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ARTICLE

Polymorphisms in Toll-like receptor 4 (*TLR4*) are associated with protection against leprosy

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Abstract Accumulating evidence suggests that polymorphisms in Toll-like receptors (TLRs) influence the pathogenesis of mycobacterial infections, including leprosy, a disease whose manifestations depend on host immune responses. Polymorphisms in *TLR2* are associated with an increased risk of reversal reaction, but not susceptibility to leprosy itself. We examined whether polymorphisms in *TLR4* are associated with susceptibility to leprosy in a cohort of 441 Ethiopian leprosy patients and 197 healthy controls. We found that two single nucleotide polymorphisms (SNPs) in *TLR4* (896G>A [D299G] and 1196C>T [T399I]) were associated with a protective effect against the disease. The 896GG, GA and AA genotypes were found in 91.7, 7.8 and 0.5% of leprosy cases versus 79.9, 19.1 and 1.0% of controls, respectively (odds ratio [OR]=0.34, 95% confidence interval [CI] 0.20–0.57, $P<0.001$, additive model). Similarly, the 1196CC, CT and TT genotypes were found in 98.1, 1.9 and 0% of leprosy cases versus 91.8, 7.7 and 0.5% of controls, respectively (OR=0.16, 95% CI 0.06–.40, $P<0.001$, dominant model). We found that *Mycobacterium leprae* stimulation of monocytes partially inhibited their subsequent response to lipopolysaccharide (LPS) stimulation. Our data suggest that *TLR4* polymorphisms are associated with susceptibility to leprosy and that

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this effect may be mediated at the cellular level by the modulation of TLR4 signalling by *M. leprae*.

Introduction

Leprosy remains an important public health concern in developing countries, with an estimated 250,000 new patients each year [1]. Infection with *Mycobacterium leprae* is characterised by a polarised spectrum of clinical manifestations that correlate with the level and type of cell-mediated immunity [2, 3]. At one end of the spectrum, tuberculoid leprosy patients have localised lesions that contain few bacilli and a Th1 T-cell-mediated cellular immune response [3–5]. At the opposite end, lepromatous leprosy patients have disseminated infection with extensive lesions containing numerous intra-cellular bacilli and a Th2 T-cell-mediated cellular immune response. This wide range of clinical and immunological presentations makes leprosy a prototypical disease to understand how polymorphisms in innate immunity genes influence adaptive immune responses. Furthermore, insights into leprosy pathogenesis may help elucidate mechanisms of protective immunity to other mycobacteria such as *M. tuberculosis*, a leading cause of morbidity and mortality worldwide.

Toll-like receptors (TLRs) constitute a family of transmembrane proteins that play an important role in initiating the host immune response to various pathogens, including the pathogenesis of mycobacterial infections [6–8]. The interaction of specific mycobacterial agonists with TLRs leads to the production of inflammatory mediators that are critical for the activation of innate and adaptive immune responses [9, 10]. Among the members of the TLR family, TLR2 (as a heterodimer with TLR1 or TLR6), TLR4 and TLR9 have been shown to interact with mycobacterial agonists [9–11]. A number of studies have established a role for the TLR2-mediated recognition of *M. tuberculosis*, *M. leprae* and other non-tuberculous mycobacteria [10, 12]. In vitro data also supported a role for the TLR4-mediated recognition of an *M. tuberculosis* ligand that was stimulatory only when isolated from live organisms [13].

For decades, host genetic factors have been known to play an important role in leprosy. Genome-wide studies have identified several loci that are associated with the disease or its clinical presentation (reviewed in [14, 15]). In addition, studies have reported associations between leprosy and/or leprosy type and polymorphisms in numerous candidate genes, including the tumour necrosis factor α (TNF-α) gene, the interleukin-10 gene, the transporter associated with antigen processing 1 and 2 (*TAP1* and *TAP2*) genes, the natural resistance associated macrophage protein 1 (*NRAMP1*) gene and the vitamin D receptor gene (reviewed in [14, 16–18]).

Human genetic studies suggest that polymorphisms in TLRs influence susceptibility to a variety of infections [6, 19]. We recently demonstrated that a microsatellite polymorphism in *TLR2* was significantly associated with the occurrence of reversal reaction among leprosy patients [20]. However, this polymorphism did not strongly influence susceptibility to leprosy itself, nor leprosy type. Therefore, we hypothesised that polymorphisms in TLRs other than *TLR2* may influence susceptibility to the disease. Two *TLR4* polymorphisms (896G>A [D299G] and 1196C>T [T399I]) are emerging as major variants in TLR genes that influence human susceptibility to a number of infectious diseases, including tuberculosis [21] and invasive aspergillosis [22, 23]. In the present study, we investigated the role of *TLR4* polymorphisms in susceptibility to leprosy and its clinical manifestations in an Ethiopian cohort.

