1.5).

To identify gene sets upregulated in each clinical state, we employed GSEA (35). Numerous GO functions representing diverse immune processes were upregulated in samples from children during mild malaria. The top pathways are presented in Table 2 ($P \leq 0.001$; false-discovery rate [FDR] of <0.01), with a complete list ($n = 116$) given in Table S2 in the supplemental material ($P \leq 0.001$; FDR of <0.05). These GO functions included immune response (GO 0006955), regulation of inflammation (GO 0050727), the type I interferon signaling pathway (GO 0060337), regulation of leukocyte proliferation (GO 00700663), T cell acti-

TABLE 1 Clinical and laboratory characteristics of children with severe malaria and a subsequent bout of mild malaria[a]

Characteristic	Patient				
	1	2	3	4	5
Demographics					
Age (mo.)	8	45	42	23	8
Sex (M or F)[b]	F	M	F	M	M
Severe presentation					
Temp (°C)	38.0	38.8	39.8	39.5	36.7
Pulse	104	183	179	157	152
Systolic BP[c]	99	92	106	88	111
Respirations	56	54	44	52	52
Retinopathy	Yes	Yes	Yes	Yes	No
Glucose (mmol/liter)	9.8	5.1	7.2	5.4	4.4
Lactate (mmol/liter)	17.2	10.7	1.8	10.9	15.3
Hematocrit (%)	9.9	14.9	17.0	9.7	13.9
White blood count ($10^3/\mu$l)	75.3	19.3	8.1	8.7	45.4
Platelet count ($10^3/\mu$l)	299	137	48	52	103
Parasitemia (no. of parasites/μl)	49980	26240	12660	400400	2270
Parasite clearance time (h)	36	42	30	48	30
WHO diagnosis	CM/An[e]	CM/An	CM	CM/An	CM/An
Mild presentation					
Temp (°C)	37	39.5	39.0	39.0	38.4
Hematocrit (%)	18	36	N/A	17	31
Parasitemia[d]	4+	4+	2+	3+	4+

[a] Host demographic and selected physical findings and laboratory values of five infected children obtained at presentation with severe malaria and 1 month later with mild malaria.
[b] M, male; F, female.
[c] BP, blood pressure.
[d] Mild malaria parasite loads were estimated using a scoring system of 1 to 4+, with 4+ representing high parasitemia.
[e] An, severe anemia (hematocrit of <15%).

vation (GO 00042110), cell adhesion (GO 007155), and others. The GO functions associated with severe malaria contained G protein-coupled receptor families (GO 0004930; G protein-coupled receptor activity). The high degree of sequence homology of these gene families to GPR89B are consistent with cross-hybridization and thus are not further analyzed.

DISCUSSION

Understanding the factors leading to the acquisition of protection against severe clinical presentations of malaria in areas of endemicity may illuminate new approaches to both prevention and treatment. This is the first report that compares the transcriptomes of peripheral blood cells from children with severe malaria with the transcriptomes of cells from the same children during a subsequent mild malaria infection. These data identify an association of mild malaria with a type I IFN response.

In areas of high transmission, despite episodes of reinfection older children and adults generally suffer only mild or asymptomatic malaria infections and rarely develop severe disease (4, 9). To investigate the mechanism of these field observations and the immune responses associated with variation in disease severity, we carried out a subanalysis on patients enrolled in the Blantyre Ma-

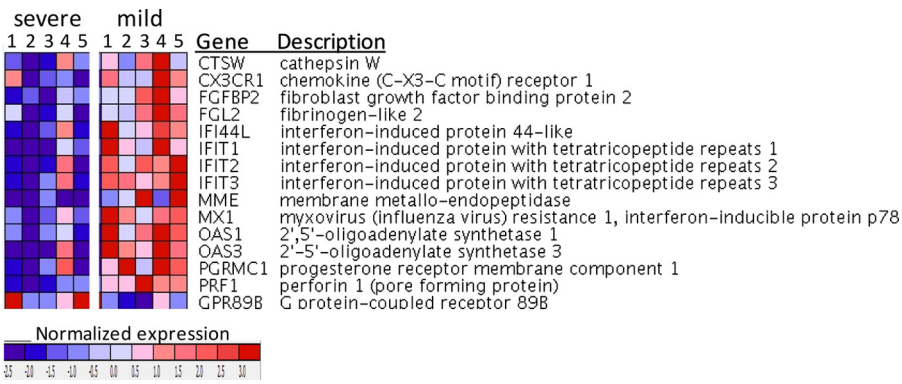

FIG 1 A heat map of genes associated with mild and severe malaria transcripts significantly differentially abundant between severe and mild presentations is shown (paired Student's *t* test, $P < 0.05$; ≥2-fold difference between averages of groups). Many genes associated with mild disease are in the interferon pathway and associated with T cell biology. Data are row normalized.

