MAJOR ARTICLE

Heterozygosity for the S180L Variant of *MAL/TIRAP*, a Gene Expressing an Adaptor Protein in the Toll-Like Receptor Pathway, Is Associated with Lower Risk of Developing Chronic Chagas Cardiomyopathy

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Background. Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*. Among *T. cruzi*–infected individuals, only a subgroup develops severe chronic Chagas cardiomyopathy (CCC); the majority remain asymptomatic. *T. cruzi* displays numerous ligands for the Toll-like receptors (TLRs), which are an important component of innate immunity that lead to the transcription of proinflammatory cytokines by nuclear factor–κB. Because proinflammatory cytokines play an important role in CCC, we hypothesized that single-nucleotide polymorphisms (SNPs) in the genes that encode proteins in the TLR pathway could explain differential susceptibility to CCC among *T. cruzi*–infected individuals.

Methods. For 169 patients with CCC and 76 *T. cruzi*–infected, asymptomatic individuals, we analyzed SNPs by use of polymerase chain reaction–restriction fragment length polymorphism analysis for the genes *TLR1, TLR2, TLR4, TLR5, TLR9,* and *MAL/TIRAP,* which encodes an adaptor protein.

Results. Heterozygous carriers of the *MAL/TIRAP* variant S180L were more prevalent in the asymptomatic group (24 [32%] of 76 subjects) than in the CCC group (21 [12%] of 169) ($\chi^2 = 12.6$; $P = .0004$ [adjusted P (P_c) $= .0084$]; odds ratio [OR], 0.31 [95% confidence interval {CI}, 0.16–0.60]). Subgroup analysis showed a stronger association when asymptomatic patients were compared with patients who had severe CCC (i.e., patients with left-ventricular ejection fraction ⩽40%) ($\chi^2 = 11.3$; $P = .0008$ [$P_c = .017$]; OR, 0.22 [95% CI, 0.09–0.56]) than when asymptomatic patients were compared with patients who had mild CCC (i.e., patients with left-ventricular ejection fraction >40%) ($\chi^2 = 7.7$; $P = .005$ [$P_c = .11$]; OR, 0.33 [95% CI, 0.15–0.73]).

Conclusion. *T. cruzi*–infected individuals who are heterozygous for the *MAL/TIRAP* S180L variant that leads to a decrease in signal transduction upon ligation of TLR2 or TLR4 to their respective ligand may have a lower risk of developing CCC.

Chagas disease is caused by the intracellular protozoan parasite *Trypanosoma cruzi*. This parasite causes life-threatening chronic Chagas cardiomyopathy (CCC) in nearly one-third of *T. cruzi*–infected individuals. The remaining two-thirds of infected individuals are asymptomatic and free from heart disorders. Chagas disease remains a major threat to human health in Latin America despite efficient control of the transmitting vector, the reduviid bug. More than 18 million people are estimated to be chronically infected with *T. cruzi* and ~200,000 new cases are reported each year [1]. After acute infection, a strong immune response holds the parasite in check, but a low-grade parasitemia persists throughout the chronic phase.

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Although the underlying mechanisms that lead to the development of CCC remain poorly understood, inflammation is well documented in its pathogenesis. The survival time of patients with severe CCC is significantly shorter than that of patients with noninflammatory cardiomyopathy [2], which suggests that inflammation plays a significant pathogenic role. Our understanding of the innate immune system, the earliest host response to invading pathogens, has been enriched by the discovery of the Toll-like receptors (TLRs) [3]. TLRs, a family of pathogen-recognition receptors, play an important role in innate immunity by acting as sensors for invading pathogens through the recognition of distinct pathogen-associated molecular patterns. The intracellular signaling of TLRs is mediated by at least 5 adaptor proteins, including MyD88, MAL/TIRAP, TRIF, TICAM, and SARM [4]. The adaptor proteins link to the Toll–interleukin-1 (IL-1) receptor (TIR) domain of the TLR, which is located in the cytosol, and bind to downstream protein kinases that ultimately lead to the activation of transcription factors, such as nuclear factor–κB (NF-κB) and members of the interferon (IFN)–regulatory factor family, when TLRs sense pathogen-associated molecular patterns. The inducement of specific downstream signaling, such as promotion of the transcription of proinflammatory cytokines by NF-κB, provides the host with immediate first-line defense while also shaping the acquired immune response against invading pathogens.