Methods

Human subjects

The enrolment procedures and clinical definitions have been described previously [20, 24]. Briefly, 441 leprosy patients were drawn from the All Africa Leprosy Rehabilitation and Training (ALERT) Multi-Drug Therapy (MDT) Field Evaluation Study (AMFES) [24] and 197 unrelated, healthy controls were collected from the local population. Self-reported ethnicity included three major ethnic groups (Oromo, Amhara and Gurage). Leprosy types were established on clinical grounds according to the simplified Ridley/Jopling classification, which adds the rarely occurring borderline borderline (BB) patients to the borderline lepromatous (BL) patients [25]. Twenty-five additional patients were classified as multibacillary (MB) or paucibacillary (PB) according to the WHO classification. Leprosy reactions were reported only in a subgroup of 216 patients and diagnosed as reported elsewhere [26]. Informed consent was obtained from each study participant. The study was approved by human subject review boards from the Armauer Hansen Research Institute (Addis Ababa, Ethiopia), the Rockefeller University (New York, NY), the Public Health Research Institute (Newark, NJ), the University of Washington (Seattle, WA) and the Western Institutional Review Board (Olympia, WA).

DNA sequencing and genotyping

Genomic DNA was extracted from whole blood using standard protocols [20]. To determine whether there were any polymorphisms that are unique to Ethiopians, we sequenced the coding region of 28 individuals (9 controls and 19 cases). The polymerase chain reaction (PCR) products were sequenced with Big Dye Terminator v3.0 and analysed on an ABI PRISM 3730 capillary sequencer (Applied Biosystems). Sequences were aligned and ana-

lysed with the programs PHRED/PHRAP and CONSED [27]. Genotyping among the whole cohort was carried out by the MassARRAY™ technique (Sequenom, San Diego, CA) using a chip-based matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer as previously described [28]. Single nucleotide polymorphism (SNP) selection included four SNPs, three previously reported SNPs (896G>A, 1196C>T and 1530G>T [Q510H] [6, 29]) and a previously unreported SNP that was discovered by sequencing (1976A>G [M658G]).

Statistics

Statistical analyses were performed using Stata 9 (StataCorp, College Station, TX). All SNPs were tested for Hardy-Weinberg equilibrium using the genhw program. Pairwise linkage disequilibrium was assessed using the pwld program. Haplotypes were inferred separately for each ethnic group using the expectation-maximisation algorithm implemented in the DECIPHER program (S.A.G.E.) [30]. Haplotypes with frequencies <1.5% were grouped together. The association of *TLR4* SNPs with susceptibility to leprosy and leprosy type was first assessed in a multivariate logistic regression model (general model) that did not assume any particular mode of inheritance and accounted for the presence of each genotype versus the wild type genotype (0/1 and 1/1, each versus 0/0). For statistically significant associations, we performed likelihood ratio tests for three different models versus the general model. Each of these three models assumed one of the following modes of inheritance: dominant (comparing the presence of one or two copies of the minor allele versus no copies), recessive (comparing the presence of two copies of the minor allele versus no or one copies) and additive (no, one or two copies of the minor allele were coded 0, 1 and 2, respectively, assuming greater effect with increased copy number of the minor allele). The best fitting model was assumed in the final presentation of the results. The association of *TLR4* haplotypes with susceptibility to leprosy was calculated in a logistic regression model including all haplotypes (each coded 0, 1 or 2 for the presence of no, one or two copies of the haplotype), using haplotype 1 as a reference. All analyses were adjusted for age groups, sex and ethnicity (when applicable). Since the sample size was limited, especially for rare SNPs, the statistical analyses were initially performed in the whole population. The consistency of significant results was then verified within each ethnic group.

Bacterial strains

M. leprae strain Thai-53 was provided by Dr. James Krahenbuhl of Lousiana State University and was propagated and collected from the mouse footpad model as described previously [31]. *M. leprae* was irradiated as described previously [31]. Since *M. leprae* grows optimally at 30–33°C, which is suboptimal for cell culture, heat-killed *M. leprae* were used as described previously. Heat-killed and viable *M. leprae* were compared previously and were shown to induce similar relative cytokine response from infected monocytes [32, 33, and G. Kaplan, unpublished data].

Human monocyte and peripheral blood mononuclear cell stimulations

Human peripheral blood monocytes and peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors (New Jersey Blood Center, East Orange, NJ) by Ficoll-Paque separation and plated at a density of 3×10^6 PBMCs per well in a 24-well tissue culture plate. For the preparation of monocytes, isolated PBMCs were resuspended in RPMI 1640 and supplemented with 1% human AB serum (Gemini Bioproducts, Calabasas, CA), allowed to adhere for 2 h and then washed to remove non-adherent cells. Both adherent monocytes and PBMCs were cultured in RPMI 1640 with 20% human serum prior to stimulation at a multiplicity of infection (MOI) of 5:1 with heat-killed *M. leprae* (ML) or stimulation with ultrapure lipopolysaccharide (LPS; 100 ng/mL, InvivoGen, San Diego, CA). Supernatants were collected at 48 h post-stimulation and analysed by Luminex for the presence of multiple cytokines (Bio-Rad, Hercules, CA).

