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Role of Toll-Like Receptors in Tuberculosis Infection

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Abstract

Background: One-third of the world's population is infected with *Mycobacterium tuberculosis*. Investigation of Toll-like receptors (TLRs) has revealed new information regarding the immunopathogenesis of this disease. Toll-like receptors can recognize various ligands with a lipoprotein structure in the bacilli. Toll-like receptor 2 and TLR-4 have been identified in association with tuberculosis infection.

Objectives: The aim of our study was to investigate the relationship between TLR polymorphism and infection progress.

Methods: Twenty-nine patients with a radiologically, microbiologically, and clinically proven active tuberculosis diagnosis were included in this 25-month study. Toll-like receptor 2 and TLR-4 polymorphisms and allele distributions were compared between these 29 patients and 100 healthy control subjects. Peripheral blood samples were taken from all patients. Genotyping of TLR-2, TLR-4, and macrophage migration inhibitory factor was performed. The extraction step was completed with a Qiagen mini blood purification system kit (Qiagen, Ontario, Canada) using a peripheral blood sample. The genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism.

Results: In total, 19 of the 29 patients with tuberculosis infection had a TLR-2 polymorphism, and 20 of the 100 healthy subjects had a TLR-2 polymorphism ($P < 0.001$). The TLR-4 polymorphism and interferon-γ allele distributions were not statistically correlated.

Conclusions: Toll-like receptor 2 polymorphism is a risk factor for tuberculosis infection. The limiting factor in this study was the lack of investigation of the interferon-γ and tumor necrosis factor-α levels, which are important in the development of infection. Detection of lower levels of these cytokines in bronchoalveolar lavage specimens, especially among patients with TLR-2 defects, will provide new data that may support the results of this study.

Keywords: Toll-Like Receptor, Infection, Genetic Polymorphism, *Mycobacterium tuberculosis*

1. Background

Tuberculosis is a granulomatous infectious disease caused by *Mycobacterium tuberculosis*. Infection with *M. tuberculosis* involves the participation of more than 90 antigens and various virulence factors, and results from the interaction between the pathogen and the host's mononuclear phagocytes and T lymphocytes. According to the world health organization, 32% of the world's population is infected with *M. tuberculosis*. Approximately 8.4 million people are infected each year, and 2.0 million die of the disease. Tuberculosis accounts for 2.5% of all diseases worldwide and 26.0% of evitable deaths (1).

Tuberculosis bacilli can stay dormant for months to years without causing disease. The immune response can keep the pathogen inactive during this latent period. The pathogenesis of the disease is based on the interaction between the bacilli and the host. Although one-third of the world's population is infected with *M. tuberculosis*, the infection usually does not progress to active disease. The pathogen remains in latent form in about 90% of infected individuals, who show no clinical features of the disease (2).

About 3-4% of the individuals infected with tuberculosis bacilli can develop infection within 1 year after exposure, and 5-15% may show active disease during one or more of the immune response phases. The immune response to this infection is not successful in destroying the organism's pathogenicity, and acute active disease is detected in a small number of affected individuals. This may occur because of delayed activation of the immune re-

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sponse. Cellular-mediated immunity and the delayed immune response are activated in the early phase; why the immune system is less effective in some infected individuals remains unclear.

Both natural immunity and acquired immunity play important roles in tuberculosis. The host response is mediated by pro-inflammatory and anti-inflammatory cytokines and chemokines. Mediators are secreted by macrophages and dendritic cells. This immediate response avoids bacterial proliferation and helps to suppress the infection. Phagocytic cells play an important role in antigen presentation and T-cell-mediated immunity. The bacilli develop antagonizing and immune response-avoiding mechanisms to protect themselves from the immune response. Toll-like receptors (TLRs) were first described in *Drosophila* species; later, in 1997, human analogues were found to be an important component of natural immunity. Eleven TLRs have been described to date. Every TLR has different ligand specificity (3).