In humans, 10 functional TLRs have been described. TLR1, TLR2–TLR6, and TLR10 are found on the extracellular surface of cells, whereas TLR3 and TLR7-TLR9 are located intracellularly within the endosomes. The TIR domain that contains the adaptor protein (TIRAP), also known as MAL, plays an essential role in signaling mediated by TLR1, TLR2, TLR4, and/or TLR6. MAL/TIRAP is the immediate downstream adaptor for TLR2 and TLR4 and acts as a bridging adaptor protein recruiting MyD88 to TLR2 and TLR4. MAL/TIRAP-deficient mice show impaired cytokine responses and NF-κB activation when stimulated with ligands for TLR2, TLR4, or TLR2 with its coreceptors TLR1 and TLR6 [5, 6].

T. cruzi contains a variety of ligands for several TLRs. *T. cruzi*–derived glycosylphosphatidylinositol (GPI) anchors activate the NF-κB pathway and induce proinflammatory cytokines in Chinese hamster cells transfected with *TLR2* genes [7]. Macrophages from TLR2-deficient mice show a low response to *T. cruzi*–derived GPI anchors, compared with macrophages from wild-type mice [7]. TLR2 functions as a heterodimer with TLR1 or TLR6. Macrophages from TLR6-deficient mice do not respond to *T. cruzi*–derived GPI anchors [8]. *T. cruzi*–derived glycoinositolphospholipids containing ceramide (GIPL-ceramide) triggered the production of chemokines in wild-type mice, but not in TLR4-deficient mice [9]. *T. cruzi*–derived protein Tc52 induced the synthesis of proinflammatory cytokines through TLR2 [10]. It has also been reported that TLR2 regulates interleukin-1β–dependent cardiomyocyte hypertrophy triggered by *T. cruzi,* which is an important pathogenic event [11]. The proinflammatory activity of *T. cruzi* DNA is mediated by TLR9 [12]. Experimental infection of mice deficient for TLR2, TLR4, TLR9, and MyD88 with *T. cruzi* showed an increased mortality rate and earlier death, compared with wild-type mice [13]

The studies mentioned above provide evidence that the TLR pathway plays an important role in determining mortality rate and parasitemia during the acute phase of experimentally induced *T. cruzi* infection. However, the role of the TLR in the pathogenesis of Chagas disease remains unexplored for chronic Chagas disease in humans. Although studies involving TLR-deficient mice showed a crucial role for TLR signaling in resistance to *T. cruzi* infection, mice deficient for a single TLR did not show either a marked increase in susceptibility or an increase in mortality rate, which suggests that several TLRs may act synergistically to provide resistance. Indeed, mice deficient for both TLR2 and TLR9 are more susceptible to *T. cruzi* infection than are mice deficient for a single TLR [12].

In humans, the fact that only a subset of *T. cruzi*–infected individuals develop CCC and the observation of familial aggregation of patients with CCC in CCC-endemic areas [14] suggest that host genetic factors may play a role in the underlying mechanisms of disease pathogenesis. Given the important role of the T helper 1–type inflammatory process in the pathogenesis of CCC heart lesions, it is likely that the quality and intensity of the innate response may impact disease progression. We thus hypothesized that common genetic variations in the *TLR* and *TIRAP* genes may confer differential susceptibility in *T. cruzi*–infected individuals, some of whom develop CCC and others of whom remain asymptomatic.