Results

The study included 441 leprosy cases and 197 controls (Table 1). The majority of participants were males (71.6% of cases and 73.5% of controls). Self-reported ethnicities were Oromo (43.8% of cases and 46.7% of controls), Amhara (28.6% of cases and 22.3% of controls) and Gurage (25.4% of cases and 25.9% of controls). Age categories were not equally represented among cases and controls (mean age 39.0 ± 15.1 years in cases versus 29.1 ± 12.4 years in controls, $P<0.001$). A total of 298 cases (68.1%) were grouped into the lepromatous pole, including 199 borderline lepromatous (BL), 81 lepromatous lepromatous (LL) and 18 multibacillary (MB, WHO classification). Among the 138 cases grouped into the tuberculoid pole (31.9%), 128 were borderline tuberculoid (BT), three polar tuberculoid (TT) and seven paucibacillary (PB, WHO classification). Among the 441 patients, 216 had complete data on leprosy complications: neuritis occurred in 133 patients (61.6%), reversal reaction in 66 (30.6%) and erythema nodosum leprosum (ENL) in 17 (7.9%).

Table 1 Baseline characteristics of leprosy cases and control subjects

Characteristics	Total leprosy cases		Controls		*P*-value[a]
	n	(%)	*n*	(%)	
Number of cases	441	(100.0)	197	(100.0)	
Male gender	302	(71.6)	144	(73.5)	0.700
Ethnicity[b]					
Oromo	193	(43.8)	92	(46.7)	0.337
Amhara	126	(28.6)	44	(22.3)	
Gurage	112	(25.4)	51	(25.9)	
Age groups					
0–19	41	(9.9)	43	(22.2)	<0.001
20–39	159	(38.5)	109	(56.2)	
40–59	168	(40.7)	37	(19.1)	
≥60	45	(10.9)	5	(2.6)	
Leprosy type[c]					
Lepromatous lepromatous (LL)	81	(19.7)			
Borderline lepromatous (BL)	199	(48.4)			
Borderline tuberculoid (BT)	128	(31.1)			
Tuberculoid (TT)	3	(0.7)			
Leprosy complications[d]					
Neuritis	133	(61.6)			
Reversal reaction	66	(30.6)			
Erythema nodosum leprosum	17	(7.9)			

[a] Double-sided Fisher's exact test for the distribution of characteristics among all cases versus controls

[b] Twenty patients had other (5 cases and 9 controls) or missing (5 cases and 1 control) ethnicity

[c] Leprosy type was missing in five patients; 25 patients classified according the WHO as multibacillary (MB, 18 cases) or paucibacillary (PB, 7 cases) are not shown. Borderline borderline patients were classified together with BL (see Methods section). A total of 298 patients were lepromatous (81 LL + 199 BL + 18 MB) and 138 were tuberculoid (128 BT + 3 TT + 7 PB, see Table 4)

[d] Complications data were available in 216 patients only

Note that the characteristics of patients and controls have already been reported [20]. They are presented here to provide the reader with an overview of the study population.

The *TLR4* SNPs were at Hardy-Weinberg equilibrium and their frequencies were similar among the three ethnic groups, except for 1530G>T among Oromo (P=0.02) (Table 2). While both 896G>A and 1196C>T are in strong linkage disequilibrium among Caucasians (minor allele frequency ~7%, R^2>0.95), we found that this was not the case in the present study (R^2=0.32) and that 896A was ~3-fold less frequent than 1196T, as expected in an African population (Table 3) [34]. To investigate whether polymorphisms in *TLR4* were associated with susceptibility to leprosy, we compared the frequencies of *TLR4* SNPs among leprosy cases and controls, adjusting for sex, age groups and ethnicity (Table 4). Both *TLR4* SNPs 896G>A and 1196C>T were less frequent among leprosy cases than controls. The 896GG, GA and AA genotypes were found in 91.7, 7.8 and 0.5% of leprosy cases versus 79.9, 19.1 and 1.0% of controls, respectively (odds ratio [OR]=0.34, 95% confidence interval [CI] 0.20–0.57, P<0.001, additive model, Table 5). These results were still significant when the three different ethnic groups were analysed separately (OR=0.43, 95% CI 0.21-–.89, P=0.02 in Oromo; OR=0.34, 95% CI 0.13-0–94, P=0.04 in Amhara and OR=0.18, 95% CI 0.05–0.65, P=0.008 in Gurage). Similarly, the 1196CC, CT and TT genotypes were found in 98.1, 1.9 and 0% of leprosy cases versus 91.8, 7.7 and 0.5% of controls, respectively (OR=0.16, 95% CI 0.06–0.40, P<0.001, dominant model, comparing the presence of one or two copies of the minor allele versus no copies). Again, these results were consistent among the three different ethnic groups, although the significance level was not reached in the Gurage population (P=0.02 in Oromo; P=0.04 in Amhara and P=0.3 in Gurage, two-sided Fisher's exact test). A third *TLR4* SNP (1530G>T) also tended to be less frequent among leprosy cases than controls (1530GG, GT and TT genotypes were found in 98.0, 1.8 and 0.0% of leprosy cases versus 93.6, 5.8 and 0.6% of controls, respectively, OR=0.38, 95% CI 0.14–1.01, P=0.05, dominant model). However, the assessment of this third SNP

