MAJOR ARTICLE

Variants in the Toll-Like Receptor Signaling Pathway and Clinical Outcomes of Malaria

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Background. Malaria is one of the most significant infectious diseases in the world and is responsible for a large proportion of infant deaths. Toll-like receptors (TLRs), key components of innate immunity, are central to countering infection. Variants in the TLR-signaling pathway are associated with susceptibility to infectious diseases.

Methods. We genotyped single nucleotide polymorphisms (SNPs) of the genes associated with the TLR-signaling pathway in patients with mild malaria and individuals with asymptomatic *Plasmodium* infections by means of polymerase chain reaction.

Results. Genotype distributions for the *TLR-1 I602S* differed significantly between patients with mild malaria and persons with asymptomatic infection. The *TLR-1 602S* allele was associated with an odds ratio (OR) of 2.2 ($P = .003$; $P_{corrected} = .015$) for malaria among patients with mild malaria due to any *Plasmodium* species and 2.1 ($P = .015$; $P_{corrected} = .75$) among patients with mild malaria due to *Plasmodium falciparum* only. The *TLR-6 S249P* SNP showed an excess of homozygotes for the *TLR-6 249P* allele in asymptomatic persons, compared with patients with mild malaria due to any *Plasmodium* species (OR 2.1; 95% confidence interval [CI], 1.1–4.2; $P = .01$; $P_{corrected} = .05$), suggesting that the *TLR-6 249S* allele may be a risk factor for malaria (OR, 2.0; 95% CI, 1.1–3.7; $P = 0.01$; $P_{corrected} = .05$). The *TLR-9 -1486C* allele showed a strong association with high parasitemia ($P < .001$).

Conclusions. Our findings indicate that the *TLR-1* and *TLR-6* variants are significantly associated with mild malaria, whereas the *TLR-9 -1486C/T* variants are associated with high parasitemia. These discoveries may bring additional understanding to the pathogenesis of malaria.

Malaria, a vector-borne disease, is caused by protozoan parasites of the *Plasmodium* genus, which are transmitted by *Anopheles* mosquitoes. *Plasmodium falciparum* malaria affects 300–500 million people annually and is the leading cause of death among young children in sub-Saharan Africa. It is estimated that 1 million children die from malaria each year [1], and nearly 2.5 billion and 2.6 billion people, respectively, are possibly at risk for acquisition of *P. falciparum* and *Plasmodium vivax* [2].

P. falciparum-infected individuals show a wide spectrum of clinical manifestations, which range from asymptomatic infection to severe life-threatening forms, such as hyperparasitemia, hypoglycemia, cerebral malaria, respiratory distress, and vital organ dysfunction. Elevated levels of circulating tumor necrosis factor–α (TNF-α), interleukin-6 (IL-6), IL-12, IL-1β, and IL-10 seemed to correlate with the severity of the disease and with fatal outcomes [3]. Autopsy studies of human brain tissue from patients with cerebral malaria showed increased expression of TNF-α and IL-1β [4–6].

The host-pathogen interaction has several layers of complexity, including the genetic make-up of the host, the genotype of the parasite, the malaria vector *Anopheles,* and an environment favorable for the development of the disease. However, not all individuals infected by *Plasmodium* organisms develop severe and complicated disease. Only a very small subset (1%–2%) of *Plasmodium*-infected individuals progress to the life-

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threatening forms of malaria [1]. This has led to the pertinent question of why only a very small subset of *Plasmodium*-infected individuals develop severe and complicated symptoms, with others remaining asymptomatic or developing mild malaria. The development of severe and complicated disease in only 1%–2% of *P. falciparum*–infected children is probably a reflex of differential susceptibility. Indeed, numerous studies reported that host genetic characteristics are strong determinants in the differential susceptibility to human infection [7]. Furthermore, interindividual variations in the expression of cytokines appear to be under the influence of genetic factors, and individuals may be classified as having a high or low inflammatory response [8, 9]. Better control of *Plasmodium* infection by the host implies the presence of a finely balanced immune response sufficient to contain the parasite but tightly controlled to avoid damage to the host.