Mycobacterial components are recognized by TLR-2 and TLR-4. Lipoarabinomannan, the 19-kDa *M. tuberculosis* lipoprotein, lipomannan, and phosphatidyl-myo-inositol mannoside are ligands for TLRs. Toll-like receptor 2 and TLR-4 are overexpressed during infection. Pathogen-associated molecules combine with TLRs and the Toll-interleukin 1 (IL-1) receptor (TIR) domains of myeloid differentiation protein 88 (MyD88). This interaction activates IL-1 receptor-related kinase (IRAK-1), tumor necrosis factor-α (TNF-α)-related factor (TRAF), and interferon-β (IFN-β)-induced TIR-carrying molecule (TIRAK) and conveys TLR activation toward the nucleus. Nuclear factor-κB, TNF-α, and IL-1 then initiate the immune response to *M. tuberculosis* in the nucleus (3-6).

2. Objectives

In the present study, we investigated the role of TLRs in the immune response to tuberculosis bacilli by detecting TLR polymorphisms. We also assessed the disease severity in patients with mutation, compared the clinical and laboratory data between patients with and without mutation, and investigated the effect of mutations on the patients' clinical presentation.

3. Methods

3.1. Patients

During the 25-month study period, 29 patients with a diagnosis of active tuberculosis, as determined by radiologic, microbiologic, and clinical examinations, were evaluated. One hundred healthy subjects with no respiratory symptoms comprised the control group. All control subjects underwent chest X-ray examinations to rule out tuberculosis, and those with suspicious lesions were excluded from the study. Written informed consent for inclusion in the study was obtained from all participants in both the patient and control groups.

Tuberculosis was diagnosed by clinical examination, identification of acid-fast bacilli (AFB) in sputum, growth of *M. tuberculosis* on appropriate media, and radiologic findings. Only patients with *M. tuberculosis* results obtained by the automated BACTEC 460 TB system (Becton Dickinson, Franklin Lakes, NJ, USA) and conventional methods were included in the study. Participants without microbiological proof of M. tuberculosis were excluded.

3.2. Toll-Like Receptor Mutation Detection

Peripheral blood samples were taken from all patients and transported to the laboratory within 1 hour. The vaccination status, tuberculin skin test positivity, acid-resistance staining results, culture results, underlying diseases, immunosuppression status, and presence of extrapulmonary tuberculosis with radiological symptoms and resistance patterns, were recorded. Strains with multidrug resistance were not included.

3.3. Toll-Like Receptor 2 Genotyping

The extraction step was performed with a DNA blood mini kit (Qiagen, Toronto, Ontario, Canada) using a peripheral blood sample. The genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP); Arg753Gln polymorphism was detected using Arg753Gln restriction enzyme. The primers are listed in Table 1 (7). The amplification step was performed with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The master mix included 1 mL DNA solution, GeneAmp Gold Buffer (15 mmol/L Tris HCl; pH 8.0), 50 mmol KCl, 1.5 mmol $MgCl_2$, 50 mmol/L each of dGTP, dATP, dTTP, and dCTP (Promega, Madison, WI, USA), 25 pmol forward primer, 25 pmol reverse primer, and 1.0 U AmpliTaq Gold polymerase (Applied Biosystems).

The PCR reaction was carried out under the following conditions: pre-PCR at 95.0°C for 10 minutes, annealing at 60°C for 45 minutes, extension at 72°C for 45 minutes, and final extension at 72°C for 7 minutes. The PCR gel image was visualized in 2% ethidium bromide containing agar (117 mA and 102 mV) (OWL separation systems, Inc., Portsmouth, NH, USA) under ultraviolet (UV) light (InGenius; Syngene, Cambridge, UK). The PCR products then underwent restriction by AciI enzyme (New England Biolabs, Beverly, MA, USA) to a final volume of 10 μL at 37°C overnight incubation. The restriction took place in a 1-mL solution with 2

and 4 U of Acil enzyme. The restriction products were visualized in 3% ethidium bromide-containing gel under UV light (100 mV, 60 minutes).

3.4. Toll-Like Receptor 4 Genotyping

The extraction step was performed with a DNA blood mini kit (Qiagen) using a peripheral blood sample. The PCR-RFLP method was used to identify polymorphisms. Asp299Gly polymorphism was present at the Ncol enzyme restriction site. Thr399lle polymorphism was present at the Hinf 1 restriction site. The primers are listed in Table 1 (7). The amplification step was performed with the GeneAmp PCR system 9700 (Applied Biosystems).