Table 2 Allelic and haplotypic frequencies of Toll-like receptor 4 (*TLR4*) single nucleotide polymorphisms (SNPs) in the three Ethiopian populations

SNP	Amino acid change	rs number	Oromo		Amhara		Gurage		*P*-value[b]
			n=285		*n*=170		*n*=163		
			MAF	HWE[a]	MAF	HWE[a]	MAF	HWE[a]	
896G>A	D299G	4986790	0.069	0.12	0.064	0.50	0.052	1.00	0.604
1196C>T	T399I	4986791	0.022	1.00	0.021	0.06	0.016	1.00	
1530G>T	Q510H	5030719	0.030	0.02	0.010	1.00	0.007	1.00	
1976A>G	M658G	N/A[c]	0.024	1.00	0.019	1.00	0.010	1.00	
Haplotype[d]									
1) ACGA	-	-	0.907	-	0.923	-	0.941	-	0.235
2) GCGA	-	-	0.024	-	0.030	-	0.028	-	
3) GTGA	-	-	0.020	-	0.021	-	0.016	-	
4) ACGG	-	-	0.020	-	0.015	-	0.006	-	
5) GCTA	-	-	0.024	-	0.009	-	0.003	-	

MAF = minor allele frequency; HWE = Hardy-Weinberg equilibrium test

[a] Hardy-Weinberg equilibrium was calculated for each locus and the *P*-value is reported. A *P*-value of <0.05 indicates that the alleles are not in HWE

[b] Double-sided Fisher's exact test for the overall distribution of alleles/haplotypes among the three ethnic groups

[c] SNP 1976A>G has not been previously reported

[d] Haplotypes with an MAF <1.5% in the whole population are not shown

TLR4 SNPs were not in linkage disequilibrium with any of the *TLR2* polymorphisms that have been previously analysed in this population [20]

among the different ethnic groups was limited by the small sample size. In the analyses by haplotypes, all haplotypes that contained the 896A, the 1196C and/or the 1530T alleles were associated or tended to be associated with protection against leprosy (OR=0.48, 95% CI 0.21–1.08, *P*=0.08 for haplotype 2, OR=0.12, 95% CI 0.05–0.34 for haplotype 3 and OR=0.23, 95% CI 0.08–0.69, *P*=0.008 for haplotype 5, compared to haplotype 1, Table 6). Together, these results suggested that *TLR4* polymorphisms are associated with susceptibility to leprosy.

Table 3 Linkage disequilibrium between the *TLR4* SNPs in all ethnic groups

SNPs	896G>A	1196C>T	1530G>T	1976A>G
896G>A	*0.06*			
1196C>T	0.32	*0.02*		
1530G>T	0.26	0.00	*0.02*	
1976A>G	0.00	0.00	0.00	*0.02*

Pairwise linkage disequilibrium (LD) were calculated with the pwld program developed in Stata 9 (StataCorp, College Station, TX). Off-diagonal elements are estimates of R^2, assuming Hardy-Weinberg equilibrium. The diagonal elements are minor allele frequencies (in *italics*). LD between 896G>A and 1196C>T was similar among the three ethnic groups (R^2 = 0.34 in Oromo, R^2 = 0.32 in Amhara and R^2 = 0.27 in Gurage)

Then, we compared frequencies of the *TLR4* SNPs among leprosy patients with lepromatous (LL and BL) and tuberculoid (BT and TT) leprosy, adjusting for sex, age groups and ethnicity (Table 4). There was an association at the limit of the significant level between the 1196C>T SNP and lepromatous leprosy. The 1196CC, CT and TT genotypes were found in 98.9, 1.1 and 0% of lepromatous leprosy patients versus 96.2, 3.8 and 0% of tuberculoid patients, respectively (OR=0.23, 95% CI 0.05–0.99, *P*=0.05, dominant model). No significant association was found when lepromatous lepromatous patients were compared with the other leprosy patients. Together, these results suggested that *TLR4* SNPs are not strongly associated (if at all) with the type of leprosy.

Next, we investigated whether *TLR4* polymorphisms were associated with the occurrence of leprosy complications. No association was found between *TLR4* SNPs and neuritis. The 1530T allele was more frequent in patients with reversal reaction (2/15 [13%] in patients with reversal reaction versus 3/177 [1.7%] in those without reversal reaction, *P*=0.05, Fisher's exact test) and ENL (4/58 [6.9%] in patients with ENL versus 1/133 [0.7%] in those without ENL, *P*=0.03). However, the interpretation of these results is limited by the small sample size, which prevented stratification into separate ethnic groups and the use of multivariable models.

