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TLR9 Polymorphisms Are Associated with Altered IFN-γ Levels in Children with Cerebral Malaria

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Abstract. Toll-like receptor (TLR) polymorphisms have been associated with disease severity in malaria infection, but mechanisms for this association have not been characterized. The TLR2, 4, and 9 single nucleotide polymorphism (SNP) frequencies and serum interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) levels were assessed in Ugandan children with cerebral malaria (CM, $N = 65$) and uncomplicated malaria (UM, $N = 52$). The TLR9 C allele at −1237 and G allele at 1174 were strongly linked, and among children with CM, those with the C allele at −1237 or the G allele at 1174 had higher levels of IFN-γ than those without these alleles ($P = 0.03$ and 0.008, respectively). The TLR9 SNPs were not associated with altered IFN-γ levels in children with UM or altered TNF-α levels in either group. We present the first human data that TLR SNPs are associated with altered cytokine production in parasitic infection.

INTRODUCTION

Cerebral malaria (CM) affects over half a million African children per year and has a case fatality rate of 15% to 40%.[1,2] Cerebral malaria is defined as coma in the presence of *Plasmodium falciparum* parasitemia, with no other cause of coma identified.[3] For children who survive, 10–17% may suffer from neurological sequelae such as epilepsy, cerebral palsy, cortical blindness, and deafness,[2] and up to 21% may suffer cognitive impairment.[4]

Excessive serum levels of pro-inflammatory cytokines, particularly interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), have been implicated in the pathogenesis of CM in animal and human studies.[5–12] Human studies have shown an association between CM and elevated IFN-γ[13] or TNF-α[6,8,14,15] levels, and our previous studies documented an association between elevated IFN-γ levels and mortality in CM.[13] In addition, some human studies[16] and several animal studies[5,12,17] have provided pathological evidence that one or both cytokines are involved in CM pathogenesis.

Toll-like receptors (TLRs) are mammalian pattern recognition receptors that are found primarily in monocytes, macrophages, and dendritic cells. These antigen-presenting cells recognize and signal in response to microbial ligands that are bound by the TLRs. As a result, the innate immune system is triggered to produce specific cytokines to contain or eliminate the microbial infection. *In vitro* evidence suggests that TLR2, TLR4, and TLR9 recognize and signal in response to *P. falciparum* ligands. Murine studies suggest that TLR2, and to a lesser extent, TLR4 recognition of *P. falciparum* glycophosphatidylinositol, are associated with increased production of TNF-α,[18] and that TLR9 signals in response to hemozoin, a by-product of host hemoglobin digestion by malaria parasites, and upregulates TNF-α, IL-12p40, monocyte chemoattractant protein (MCP)-1, and IL-6 production by dendritic cells.[19] Hemozoin acts as a carrier to facilitate entry of malarial DNA into the host cell, where the latter can bind to, and stimulate TLR9.[20] Human studies have shown that ligand in *P. falciparum* schizont extract stimulates human plasmacytoid dendritic cells to upregulate populations of γδ T cells, which increase IFN-γ production by a TLR9-dependent pathway.[21]

Toll-like receptor single nucleotide polymorphisms (SNPs) or corresponding amino acid substitutions have been associated with malaria manifestations and parasitemia in several recent studies.[22–25] We focused on two studies, because their results suggested an increase in clinical malaria severity and malaria-related complications in *P. falciparum*-infected patients in Africa, where we have two study sites. In the first study, TLR4 Asp299Gly was associated with an increased risk of maternal anemia, and TLR4 Asp299Gly and the C allele at TLR9 −1486 were associated with subsequent infant low-birth weight in pregnant Ghanaian women with malaria.[22] In the second, the risk of severe malaria in Ghanaian children was increased 1.5-fold and 2.6-fold with TLR4 Asp299Gly and TLR4 Thr399Ile, respectively.[23] Another study by Campino and others,[24] based in The Gambia and Malawi, studied the effect of TLR9 genetic variation on severe malaria. The TLR9 SNPs included all 3 SNPs investigated in this study, plus the G2848A SNP on exon 2 of the TLR9 gene. Although there was "no convincing association" between TLR9 SNPs and malaria severity in that study, the authors concluded that "*TLR9* expression is potentially modulated through *cis*-regulatory variants, which may lead to differential inflammatory responses to infection between individuals."

These studies provided intriguing new information about TLR SNPs and malaria disease severity, but did not further investigate the potential mechanisms by which TLR SNPs may mediate pathogenesis.

In this study, we hypothesized that TLR SNPs affect malaria severity through inappropriate TLR signaling, causing downstream elevations in pro-inflammatory cytokine levels that in turn result in more severe manifestations of malaria. To test the relationship between TLR SNPs, cytokine production, and disease severity, TLR2, 4, and 9 SNP frequencies and serum levels of IFN-γ and TNF-α, were compared in Ugandan children 3–12 years of age with CM and uncomplicated malaria (UM).