The PCR master mix comprised 1 mL DNA solution, GeneAmp Gold buffer (15 mmol/L Tris HCl; pH 8.0), 50 mmol KCl, 1.5 mmol $MgCl_2$, 50 mmol/L each of dGTP, dATP, dTTP, and dCTP (Promega), 25 pmol forward and reverse primers, and 1.0 U AmpliTaq Gold polymerase (Applied Biosystems). The PCR reaction was performed at a 25-μL volume and involved denaturing at 95°C for 10 minutes, annealing at 59°C for 45 minutes, extension at 72°C for 45 minutes, and a final extension at 72°C for 7 minutes. The PCR gel image was visualized in 2% ethidium bromide-containing agar (117 mA, 102 mV) (OWL separation systems) under UV light (InGenius; Syngene). The PCR products then underwent restriction by restriction enzymes (New England Biolabs) to a final volume of 10 μL at 37°C overnight incubation. The restriction took place in a 1-mL solution with 2 and 4 U of Acil enzyme. The restriction products were visualized in 3% ethidium bromide-containing gel under UV light (100 mV, 60 minutes).

3.5. Macrophage Migration Inhibitory Factor Genotyping

The extraction step was performed with a DNA blood mini kit (Qiagen) using a peripheral blood sample. The primers are listed in Table 1 (7). The master mix and amplification step were as described for TLR-2 and TLR-4 genotyping. The restriction step was performed as described for TLR-4 genotyping.

3.6. Statistical Analysis

The differences between the two groups were evaluated with Fisher's exact test. A P value of < 0.05 was considered statistically significant.

4. Results

In total, 23 of the 29 patients with tuberculosis were male, and 6 were female. The patients' mean age was 50.8 $\pm$ 15.0 years (range: 22 - 77 years). In total, 79 of the 100 subjects in the control group were male, and 21 were female. The control subjects' mean age was 46.0 $\pm$ 12.0 years (range: 18 - 67 years). Two of the twenty-nine patients with tuberculosis showed a TLR-2 mutation; none of the control subjects had this mutation. A TLR-2 heterozygote mutation was present in 17 patients with tuberculosis and 12 control subjects. In total, 19 (65%) of the 29 patients and 12 (12%) of the 100 control subjects had a heterozygote or mutant TLR-2 polymorphism; the difference between the two groups was statistically significant ($P < 0.001$) (Table 2).

The TLR-4 nucleotide Asp299Gly polymorphism was normal in 28 of the 29 patients with tuberculosis; the remaining patient had a heterozygote-type change. Three control subjects had a heterozygote change, and one showed a mutation. There was no statistically significant difference between the two groups (P = 0.80). Similarly, 1 patient with a Thr399lle polymorphism had a mutation; the remaining 28 patients were normal. Five control subjects had heterozygote changes, and one showed a mutation. There was no statistically significant difference between the two groups (P = 0.60) (Table 2).

A GG, GC, and CC IFN-γ allele distribution was detected in 18, 9, and 2 patients, respectively. These same allele distributions were detected in 80, 20, and 10 control subjects, respectively. No statistically significant correlation was detected between the allele distributions in the patients and controls (P = 0.19) (Table 3). The TLR-2 and TLR-4 mutations and IFN-γ allele distributions in patients with tuberculosis are shown in Table 4. There were no statistically significant relationships among the patients' TLR-2 and TLR-4 mutations, IFN-γ allele distributions, age, and sex. In total, 11 of the 29 patients with tuberculosis were smokers, and 22 of these patients had received the bacillus Calmette-Guerin vaccine. There were no statistically significant relationships among smoking status, vaccination status, TLR-2 and TLR-4 mutations, and IFN-γ allele distribution.