Several single-nucleotide polymorphisms (SNPs) in the *TLR* family influence the inflammatory pathways and are associated with susceptibility to infection [15]. Two variants in the *TLR4* gene, *TLR-4* D299G (rs4986790) and *TLR-4* T399I (rs4986791), are associated with resistance to Legionnaires disease [16]. Heterozygosity for the *TLR-2* R753Q variant (rs5743708) has been suggested to impair immune activation by *Borrelia burgdorferi* and to protect individuals from late-stage Lyme disease [17]. With respect to the promoter polymorphisms of *TLR9, TLR9* −1237C/T (rs5743836) is reported to confer susceptibility to asthma [18], whereas *TLR9* −1486C/T (rs1870884) is associated with placental malaria [19]. The *TLR1* variant I602S (rs5743618) is associated with susceptibility to inflammatory bowel disease confined to the ileum, mild malaria [20, 21], and protection against leprosy [22]. *TLR6* S249P (rs5743810) is associated with asthma [23]. The *TLR5* SNP transition of a cytosine to thymidine at base pair 1174 that changes an arginine at codon 392 to a stop codon (*TLR5* R392stopCodon) is associated with susceptibility to Legionnaires disease [24]. Recently, it was reported that SNPs of *MAL/TIRAP* are associated with infectious diseases and inflammatory bowel disease [25, 26]. It is worth noting that *TLR1* and *TLR6* are situated on chromosome 4p14, *TLR2* on

Table 1. Primers and polymerase chain reaction (PCR) protocols for the different polymorphisms studied in *Trypanosoma cruzi*–infected subjects.

Target, primer	Sequence (5'→3')	PCR protocol
TLR1 T1805G (I602S)		95°C for 5 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; followed by 72°C for 7 min
Forward	GGAAAGTTATAGAGGAACCCT	
Reverse	CTTCACCCAGAAAGAATCGTGCC	
TLR2 R753Q		95°C for 5 min; 35 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s; followed by 72°C for 7 min
Forward	GCCTACTGGGTGGAGAACCT	
Reverse	GGCCACTCCAGGTAGGTCTT	
TLR4 896A/G (D229G)		95°C for 5 min; 35 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s; followed by 72°C for 7 min
Forward	GATTAGCATACTTAGACTACTACCTCCATG	
Reverse	GATCAACTTCTGAAAAAGCATTCCCAC	
TLR5 R392stopCodon		95°C for 5 min; 35 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s; followed by 72°C for 7 min
Forward	GGTAGCCTACATTGATTTGC	
Reverse	GAGAATCTGGAGATGAGGTACCCG	
TLR9 −1237		95°C for 5 min; 35 cycles at 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s; followed by 72°C for 7 min
Forward	CTGCTTGCAGTTGACTGTGT	
Reverse	ATGGGAGCAGAGACATAATGGA	
TLR9 −1486		95°C for 5 min; 35 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s; followed by 72°C for 7 min
Forward	TATCGTCTTATTCCCCTGCTGGAATGT	
Reverse	TGCCCAGAGCTGACTGCTGG	
TIRAP 975C/T (S180L)		95°C for 5 min; 35 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s; followed by 72°C for 7 min
Forward	TGCTCATCACGCCGGGCTTCCTT	
Reverse	TAGGCAGCTCTGCTGAGGTCC	

chromosome 4q31.3, *TLR4* on chromosome 9q32–q33, *TLR5* on chromosome 1q41–1q42, *TLR9* on chromosome 3p21.3, and *MAL/TIRAP* on chromosome 11q24.2.

These studies suggest that the genes involved in the TLR pathway play an important role in the host defense during infection. In the present study, we investigated whether SNPs in *TLR1, TLR2, TLR4, TLR5, TLR9,* and*MAL/TIRAP,* which encodes an adaptor protein, may be associated with clinical outcomes in *T. cruzi*–infected individuals by conducting a case-control study. As control subjects, we used asymptomatic *T. cruzi*–infected individuals whose disease did not progress to CCC.

SUBJECTS, MATERIALS, AND METHODS

Subjects and study design. The 245 *T. cruzi*–infected individuals included in the present study were monitored at the Heart Institute (InCor) of the University of São Paulo School of Medicine. Subjects provided written informed consent in accordance with the guidelines of the hospital's internal review board. The criteria used to classify *T. cruzi*–infected individuals as patients with CCC or asymptomatic patients, as well as details about the subjects' characteristics, are provided elsewhere [27]. In brief, 169 *T. cruzi*–infected individuals were classified as patients with CCC, and the remaining 76 were classified as asymptomatic. Of the 169 patients with CCC, 93 (55%) were men (mean age [± standard deviation {SD}] , 51.4 ± 12.8 years), and 76 (45%) were women (mean age [± SD], 54.5 ± 10.3 years). Normal left ventricular function (left-ventricular ejection fraction [LVEF], >40%) was observed in 83 patients with CCC; 32 (39%) of these patients were men, and 51 (61%) were women. Severe ventricular dysfunction (LVEF, ⩽40%) was observed in 75 patients with CCC; 53 (71%) of these patients were men, and 22 (29%) were women. LVEF data were not available for the remaining 11 patients. Of the 76 asymptomatic subjects, 27 (36%) were men (mean age [±SD], 53.4 ± 7.87 years), and 49 (64%) were women (mean age [±SD], 56.7 ± 9.74 years).