Table 4 Association of *TLR4* SNPs with leprosy and leprosy type

SNP	Leprosy *n*=441		Controls *n*=197		*P*-value	Lepromatous, *n*=298		Tuberculoid, *n*=138		*P*-value	Lepromatous Lepromatous, *n*=81		Other, *n*=355		*P*-value
	n	(%)	*n*	(%)		*n*	(%)	*n*	(%)		*n*	(%)	*n*	(%)	
896G>A															
G/G	375	(91.7)	155	(79.9)	ref.	256	(91.8)	119	(91.5)	ref.	73	(91.3)	302	(91.8)	ref.
G/A	32	(7.8)	37	(19.1)	<0.001	22	(7.9)	10	(7.7)	0.95	7	(8.8)	25	(7.6)	0.86
A/A	2	(0.5)	2	(1.0)	0.08	1	(0.4)	1	(0.8)	-	0	(0.0)	2	(0.6)	-
1196T>C															
T/T	407	(98.1)	179	(91.8)	ref.	282	(98.9)	125	(96.2)	ref.	78	(98.7)	329	(97.9)	ref.
T/C	8	(1.9)	15	(7.7)	<0.001	3	(1.1)	5	(3.8)	0.05	1	(1.3)	7	(2.1)	0.58
C/C	0	(0.0)	1	(0.5)	-	0	(0.0)	0	(0.0)	-	0	(0.0)	0	(0.0)	-
1530G>T															
G/G	391	(98.0)	162	(93.6)	ref.	271	(98.2)	120	(97.6)	ref.	75	(98.7)	316	(97.8)	ref.
G/T	7	(1.8)	10	(5.8)	0.06	5	(1.8)	2	(1.6)	0.72	1	(1.3)	6	(1.9)	0.98
T/T	1	(0.3)	1	(0.6)	0.56	0	(0.0)	1	(0.8)	-	0	(0.0)	1	(0.3)	-
1976A>G															
A/A	386	(96.5)	164	(94.8)	ref.	267	(95.7)	119	(98.3)	ref.	73	(94.8)	313	(96.9)	ref.
A/G	14	(3.5)	9	(5.2)	0.64	12	(4.3)	2	(1.7)	0.29	4	(5.2)	10	(3.1)	0.62

P-values are calculated in a logistic regression model that did not assume any particular mode of inheritance and accounted for the presence of each genotype (0/1 and 1/1) versus the wild type genotype (0/0), adjusted for age group, sex and ethnicity (general model). For statistically significant associations, we performed a likelihood ratio tests for three different models (dominant, recessive and additive) versus the general model. The best fitting models are shown in Table 5. The numbers of individuals with assessable genotypes may differ slightly for the different SNPs

Finally, we examined the cellular mechanism of how *M. leprae* modulates TLR4-mediated immune responses. We previously demonstrated that irradiated *M. leprae* or its cell wall extracts stimulate TNF-α secretion through TLR2, but not TLR4, in bone marrow-derived macrophages [35]. In HEK293 cells transfected with an NF-κB luciferase construct, we also found that TLR2, but not TLR4, mediates signalling in response to stimulation with irradiated *M. leprae* or its cell wall extracts (data not shown and [35]). Together, these results suggested that *M. leprae* does not directly stimulate an immune response through TLR4, which is consistent with the absence of LPS in its cell wall.

We hypothesised that *M. leprae* modulates TLR4-mediated immune responses by regulating shared components of the downstream TLR signalling pathway. To test this hypothesis, we isolated and stimulated human monocytes with LPS and *M. leprae* and measured cytokine production in culture supernatants. In contrast to LPS, *M. leprae* did not induce IL-1β, IL-6 or IL-12p70 in monocytes (Fig. 1A, B, C). To examine whether *M. leprae* inhibits or merely fails to activate cytokine production, we examined cytokine production with mixed stimulations. In monocytes that were concurrently co-stimulated with *M. leprae* and LPS, cytokine production was similar to that of LPS alone. In contrast, when cells were pre-stimulated with *M. leprae* for 5 or 24 h and then stimulated with LPS, IL-1β production was lower in comparison to stimulation with LPS alone (Fig. 1A). A similar trend was observed for IL-6 production after 24 h of *M. leprae* pre-stimulation, but the differences were not statistically significant (Fig. 1B). In contrast to IL-6 and IL-1β, there was no difference in IL-12p70 production among the different conditions tested (Fig. 1C). As a control, we repeated the experiment in the opposite stimulation order (LPS followed by *M. leprae*) and found no difference in cytokine levels. Next, we examined whether *M. leprae* modulated LPS-induced signalling in PBMCs. Similar to monocytes, we found that the pre-stimulation of PBMCs with *M. leprae* for 24 h led to decreased LPS-induced secretion of IL-6 in comparison to stimulation with LPS alone (Fig. 1E). IL-1β levels were also lower in the PBMCs that were pre-treated with *M. leprae* for 24 h, although the difference was not statistically significant (Fig. 1D). Similar to monocytes, no differences in IL-6 or IL-1β levels were observed when the cells were stimulated with *M. leprae* after LPS. There was no difference among the different stimulation conditions when comparing levels of IL-12p70 or several T cell cytokines (IFN-γ, IL-4 or IL-17). Together, these results suggest that *M. leprae* inhibits the LPS induction of IL-1β and IL-6 secretion from monocytes. Furthermore, this effect was selective and not found for T-cell cytokines or other monocyte cytokines, such as IL-12p70.