Severe malaria is characterized by marked changes in cytokine expression that result from the individual's immune response to infection. A robust innate immune response in a susceptible host either to *Plasmodium* organisms or to the metabolites released from ruptured red blood cells may lead to production of high levels of proinflammatory cytokines, which in turn triggers the major malarial symptoms, such as high fever [10]. Innate immunity is the first line of host defense in response to invading pathogens. During the past decade, Toll-like receptors (TLRs), a new family of pathogen-recognition receptors, have been identified [11]. TLRs recognize invading pathogens through distinct pathogen-associated molecular patterns and play a key role in the innate immunity of the host. In humans, 10 functional TLRs are described. On recognition of their respective ligands, TLRs trigger the production of proinflammatory cytokines through nuclear factor-κB–dependent or interferon regulatory factor–dependent signaling pathways. The intracellular signaling of TLRs is mediated by at least 5 adaptor proteins, MyD88, MAL/TIRAP, TRIF, TICAM, and SARM [12]. TLR1, 2, 4, 5, 6, and 10 are found on the extracellular surface of cells, whereas TLR3, 7, 8, and 9, each of which is a nucleic-acid sensor, are located within the endoplasmic reticulum and cytoplasmic vesicles, such as endosomes. TLR2 functions as a heterodimer with TLR1 or TLR6 and recognizes a wide variety of ligands, one of which is *P. falciparum* glycosylphosphatidylinositol (GPI) [13]. *P. falciparum* GPI is also one of the ligands recognized by TLR4 [13]. TLR9, a receptor for double-stranded DNA (CpG DNA), is activated by *Plasmodium* DNA [14]. Mice immunized with synthesized GPI and challenged with *Plasmodium berghi* are, in contrast to nonimmunized mice challenged with *P. berghi,* protected against malaria-related acidosis and pulmonary edema, suggesting that GPI may contribute to malaria mortality and pathogenesis [15]. In vitro GPI influences the expression of adhesion molecules and the proinflammatory cytokines IL-1 and TNF-α [16], all of which are known to cause severe malaria [17, 18].

MyD88 is the central mediator of TLRs and IL-1 signaling. In mice deficient for MyD88 and infected with *P. berghi,* a decrease in IL-12 production with no sign of liver damage is observed, contrasting with findings for wild-type mice [19]. Several single-nucleotide polymorphisms (SNPs) in the TLR family influence the inflammatory pathways and are associated with susceptibility to infection [20, 21]. Two variants in the TLR4 gene, *TLR-4 D299G* (rs4986790) and *TLR-4 T399I* (rs4986791), have been reported [22]. The *TLR-4 D299G* variant is associated with susceptibility to bacterial infection [23]. The *TLR-2 R753Q* variant (rs5743708) has been suggested to be a predisposing factor for staphylococcal infection [24]. The promoter polymorphisms of *TLR-9, TLR-9 -1237C/T* (rs5743836), is cited to confer susceptibility to asthma [25], whereas *TLR-9 -1486C/T* (rs1870884) is associated with placental malaria [26]. The *TLR-1 I602S* variant is associated with susceptibility to inflammatory bowel disease confined to the ileum [27] and with protection against leprosy [28]. *TLR-6 S249P* (rs5743810) is associated with asthma [29]. The adaptor protein MAL encoded by *TIRAP* on chromosome 11q24.2 contained a variant, *TIRAP S180L* (rs8177374), that is associated with protection against several infectious diseases, including malaria [30]. Of note, *TLR-1* and *TLR-6* are situated on chromosome 4p14, *TLR-2* on chromosome 4q31.3, *TLR-4* on chromosome 9q32-q33, and *TLR-9* on chromosome 3p21.3.

Here, we investigated whether SNPs in TLR genes and the adaptor protein gene *MAL/TIRAP* may be associated with clinical outcomes of malaria in *Plasmodium*-infected individuals, because *Plasmodium* organisms display a variety of ligands for TLRs. To this end, we conducted a case-control study to compare patients who had mild malaria with patients who had asymptomatic *Plasmodium* infection. We report that *TLR-1 I602S* and *TLR-6 S249P* are associated with mild malaria, whereas the promoter SNPs of *TLR-9* are associated with high levels of *Plasmodium* parasitemia.

SUBJECTS, MATERIALS, AND METHODS

Study area and population. The study population was from 3 areas of endemicity in Amazonian region of Brazil and consisted of 304 unrelated *Plasmodium*-infected individuals. A total of 265 persons were from Peixoto de Azevedo, Matto Grosso; 23 were from the community of the Machado River, a tributary of the Madeira River 180 km downstream from Porto Velho, Rondonian [31]; and 16 were from the community of the Padauiri River, a left-margin affluent of the Negro River in the Amazon basin [32]. The study population was predominately Amerindian, with a few individuals who were white or black. The climates of the 3 areas are similar, with a rainy season from October through April and a dry season from May through September. The temperature ranges from 15°C–38°C, with a relative humidity of >90%. The study was approved by the Ethical Committee of the Hospital das Clinicas da Faculdade de Medicina da Uni-

Table 1. Primers and polymerase chain reaction (PCR) conditions for the different polymorphisms studied.