MATERIALS AND METHODS

Study population and recruitment. The study was conducted at our study site at the Mulago Hospital, in Kampala, Uganda,

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from November 2003 to July 2004. This time period spanned one major rainy season (March through May) and one minor rainy season (November to December.) Children 3–12 years of age were recruited as part of two studies assessing the complications of CM. Eighty-six children with CM and 76 children with UM were enrolled. Children with CM were enrolled if they were admitted to Mulago Hospital and met the World Health Organization (WHO) criteria for CM: coma (Blantyre coma scale ≤ 2 or Glasgow coma scale ≤ 8), *P. falciparum* on blood smear, and no other cause for coma. Lumbar punctures were performed to rule out meningitis and encephalitis. Children with UM were enrolled from the hospital's acute care clinic or another outpatient malaria clinic at the hospital. Children were considered to have UM if they had signs and symptoms of malaria (fever, chills, vomiting, headache), *P. falciparum* infection on blood smear, and no evidence of malaria complications (e.g., seizures, respiratory distress, severe anemia, or coma) or other acute illness. Genetic testing was requested from study participants on study follow-up testing 2 years after enrollment. If consent was obtained from the study participant's parent or guardian, testing for TLR SNPs was performed on the filter paper samples collected at initial enrollment. Sixty-five children with CM and 52 children with UM consented to genetic studies.

Blood samples of 5 mL were obtained on enrollment. Serum was frozen at −70°C until testing was performed. Blood was collected on Whatman FTA filter paper (Whatman Corporation, Florham Park, NJ) for future DNA extraction and testing. Presence of *P. falciparum* was determined by light microscopy of thick and thin blood smears, with two independent readings, and a third independent reading if necessary to resolve any discrepancies between the two initial readings.

Written informed consent was obtained from the parents or guardians of study participants. Ethical approval for the study was granted by the Institutional Review Boards for Human Studies at Makerere University Faculty of Medicine, Case Western Reserve University, Indiana Wesleyan University, and the University of Minnesota.

DNA extraction. Genomic DNA was extracted from filter paper samples according to instructions from the QIAamp DNA mini kit (QIAGEN, Valencia, CA) We assessed for common TLR2, TLR4, and TLR9 SNPs (Table 1). These SNPs were selected because they have been associated with malaria severity in African populations[22,23] and with other infectious and autoimmune diseases in different populations.[26–32]

Polymerase chain reaction. Polymerase chain reaction (PCR) was performed using a master mix consisting of 1× PCR buffer, 125 μM dNTPs, 2.5 mM $MgCl_2$, 125 nM primers, and 0.8 units of *Taq* polymerase in a reaction volume of 25 μL. The PCR primers and amplification conditions are listed in Supplemental Table 1 (available online at www.ajtmh.org). The PCR products for SNP detection were analyzed on a 2% agarose gel before the ligase detection reaction-fluorescent microsphere assay (LDR-FMA).

Table 1

TLR2, TLR4, and TLR9 single nucleotide polymorphism (SNPs) genotyped

SNP	Location	Nucleotide change	GenBank accession no.
TLR2	4q32		
Pro631His	Exon 3	C → A	rs5743704
Arg753Gln	Exon 3	G → A	rs5743708
TLR4	9q32–33		
Asp299Gly	Exon 4	A → G	rs4986790
Thr399Ile	Exon 4	C → T	rs4986791
TLR9	3p21.3		
T1486C	5′ Promoter	T → C	rs187084
T1237C	5′ Promoter	T → C	rs5743836
G1174A	Intron 1	G → A	rs352139

Cloning and sequencing. The PCR amplification products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA). Purified PCR products were sent to MWG Biotech (High Point, NC) for sequencing. Sequences were analyzed using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI). Sequenced DNA templates were subjected to PCR and used in the LDR-FMA as positive controls.

SNP genotyping. Polymerase chain reaction products were analyzed in an LDR-FMA divided into three steps: 1) ligation of oligonucleotides to the SNP, 2) FlexMAP (Luminex Corp., Austin, TX) microsphere hybridization, and 3) detection using the Bioplex suspension array system, which includes a fluorescence reader and the Bio-Plex Manager analytical software (Bio-Rad Laboratories, Hercules, CA). This procedure is described in detail elsewhere.[33] Common/conserved and allele-specific probe sequences used in this assay are listed in Supplemental Supplemental Table 2 (available online at www.ajtmh.org). A multiplex assay was used for simultaneous detection of TLR2 and TLR9 SNPs. The TLR4 SNPs were evaluated in a separate multiplexed LDR. Equal volumes of each PCR product were mixed, and 1 μL was added to the LDR. Conditions for the LDR step of this assay are described elsewhere.[33] Mean fluorescence intensity (MFI) values were used to calculate the allelic ratio for each SNP by dividing the allele-specific MFI value by the sum of the MFI values for that SNP (allele A/A + B = A_n and allele B/A + B = B_n), where A and B are the 2 alleles of a SNP. To be homozygous for a particular allele, the allelic ratio must be > 0.75. To be heterozygous, the ratio of the two alleles must be between 0.25 and 0.75. Consequently, an allele included in a ratio of < 0.25 is considered not present. Normalized values for A_n and B_n were divided, and the quotient was log-transformed.