Table 5 Association of *TLR4* SNPs with leprosy in three ethnic groups

	All ethnicities				Oromo				Amhara				Gurage			
	Leprosy, $n = 441$		Controls, $n = 197$		Leprosy, $n = 193$		Controls, $n = 92$		Leprosy, $n = 126$		Controls, $n = 44$		Leprosy, $n = 112$		Controls, $n = 51$	
SNP	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
896G>A																
G/G	375	(91.7)	155	(79.9)	162	(90.5)	72	(80.9)	108	(90.8)	35	(79.5)	97	(94.2)	41	(80.4)
G/A	32	(7.8)	37	(19.1)	15	(8.4)	16	(18.0)	11	(9.2)	8	(18.2)	6	(5.8)	10	(19.6)
A/A	2	(0.5)	2	(1.0)	2	(1.1)	1	(1.1)	0	(0.0)	1	(2.3)	0	(0.0)	0	(0.0)
Add. model	OR = 0.34 (0.20–0.57), $P < 0.001$				OR = 0.43 (0.21–0.89), $P = 0.02$				OR = 0.34 (0.13–0.94), $P = 0.04$				OR = 0.18 (0.05–0.65), $P = 0.008$			
1196T>C																
T/T	407	(98.1)	179	(91.8)	175	(97.8)	82	(91.1)	121	(98.4)	40	(90.9)	103	(98.1)	48	(94.1)
T/C	8	(1.9)	15	(7.7)	4	(2.2)	8	(8.9)	2	(1.6)	3	(6.8)	2	(1.9)	3	(5.9)
C/C	0	(0.0)	1	(0.5)	0	(0.0)	0	(0.0)	0	(0.0)	1	(2.3)	0	(0.0)	0	(0.0)
Dom. model[a]	OR = 0.15 (0.06–0.39), $P < 0.001$				($P = 0.02$)[b]				($P = 0.04$)[b]				($P = 0.3$)[b]			
1530G>T																
G/G	391	(98.0)	162	(93.6)	167	(97.1)	72	(90.0)	115	(99.1)	37	(94.9)	101	(98.1)	45	(100.0)
G/T	7	(1.8)	10	(5.8)	4	(2.3)	7	(8.8)	1	(0.9)	2	(5.1)	2	(1.9)	0	(0.0)
T/T	1	(0.3)	1	(0.6)	1	(0.6)	1	(1.3)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Dom. model	OR = 0.38 (0.14–1.01), $P = 0.05$				($P = 0.03$)[b]				($P = 0.2$)[b]						-	

We performed a likelihood ratio test for three different models (dominant, recessive and additive) versus the general model. The best fitting models are shown.

[a] Indicates that the best fitting models could not be determined by likelihood ratio testing, since homozygous cases were missing in certain groups; the dominant model is shown

[b] For small groups, rare allele carriers (0/1 or 1/1) were compared to non-carriers (0/0) using a two-sided Fisher's exact test. Numbers of individuals with assessable genotypes may differ slightly for the different SNPs. The effect of *TLR4* 896G>A and 1196T>C were independent from the presence of *TLR2* polymorphisms previously associated with reversal reaction [54] (not shown)

Table 6 Association of *TLR4* haplotypes with leprosy

Haplotype	Leprosy (n = 441) vs. controls (n = 197)	
	OR (95% CI)	P-value)
1) ACGA	ref.	-
2) GCGA	0.48 (0.21–1.08)	0.08
3) GTGA	0.12 (0.05–0.34)	<0.001
4) ACGG	0.62 (0.23–1.67)	0.3
5) GCTA	0.23 (0.08–0.69)	0.008

P-values are calculated in a logistic regression model including all haplotypes (each coded 0, 1 or 2, for the presence of no, one or two copies of the haplotype), using haplotype 1 as a reference. The results are adjusted for age group, sex and ethnicity

Discussion

In this paper, we showed that two SNPs in *TLR4* (896G>A [D299G] and 1196C>T [T399I]) were associated with protection against leprosy in an Ethiopian cohort of leprosy patients and healthy controls. We also demonstrated that *M. leprae* downregulates TLR4-mediated cytokine production in monocytes.

Recent studies have also found associations of TLR SNPs and leprosy susceptibility. An SNP in *TLR1* (T1805G [I602S]) was associated with *M. leprae* infection [36–38]. Another study reported that a *TLR2* SNP (C2029T [R677W]) located in a critical region of the Toll-interleukin receptor (TIR) domain was associated with lepromatous leprosy in

