Toll-Like Receptor 4 Gene (TLR4), but Not TLR2, Polymorphisms Modify the Risk of Tonsillar Disease Due to Streptococcus pyogenes and Haemophilus influenzae

Kyriaki Liadaki,1 Efthimia Petinaki,2 Charalampos Skoulakis,3 Paraskevi Tsirevelou,3 Dimitra Klapsa,2 Anastasios E. Germenis,1 and Matthaios Speletas1*

Department of Immunology and Histocompatibility, University of Thessaly Medical School, 41110 Biopolis, Larissa, Greece;2 Department of Microbiology, University of Thessaly Medical School, 41110 Biopolis, Larissa, Greece;3 and ENT Department, General Hospital of Volos, 38222 Volos, Greece3

Received 15 October 2010/Returned for modification 15 November 2010/Accepted 8 December 2010

Tonsillar disease (recurrent tonsillitis and/or tonsillar hypertrophy) is one of the most common human disorders, with Streptococcus pyogenes (group A beta-hemolytic streptococcus [GAS]) and Haemophilus influenzae representing the most common pathogens. Until now, no study has investigated why some individuals are more susceptible to tonsillar infections caused by specific bacteria than others. The aim of this study was to uncover possible associations between common Toll-like receptor gene (TLR) polymorphisms and tonsillar disease. The TLR2-R753Q, TLR4-D299G, and TLR4-T399I polymorphisms were determined in a cohort of 327 patients subjected to tonsillectomy due to recurrent tonsillitis (n = 245) and tonsillar hypertrophy (n = 82) and 245 healthy bone marrow donors. Associations of the aforementioned polymorphisms with the isolated bacterial strains after tonsillectomy were also investigated. Interestingly, carriers of the TLR4 polymorphisms displayed an approximately 3-fold increased risk for GAS infections (for TLR4-D299G, odds ratio [OR] = 2.81, 95% confidence interval [CI] = 1.16 to 6.79, P = 0.038; for TLR4-T399I, OR = 3.01, 95% CI = 1.29 to 7.02, P = 0.023), and this association was more profound in patients with recurrent tonsillitis. On the contrary, the presence of the TLR4-T399I polymorphism was associated with a 2-fold decreased risk of Haemophilus influenzae carriage (OR = 0.38, 95% CI = 0.15 to 0.96, P = 0.038). In the end, no significant differences were observed, considering the genotype and allele frequencies of the above-mentioned polymorphisms, between patients and controls. Our findings indicate that, regarding tonsillar infections, TLR4 polymorphisms predispose individuals to GAS infection, while they are protective against Haemophilus influenzae infection. This result further elucidates the role that host immune genetic variations might play in the susceptibility to common infections and tonsillar disease.

Tonsillar disease is one of the most common human disorders and is accompanied by symptoms such as recurrent acute tonsillitis, peritonsillar abscess, and tonsillar hypertrophy (TH). Tonsillitis is indicated in cases of recurrent or persistent symptoms of tonsillar infection or hypertrophy and has functional consequences (dyspnea or dysphagia, mouth breathing, obstructive sleep apnea, etc.), and it represents one of the most frequent operations performed, especially in children (10, 34). Several studies have demonstrated that Streptococcus pyogenes (group A beta-hemolytic streptococci [GAS]) and Haemophilus influenzae, isolated in 14 to 37% and 16 to 32% of the patients, respectively, are the most common bacteria causing tonsillar disease (10, 15, 19, 30, 34). The same bacteria, however, have also been isolated from the tonsils of healthy individuals (34). In general, no significant variations in microbial flora between patients with tonsillar disease and healthy controls have been observed (32, 34). Therefore, the emerging and still unanswered questions are why are some individuals more susceptible to tonsillar infections, as well as why do some of them suffer from infections by specific bacteria? Although environmental factors, such as day care attendance or the number of siblings, influence the morbidity from common bacteria (1), it is noteworthy that a genetic predisposition for tonsillar disease has also been observed (14).