Cytokine testing. We compared serum IFN-γ and TNF-α levels in children with TLR SNP variants because these cytokines have been implicated in the development of CM in previous studies (see Introduction). Serum samples for cytokine measurement were obtained at the time of admission (CM), outpatient treatment (UM), or enrollment (CC). Levels of IFN-γ and TNF-α were also determined by microbead suspension array technology using the Bioplex-Luminex system (Austin, TX).[13] Results were interpolated from 5-parameter-fit standard curves generated with the relevant recombinant human proteins (R&D Systems, Minneapolis, MN). Samples were tested at a 1:8 dilution. Cytokine assay results in the full cohort have previously been published.[13]

Statistical analysis. Statistical analysis was performed with Stata 10 software (StataCorp, College Station, TX) and R version 2.8 (http://www.r-project.org). Genotype frequencies in children with CM and UM were compared by the χ^2 test or Fisher's exact test as appropriate. Cytokine levels across groups (common allele homozygotes, heterozygotes, rare allele homozygotes) were compared using a log-additive model (in R, linear model [lm] function). Linkage disequilibrium was assessed by Lewontin's D' and the correlation coefficient *r* statistics, calculated from estimates provided in Devlin and Risch[34] requiring estimation of the two loci haplotype. The χ^2 test was calculated from the statistic provided in Weir.[35]

TABLE 2
Patient demographic and clinical characteristics*

	CM (*N* = 65)	UM (*N* = 52)	*P*
Age, years: mean (SD)	6.2 (2.4)	7.5 (2.7)	0.007†
Sex: male (%)	63.1	42.3	0.02‡
Weight, kg: mean (SD)	19.3 (7.1)	21.6 (6.6)	0.08†
Pre-presentation illness/fever duration (days): mean (SD)	4.3 (3.0)	4.4 (5.0)	0.89†
Children with any antimalarial pretreatment (%)	72.3	65.4	0.29‡
With chloroquine	41.5	36.5	0.51‡
No antimalarials/not sure	27.7	34.6	
Parasite density/μL, median (interquartile range)	28,120 (147,400)	32,200 (92,840)	0.35†

* CM = cerebral malaria; UM = uncomplicated malaria.
† Wilcoxon ranksum test.
‡ χ^2 test.

The 2-locus haplotype for each person in the dataset and the posterior probability of that haplotype were calculated using the EM algorithm,[36] and associations between haplotype and cytokine levels assessed by the methods of Schaid and others.[37] To control the experiment-wise error rate, *P* values were adjusted by the Bonferroni correction for multiple comparisons. The primary analysis, comparison of IFN-γ levels according to the 3 TLR9 SNPs, is a single experiment. With this correction, at a false positive rate of 5%, *P* values of < 0.017 were considered statistically significant.

RESULTS

Study participant characteristics. Sixty-five children with CM and 52 with UM consented to genetic testing and had samples from enrollment available for SNP genotyping. Clinical and demographic characteristics of the study participants are outlined in Table 2. As compared with children with UM, children with CM were younger and more often male. Fifty-nine of 65 children with CM and 47 of 52 children with UM had adequate serum for cytokine testing.

TLR2, 4, and 9 SNP frequencies. The TLR2 SNPs showed no rare alleles (i.e., all children were CC for Pro631His and GG for Arg753Gln). Rare alleles of the TLR4 SNPs (Asp299Gly and Thr399Ile) occurred at a genotype frequency of < 13% in children with CM or UM (Table 3). In contrast, for the three TLR9 SNPs assessed, rare allele genotype frequencies (C at −1486, C at −1237, A at 1174) exceeded > 40% in children with CM or UM (Table 4). Overall, frequency of the C allele at −1237 did not differ between children with CM and children with UM, but children with CM were more frequently homozygotes for the C allele (CC) than children with UM (18.5% versus 5.8%, $P = 0.05$). In contrast, for the intronic A allele at 1174 and the C allele at −1486, neither allele nor homozygote frequency differed significantly between children with CM and those with UM (Table 4).

IFN-γ and TNF-α levels in children with CM according to TLR4 and TLR9 SNPs. The TLR4 Arg299Trp, which had a genotype frequency of 12.3% in children with CM, was the only TLR4 SNP variant with > 2% frequency in children with CM. There were no homozygotes for this variant, and there was no difference in IFN-γ or TNF-α levels in individuals with or without the variant (data not shown). The TLR9 SNP variants or rare alleles occurred in frequencies high enough to allow comparison of IFN-γ and TNF-α levels according to SNP variant. Children with CM and the rare C allele at −1237 had higher levels of IFN-γ than those without this allele, and children with CM and the common G allele at 1174 had higher levels of IFN-γ than those without this allele (Figure 1). For both the C allele at −1237 and the G allele at 1174, there was a "dose-response" relationship, with increasing IFN-γ levels in homozygotes as compared with heterozygotes (Figure 1). Fitting a log-additive model to test the association between each of these polymorphisms with the response yields significance levels of 0.03 for the C allele at −1237 and 0.008 for the G allele at 1174.