In total, 16 patients had underlying diseases: 7 had diabetes mellitus (DM), 4 had malignancies, 2 had atherosclerotic disorders, and 3 had other systemic diseases. No statistical correlation was found among TLR-2 and TLR-4 mutations, the IFN-γ allele distribution, and underlying diseases. Twelve patients were immunosuppressed due to steroid use and DM. No statistically significant relationship was found between the TLR mutations. Nine patients with tuberculosis had a negative tuberculin skin test result, and twenty patients had a result of $\geq$ 10 mm. No statistical correlation was detected among tuberculin skin test positivity, TLR-2 and TLR-4 mutations, and the IFN-γ allele distribution.

Chest X-rays revealed involvement of a single lung zone in 13 patients, multiple zones in 10, and bilateral zones in 6. Cavitation was present in 14 patients. No statisti-

Table 1. The List of Primers that Are Used

	Forward Primer	Reverse Primer
TLR-2	5'GGGACTTCATTCCTGGCAAGT3'	5'GGCCACTCCAGGTAGGTCTTC3'
TLR-4 (Asp299Gly)	5'GATTAGCATACTTAGACTTACTACCTCCATG3'	5'GATCAACTTCTGAAAAAGCATTCCCAC3'
TLR-4 (Thr399lle)	5'GGTTGCTGTTCTCAAAGTGATTTTGGGAGA3'	5'CCTGAAGACTGGAGAGTGAGTTAAATGCT3'
MIF	5'ACTAAGAAAGACCCGAGGC3'	5'GGGGCACGTTGGTGTTTAC3'

Table 2. Polymorphism Distribution of Toll-Like Receptors

	TLR-2 Polymorphism Distribution		
P < 0.001	**Heterozygote + Mutant, No. (%)**	**Normal, No. (%)**	**Total, No. (%)**
Patient	19 (65.5)*	10 (34.5)	29 (100)
Control	12 (12.0)	88 (88.0)	100 (100)
Total	31 (24.0)	98 (76.0)	129 (100)
	TLR-4 Thr399lle Polymorphism Distribution		
Patient	1 (3.4)	28 (96.6)	29 (100)
Control	6 (6.0)	94 (94.0)	100 (100)
Total	7 (5.4)	122 (94.6)	129 (100)
	TLR-4 Asp299Gly Polymorphism Distribution		
Patient	1 (3.4)	28 (96.6)	29 (100)
Control	4 (4.0)	96 (96.0)	100 (100)
Total	5 (3.9)	124 (96.1)	129 (100)

Table 3. IFN-γ Allel Distribution

	Guanin-Cytosine (GC), No. (%)	**Guanin-Guanin (GG), No. (%)**	**Cytosine-Cytosine (CC), No. (%)**
Patient	9 (31.0)	18 (62.1)	2 (6.9)
Control	20 (20.0)	80 (80.0)	0 (0)
Total	29 (22.5)	98 (76.0)	2 (1.6)

cally significant correlation was detected between the mutations and the presence of cavitation. AFB positivity was detected in 25 patients, and 4 had a negative microscopic examination. Cultures were positive in 26 patients. The three culture-negative cases were diagnosed histopathologically. No statistically significant relationship was detected among AFB positivity, culture positivity, TLR-2 and TLR-4 mutations, and the IFN-γ allele distribution (Table 5). Drug resistance and extrapulmonary tuberculosis were detected in only one patient. This patient had no mutations.

5. Discussion

Toll-like receptors are transmembrane proteins that induce a natural immune response to many pathogens. They are characteristically formed from leucine-rich repeats and intracellular TIR domains. The TLR-mediated signal pathway is triggered when exposed to specific molecules that accompany pathogens. Antimicrobial proteins and inflammatory cytokines are then synthesized. Eleven TLRs have been described to date. Tuberculosis bacilli stimulate the expression of TLR-2 and TLR-4. No further TLRs that recognize tuberculosis bacilli have yet been identified. The mycobacterial ligands recognized by TLRs are lipoarabinomannan, lipomannan, phosphatidylinositol mannoside, and the 19-kDa lipoprotein. After recognition of these receptors, the TLR signal pathway is activated by binding of the TIR domain to MyD88 adaptor protein. IRAK-1, Toll/IL-1 receptor domain-containing adapter protein, and TIR-domain-containing adapter-inducing IFN-β adaptor protein then participate in the activation of mitogen-activated protein kinase and nuclear factor-B in the nucleus. Increasing levels of inflammatory cytokines, especially TNF-α, then initiate the natural immune response to bacteria (3-5, 8).