Primer sequence, 5'–3'	PCR protocol	Restriction enzyme	Allele; length in bp
TLR-1 (I620S)	95°C for 5 min, 35 × (95°C for 30 s, 55°C for 30 s, 72°C for 30 s), 72°C for 7 min	*Alu*I	. . .
Forward: GGAAAGTTATAGAGGAACCCT	. . .	. . .	*TLR-1 602S;* 129 + 151
Reverse: CTTCACCCAGAAAGAATCGTGCC	. . .	. . .	*TLR-1 602I;* 280
TLR-4 D299G	95°C for 5 min, 35 × (95°C for 30s, 62°C for 30 s, 72°C for 30 s), 72°C for 7 min	*Nco*I	. . .
Forward: GATTAGCATACTTAGACTACTACCTCCATG	. . .	. . .	*TLR-4 229G;* 30 + 219
Reverse: GATCAACTTCTGAAAAAGCATTCCCAC	. . .	. . .	*TLR-4 299D;* 249
TLR-6 S249P	95°C for 5 min, 35 × (95°C for 30 s, 63°C for 30 s, 72°C for 30 s), 72°C for 7 min	*Ava*II	. . .
Forward: GCATTTCCAAGTCGTTTCTATGT	. . .	. . .	*TLR-6 249P;* 50 + 160210
Reverse: GCAAAAACCCTTCACCTTGTT	. . .	. . .	*TLR-6 249S;*
TLR-9 -1237C/T	95°C for 5 min, 35 × (95°C for 30 s, 59°C for 30 s, 72°C for 30 s), 72°C for 7 min	*Bst*NI	. . .
Forward: CTGCTTGCAGTTGACTGTGT	. . .	. . .	*TLR-9 -1237C;* 27 + 48 + 60
Reverse: ATGGGAGCAGAGACATAATGGA	. . .	. . .	*TLR-9 -1237T;* 27 + 108
TLR-9 -1486C/T	95°C for 5 min, 35 × (95°C for 30 s, 62°C for 30 s, 72°C for 30 s), 72°C for 7 min	*Afl*II	. . .
Forward: TATCGTCTTATTCCCCTGCTGGAATGT	. . .	. . .	*TLR-9 -1486T;* 34 + 111
Reverse: TGCCCAGAGCTGACTGCTGG	. . .	. . .	*TLR-9 -1486C;* 145
TIRAP S180L	95°C for 5 min, 35 × (95°C for 30 s, 62°C for 30 s, 72°C for 30 s), 72°C for 7 min	*Hpy188*I	. . .
Forward: TGCTCATCACGCCGGGCTTCCTT	. . .	. . .	*TIRAP 180S;* 21 + 106
Reverse: TAGGCAGCTCTGCTGAGGTCC	. . .	. . .	*TIRAP 180L;* 127

versidade de São Paulo, and all study participants provided informed consent.

Blood was collected from all participants at the time of enrollment, and *Plasmodium* infection was confirmed on the basis of positive results of polymerase chain reaction (PCR) analysis or a thick blood smear film, performed as described below. *Plasmodium*-infected individuals who presented with malaise, fever, or muscular pain and headache were considered symptomatic and classified as having mild malaria, whereas infected persons without symptoms for 60 consecutive days were classified as asymptomatic.

Thick blood smear films. For each individual, thick blood films were prepared and stained with Giemsa for microscopic identification of *Plasmodium* parasites. Well-trained microscopists examined 200 fields of Giemsa-stained thick blood smears under immersion oil (original magnification, ×1000). The density of parasites was determined by counting the number of asexual forms per 200 leukocytes, assuming a leukocyte number of 6000 cells/mL.

PCR analysis. Genomic DNA was isolated from blood cells collected in EDTA tubes by the phenol-chloroform method. Detection of *Plasmodium* species was performed as described elsewhere [33, 34]. Briefly, nested PCR is performed with genus-specific primers for the target 18S rRNA gene, followed by a second round of PCR with primer-specific for the target species (i.e., *P. falciparum*, *P. vivax*, and *Plasmodium malariae*).