The C allele at −1237 was in linkage disequilibrium with the G allele at 1174 in children with CM (Lewontin's D' 0.885, correlation coefficient 0.60, $\chi^2 = 23.35$ $P < 0.0001$) or UM (Lewontin's D' 0.907, correlation coefficient 0.55, $\chi^2 = 16.08$ $P < 0.0001$). Genotype frequencies and IFN-γ levels for the various −1237 and 1174 genotype combinations in children who had IFN-γ measured are shown in Table 5. To assess if the −1237 and 1174 SNPs were independently associated with IFN-γ levels, the 2-locus haplotype was first estimated for each child. In children with CM, −1237/1174 haplotype frequencies were 0.019 (C/A), 0.381 (C/G), 0.389 (T/A), and 0.211 (T/G).

TABLE 3
TLR4 SNP genotype frequencies in children with CM vs. UM*

TLR4 SNP		CM (*N* = 65) No. (%)	UM (*N* = 52) No. (%)	*P*†
Asp299Gly (A → G)	AA	57 (87.7)	47 (90.4)	
	AG	8 (12.3)	5 (9.6)	
	GG	0 (0)	0 (0)	0.77
Thr399Ile (C → T)	CC	64 (98.5)	52 (100)	
	CT	1 (1.5)	0 (0)	
	TT	0 (0)	0 (0)	1.0

* SNP = single nucleotide polymorphism; CM = cerebral malaria; UM = uncomplicated malaria.
† Fisher's exact test, comparing proportions in the three groups.

TABLE 4
TLR9 SNP genotype frequencies in children with CM vs. UM*

TLR9 SNP		CM (*N* = 65) No. (%)	UM (*N* = 52) No. (%)	*P*†
T1486C	TT	34 (52.3)	28 (53.8)	
	TC	27 (41.5)	21 (40.4)	
	CC	4 (6.2)	3 (5.8)	1.0
T1237C	TT	25 (38.4)	24 (46.2)	
	TC	28 (43.1)	25 (48.1)	
	CC	12 (18.5)	3 (5.8)	0.12
G1174A	GG	24 (36.9)	16 (32.1)	
	GA	29 (44.6)	22 (42.3)	
	AA	12 (18.5)	14 (26.9)	0.52

* SNP = single nucleotide polymorphism; CM = cerebral malaria; UM = uncomplicated malaria.
† Fisher's exact test, comparing proportions in the three groups.