Early studies have shown that protection against infections is linked to the ability of the host to generate sufficient immune responses against essential bacterial elements (11, 13). To this end, the family of Toll-like receptors (TLRs) plays a central role, recognizing evolutionarily conserved pathogen-associated molecular patterns (PAMPs) present on most types of microorganisms (9). Among them, TLR2 and TLR4 are located on the cell surface and recognize bacterial products unique to the invading organisms, including GAS and Haemophilus influenzae (20, 24, 31). Recently, single nucleotide polymorphisms (SNPs) of TLR2 and TLR4 have been associated with receptor hyporesponsiveness and immunopathology, including susceptibility to bacterial, fungal, and viral infections (23). However, the possible contribution of these SNPs toward tonsillar immunopathology is completely unknown.

The aim of this study was to investigate whether common SNPs of TLR2 and TLR4, in particular, TLR2-R753Q (SNP database [dbSNP] accession no. rs5743708), TLR4-D299G (dbSNP accession no. rs4986790), and TLR4-T399I (dbSNP database [dbSNP] accession no. rs11549), are located on TLR2 and TLR4, respectively, are the most common bacteria causing tonsillar disease (10, 34). In general, no significant variations in microbial flora between patients with tonsillar disease and healthy controls have been observed (32, 34). Therefore, the emerging and still unanswered questions are why are some individuals more susceptible to tonsillar infections, as well as why do some of them suffer from infections by specific bacteria? Although environmental factors, such as day care attendance or the number of siblings, influence the morbidity from common bacteria (1), it is noteworthy that a genetic predisposition for tonsillar disease has also been observed (14).

Early studies have shown that protection against infections is linked to the ability of the host to generate sufficient immune responses against essential bacterial elements (11, 13). To this end, the family of Toll-like receptors (TLRs) plays a central role, recognizing evolutionarily conserved pathogen-associated molecular patterns (PAMPs) present on most types of microorganisms (9). Among them, TLR2 and TLR4 are located on the cell surface and recognize bacterial products unique to the invading organisms, including GAS and Haemophilus influenzae (20, 24, 31). Recently, single nucleotide polymorphisms (SNPs) of TLR2 and TLR4 have been associated with receptor hyporesponsiveness and immunopathology, including susceptibility to bacterial, fungal, and viral infections (23). However, the possible contribution of these SNPs toward tonsillar immunopathology is completely unknown.

The aim of this study was to investigate whether common SNPs of TLR2 and TLR4, in particular, TLR2-R753Q (SNP database [dbSNP] accession no. rs5743708), TLR4-D299G (dbSNP accession no. rs4986790), and TLR4-T399I (dbSNP database [dbSNP] accession no. rs11549), are located on TLR2 and TLR4, respectively, are the most common bacteria causing tonsillar disease (10, 34). In general, no significant variations in microbial flora between patients with tonsillar disease and healthy controls have been observed (32, 34). Therefore, the emerging and still unanswered questions are why are some individuals more susceptible to tonsillar infections, as well as why do some of them suffer from infections by specific bacteria? Although environmental factors, such as day care attendance or the number of siblings, influence the morbidity from common bacteria (1), it is noteworthy that a genetic predisposition for tonsillar disease has also been observed (14).

Early studies have shown that protection against infections is linked to the ability of the host to generate sufficient immune responses against essential bacterial elements (11, 13). To this end, the family of Toll-like receptors (TLRs) plays a central role, recognizing evolutionarily conserved pathogen-associated molecular patterns (PAMPs) present on most types of microorganisms (9). Among them, TLR2 and TLR4 are located on the cell surface and recognize bacterial products unique to the invading organisms, including GAS and Haemophilus influenzae (20, 24, 31). Recently, single nucleotide polymorphisms (SNPs) of TLR2 and TLR4 have been associated with receptor hyporesponsiveness and immunopathology, including susceptibility to bacterial, fungal, and viral infections (23). However, the possible contribution of these SNPs toward tonsillar immunopathology is completely unknown.
acquisition no. rs4986791), are associated with GAS and *Haemophilus influenzae* infections, influencing their virulence and the ability to cause tonsillar disease.