TABLE 2 Top gene sets enriched in mild malaria[a]

Group and GO identification no.	Description	Size	*P* value	FDR
Cytokines				
0071357	Cellular response to type I interferon	20	<0.001	0
0060337	Type I IFN-mediated signaling pathway	20	<0.001	0
0034340	Response to type I IFN	20	<0.001	0
0019221	Cytokine-mediated signaling pathway	47	<0.001	0
0071345	Cellular response to cytokine stimulus	51	<0.001	0
0009615	Response to virus	44	<0.001	0.01
0034097	Response to cytokine stimulus	66	<0.001	0.01
Cellular				
0002521	Leukocyte differentiation	29	<0.001	0.01
0002694	Regulation of leukocyte activation	50	<0.001	0
0051249	Regulation of lymphocyte activation	46	<0.001	0
0042110	T cell activation	50	<0.001	0.01
0050863	Regulation of T cell activation	38	<0.001	0
0050865	Regulation of T cell activation	52	<0.001	0.01
Other				
0010647	Positive regulation of cell communication	61	<0.001	0.01
0023056	Positive regulation of signaling	63	<0.001	0.01
0048585	Negative regulation of response to stimulus	59	<0.001	0.01
0005102	Receptor binding	82	<0.001	0.01
0006955	Immune response	199	<0.001	0.01
0050776	Regulation of immune response	94	<0.001	0.01

[a] The top GO pathways enriched in mild malaria samples compared to levels in severe malaria ($P < 0.001$; FDR of ≤ 0.01) are listed. These were identified using gene set enrichment analysis of a preranked list of genes based on the paired Student *t* test statistic comparing blood samples obtained during the severe and mild presentations.

laria Research Project. This study protocol provided an opportunity to identify and study the same children during an episode of severe malaria and, 1 month later, with mild malaria.

During the initial severe disease presentation, the five patients who were part of this study displayed a broad range of abnormal laboratory findings and manifested clinical features characteristic of severe malaria (13, 20). During the second observed infection, patients were dramatically less ill, having no clinical evidence of severe disease. The illness in some was limited to a history of recent fever and vomiting, and they presented with an otherwise normal exam. No complete blood count was done during the outpatient visit, but the semiquantitative estimates of parasitemia revealed high-density parasitemias in several of the children. This may suggest the ability of these patients to mount a more effective host response despite high antigen loads during the mild malaria presentation.

Many of the genes upregulated during the subsequent mild malaria disease were related to the type I IFN pathway. The transcripts OAS1 and OAS3 that were upregulated in mild disease are part of the oligoadenylate synthase family of IFN-induced RNA polymerases (18). Other genes associated with mild disease included IFIT1, IFIT3, and MX1, which are also in the IFN-α/β signaling pathway (Ingenuity Pathway Analysis, version 9.0). The type I interferons IFN-α and IFN-β were originally described as central to effective antiviral immune responses (11). More recently, it has been shown that type I IFNs are also produced in response to *Trypanosoma cruzi* infection (34, 40). Additionally, a dominant type I IFN signature was identified from whole-blood transcriptional profiling in patients with active *Mycobacterium tuberculosis* (5).

Genetic studies of malaria-infected patients have found an association between mutations within IFN-α receptor (IFN-αR) and severe disease susceptibility, suggesting that the type I IFN pathway may play a role in severe disease (3, 17). IFN-α has been identified in a subset of malaria-infected patient sera (25, 29). A recent report finds that the mechanism of type I IFN immune induction is through host cell recognition of AT-rich parasite DNA motifs (30). However, the role of type I IFN in protection from severe malaria disease in human studies is unknown. Studies in the rodent model of experimental CM provide conflicting results regarding the role of type I IFN in disease outcomes. *Plasmodium berghei* ANKA-infected mice treated with 800,000 U of IFN-β demonstrated a 60% improvement in survival compared to untreated mice, without any differences in parasitemia (22). This IFN-β treatment led to reduction in CXCL9 and intracellular adhesion molecule-1 (ICAM-1) transcript levels in the brain, reduction of T-cell CXCR3 expression, and downregulation of serum tumor necrosis factor alpha (TNF-α), suggesting that the anti-inflammatory properties potentially accounted for improved survival. IFN-α has also been demonstrated to have a protective role in the rodent model of severe malaria. Administration of 25 days of IFN-α reduced the CM death rate in C57BL/6 *P. berghei* ANKA-infected mice from 87% to 6% (41). This protection was associated with reduction in peripheral parasitemia and also reduced upregulation of ICAM-1 expression in brain endothelial cells. In contrast, mice lacking the type I IFN receptor (IFN-αR1$^{-/-}$) were protected against CM in the *P. berghei* ANKA model in two studies (15, 30). These conflicting data may be due to the approaches taken; however, they suggest that this pathway is involved in mediating disease outcomes. Our data suggest that the type I IFN pathway is associated with a reduction in disease severity in the *P. falciparum*-infected human host, but more studies are needed. Identification of plasma α/β cytokines accompanying the type I IFN transcriptional signature during mild malaria would provide further evidence that this pathway is central to the host response during mild malaria infection compared to a severe disease presentation.