Many studies have evaluated various mutations and functional disorders since recognition of the roles of TLR-2 and TLR-4 in the immune response to tuberculosis. The most important finding in our study was detection of the higher ratio of TLR-2 polymorphisms in the patients with tuberculosis than in the healthy controls. This finding was similar for TLR-4 polymorphisms, but it was not statistically significant because of the limited number of patients with tuberculosis.

Branger et al. (9) compared mice with and without TLR-4. All mice were intranasally inoculated with a mycobacterial suspension. After infection, the liver, lung, and spleen were extracted and cytokine levels were measured. Cultures were performed, and all tissues were examined histologically. Mice with TLR-4 were still alive at week 15 of the study. However, 7 of the 12 TLR-4-deficient mice died, and this rate was statistically significant ($P < 0.002$). No difference was observed when the mice were infected with

Table 4. TLR-2, TLR-4 Polymorphism and Interferon Allel Distribution of the Patients with Tuberculosis

Patient	IFN-γ allel Distribution	TLR-2 Polymorhism	TLR-4 Asp299Gly Polymorphism	TLR-4 Thr399Ile Polymorphism
1	GC	Normal	Normal	Normal
2	GG	Normal	Normal	Normal
3	GG	Mutant	Normal	Normal
4	GC	Mutant	Normal	Normal
5	CC	Heterozygote	Normal	Normal
6	CC	Heterozygote	Normal	Normal
7	GG	Normal	Heterozygote	Normal
8	GG	Heterozygote	Normal	Mutant
9	GC	Heterozygote	Normal	Normal
10	GG	Heterozygote	Normal	Normal
11	GG	Normal	Normal	Normal
12	GC	Heterozygote	Normal	Normal
13	GG	Normal	Normal	Normal
14	GG	Normal	Normal	Normal
15	GC	Heterozygote	Normal	Normal
16	GC	Heterozygote	Normal	Normal
17	GC	Normal	Normal	Normal
18	GG	Normal	Normal	Normal
19	GG	Heterozygote	Normal	Normal
20	GG	Heterozygote	Normal	Normal
21	GG	Heterozygote	Normal	Normal
22	GG	Heterozygote	Normal	Normal
23	GG	Heterozygote	Normal	Normal
24	GG	Normal	Normal	Normal
25	GC	Heterozygote	Normal	Normal
26	GC	Normal	Normal	Normal
27	GG	Heterozygote	Normal	Normal
28	GC	Heterozygote	Normal	Normal
29	GG	Heterozygote	Normal	Normal

Table 5. The Relationship Between AFB Direct Microscopic Examination, Culture Positivity and TLR-2 Polymorphism

TLR-2 Polmorphism	AFB Positivity		AFB Culture Positivity	
	(+)	(-)	(+)	(-)
Normal	8	2	9	1
Heterozygote	15	2	15	2
Mutant	2	-	2	-
Total	25	4	26	3

higher numbers of bacilli. Measurement of the mycobacterial load and bacterial growth in the lung showed that

the TLR-4-deficient mice had a 3-fold higher bacterial load ($P < 0.004$). Cytokine levels were also significantly lower in the TLR-4-deficient mice; this may have been related to a decreased inflammatory response. All of these results indicate a protective role of TLR-4 in pulmonary tuberculosis of mice.

Kamath et al. (10) also evaluated TLR-4-deficient mice and normal mice. A tuberculosis bacilli suspension was administered intranasally. Bronchoalveolar lavage was then performed, and the TNF-α, IL-12, and IFN-γ levels were measured. The TNF-α, IL-12 and IFN-γ levels did not differ between the two groups. The survival rates were also similar between the two groups. The TLR-4-defective mice showed no tendency to develop pulmonary tuberculosis.