**MATERIALS AND METHODS**

**Collection of specimens.** Three-hundred twenty-seven patients (173 males, 154 females; mean age, 12.6 years; age range, 3 to 59 years) who underwent a total or partial tonsillectomy between January 2006 and January 2010 were enrolled in the study. The patients were categorized into two groups: the recurrent tonsillitis (RT) group (n = 245 patients; 131 males, 114 females) and the TH group (n = 82 patients; 42 males, 40 females). Patients with recurrent tonsillitis who had had at least three episodes in each of the prior 3 years, five episodes in each of 2 years, or seven episodes in 1 year were categorized into group 1, while patients with tonsillar hypertrophy accompanied by snoring, dysphagia, mouth breathing, and sleep apnea syndrome but without recurrent tonsillitis were categorized into group 2. Patients receiving antibiotics 2 weeks prior to surgery were excluded. Surgery was performed during periods when the patients were free from symptoms. Throat swab specimens were again taken from each patient prior to surgery. Tonsillar tissues extracted during surgery were immediately transferred into sterile dry containers and shipped to the laboratory. Tonsillar tissues were cut into several pieces (2 to 4 mm), divided into three parts, and stored at −80°C for conventional culture, molecular analyses, and archival material.

All samples came from unrelated individuals who were ethnic Greeks, as assessed by questionnaire. The study was approved by the Institutional Review Board of the University Hospital of Larissa, and written informed consent was obtained from each individual or an accompanying relative, in the case of patients whose consent was not legally applicable (e.g., with children).

**Conventional cultures.** For each patient, throat swabs obtained prior to the operation, as well as deep tonsillar tissue pieces obtained post-surgery, were inoculated onto 5% sheep blood Columbia and chocolate agar (bioMérieux, Marcy l’Etoile, France) and incubated at 37°C in a 5% CO₂ atmosphere and an anaerobic atmosphere for 10 days. Gram stain was performed on all specimens in order to evaluate whether leukocytes and microbial flora were present. The isolates in pure bacterial cultures, obtained by picking isolated colonies, were identified with commercially available biochemical assays and, when needed, with molecular identification methods (18, 22).

**Detection of bacterial DNA and RNA.** Four individual pieces of deep tonsillar tissue from each patient were chosen for simultaneous extraction of DNA and RNA, using commercial kits (Invitrogen, CA), according to the manufacturer’s instructions. In addition, the identity of the RNA extract was confirmed by electrophoresis, after a 10-min digestion at 37°C with DNase-free RNase (Qiagen, Crawley, United Kingdom). The efficiency of DNA extraction and the possible presence of inhibitors in the sample were confirmed by the detection of the β₂-microglobulin gene (26). The PCR primers for the β₂-microglobulin gene were forward primer 5′-GAAGCGCAAGGACAGGTA C-3′ and reverse primer 5′-CAACCTTACACGGTTCACC-3′, and the PCR conditions were 4 min at 94°C, followed by 35 cycles of 94°C for 60 s, 58°C for 60 s, and 72°C for 60 s and 10 min at 72°C after the last cycle. Both DNA and RNA were stored at −80°C.

The presence of bacterial DNA was determined by PCR targeting the 16S rRNA gene as previously described (22), with positive and negative controls included in the reaction according to standard procedures. The universal PCR primers used were forward primer 5′-TATGTCCAGAGGCTGGGAA-3′ and reverse primer 5′-TGAACAATCCCAAGAACAGTAC-3′, and the PCR conditions were 2 min at 94°C, followed by 38 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 45 s and 5 min at 72°C after the last cycle. The presence of undigested PCR products was indicative of wild-type samples, whereas the presence of the polymorphism resulted in the digestion of the PCR products to 375- and 32-bp fragments. For the *TLR1* and *TLR4* SNPs, a 249-bp fragment was amplified by PCR and subjected to Ncol digestion (Invitrogen, United Kingdom) for 4 h at 37°C. The presence of undigested PCR products was indicative of wild-type samples, whereas the presence of the polymorphism resulted in the digestion of the PCR products to 375- and 32-bp fragments.