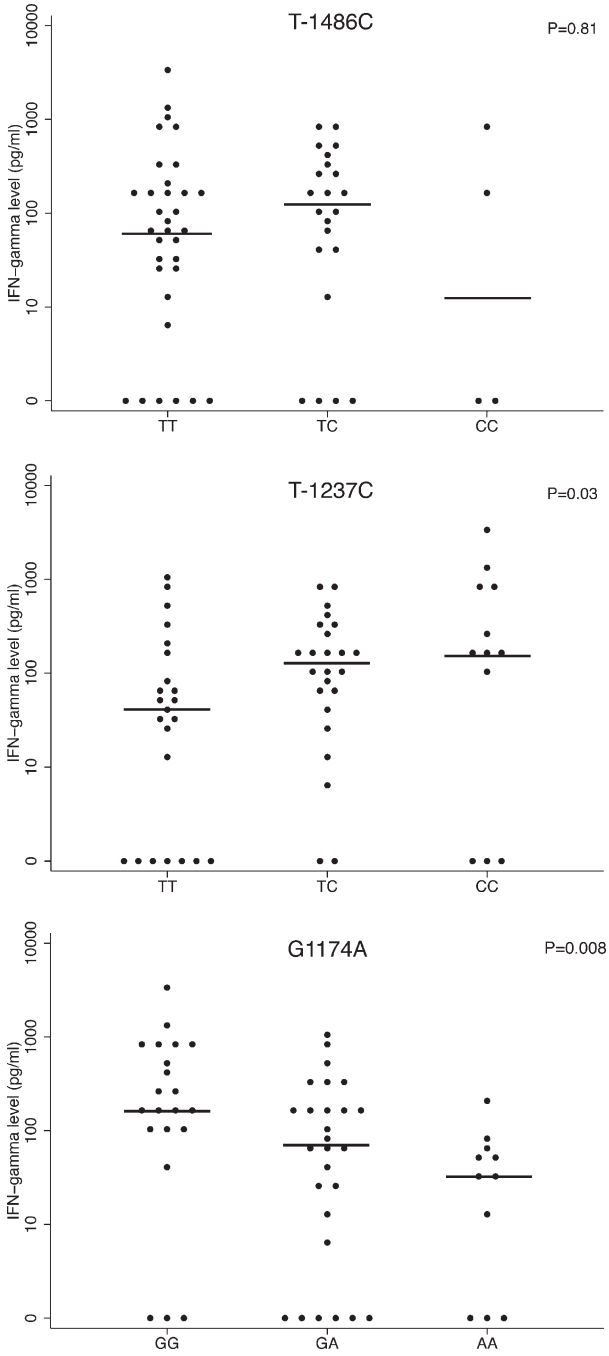

FIGURE 1. Interferon-γ (IFN-γ) levels by TLR9 single nucleotide polymorphism (SNP) in children with cerebral malaria (CM).

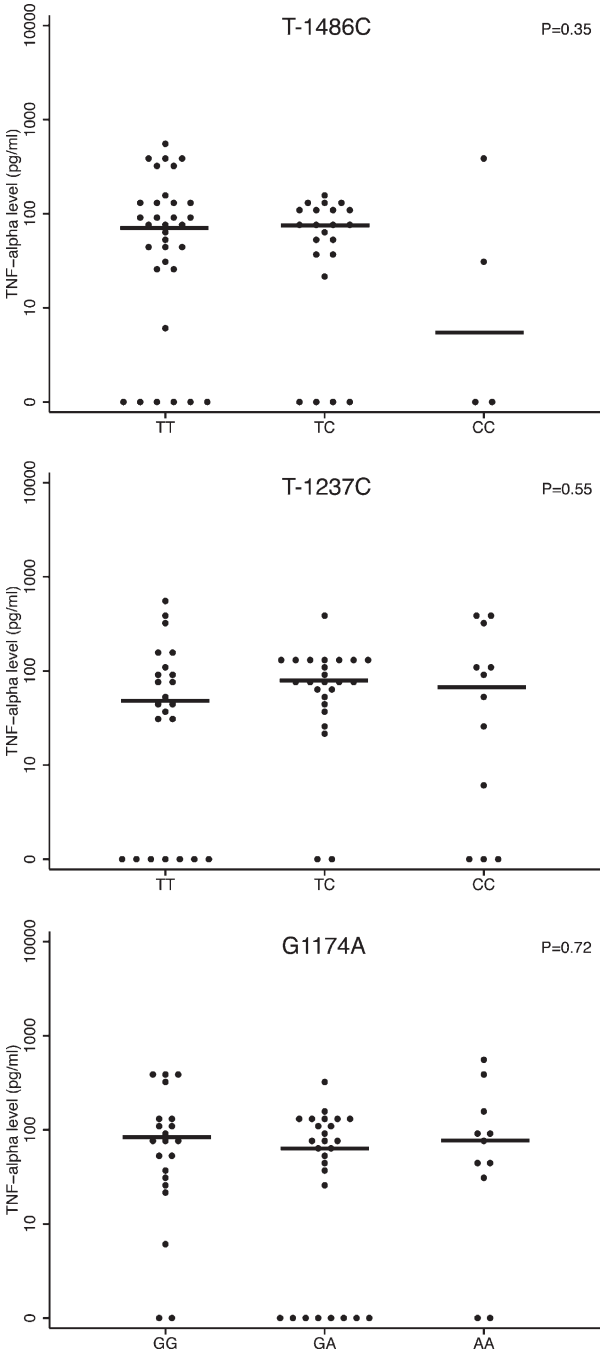

FIGURE 2. Tumor necrosis factor-α (TNF-α) levels by TLR9 single nucleotide polymorphism (SNP) in children with cerebral malaria (CM).

Using the method of Schaid and others,[37] significant differences were seen in IFN-γ levels between the four haplotypes ($\chi^2 = 8.34$, $P = 0.039$). Haplotype scores for specific –1237/1174 haplotypes were 2.56 for C/G ($P = 0.01$), –2.33 for T/A ($P = 0.02$), and 0.05 for T/G ($P = 0.96$). A C/A haplotype-specific score was not calculated because this haplotype was very infrequent. The IFN-γ levels did not differ according to the presence of the C allele at –1486 in children with CM (Figure 1). The TNF-α levels did not differ according to any TLR9 SNP genotype in children with CM (Figure 2).

IFN-γ and TNF-α levels in children with UM according to TLR4 and TLR9 SNP genotypes. The IFN-γ and TNF-α levels did not differ according to any TLR9 SNP genotype in children with UM (Figures 3 and 4). Less than 10% of children with UM had the TLR4 Asp299Gly variant, and there was no difference in IFN-γ ($P = 0.57$) or TNF-α ($P = 0.84$) levels in children with as compared with those without this variant.