Various animal studies have been performed to investigate TLR-4 and tuberculosis infection; most were carried out using TLR-4-deficient mice. Some studies reported a role of TLR-4 deficiency in tuberculosis infection, while others did not support this theory. Abel et al. (11) investigated the function of TLR-4 deficiency in tuberculosis infection and showed the importance of TLR-4 in monitoring tuberculosis infection. In contrast, Shim et al. (12) hypothesized that TLR-4 does not have a role in tuberculosis infection.

Drennan et al. (13) compared TLR-2-defective and normal mice. Toll-like receptor 2-defective mice that were infected with tuberculosis bacteria by an aerosol suspension showed decreased bacterial clearance, a defective granulomatous response, and chronic pneumonia. A pulmonary immune response analysis showed that TLR-2-deficient mice had decreased levels of TNF-α, IFN-γ, and IL-12.

Sugawara et al. (14) detected low levels of TNF-α, transforming growth factor-β, IL-1β, nitric oxide synthase, and IL-2 in TLR-2-deficient mice and emphasized the role of TLR-2 in defense against tuberculosis. Newport et al. (15) reported a relationship between TLR-4 mutation and tuberculosis infection in 2004. In their study, which took place in Gambia, 320 patients with tuberculosis and 320 healthy controls were evaluated. The distribution of TLR-4 Asp299Gly mutations in both groups was compared. No statistically significant difference was detected between the two groups ($P = 0.91$). The TNF-α, IL-β, and IL-10 levels were similar between the patients with tuberculosis and healthy subjects. Comparison of ethnic populations showed similar mutation rates. The authors showed that TLR-4 Asp299Gly mutations were not associated with tuberculosis infection.

Ben-Ali et al. (16) investigated the TLR-2 Arg677Trp mutation in 33 patients with tuberculosis and 333 healthy subjects. The cytosine/tyrosine (C/T) genotype was detected in significantly more patients with tuberculosis than healthy subjects, and the authors reported that this polymorphism is a risk factor for tuberculosis. Yim et al. (17) reported that TLR-2 deficiency predisposed patients to tuberculosis infection.

Ogus et al. (18) evaluated the presence of the TLR-2 Arg753Gln polymorphism in 151 patients with tuberculosis and 116 healthy subjects. Patients with DM, immunosuppression, and malnutrition were excluded. Twenty-seven (9.3%) patients and nine (1.7%) controls had the adenosine/adenosine (A/A) allele. The A/A genotype was clearly associated with tuberculosis infection. Toll-like receptor 2 polymorphisms were not related to the localization of the disease.

In the present study, we found no relationship between TLR-4 polymorphisms and tuberculosis infection. Toll-like receptor 2 polymorphisms were statistically increased in patients with tuberculosis compared with healthy subjects. The IFN-γ allele distribution was not different between the two groups. No relationship was detected between the severity of the disease and various parameters such as chest X-ray findings and AFB positivity. Measurement of IFN-γ and TNF-α can indicate the immune response status. Toll-like receptor 2 polymorphism is a risk factor for tuberculosis infection. The limiting factor in this study was the lack of measurement of the IFN-γ and TNF-α levels, which are important in the development of infection. Detection of lower levels of these cytokines in bronchoalveolar lavage specimens, especially among TLR-2-defective patients, may provide new data in support of our findings.

Toll-like receptor agonist development, immunity, easier and more rapid polymorphism detection, and prophylaxis are developing fields of investigation. Greater numbers of patients are required for further studies, which should include evaluation of immunological parameters.

Footnotes

Authors' Contribution: Study concept and design, Oguz Oben Biyikli, Mustafa Hikmet Ozhan and Afig Berdeli; analysis and interpretation of data, Oguz Oben Biyikli, Afig Berdeli, Baysak and Gulfem Ece; drafting of the manuscript, Oguz Oben Biyikli, Aysegul Baysak, Gulfem Ece and Adnan Tolga Oz; critical revision of the manuscript for important intellectual content, Mustafa Hikmet Ozhan, Baysak, Oguz Oben Biyikli, Adnan Tolga Oz, Gulfem Ece and Afig Berdeli; statistical analysis, Oguz Oben Biyikli, Tolga Oz and Aysegul Baysak.

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