**Detection of the *TLR2* and *TLR4* SNPs.** Detection of the *TLR4-D299G* and *TLR4-T399I* SNPs was performed by allele-specific PCR, followed by restriction fragment length polymorphism (PCR-RFLP) analysis, as described previously (16, 29). The primers for the detection of the *TLR4-D299G* SNP were forward primer 5′-GATTACGATCTAGACTACT CACG(G)CATG-3′ and reverse primer 5′-GATCAACTCTGGAGAAAAAGCA TCCAC-3′. The primers for the detection of the *TLR4-T399I* SNP were forward primer 5′-GGTGCGCTGTCAAGTATTTTTGGGA(G)CAA-3′ and reverse primer 5′-GATCAACTCTGGAGAAAAAGCA TCCAC-3′. The nucleotides in parentheses were modified and changed to the underlined ones. The conditions of both PCRs were 2 min at 94°C, followed by 32 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 60 s and 5 min at 72°C after the last cycle. For the *TLR4-D299G* SNP, a 249-bp fragment was purified by amplification by PCR and subjected to Ncol digestion (Invitrogen, United Kingdom) for 4 h at 37°C. The presence of undigested PCR products was indicative of wild-type samples, whereas the presence of the polymorphism resulted in the digestion of the PCR products to 375- and 32-bp fragments. The detection of the *TLR2-R753Q* polymorphism was also performed by PCR-RFLP analysis (27). The protocol was designed on the basis of the fact that the polymorphism results in the creation of a DNA sequence recognized by the restriction enzyme SfiI (New England Biolabs). The PCR primers were forward primer 5′-TATGTCCAGAGGCTGGGAA-3′ and reverse primer 5′-TGACA AAAAGTCCCAAGAACAGTAC-3′, and the PCR conditions were 2 min at 94°C, followed by 38 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 45 s and 5 min at 72°C after the last cycle. The presence of undigested PCR products (430 bp) was indicative of wild-type samples, whereas the presence of the polymorphism resulted in the digestion of the PCR products to 307- and 123-bp fragments.

For all PCRs described here, a total of 100 to 200 ng of DNA was amplified in a 30-μl reaction mixture using 62.5 μM each deoxynucleoside triphosphate, 20 pmol of each primer, 1.5 mM MgCl₂, and 1.0 U Taq polymerase (Invitrogen, United Kingdom) in a buffer supplied by the manufacturer. All PCR and digestion procedures were carried out in the PCR engine apparatus FTC-200 (MJ Research, Watertown, MA), and the PCR and digestion products were analyzed in 2% TBE (Tris-borate-EDTA) agarose gels.

**Statistical analysis.** Pearson's chi-square test with Yates' continuity correction was used to compare the allele and genotype frequencies between BMDs, patients, and patient subgroups, while Fisher's exact test was performed when needed. Continuous variables were compared using the nonparametric Mann-Whitney U test. An association was expressed as the odds ratio (OR) with the corresponding 95% confidence interval (CI), and a variable was considered significant when P < 0.05. Finally, Spearman's correlation coefficient (r) was used to correlate the presence of SfiI with age and disease. The above analyses were performed using the Statistical Package for the Social Sciences (SPSS; version 13; Chicago, IL). Moreover, the deviations from Hardy-Weinberg equilibrium were evaluated using the freely available software Arlequin (version 3.11; http://cmpg.unibe.ch/software/arlequin3).
RESULTS