DISCUSSION

Toll-like receptor polymorphisms have been associated with an altered risk of disease or disease severity in numerous infections,[26,28–30,32,38] including malaria.[22,23] However, potential causal mechanisms for the associations between TLR SNPs

TABLE 5

Multivariate analysis: cross-tabulation of TLR9 –1,237 and 1,174 genotypes and corresponding interferon-γ (IFN-γ) levels in children with cerebral malaria (CM)*

	–1237/1174 genotypes, *N*				Median IFN-γ levels (range) (pg/mL), for all possible –1237/1174 genotypes			
T					G1174A			
1		GG	GA	AA	**Total**	GG	GA	AA
2	TT	2	10	11	**23**	TT 80.5 (0–161)	52.5 (0–500.7)	32.8 (0–200.2)
3	TC	9	15	0	**24**	TC 285.5 (0–771.6)	92.8 (0–335.8)	–
7	CC	10	2	0	**12**	CC 223.3 (0–3369.6)	0 (0–0)	–
C	**Total**	**21**	**27**	**11**	**59**			

* IFN-γ levels shown on the right half of the table are median (range) values for individuals with that genotype (e.g., first row, second column, individuals with a 1174 GA and –1237 TT genotype have a median IFN-γ level of 52.5 pg/mL, with a range from 0 to 500.7 pg/mL). For genotypes where there are only two individuals (e.g., 1174 GG/–1237 TT), the median value given the value halfway between the two values for that genotype.

and disease severity in malaria have not been described. In this study, we documented that TLR9 SNP genotypes are associated with altered serum IFN-γ levels in children with CM. This study is the first to report TLR SNP-related alterations in cytokine levels in humans in response to a parasitic infection, and suggests a mechanism by which TLR SNPs may relate to disease severity in *P. falciparum* infection. Previous studies have documented elevated IFN-γ levels in children with severe as compared with uncomplicated malaria,[13,39] and an earlier report of the cohort of children described here[13] showed a further increase in IFN-γ levels in children with CM who die as compared with survivors. In a human study, it is difficult to assess definitively whether IFN-γ levels are part of pathogenesis of disease or an epiphenomenon, but several studies have implicated a causal role for IFN-γ in a mouse model of CM pathogenesis.[7,10,17,40,41] If elevated IFN-γ levels contribute to the pathogenesis of human CM, then children with the C allele at TLR9 –1237 or the G allele at TLR9 1174 may be more likely to develop CM because these alleles are associated with increased IFN-γ levels in severe *P. falciparum* infection. The lack of association between these TLR9 SNPs and altered IFN-γ levels in children with UM suggests that an additional factor may be required to alter IFN-γ levels in infected children.

Hemozoin is a potential co-factor that could functionally affect TLR9 response to *P. falciparum* infection. The TLR9 is located in the endosomal/lysosomal compartment of dendritic and other cell types, and typically recognizes nucleic acids common in microbes, particularly CpG DNA.[42,43] *Plasmodium falciparum*, although possessing an AT-rich genome,[44] has potentially stimulatory CpG motifs and may require a carrier to transport its DNA to the endosomal compartment. A recent study by Parroche and others[20] suggested that hemozoin is not a primary ligand for TLR9 but functions as a carrier for *P. falciparum* DNA. Greater levels of hemozoin may for this reason lead to increased TLR9 activation. Several studies have documented that hemozoin load is higher in children with severe malaria as compared with uncomplicated malaria.[45–47] A difference in hemozoin level may have allowed greater endosomal transport of parasite DNA and TLR9 activation in children with CM relative to those with UM. Unfortunately, microscope slides were not placed in long-term storage so we were not able to assess hemozoin load and test this hypothesis. Future studies will assess the interactions between TLR9 SNPs, hemozoin load, and IFN-γ levels.

Evidence from transfected animal and human cells shows that TLR SNPs can affect signaling and ultimately, cytokine production in response to infection.[26,38,48,49] These exaggerated responses may involve several mechanisms, including changes in ligand binding sites on the receptor, thereby affecting ligand affinity and strength of stimulation, changes in transcription factor binding sites on the *TLR* promoter,[50] or qualitative/quantitative changes in the TLR protein. In regard to TLR9 SNP genotypes, the C allele at –1237, in the promoter region, has been shown to affect promoter activity,[51] most likely by modifying a potential binding site for the transcription factor NF-κB.[50] NF-κB is a complex of proteins that remains in cells in an inactive state and is rapidly activated by a series of cascade events after ligands are bound to TLRs. Qualitative or quantitative changes in NF-κB activation may in turn lead to altered transcription regulation of inflammatory cytokine genes, which could lead to alterations in the production of cytokines such as IFN-γ. The 1174 SNP is located in an intron in the TLR9 gene. Though located in a non-coding region, variants in such an SNP could affect signaling by creating alternative splicing sites and consequently, affecting the protein product. In this study, the rare C allele at –1237 and the common G allele at 1174 were associated with increasing IFN-γ levels, and haplotype analysis suggested that both genotypes contributed to the differences in IFN-γ levels. TLR9 may also affect signaling to dendritic cells and thus affect activation of T regulatory cells (Tregs), as has been recently demonstrated in a murine model.[52] A recent study showed that individuals infected with malaria have upregulation of TLR-9 and increased IFN-γ, and that TLR-9 knockout mice have significantly reduced levels of IFN-γ in response to *Plasmodium chabaudi* infection as compared with wild-type mice,[53] supporting the importance of TLR-9 in IFN-γ production in malaria infection. Further studies are required to determine the specific effects of these SNP genotypes on TLR9 signaling.