SNP genotyping. We studied the following nonsynonymous SNPs: *TLR1* T1805G (I602S), *TLR2* R753Q, *TLR4* 896A/G (D229G), *TLR5* R392stopCodon, and *TIRAP* 975C/T (S180L). We also studied the 2 promoter SNPs of *TLR9* at positions T-1237C and T-1486C. The different SNPs were identified by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. The primers and PCR proto-

Table 2. Polymerase chain reaction products and restriction endonucleases (REs) used.

Gene, product	RE	Allele length, bp
TLR1	*Alu*I	
602S		129, 151
602I		280
TLR2	*Aci*I	
753Arg		227, 75, 38
753Glu		265, 75
TLR4	*Nco*I	
229G		30, 219
299D		249
TLR5	*Dde*I	
392R		277
392stop		91, 186
TLR9		
−1237	*Bst*NI	
C		27, 48, 60
T		27, 108
−1486	*Afl*II	
T		34, 111
C		145
TIRAP	*Hpy*188I	
180S		21, 106
180L		127

cols used are shown in table 1. The PCR reaction for each SNP involved 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), 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 assays were performed under their respective PCR cycling conditions for 35 cycles. Ten μL of PCR product was digested with 5 U of the appropriate restriction endonuclease (New England Biolabs) in a final volume of 20 μL that contained 2 μL of 10× enzyme buffer (table 2). The resulting fragments were separated by electrophoresis in either a 3% agarose gel or a 12% polyacrylamide gel. 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 (version 4.0; GraphPad). Allele and genotype frequencies were calculated by direct counting. Associations, both between disease groups and a specific allele and between disease groups and genotypes, were analyzed by use of the 2-tailed χ^2 test; odds ratios (ORs) and 95% confidence intervals (CIs) were also calculated. Hardy-Weinberg equilibrium (HWE) was determined by comparing the observed number of different genotypes with the expected number under the HWE for the estimated allele frequency. Bonferroni corrections were applied by multiplying the *P* values by the number of comparisons.

RESULTS

We investigated groups of *T. cruzi*–infected individuals for each of the variants; the number in each group was as follows: 196 subjects were evaluated for *TLR1* I602S, 240 subjects for *TLR2* N753G, 239 subjects for *TLR4* D229G, 241 subjects for *TLR5* 392stop codon, 215 subjects for *TLR9* T−1237C, 219 subjects for *TLR9* T−1486C, and 238 subjects for *TIRAP* S180L. The number in each group varies because there was not adequate DNA available to test all subjects for each genotype. Table 3 shows the frequency of the genotypes and alleles of the different SNP studied. All the SNPs were in HWE in both asymptomatic subjects and subjects with CCC, except for the *MAL/TIRAP* S180L variant, which deviated slightly from HWE in the CCC group ($P = .02$). Comparison of the asymptomatic and CCC groups with respect to genotype distribution for each SNP showed a statistically significant difference only for the *MAL/TIRAP* S180L variant that was assessed in 75 asymptomatic subjects and 163 subjects with CCC ($2 \times 3\ \chi^2 = 12.97$; $P = .0015$) (table 4).