TLR polymorphism analysis in patients with tonsillar disease and BMDs. None of the patients with tonsillar disease showed homozygosity for any SNP or double heterozygosity of the TLR2-R753Q and TLR4-D299G or TLR4-T399I polymorphisms. Moreover, the G allele of the TLR4-D299G SNP has a high level of linkage disequilibrium with allele C of the TLR4-T399I SNP for both patients and BMDs, as reported previously (28). When the allele frequencies of the TLR4-D299G, TLR4-T399I, and TLR2-R753Q polymorphisms are considered, no significant differences were observed between patients and controls (5.05% and 6.94%, respectively, for TLR4-D299G [P = 0.203]; 5.51% and 6.74%, respectively, for TLR4-T399I [P = 0.452]; and 1.07% and 0.61%, respectively, for TLR2-R753Q [P = 0.339]). Moreover, no significant differences in genotype and allele frequencies of the aforementioned SNPs and age in the different subgroups analyzed in the study. Heter, heterozygous; Homoz, homozygous.

Among patients below age 14 years than adult patients (Table 1). This finding was also profound even for carriers of both TLR4 polymorphisms (allele frequency, 21.6% versus 8.4%, odds ratio = 3.00, 95% CI = 1.23 to 7.28, P = 0.019; allele frequency, 10.8% versus 4.2%, odds ratio = 2.94, 95% CI = 1.21 to 7.13, P = 0.021). On the other hand, no association of the TLR2-R753Q polymorphism with GAS was found (P = 0.418 and P = 0.419 for genotype and allele frequencies, respectively). Moreover, we also observed that GAS infections were more prominent in patients below age 14 years than adult patients (Table 1).

Further analysis regarding the different tonsillar groups demonstrated a strong association between both TLR4 SNPs and GAS infection only for the RT group. In particular, patients with TLR4-T399I and recurrent tonsillitis exhibited a 2.6-fold increased risk for GAS infections (P = 0.043, 95% CI = 1.12 to 6.25), while the presence of the TLR4-D299G polymorphism was also associated with a 2.2-fold increased risk, but the difference did not reach statistical significance (P = 0.075, 95% CI = 0.94 to 6.32). In the end, carriers of both TLR4 SNPs displayed a 2.5-fold increased risk for GAS infections; however, the difference was not significant (odds ratio = 2.55, 95% CI = 0.98 to 6.63, P = 0.068).

On the other hand, the presence of the TLR4-D299G and TLR4-T399I polymorphisms was not associated with GAS infections in the TH group (P = 0.273 and P = 0.362, respectively). In the end, no association of the TLR2-R753Q SNP with GAS infections was observed for either of the two groups.

Interestingly, as presented in Table 2, the presence of the TLR4-T399I polymorphism was associated with an almost 2-fold decreased risk of Haemophilus influenzae infection, as indicated by genotype and allele frequency analyses. Interestingly, an almost similar association was observed with the TLR4-D299G polymorphism, but it failed to reach statistical significance (Table 2). Moreover, the carriers of both TLR4 SNPs displayed a 2-fold decreased risk of Haemophilus influenzae infections. Among patients with tonsillar disease, Haemophilus influenzae was isolated from 105 patients (32.1%) and GAS was isolated from 38 (11.6%), while both bacteria were identified in 10 of these patients. Consequently, we compared the prevalence of TLR polymorphisms in subgroups of patients according to the cause of tonsillar disease. As shown in Table 1, the TLR4-D299G and TLR4-T399I polymorphisms were strongly associated with GAS, since the carriers displayed an approximately 3-fold increased risk for GAS infection (Table 1). This finding was also profound even for carriers of both TLR4 polymorphisms (genotype frequency, 21.6% versus 8.4%, odds ratio = 3.00, 95% CI = 1.23 to 7.28, P = 0.019; allele frequency, 10.8% versus 4.2%, odds ratio = 2.94, 95% CI = 1.21 to 7.13, P = 0.021). On the other hand, no association of the TLR2-R753Q polymorphism with GAS was found (P = 0.418 and P = 0.419 for genotype and allele frequencies, respectively). Moreover, we also observed that GAS infections were more prominent in patients below age 14 years than adult patients (Table 1). Further analysis regarding the different tonsillar groups demonstrated a strong association between both TLR4 SNPs and GAS infection only for the RT group. In particular, patients with TLR4-T399I and recurrent tonsillitis exhibited a 2.6-fold increased risk for GAS infections (P = 0.043, 95% CI = 1.12 to 6.25), while the presence of the TLR4-D299G polymorphism was also associated with a 2.2-fold increased risk, but the difference did not reach statistical significance (P = 0.075, 95% CI = 0.94 to 6.32). In the end, carriers of both TLR4 SNPs displayed a 2.5-fold increased risk for GAS infections; however, the difference was not significant (odds ratio = 2.55, 95% CI = 0.98 to 6.63, P = 0.068).