This study measured serum levels of IFN-γ rather than IFN-γ produced by parasite-stimulated or antigen-stimulated mononuclear cells. Serum levels may be considered non-specific because it cannot be determined if documented IFN-γ levels are seen solely in response to *P. falciparum*. The design of this study supports the contention that the levels of IFN-γ are largely *P. falciparum* specific. First, IFN-γ levels in children with CM were measured on admission and 72 hours after antimalarial therapy was initiated. As previously reported, IFN-γ levels in children with CM decreased after treatment with quinine to those of healthy community children (median level 0 pg/mL).[13] This finding showed that serum IFN-γ levels in children with CM did not appear to be elevated for reasons other than *P. falciparum* infection: once *P. falciparum* infection was treated, serum IFN-γ returned to very low or undetectable levels. Second, IFN-γ levels in children with CM were compared with those in children with UM and age-matched healthy community controls; the levels were higher in children with CM

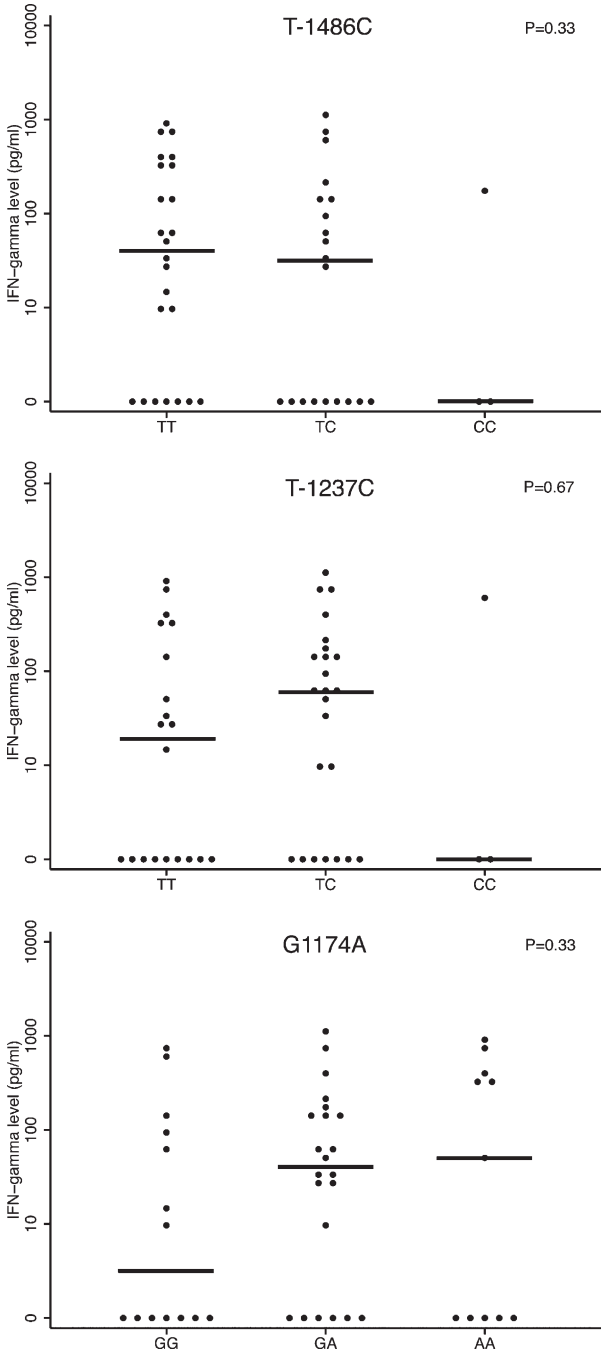

FIGURE 3. Interferon-γ (IFN-γ) levels by TLR9 single nucleotide polymorphism (SNP) in children with uncomplicated malaria (UM).