SNP genotyping. The nonsynonymous SNPs *TLR-1 T1805G (I602S)*, *TLR-4 896A/G (D229G)*, *TLR-6 745C/T (S249P)*, and *TIRAP 975C/T (S180L)*, as well as the 2 promoter SNPs of *TLR-9* at positions *-1237C/T* and *-1486C/T*, were studied. The different SNPs were identified by PCR-restriction fragment length polymorphism analysis. The primers and PCR cycling conditions are shown in table 1. The PCR reaction for each SNP was 1 μL of genomic DNA (50 ng) added to 24 μL of amplification mix containing 2 U of Taq polymerase in buffer that contained 100 mmol/L Tris-HCl (pH 8.3) and 500 mmol/L KCL, 1.5 mmol/L $MgCl_2$, 40 mmol/L dNTPs, and 0.25 pmol/L each of forward and reverse primer. The PCR was performed under their respective PCR cycling conditions for 35 cycles. A total of 10 μL of PCR product was digested with 5 U of respective restriction endonuclease from New England Biolabs in a final volume of 20 μL that contained 2 μL of 10× enzyme buffer according to the manu-

Table 2. Baseline characteristics of persons infected with *Plasmodium* species.

Characteristic	Overall ($n = 304$)	Mild malaria ($n = 230$)	Asymptomatic infection ($n = 74$)	P
Age, median, years	30	29	35.5	<.001
Sex				
Male	251	197 (86)	54 (73)	.02
Female	53	33 (14)	20 (27)	.02
Infecting *Plasmodium* species				
P. falciparum	147	124 (54)	23 (31)	.001
P. vivax	53	27 (12)	26 (35)	<.001
P. malariae	1	0 (0)	1 (1)	
P. falciparum + *P. vivax*	82	62 (27)	20 (27)	.88
P. falciparum + *P. malariae*	2	2 (1)	0 (0)	
P. vivax + *P. malariae*	1	1 (0.5)	0 (0)	
P. falciparum + *P. vivax* + *P. malariae*	18	14 (6)	4 (5)	

NOTE. Data are no. (%) of subjects, unless otherwise indicated.

facturer instructions. The resulting fragments were separated by electrophoresis in either a 3% agarose gel or a 12% polyacrylamide gel and were visualized under UV light by staining with ethidium bromide. To ensure the validity of our genotyping methods, a known genotype for each SNP was used as a control.

Statistical analysis. Statistical analysis was performed using Prism software, version 4.0 (GraphPad). Allele and genotype frequencies were calculated by direct counting. Associations between disease groups and a specific allele, as well as between disease groups and genotypes, were analyzed using the χ^2 test. In addition, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. The Hardy-Weinberg expectation (HWE) was determined by comparing the observed number of different genotypes with those expected under the HWE for the estimated allele frequency. Statistical comparison also was performed using the Fisher exact test whenever a variable in the contingency table was <5. Bonferroni corrections were calculated by multiplying the P values by the number of gene studied.

RESULTS

The baseline characteristics of the 304 *Plasmodium*-infected individuals are shown in table 2. A total of 230 individuals had mild malaria symptoms, and 74 were asymptomatic. The median age was 29 years for patients with mild malaria and 35.5 years for patients with asymptomatic infection ($P < .001$). A total of 202 patients with mild malaria were infected with *P. falciparum,* compared with 47 individuals with asymptomatic infection. However, *P. falciparum* was the only *Plasmodium* species detected in 124 patients with mild malaria and 23 patients with asymptomatic infection. For clarity of comparison between individuals with mild malaria and those with asymptomatic infection, participants were stratified as having infection due to *P. falciparum* infection only or as having infection due to any *Plasmodium* species.