On the other hand, the presence of the TLR4-D299G and TLR4-T399I polymorphisms was not associated with GAS infections in the TH group (P = 0.273 and P = 0.362, respectively). In the end, no association of the TLR2-R753Q SNP with GAS infections was observed for either of the two groups. Interestingly, as presented in Table 2, the presence of the TLR4-T399I polymorphism was associated with an almost 2-fold decreased risk of Haemophilus influenzae infection, as indicated by genotype and allele frequency analyses. Interestingly, an almost similar association was observed with the TLR4-D299G polymorphism, but it failed to reach statistical significance (Table 2). Moreover, the carriers of both TLR4 SNPs displayed a 2-fold decreased risk of Haemophilus influenzae infections. Among patients with tonsillar disease, Haemophilus influenzae was isolated from 105 patients (32.1%) and GAS was isolated from 38 (11.6%), while both bacteria were identified in 10 of these patients. Consequently, we compared the prevalence of TLR polymorphisms in subgroups of patients according to the cause of tonsillar disease. As shown in Table 1, the TLR4-D299G and TLR4-T399I polymorphisms were strongly associated with GAS, since the carriers displayed an approximately 3-fold increased risk for GAS infection (Table 1). This finding was also profound even for carriers of both TLR4 polymorphisms (genotype frequency, 21.6% versus 8.4%, odds ratio = 3.00, 95% CI = 1.23 to 7.28, P = 0.019; allele frequency, 10.8% versus 4.2%, odds ratio = 2.94, 95% CI = 1.21 to 7.13, P = 0.021). On the other hand, no association of the TLR2-R753Q polymorphism with GAS was found (P = 0.418 and P = 0.419 for genotype and allele frequencies, respectively). Moreover, we also observed that GAS infections were more prominent in patients below age 14 years than adult patients (Table 1). Further analysis regarding the different tonsillar groups demonstrated a strong association between both TLR4 SNPs and GAS infection only for the RT group. In particular, patients with TLR4-T399I and recurrent tonsillitis exhibited a 2.6-fold increased risk for GAS infections (P = 0.043, 95% CI = 1.12 to 6.25), while the presence of the TLR4-D299G polymorphism was also associated with a 2.2-fold increased risk, but the difference did not reach statistical significance (P = 0.075, 95% CI = 0.94 to 6.32). In the end, carriers of both TLR4 SNPs displayed a 2.5-fold increased risk for GAS infections; however, the difference was not significant (odds ratio = 2.55, 95% CI = 0.98 to 6.63, P = 0.068).

On the other hand, the presence of the TLR4-D299G and TLR4-T399I polymorphisms was not associated with GAS infections in the TH group (P = 0.273 and P = 0.362, respectively). In the end, no association of the TLR2-R753Q SNP with GAS infections was observed for either of the two groups. Interestingly, as presented in Table 2, the presence of the TLR4-T399I polymorphism was associated with an almost 2-fold decreased risk of Haemophilus influenzae infection, as indicated by genotype and allele frequency analyses. Interestingly, an almost similar association was observed with the TLR4-D299G polymorphism, but it failed to reach statistical significance (Table 2). Moreover, the carriers of both TLR4 SNPs displayed a 2-fold decreased risk of Haemophilus influenzae infections.
enzae infection, but this difference also failed to be significant (odds ratio = 0.45, 95% CI = 0.16 to 1.25, \( P = 0.131 \)). On the other hand, the presence of the TLR2-753Q SNP did not affect the possibility of Haemophilus influenzae infection (Table 2). A further analysis regarding the RT and TH groups did not reveal any significant association between TLR polymorphisms and Haemophilus influenzae infections (data not shown).