The frequency of homozygosity for the *MAL/TIRAP* 180S allele was significantly higher among patients with CCC (137 [85%] of 162) than among asymptomatic patients (48 [64%] of 75) ($2 \times 2\ \chi^2 = 12.66$; $P = .0004$; OR, 3.1 [95% CI, 1.6–5.8]), whereas while the percentage of subjects homozygous for the *MAL/TIRAP* 180L allele was similar in both groups. The percentage of subjects heterozygous for *MAL/TIRAP* S180L among patients with CCC was 13% (21 of 162) compared with 32% (24 of 75) among asymptomatic patients, and this difference was highly significant ($2 \times 2\ \chi^2 = 12.62$; $P = .0004$; OR, 0.31 [95% CI, 0.16–0.60]). After adjustments for multiple testing (7 SNPs studied) and comparisons (3 genotypes studied), the difference was still significant ($P_c = .0084$ [$.0004 \times 7 \times 3$]). The differences between patients with CCC and asymptomatic patients with respect to allele frequencies were also statistically significant ($2 \times 2\ \chi^2 = 11.49$; $P = .0007$ [$P_c = .015$]; OR, 0.39 [95% CI, 0.23–0.68]), which suggests that the *TIRAP* 180L allele may be associated with a lower risk of developing CCC.

We stratified the patients with CCC according to their LVEF status to determine whether the *TIRAP* S180L variant had any influence on severity of disease. As shown in table 3, the percentage of patients with CCC who were homozygous for the *TIRAP* 180S allele was similar, regardless of whether their LVEF was ⩽40% (63 [88%] of 72) or >40% (67 [84%] of 80). Heterozygosity was slightly more common among patients with CCC who had LVEF >40% (11 [14%] of 80]) than among patients with LVEF ⩽40% (7 [10%] of 72), but this difference did not reach statistical significance. There was also no statistically significant difference between patients with CCC who had LVEF >40% and patients with CCC who had LVEF ⩽40% with respect to the allele frequencies of the *TIRAP* S180L variant, which suggests that it probably has no influence on severity of disease. Compar-

Table 3. Genotype and allele frequencies for the different single-nucleotide polymorphisms studied in *Trypanosoma cruzi*– infected subjects.

Gene or locus, position	Patients with CCC, no. (%)			Asymptomatic patients, no. (%)
	LVEF ⩽40%	LVEF >40%	All[a]	
TLR1, aa 602				
Genotype				
I602I	25 (40)	35 (52)	65 (48)	29 (48)
I602S	27 (44)	24 (36)	52 (39)	24 (39)
S602S	10 (16)	8 (12)	18 (13)	8 (13)
Allele				
602S	47 (38)	40 (30)	88 (33)	40 (33)
602I	77 (62)	94 (70)	182 (67)	82 (67)
TLR2, aa 735				
Genotype				
R753R	68 (97)	81 (98)	160 (98)	75 (99)
R753Q	2 (3)	2 (2)	4 (2)	1 (1)
TLR4, aa 299				
Genotype				
D299D	65 (93)	76 (93)	151 (93)	71 (93)
D299G	5 (7)	6 (7)	12 (7)	5 (7)
TLR5, 392				
Genotype				
R392R	62 (89)	77 (92)	150 (91)	69 (91)
R392StopCodon	8 (11)	7 (8)	15 (9)	6 (8)
StopCodonStopCodon	. . .	. . .	. . .	1 (1)
TLR9, nt −C1237T				
Genotype				
TT	35 (55)	41 (55)	78 (54)	30 (42)
CT	21 (34)	30 (40)	55 (38)	37 (52)
CC	7 (11)	4 (5)	11 (8)	4 (6)
Allele				
−1237C	35 (28)	38 (25)	77 (27)	45 (32)
−1237T	91 (72)	112 (75)	211 (73)	97 (68)
TLR9, nt −T1486C				
Genotype				
CC	5 (8)	6 (8)	11 (7)	7 (10)
CT	32 (51)	36 (48)	73 (49)	26 (37)
TT	26 (41)	33 (44)	64 (43)	38 (53)
Allele				
−1486T	84 (67)	102 (68)	201 (68)	102 (72)
−1486C	42 (33)	48 (32)	95 (32)	40 (28)
TIRAP, aa 180				
Genotype				
L180L	2 (3)	2 (2)	4 (3)	3 (4)
S180L	7 (10)	11 (14)	21 (12)	24 (32)
S180S	63 (88)	67 (84)	137 (85)	48 (64)
Allele				
180S	133 (92)	145 (91)	295 (91)	120 (80)
108L	11 (8)	15 (9)	29 (9)	30 (20)

NOTE. Patients were stratified according to whether they had chronic Chagas cardiomyopathy (CCC) or were asymptomatic. Patients with CCC were further stratified according to whether their left-ventricular ejection fraction (LVEF) was ⩽40% or >40%. The *n* value for each variant varies because there was not adequate DNA available to test all subjects for each genotype.