Overall, we investigated 298 *Plasmodium*-infected individuals for the *TLR-1 I602S* SNP, 304 for the *TLR-4 D229G* SNP, 290 for the *TLR-6 S249P* SNP, 290 for the *TLR-9 -1237C/T* SNP, 298 for the *TLR-9 -1486C/T* SNP, and 302 for the *TIRAP S180L* SNP; multiple genotypes were missing because of a lack of DNA. All of the SNPs were in HWE in the asymptomatic group, whereas the *TLR-1 I602S* and *TIRAP S180L* SNPs deviated slightly and significantly, respectively, in patients with mild malaria. To ensure that there was no error in genotyping for both polymorphisms, typing was repeated with the inclusion of control DNA with a known genotype for both SNPs. No discordance was observed. The genotype distributions and allele frequencies for all SNPs are shown in table 3. To determine whether there was any difference in the genotype distribution for each SNP, comparison of the patients with mild malaria group with those in the asymptomatic group was performed initially for persons with *P. falciparum* infection and then for persons infected with any *Plasmodium* species. Only the *TLR-1 I602S* genotype distribution was significantly different between the patients with mild malaria and those with asymptomatic infection (persons with any *Plasmodium* infection: 2×3 $\chi^2 = 9.6$ and $P = .008$; persons with *P. falciparum* infection: 2×3 $\chi^2 = 6.9$ and $P = .032$). In the asymptomatic group, homozygosity for the *TLR-1 602I* allele was predominant, regardless of the infecting *Plasmodium* species (I/I vs. I/S + S/S: OR, 2.6; 95% CI, 1.4–4.9; 2×2 $\chi^2 = 9.5$; $P = .001$; P corrected for the number of genes studied [$P_{corrected}$] = .005). A similar trend was also observed in the group infected with *P. falciparum* (OR, 2.6; 95% CI, 1.2– 5.6; 2×2 $\chi^2 = 6.6$; $P = .005$; $P_{corrected} = .025$). Heterozygotes were more prevalent among patients with mild malaria than among patients with asymptomatic infection, both among individ-

Table 3. Frequencies of genotypes and alleles of polymorphisms studied in subjects with mild malaria or asymptomatic *Plasmodium* infection, by infecting *Plasmodium* species.

Protein, genotype or allele	*P. falciparum* infection only, no. (%) of subjects				Any *Plasmodium* infection, no. (%) of subjects			
	Mild malaria	Asymptomatic	OR (95% CI)	*P* ($P_{corrected}$)	Mild malaria	Asymptomatic	OR (95% CI)	*P* ($P_{corrected}$)
TLR1								
Genotype								
I/I	114 (0.58)	36 (0.78)	2.6 (1.2–5.6)	.005 (.025)[a]	134 (0.59)	58 (0.80)	2.6 (1.4–4.9)	.001 (.005)[a]
I/S	79 (0.40)	9 (0.20)	2.8 (1.3–6.1)	.004 (.02)[b]	87 (0.39)	14 (0.19)	2.7 (1.4–5.1)	.001 (.005)[b]
S/S	4 (0.02)	1 (0.02)	. . .		4 (0.02)	1 (0.01)	. . .	
Allele								
I	307 (0.78)	81 (0.88)	2.1 (1.1–4.1)	.015 (.75)	355 (0.79)	130 (0.89)	2.2 (1.2–3.8)	.003 (.015)
S	87 (0.22)	11 (0.12)	. . .		95 (0.21)	16 (0.11)	. . .	
TLR4								
Genotype								
D299D	184 (0.91)	40 (0.87)	. . .		211 (0.92)	67 (0.91)	. . .	
D299G	16 (0.08)	6 (0.13)	. . .		17 (0.07)	7 (0.09)	. . .	
G299G	2 (0.01)	0 (0.00)	. . .		2 (0.01)	0 (0.00)	. . .	
Allele								
D	384 (0.95)	86 (0.93)	. . .		439 (0.95)	141 (0.95)	. . .	
G	20 (0.05)	6 (0.07)	. . .		21 (0.05)	7 (0.05)	. . .	
TLR6								
Genotype								
P249P	130 (0.68)	35 (0.78)	. . .	NS[c]	148 (0.67)	58 (0.82)	2.1 (1.1–4.2)	.01 (.05)[c]
P249S	55 (0.28)	9 (0.20)	. . .	NS[d]	63 (0.29)	12 (0.17)	2.2 (1.0–4.1)	.02 (.10)[d]
S249S	7 (0.04)	1 (0.02)	. . .		8 (0.04)	1 (0.01)	. . .	
Allele								
249P	315 (0.82)	79 (0.88)	1.6 (0.8–3.1)	.10 (.5)	359 (0.82)	128 (0.90)	2.0 (1.1–3.7)	.01 (.05)
249S	69 (0.18)	11 (0.12)	. . .		79 (0.18)	14 (0.10)	. . .	
TLR9								
Genotype								
1237T/T	140 (0.71)	32 (0.70)	. . .		159 (0.73)	56 (0.79)	. . .	
1237T/C	50 (0.25)	12 (0.26)	. . .		52 (0.24)	13 (0.18)	. . .	
1237C/C	8 (0.04)	2 (0.04)	. . .		8 (0.04)	2 (0.03)	. . .	
Allele								
1237T	330 (0.83)	76 (0.83)	. . .		370 (0.84)	125 (0.88)	. . .	
1237C	66 (0.17)	16 (0.17)	. . .		68 (0.16)	17 (0.12)	. . .	
TLR9								
Genotype								
1486T/T	70 (0.35)	18 (0.40)	. . .		82 (0.36)	23 (0.32)	. . .	
1486T/C	98 (0.49)	23 (0.51)	. . .		107 (0.47)	37 (0.52)	. . .	
1486C/C	31 (0.16)	4 (0.09)	. . .		38 (0.17)	11 (0.16)	. . .	
Allele								
1486T	238 (0.60)	59 (0.66)	. . .		271 (0.60)	83 (0.58)	. . .	
1486C	160 (0.40)	31 (0.34)	. . .		183 (0.40)	59 (0.42)	. . .	
TIRAP								
Genotype								
S180S	156 (0.78)	32 (0.69)	. . .		177 (0.78)	49 (0.68)	. . .	
S180L	34 (0.17)	10 (0.22)	. . .		40 (0.18)	18 (0.25)	. . .	
L180L	10 (0.05)	4 (0.09)	. . .		11 (0.05	5 (0.07)	. . .	
Allele								
180S	346 (0.87)	74 (0.80)	. . .		394 (0.86)	116 (0.81)	1.5 (0.94–2.5)	.043 (.25)
180L	54 (0.13)	18 (0.20)	. . .		62 (0.14)	28 (0.19)	. . .	