As mentioned above, both bacterial strains were isolated from 10 patients (5 males, 5 females; mean ± standard deviation [SD] age, 9.1 ± 6.3 years; 8 patients with RT and 2 with TH). Interestingly, in this group the genotype and allele frequencies of both TLR4 polymorphisms were extremely high (30% and 15%, respectively); however, due to the low number of these patients, further statistical analysis was not performed.

### DISCUSSION

To the best of our knowledge, this is the first study presenting strong associations between common TLR polymorphisms and tonsillar disease. Analyzing a large cohort of patients subjected to tonsillectomy due to recurrent tonsillitis and/or tonsillar hypertrophy, clear evidence demonstrating that TLR4 polymorphisms are associated with an increased risk of recurrent tonsillitis due to GAS infections is provided. Simultaneously, their presence, especially the presence of the TLR4-T399I SNP, seems to provide protection against Haemophilus influenzae infection and/or carriage. On the contrary, no association between the TLR2-753Q polymorphism and a predisposition to tonsillar disease was found.

GAS is an important human Gram-positive pathogen responsible for a wide spectrum of infections, ranging from mild diseases (e.g., acute tonsillitis) to serious illnesses (e.g., recurrent tonsillitis, necrotizing fasciitis, sepsis, and severe post-streptococcal sequelae) (3, 4). The factors that provoke pathogen virulence, enabling GAS to escape immune surveillance or, on the contrary, inducing an overreaction of the immune system, are not clear (3, 4). Recent studies have demonstrated that a multimodal recognition of GAS by TLRs, including TLR2 and TLR4, takes place, in which a combination of different TLR-mediated signals is essential for a rapid and effective response to the pathogen (5, 31, 35). The two TLR4 SNPs analyzed in this study (D229G and T399I) are located in the third exon of the gene and alter the extracellular domain of the receptor (2). They have been associated with receptor hyporesponsiveness in macrophages, epithelial cells, and peripheral blood mononuclear cells, resulting in impaired TLR4 signaling (2). Thus, we could expect that the presence of TLR4 SNPs

### TABLE 1. Association of TLR polymorphisms with Streptococcus pyogenes (GAS) infections in patients with tonsillar disease

| Variable | GAS-positive patients | GAS-negative patients | \( P \) valueb | OR (95% CI) |
|----------|-----------------------|-----------------------|---------------|-------------|
| No. of patients | 38 | 289 |
| Mean ± SD age (yr) | 9.2 ± 7.13 | 13.1 ± 11.1 | 0.430 |
| No. (%) below age 14 yr | 32 (84.2) | 182 (64.6) | 0.011 |
| No. (%) above age 14 yr | 6 (15.8) | 104 (36.4) |
| Sex (no. male/no. female) | 19/19 | 154/135 | 0.732 |
| **TLR4-D229G** | | | | |
| No. (%) of patients with genotype: | | | | |
| A/A (wt) | 30 (78.9) | 264 (91.3) | 0.038 |
| A/G (het) | 8 (21.1) | 25 (8.7) |
| G/G (hom) | 0 (0) | 0 (0) |
| No. (%) of patients with allele with: | | | | |
| A | 68 (89.4) | 553 (95.6) | 0.044 |
| G | 8 (10.5) | 25 (4.3) |
| **TLR4-T399I** | | | | |
| No. (%) of patients with genotype: | | | | |
| C/C (wt) | 29 (76.3) | 262 (90.7) | 0.023 |
| C/T (het) | 9 (23.7) | 27 (9.3) |
| T/T (hom) | 0 (0) | 0 (0) |
| No. (%) of patients with allele with: | | | | |
| C | 67 (88.1) | 551 (95.3) | 0.027 |
| T | 9 (11.8) | 27 (4.6) |
| **TLR2-R753Q** | | | | |
| No. (%) of patients with genotype: | | | | |
| G/G (wt) | 38 (100) | 282 (97.6) | 0.418 |
| G/A (het) | 0 (0) | 7 (2.4) |
| A/A (hom) | 0 (0) | 0 (0) |
| No. (%) of patients with allele with: | | | | |
| G | 76 (100) | 571 (99.7) | 0.419 |
| A | 0 (0) | 7 (1.21) |