Genes and gene sets involved in a T cell response, such as T cell activation (GO 0042110), are also found to be associated with mild malaria. Cytotoxic T lymphocyte (CTL) granules contain perforin and granzyme A (27). These are key effector molecules for CTL-mediated cytolysis, and their transcripts perforin 1 (PRF1) and granzyme A (GZMA) are upregulated in mild malaria. Also upregulated in mild malaria is cathepsin W, a cysteine protease that is expressed in natural killer and CD8$^+$ T cells and is released in target cell killing (33). Studies in the rodent model have identified T cell responses to be important in the liver stage of infection and in the control of the asexual stages (31, 39). Less is known about the role of T cells in human malaria infections. Recently, age-related qualitative differences in the T cell response to merozoite surface protein 1 were shown, which may contribute to age-related malarial immunity (10). Further investigation in the role of T cells in protection from severe disease in humans is needed.

There have been a number of studies that have identified biomarkers associated with severe malaria, including TNF-α, endo-

thelial microparticles, and intracellular adhesion molecule-1, among many others (1). We identified six transcripts to be associated with severe disease, and some of these have been previously been reported in severe malaria. TK1 transcript was associated with severe malaria and was also found to be upregulated in peripheral whole-blood transcriptome in two CM-susceptible strains of mice (C57BL/6 and CBA/CaJ) compared to levels in CM-resistant BALB/c mice (23). Lipocalin 2, also known as human neutrophil lipocalin (HNL), is a component of the innate immune system. It is upregulated and secreted by immune cells after Toll-like receptor recognition of bacteria, with a bacteriostatic effect (12). The association of this transcript with severe malaria is consistent with a study in the Sudan that found plasma concentrations of HNL to be significantly elevated in patients with severe malaria compared to levels in patients with mild malaria (21). These results may lead to the identification of peripheral blood-based biomarkers of severe disease. A diagnostic biomarker would be clinically useful in view of the observation that the clinical diagnosis of CM can be incorrect up to 25% of the time (37).

CA1 was significantly upregulated in children during severe disease. CA1 is primarily expressed in human red blood cells, and one of its major functions is to maintain acid-base homeostasis (19). Acid-base status is often deranged in severe malaria, and four of five children had abnormally high levels of lactate during their severe disease admission (36). The upregulation of CA1 during severe malaria may reflect a host compensatory response to mediate the hyperlactemia. GPR89B was also associated with severe malaria. There are nearly 2,000 G protein-coupled receptor genes which are classified into over 100 subfamilies based on sequence, ligand structure, and function (16). The identification of peripheral blood cells expressing this protein, confirmation of protein expression, and characterization are under way to understand the role of this receptor in severe malaria.

We note that our study has potential limitations. A larger sample size may have facilitated the identification of additional genes or gene sets that are associated with severe malaria infection. The duration of infection at the time of sampling the peripheral blood transcriptomes could have differed between the severe and mild presentations. Children presenting with mild disease may have been studied earlier in the course of infection as they presented for a routine clinic visit which fortuitously detected the second infection, and the host transcriptional profile thus is related to timing of sampling rather than clinical phenotype. Studies of host response at multiple points over time postinfection could differentiate this possibility. Differences in transcriptional signatures can reflect differences in cellular composition or differences in transcript abundance. Peripheral blood contains a heterogeneous mixture of cells, and the two blood samples might differ in their cellular compositions. In a study of blood profiling of Kenyan children with mild malaria, there was a correlation of gene transcription and absolute neutrophil count (14). Thus, cell-specific transcriptomes would further define the host response on a cell phenotype level and could help determine the role of different white cell subsets in pathogenesis. Finally, through a comparison to an uninfected blood transcriptome as a baseline control, we can confirm that gene sets associated with mild malaria represent an upregulation during mild infection rather than a downregulation during severe disease.

Despite these limitations, we observed significant differences between our paired severe/mild transcriptomes. Type I IFN-related pathways and T cell biology were associated with mild malaria infection. The type I interferon data support some of the previously reported studies from animal models, and transcripts associated with severe disease replicate prior severe disease associations. To our knowledge, this is the first study comparing the transcriptional profiles of host peripheral blood cells during severe and subsequent mild disease and provides new paradigms of host response that can be explored to impact severe disease outcomes in malaria.

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