NOTE. Data are no. (%) of subjects. The χ^2 test was used to determine differences between genotype and allele frequencies. CI, confidence interval; NS, not significant; OR, odds ratio; TLR, Toll-like receptor.

[a] I/I vs. I/S + S/S.
[b] I/I vs. I/S.
[c] P/P vs. P/S + S/S.
[d] P/P vs. P/S.

Table 4. Genotype and allele frequencies for the *TLR-9 -1237C/T* and *TLR-9 -1486C/T* polymorphisms in subjects with malaria, by parasite load.

Polymorphism, genotype or allele	Parasitemia load, no. (%) of subjects		OR (95% CI)	χ^2	*P*
	High	Low			
TLR-9 -1237C/T					
Genotype					
TT	26 (0.57)	136 (0.76)	2.4 (1.2–4.7)	6.54	.005[a]
TC	17 (0.37)	38 (0.21)	2.3 (1.2–4.8)	5.7	.008[b]
CC	3 (0.06)	6 (0.03)	. . .	. . .	
Allele					
T	69 (0.75)	310 (0.86)	. . .	. . .	
C	23 (0.25)	50 (0.14)	2.1 (1.2–3.6)	6.7	.005
TLR-9 -1486C/T					
Genotype					
CC	9 (0.19)	73 (0.41)	4.2 (1.6–11.1)	9.4	.001[c]
CT	26 (0.54)	81 (0.45)	2.6 (1.1–5.9)	5.5	.009[d]
TT	13 (0.27)	25 (0.14)	3.0 (1.3–6.5)	7.96	.002[e]
Allele					
C	44 (0.46)	227 (0.63)	2.0 (1.3–3.2)	9.7	<.001
T	52 (0.54)	131 (0.37)	. . .	. . .	

NOTE. The χ^2 test was used to determine differences between genotype and allele frequencies. CI, confidence interval; OR, odds ratio.

[a] TT vs. TC + CC.
[b] TC vs. TT.
[c] CC vs. TT.
[d] CT vs. TT.
[e] CC + CT vs. TT.

ual infected with any *Plasmodium* species (39% vs. 19%; OR, 2.7; 95% CI, 1.4–5.1; 2×2 $\chi^2 = 9.5$; $P = .001$; $P_{corrected} = .005$) and among individuals infected with *P. falciparum* (40% vs. 20%; OR, 2.7; 95% CI, 1.3–6.1; 2×2 $\chi^2 = 6.9$; $P = .004$; $P_{corrected} = .020$). The frequency of homozygosity for the *TLR-1 602S* allele was similar in both groups. Overall, the *TLR-1 602S* allele seemed to be a risk factor for the development of malaria among persons infected with any *Plasmodium* species (OR, 2.2; $P = .003$; $P_{corrected} = .015$) and persons infected with *P. falciparum* (OR, 2.1; $P = .015$; $P_{corrected} = .75$).