[a] LVEF data was not available for 11 patients with CCC.

Table 4. Statistical comparisons for *TIRAP* S180L in *Trypanosoma cruzi*–infected subjects.

	All patients with CCC vs. asymptomatic patients		Patients with LVEF ≤40% vs. asymptomatic patients		Patients with LVEF >40% vs. asymptomatic patients	
Comparison	P (P_c)[a]	OR (95% CI)	P (P_c)[a]	OR (95% CI)	P (P_c)[a]	OR (95% CI)
TIRAP S180S vs. S180L + L180L	.0004 (.0084)	3.1 (1.6–5.8)	.0009 (.019)	3.9 (1.7–9.1)	.005 (.11)	2.9 (1.4–6.2)
TIRAP S180L vs. S180S	.0004 (.0084)	0.31 (0.16–0.60)	.0008 (.017)	0.22 (0.09–0.56)	.005 (.11)	0.33 (0.15–0.73)
TIRAP allele 180L vs. 180S	.0007 (.015)	0.39 (0.23–0.68)	.002 (.042)	0.33 (0.16–0.69)	.008 (.17)	0.41 (0.21–0.80)

NOTE. CCC, chronic Chagas cardiomyopathy; CI, confidence interval; LVEF, left-ventricular ejection fraction; OR, odds ratio.

[a] P_c is the corrected P value after adjustments for multiple testing (number of single-nucleotide polymorphisms studied, 7) and comparisons (i.e., $P \times 7 \times 3$).

ison of asymptomatic patients and patients with CCC who had LVEF ≤40% (i.e., heterozygous subjects vs. homozygous subjects) showed a stronger association ($P = .0008$ [$P_c = .017$]; OR, 0.22 [95% CI, 0.09–0.56]) than did comparison of asymptomatic patients and patients with CCC who had LVEF >40% ($P = .005$ [$P_c = .11$]); OR, 0.33 [95% CI, 0.15–0.73]) (table 4).

DISCUSSION

Despite tremendous achievements in the understanding of Chagas disease, the molecular mechanism that underlies the development of CCC still remains obscure. Interest in the relationship between host genetics and the outcome of *T. cruzi* infection has only taken shape in the past 2 decades. Studies of the major histocompatibility complex class I and II molecules and susceptibility to Chagas disease have been controversial [28, 29]. Recently, we described several genetic variants associated with CCC [27, 30–32]. However, no single major locus for susceptibility to CCC has been identified, which indicates that there may be several genes involved in the pathogenesis of the disease. Indeed, susceptibility to infectious diseases seems to be highly polygenic.

Several studies have reported that common genetic variations in TLRs affect host susceptibility to infectious diseases [15]. In this study, we observed that in *T. cruzi*–infected individuals, heterozygosity for the *MAL/TIRAP* S180L variant was associated with a lower risk of developing CCC. The likelihood of developing CCC was 0.31-fold lower among *T. cruzi*–infected individuals heterozygous for the *MAL/TIRAP* S180L variant, compared with homozygous carriers of the wild-type *MAL/TIRAP* 180S allele.

There are several plausible ways by which the *MAL/TIRAP* S180L variant may explain the molecular mechanism that results in a lower risk of developing CCC among certain *T. cruzi*–infected individuals. Functional studies of *MAL/TIRAP* S180L have elegantly shown that the *TIRAP* variant is associated with a decrease in signal-transduction efficiency with impaired cytokine production [26]. Because the immunopathology of CCC is caused by proinflammatory cytokines after *T. cruzi* infection, individuals homozygous for the *MAL/TIRAP* wild-type allele (S180) might be expected to respond with high levels of proinflammatory cytokine expression, leading to tissue damage, whereas homozygosity for the mutated allele *MAL/TIRAP* 180L might be expected to lead to an immune response that is insufficient to control the infection, which in turn would result in poor clearance of the parasite. In contrast, heterozygous carriers may have an intermediate state of activation of the signaling cascade that is optimal for allowing the host to hold the parasite in check while controlling the inflammatory response, as suggested by Khor et al. [26]. Our results are in line with those in the recent report by Khor et al., which showed an association between heterozygosity for the *MAL/TIRAP* variant S180L and protection against the following 4 infectious diseases: invasive pneumococcal disease, bacteremia, malaria, and tuberculosis [26]; however, some conflicting results have been reported [33]. A recent study showed that *TIRAP* S180L polymorphism is commonly a protective factor against the development of tuberculosis and systemic lupus erythematosus [34].