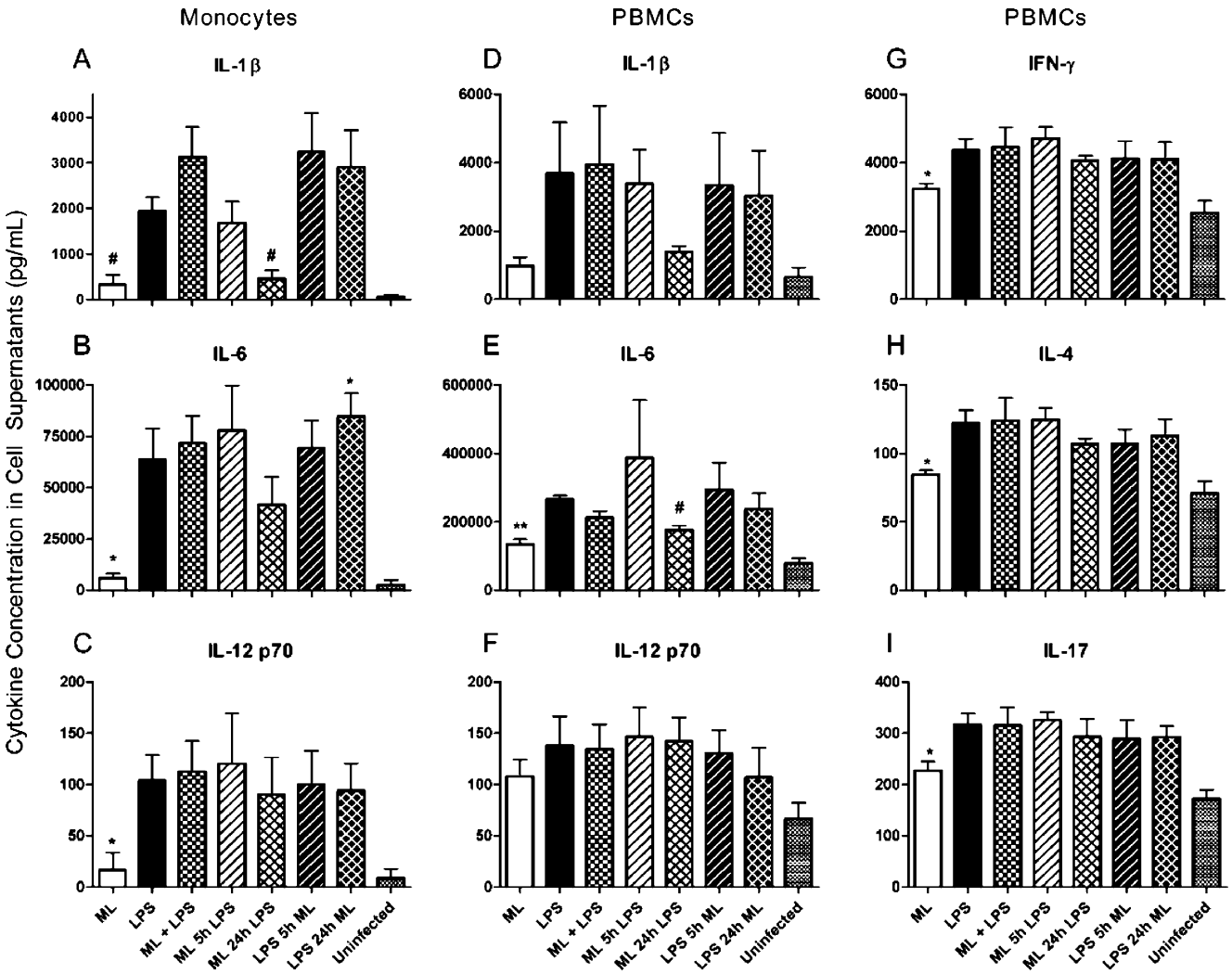

Fig. 1 Monocyte and peripheral blood mononuclear cell (PBMC) cytokine response to mixed stimulation with *Mycobacterium leprae* and lipopolysaccharide (LPS). Human peripheral blood monocytes and PBMCs were isolated and infected at a multiplicity of infection (MOI) of 5:1 with heat-killed *M. leprae* (ML), LPS (100 ng/mL), *M. leprae* and LPS simultaneously, or pre-stimulation with either *M. leprae* or LPS for 5 or 24 h, followed by treatment with the other stimulus. Supernatants were collected at 48 h post-stimulation and analysed by Luminex for the presence of multiple cytokines. The results are the means ± standard error of three experiments (donors) performed in duplicate, with the exceptions of IL-1β production from monocytes (five donors) and IL-6 production from monocytes (four donors). A two-tailed paired t-test was used for statistical analysis (*P< 0.05, **P<0.005, #P<0.001, in comparison to LPS alone)

Korea [39]. While an amino-acid substitution at this location completely abolished *TLR2* signalling in transfected cells [35], subsequent studies revealed that this supposedly functional SNP was, in fact, located in a highly homologous pseudogene region located 23 kB upstream of the *TLR2* gene [40]. More recently, we showed that a microsatellite polymorphism located in *TLR2* significantly increased the risk of leprosy reversal reaction [20]. Allelic length variations in the microsatellite may influence promoter activity and modify TLR2 function through alteration of its expression level [41]. However, the *TLR2* microsatellite polymorphism associated with leprosy reversal reaction did not strongly influence susceptibility to leprosy itself.