Comparison of the *TLR-6 S249P* allele between patients with mild malaria and those with asymptomatic infection showed that the percentage of individuals homozygous for the *TLR-6 249P* allele was greater in the asymptomatic group. A significant difference was revealed only when the comparison was restricted to persons infected with any *Plasmodium* species (P/P vs. P/S + S/S: OR, 2.1; 95% CI, 1.1–4.2; 2×2 $\chi^2 = 5.2$; $P = .01$; $P_{corrected} = .05$).

We then examined whether the SNP genotypes were associated with age and parasitemia. Patients with mild malaria were stratified as having a high parasite load ($\geq$10,000 parasites/μL of blood) or a low parasite load ($<$10,000 parasites/μL of blood). Only the *TLR-9* promoter polymorphisms showed a difference in the distribution of genotypes between both parasitemia groups (table 4; data are shown only for *TLR-9 -1237C/T* and *TLR-9 -1486C/T*). An association between the homozygous *TLR-9 -1237T* allele and a low parasite load was observed (TT vs. T/C + CC: 2×2 $\chi^2 = 6.5$; $P = .005$). Similar findings were observed for the *TLR-9 T-1486C* polymorphism (TT vs. C/T + CC: 2×2 $\chi^2 = 7.9$; $P = .001$), with the *TLR-9 -1486T* allele associated with a low parasite load and the *TLR-9 -1486C* allele strongly associated with a high parasite load ($\chi^2 = 9.7$; $P < .001$). No significant association was found with age for any of the SNPs studied. Data are shown only for TLR9 and TLR1 in table 5.

DISCUSSION

Of all diseases, malaria has the greatest effect on infant mortality, and to date there is no efficient vaccine against this disease. It is far from understood why only some *Plasmodium*-infected infants die and others remain asymptomatic or develop uncomplicated disease. However, the fact that only a small subset of *Plasmodium*-infected individuals develop life-threatening complications strongly suggests that the genetic make-up of the host plays a fundamental role, in addition to the environment and the parasite itself. The identification of these genes may pave the way for a better understanding of the disease and for designing an efficient vaccine. Increasing evidence suggests that the TLR fam-

Table 5. Age and parasitemia status among subjects with mild malaria, by Toll-like receptor 1 (TLR1) and TLR9 genotype.

Variable	Age, median (IQR), years	*P*
TLR-1 I602S		.05
I/I	29.0 (23.0–36.2)	.90[a]
I/S	29.0 (23.0–36.0)	
S/S	43.5 (37.0–58.5)	
Parasitemia status		
TLR9 -1237C/T		
High		.892
TT	23.0 (19.0–29)	
CT	24.0 (21.3–26.0)	
CC	26 (23.0-26.0)	
Low		.696
TT	30.0 (23.3–37.0)	
CT	30.0 (25.0–36.0)	
CC	33.5 (29.0–37.0)	
TLR-9 -1486C/T		
High		.575
TT	27.0 (21.3–34.8)	
TC	23.0 (19.0–28.0)	
CC	26.0 (19.0–30.3)	
Low		.779
TT	32.0 (23.0–36.3)	
TC	29.5 (25.0–39.5)	
CC	30.0 (24.5–37.0)	

NOTE. *P* values were calculated by means of the Kruskall-Wallis test, unless otherwise indicated. IQR, interquartile range.

[a] I/I vs. I/S, by the Mann-Whitney test.

ily plays an important role in innate immunity and in bridging innate and adaptive immunity [11]. TLRs orchestrate innate immune responses on recognition of invading pathogens, through the induction of chemokines and inflammatory cytokines. Several genetic variations in the TLR-signaling pathway are associated with either susceptibility or resistance to several infectious diseases [20, 21].

P. falciparum GPI is recognized by TLR2/TLR1, TLR2/TLR6, and TLR4 [13] and induces severe malaria symptoms in mice [15]. In this study, we looked for SNPs in genes encoding TLR1, 4, 6, and 9 because these TLRs are more likely to mediate recognition of invading *Plasmodium* organisms. We also looked for a SNP in TIRAP, which is a common adaptor protein for TLR2 and TLR4. We are, to our knowledge, the first to show an association between mild malaria and variants in TLR1 and TLR6. Furthermore, we observed that TLR9 promoter polymorphisms are associated with high parasitemia.