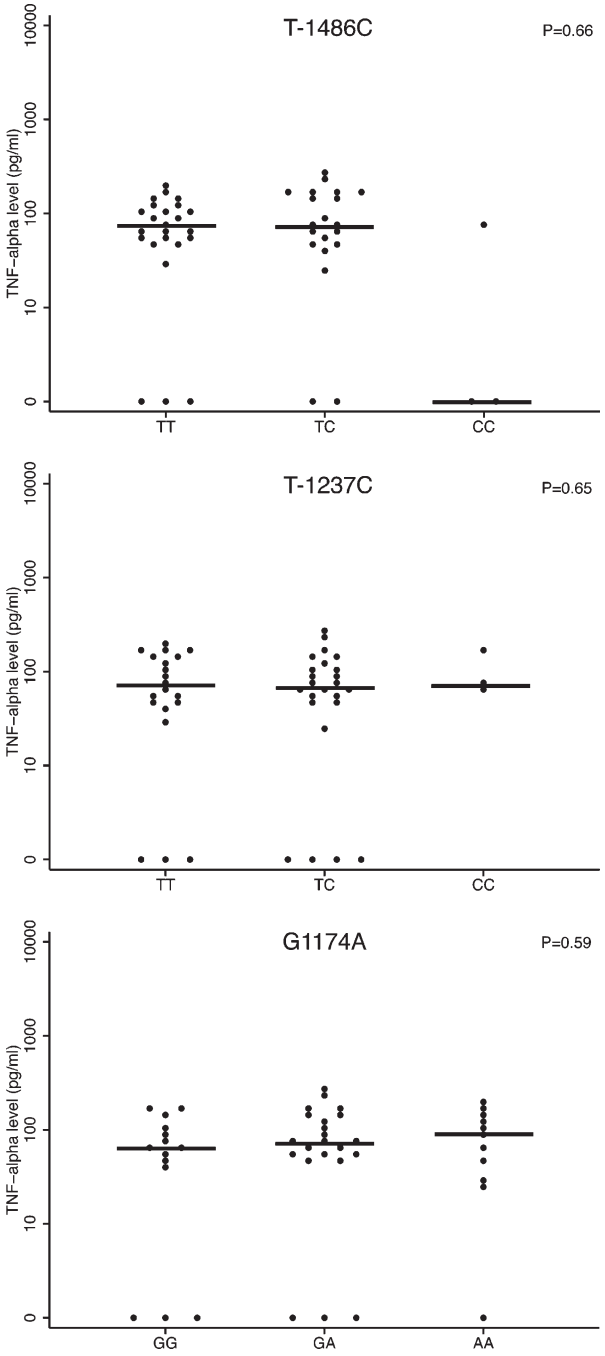

FIGURE 4. Tumor necrosis factor-α (TNF-α) levels by TLR9 single nucleotide polymorphism (SNP) in children with uncomplicated malaria (UM). Cytokine levels in Figures 1–4 were compared across the three groups using a log-additive model (see Methods). The lines shown represent the median cytokine level for each group.

than both of the latter groups, consistent with the notion that IFN-γ levels were specifically elevated in the context of CM. Third, serum IFN-γ levels, though related to severity of disease, were not related to length of the primary disease symptoms of CM (coma, fever) or UM (fever) (John CC, unpublished data). Taken together, these findings document that in children in this community, serum IFN-γ levels are very low in healthy children, are elevated with increasing severity of malaria but not with duration of symptoms, and decrease to low or undetectable levels after anti-malarial treatment of CM. Because the stimulation of TLR signaling may rely on multiple factors, including the type of cell stimulated, hemozoin level, and parasite strain (if there is variance in CpG DNA abundance between strains), a precise *in vitro* model that mimics *in vivo* disease may be difficult to construct. This study may therefore provide a more accurate reflection of the *in vivo* process than an *in vitro* cell culture-based model.

There was no relationship between TLR9 SNPs and TNF-α levels in children with CM or UM. Although TNF-α has been associated with disease severity in other studies of children with CM,[6,8,14,15] TNF-α levels in children with CM and UM were

similar in this study cohort.[13] The lack of association between TNF-α level and disease severity in this cohort may explain the lack of association of TNF-α levels with specific TLR9 SNPs. Alternatively, TNF-α levels may be associated with SNPs not yet described and not assessed in the current study. The sample size of this study could detect only large differences in TLR9 SNP allele frequency between children with CM versus UM, though the difference in homozygote frequency for the C allele at –1237 approached statistical significance. In addition, because the study assessed only CM and UM, we could not assess whether these TLR9 SNPs were associated with altered IFN-γ levels in other forms of severe malaria such as severe malarial anemia. Future studies will evaluate the frequency of TLR9 SNP alleles in a larger cohort, including individuals with severe malarial anemia, and examine whether hemozoin levels correlate with differences in IFN-γ production in children with severe versus uncomplicated malaria.

In conclusion, we presented the first human data to show that TLR SNPs are associated with altered cytokine responses in parasitic infection, and specifically documented a relationship between TLR9 SNPs and serum levels of the pro-inflammatory cytokine IFN-γ in children with CM. If confirmed by other studies, these findings may partially explain the increased risk of CM in some children and could lead to assessment of specific adjunct therapies (e.g., TLR9 antagonists). More broadly, these findings support further study of the role played by TLR SNPs in human immune responses to other infections.

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Note: Supplemental tables can be found at www.ajtmh.org.

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