Several *T. cruzi* molecules, such as GPI anchors, GIPL and GIPL-ceramide , Tc52, and *T. cruzi* DNA, are agonists of TLR2, TLR4, and TLR9. The inflammation elicited by TLRs needs to strike an appropriate balance, resulting in an inflammatory response suited to keeping the invading pathogens in check; otherwise, excess activation of the intracellular signaling pathways may lead to high levels of proinflammatory mediators, such as interferon-γ (IFN-γ), tumor necrosis factor–α (TNF-α), and reactive nitrogen intermediates, which can result in host tissue damage. Indeed, immunohistochemical analysis of heart tissue sample obtained during autopsies of patients with severe CCC showed the presence of TNF-α and IFN-γ [35, 36]; peripheral production of both TNF-α and IFN-γ have been reported to be higher in patients with CCC than in asymptomatic subjects [36, 37]. The persistence of parasites in patients with Chagas disease might also trigger ongoing inflammation. In patients with CCC, progressive inflammatory events may lead to mononuclear cell infiltration, myocarditis associated with fibrosis, and heart dysfunction [38]. Endomyocardial biopsy studies have shown that

patients with CCC who experienced heart failure were more likely to have severe myocarditis, fibrosis, and myocardial hypertrophy than were either patients who had CCC but did not experience heart failure or asymptomatic patients, which suggests a key role for myocardial inflammation [39]. All these observations support a potential role for *TIRAP* functional polymorphisms in the pathogenesis of CCC in susceptible *T. cruzi*–infected individuals. Hence, the progression to overt cardiomyopathy may result from the combined effect of high levels of proinflammatory cytokine expression triggered by parasite persistence and inadequate counterregulation of these cytokines.

Our results also indicated that common SNPs of *TLR1, TLR2, TLR4, TLR5,* and *TLR9* do not represent major risk factors for the development of CCC in *T. cruzi*–infected individuals. The functional and genetic data of the human SNPs in the extracellular domain of *TLR4* D299G and T399I, which are associated with susceptibility for other diseases [16, 17], apparently do not play a role in genetic susceptibility to CCC. The redundancy of TLR signaling by *T. cruzi* (i.e., *T. cruzi* TLR ligands, acting on TLR2–TLR6 and 9) could potentially explain why a loss-of-function mutation in a single TLR could be devoid of pathobiological consequences. Corroborating this, mice genetically deficient for both TLR2 and TLR9 had reduced control of *T. cruzi* infection, compared with mice deficient for only TLR2 or only TLR9 [12]. Furthermore, these same authors and others have demonstrated that mice genetically deficient for adaptor protein MyD88 show an increased mortality rate and earlier death [40]. Likewise, a loss-of-function polymorphism in 1 copy of the adaptor protein gene *MAL/TIRAP* may protect against progression to CCC, because this variant may be associated with a reduced *T. cruzi*–induced immune–inflammatory response.

The development of CCC in genetically susceptible *T. cruzi*–infected individuals takes 10–30 years to occur. In murine models, parasite burden during the acute phase correlates with persistence during the chronic stage [41]. Thus, it seems plausible that the complex interactions between the host and the parasite that lead to indefinite control of the parasite occur during the acute phase of infection. Compared with individuals homozygous for the wild-type allele, individuals heterozygous for the *TIRAP/MAL* variant are probably able to strike a proper balance between generating an inflammatory response to contain the parasite and counterregulating that response.

Our present findings suggest that genes involved in the TLR pathway may also be involved in the pathogenesis of CCC in *T. cruzi*–infected individuals. TLR1, TLR2, TLR4, and TLR6 interact with TIRAP for signaling. The association of the *TIRAP* S180L variant with protection against CCC among *T. cruzi*–infected individuals further provides evidence for an essential role of the TLR pathway in the pathogenesis of infectious diseases.

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