Functional studies of the *TLR-1 I602S* variant suggested that the *TLR-1 602S* allele is associated with aberrant trafficking of the receptor to the cell surface, lower levels of TNF-α [28], and impaired basal and lipopeptide-induced nuclear factor-κB signaling, compared with the 602I allele [35]. TLR2 is the major receptor for GPI and functions as a heterodimer either with TLR1 or TLR6 to attribute ligand specificity. We did not search for the *TLR-2 R753Q* polymorphism, as its frequency is very low in the Brazilian population (Ramasawmy et al., unpublished data). In leprosy caused by the intracellular bacteria *Mycobacterium leprae*, it has been reported that signaling through TLR2/TLR1 may be detrimental to the host, as the *TLR-1 602S* allele is associated with a decreased incidence of leprosy [28]. Our data suggest that the *TLR-1 602S* allele is a risk factor for malaria. A possible explanation of the difference between our findings and data from studies of leprosy (a chronic infectious disease) is that, in malaria (an acute infectious disease), it is probably necessary that the host keeps *Plasmodium* organisms in check and eliminates them as fast as possible, to avoid multiplication and migration of the organisms to the liver. We also observed borderline association between mild malaria and the *TLR-6 S249P* variant. The function of this polymorphism is not known. However, it is interesting that both TLR1 and TLR6 function as heterodimers with TLR2. It can be assumed that a defect in these receptors may impair the recognition of GPI, which in turn may lead to inefficient clearance of the parasite.

TLR1, 6, and 10 are believed to have evolved under strong purifying selection [36], and it is interesting to observe that the frequency of the *TLR-1 602S* allele differs worldwide, with prevalences of 75% among white individuals, 26% among persons of African descent, and 0% among individuals of East Asian descent [28]; the frequency is 10% in the Amazonian population, which is mainly an admixture of Amerindians, white individuals, and persons of African descent. As Johnson et al. [28] pointed out, it is probably the case that the purifying allele for *TLR-1 I602S* may be restricted by either additional genetic or environmental factors with regard to specific pathogens, because one study reported that African Americans are twice more likely than white counterparts with a similar environmental and social status to develop tuberculosis [37].

TLR-4 D229G did not reveal any association with mild malaria in our study. Recently, it has been shown that *TLR-4 D299G* is associated with severe malaria in African children [38]. The absence of an association in the present study does not exclude *TLR-4* as an important gene in malaria involvement. Our study lacks the power to detect an association, as the frequency of the minor allele *TLR-4 299G* is very low (5%) and because malaria in our patients is not complicated by other illnesses.

The promoter polymorphisms of *TLR-9* did not show any association with mild malaria, which is in line with findings of a recent study of severe malaria [38]. However, we observed an association of *TLR-9 -1486T/C* with high parasitemia. Recently, it was reported that *TLR-9 -1486T/C* is associated with placental malaria characterized by excessive accumulation of hemozoin [26]. High expression of TNF-α has been observed in hemozoin-laden macrophages from the placenta of malaria-infected

women [39]. However, the mechanisms by which TLR9 may contribute to the pathogenesis of malaria remain to be demonstrated. Of interest, mice deficient for the gene encoding TLR9 are partially resistant to *Plasmodium yoelii,* and has been suggested that malaria parasites may require TLR9 to activate regulatory T cells for immune escape [40]. Although the function of this SNP is not clear, it may be in linkage disequilibrium with an as yet unidentified causal allele, which might influence the level of transcription.

The adaptor protein MAL encoded by *TIRAP* is involved in the downward signaling of TLR2 and TLR4. Recently, it was observed that bearers of *TIRAP S180L* variants in the heterozygous state are protected from infectious diseases and that this variant attenuates TLR2 signaling [30]. We did not observe a difference in the genotype distribution of this variant between patients with mild malaria and those who were asymptomatic. However, heterozygotes were more common in the asymptomatic group, compared with the mild malaria group (25% vs. 18%), but the difference was not statistically significant. This finding suggests that asymptomatic individuals may recognize the parasites efficiently in light of the *TLR-1* and *TLR-6* polymorphisms and modulate their immune response appropriately with reference to the *TIRAP* variant.

Altogether, our data indicate that *TLR-9* promoter polymorphisms may, to some extent, control the level of *Plasmodium* parasitemia, whereas TLR1 deficiency seems to predispose to mild malaria. These genetic findings may contribute to the understanding of the pathogenesis of malaria and warrant further investigation in other populations in order to validate the genetic contribution of these loci to the pathogenesis of malaria.

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