In the present study, we examined whether polymorphisms in *TLR4* influenced susceptibility to or the clinical characteristics of leprosy in an Ethiopian cohort. We found that the minor alleles of two *TLR4* SNPs (896G>A [D299G] and 1196C>T [T399I]) were associated with a reduced risk of developing leprosy. Such a protective effect is in contrast with most studies. In fact, *TLR4* 896A and 1196T were frequently associated with increased susceptibility to pathogens, including Gram-negative bacteria, *Brucella* species, respiratory syncytial virus (RSV), *Plasmodium falciparum* and *Candida albicans* (reviewed in [6]).

Our data support the hypothesis that, in the case of specific infections, dysfunctional TLR SNPs may reduce, instead of increase, susceptibility to infections. This hypothesis is also supported by previous observations: the dysfunctional allele of *TLR1* 1805G (602S) exerted a protective effect against leprosy [37]; the dysfunctional 2258G/A (R753Q) was shown to increase the risk of tuberculosis [42] but to protect against Lyme borreliosis [43]; finally, as it is the case for leprosy, *TLR4* 896A and 1196T were shown to protect from Legionnaire's disease [44]. The reason why a specific TLR allele exerts a protective effect against some infections and an increased risk for others is unknown. These differences reflect the complexity of human immune responses to infectious agents and may result from co-evolving selective pressures in hosts and pathogens. *TLR4* 896G>A and 1196C>T have been intensively examined in functional and genetic epidemiological studies. 896A was associated with LPS hypo-responsiveness after the in vivo inhalation of endotoxin [23, 45]. In vitro cellular investigations also suggested that 299G and/or 399I was unable to mediate LPS signalling in some [23, 46] but not all cell types [47–49]. These seemingly contradictory findings may be explained by the use of small sample sizes, which resulted in differences with borderline statistical significance; comparison of different cell types; use of different doses and types of LPS; and measurement of different cytokines and inflammatory markers. Taken together, these studies suggest that SNPs 896G>A and 1196C>T partially influence inflammatory pathways under some experimental conditions.

While TLR2 has been clearly implicated in the innate immune response to *M. leprae*, the role of TLR4 is currently unknown. We previously found that irradiated *M. leprae* or its cell wall extracts stimulated TNF-α production through TLR2, but not TLR4 [35]. If live *M. tuberculosis* signals through TLR4, then it is plausible that *M. leprae* may do so similarly [13]. Alternatively, TLR4 may modulate inflammatory processes that influence leprosy disease manifestations but are not attributable to direct stimulation by *M. leprae*. Consistent with this hypothesis, we found that the stimulation of monocytes with *M. leprae* inhibited their subsequent response to TLR4 stimulation with LPS. There are several possible mechanisms of this inhibitory effect, including tolerance, a well-described phenomenon where prior exposure of cells to TLR ligands results in a transient hypo-responsive state that is refractory to additional stimulation [50]. Several mechanisms have been proposed for tolerance, including the alteration of expression levels of negative and positive regulators of the TLR signalling pathway. For example, IRAK-M is a negative regulator of the TLR signalling pathway that is downstream of both TLR2 and TLR4 and is involved in endotoxin tolerance [51]. *M. leprae* may alter the expression of molecules such as IRAK-M through TLR2 stimulation and, ultimately, modulate LPS/TLR4 responsiveness through such a shared signalling intermediate. An alternative mechanism could be from direct inhibitory effects of an *M. leprae* molecule on the TLR signalling pathway. There are several precedents for this mechanism, including YopJ inhibition of MAPK signalling by *Yersinia pestis* [52] and the recently described TIR domain containing proteins (Tcps) in *Escherichia coli* that bind MyD88 and inhibit TLR-induced signalling pathways [53]. Future studies will address these possible mechanisms of *M. leprae* inhibition of TLR4 signalling. The alteration of monocyte responses to *M. leprae* by *TLR4* polymorphisms via these different mechanisms could modulate the growth of bacilli, the nature and degree of the innate immune response, and the subsequent adaptive immune response. TLRs are known to influence Th1–Th2 pathway development in mice and such immunomodulatory effects could operate in humans as well [7]. *TLR4* polymorphisms may modulate several of these immunologic effects and, thereby, influence overall susceptibility to leprosy. Future studies will address which cellular immune responses are modulated by *TLR4* polymorphisms

This study was limited by the low frequency of the minor alleles of *TLR4*. This issue was of particular concern for the analyses of leprosy types (lepromatous versus tuberculoid), as well as for leprosy complications, for which it prevented stratification into separate ethnic groups and the use of multivariable models. However, the analyses of susceptibility to leprosy itself were performed in larger sample sizes. In this case, initial analyses were performed

for the whole population, including the three major ethnic groups. In order to limit the risk of population stratification, we also performed the analyses in the three different groups separately. Overall, most observations were consistent among the three different ethnic groups.

In summary, our data show that polymorphisms in *TLR4* are associated with susceptibility to leprosy in Ethiopian patients. It suggests that polymorphisms in individual TLRs may influence different clinical manifestations of the disease.

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