a Comparisons of genotypes were carried out between wild-type and the sum of mutated alleles (the \( \chi^2 \) two-sided test was performed for TLR4 SNPs and the \( \chi^2 \) one-sided test was performed for TLR2-R753Q). Abbreviations: wt, wild type; het, heterozygous; hom, homozygous.

b Boldface values indicate statistically significant differences.
might result in impaired signaling, induced by hyaluzonan and/or other GAS cytolysins, leading to a reduced clearance of pathogens, invasive disease, and recurrent infections. Interestingly, similar results have also been reported by Yuan et al., who showed that the genetic variability of \( TLR4 \) is associated with an increased risk of developing invasive disease by \( Strep-tococcus pneumoniae \), another Gram-positive bacterium (36). Moreover, we demonstrated that the carriers of the \( TLR4 \)-\( T399I \) SNP displayed a decreased risk for \( Haemophilus influenzae \) carriage and/or infection, namely, the opposite effect of the presumed receptor hyporesponsiveness. Similar results have also been reported recently by Hawn et al., who showed that the presence of the \( TLR4 \)-\( D299G \) SNP was associated with protection from recurrent cystitis, a condition mainly caused by Gram-negative bacteria (7). This could be considered a paradox, since \( TLR4 \) is the principal receptor of the lipopolysaccharide (LPS) of Gram-negative bacteria (21). However, it has been proposed that the carriers of the above-mentioned \( TLR4 \) SNPs may be more resistant to localized forms of endotoxin-induced inflammation (as in our case) and more susceptible to a systemic inflammatory response initiated or exacerbated by endotoxin (2). On the other hand, in the principal study of Arbour et al., not all the subjects who were hyporesponsive to LPS had \( TLR4 \) mutations, and not everyone with the \( TLR4 \) mutation was hyporesponsive to LPS (2). This suggests that \( TLR4 \) mutations might act in concert with other genetic changes or acquired factors to influence the complex immunologic response to pathogens. For example, the cocolonization of interspecies (GAS versus \( Haemophilus influenzae \)) might be common, and these species may have evolved to have specific mechanisms for targeting one another (14). Thus, the altered immune responses due to the presence of a functional polymorphism could facilitate the colonization of one species (for example, that of GAS in tonsils), preventing the colonization of the other (e.g., \( Haemophilus influenzae \)). Results from in vitro studies that could further elucidate the precise mechanisms of the actual responsiveness of a mutated \( TLR4 \) against specific cellular components of GAS and \( Haemophilus influenzae \) species are still not available.

As mentioned above, the presence of the \( TLR4\)-\( D299G \) SNP, alone or in combination with \( TLR4\)-\( T399I \), was associated with a significantly increased risk for tonsillar GAS infections and a decreased, but not significant, probability for \( Haemophilus influenzae \) ones. Although cells expressing both mutations display less \( TLR4 \) receptor expression and are less responsive to stimulation with LPS (32), initial in vitro studies

### Table 2. Association of TLR polymorphisms with \( Haemophilus influenzae \) infections and/or carriage in patients with tonsillar disease

| Variable                      | \( H. influenzae\)-positive patients | \( H. influenzae\)-negative patients | \( P \) value<sup>b</sup> | OR (95% CI)  |
|-------------------------------|--------------------------------------|--------------------------------------|---------------------------|-------------|
| No. of patients               | 105                                  | 222                                  |                           |             |
| Mean ± SD age (yr)            | 13.1 ± 11.1                          | 12.4 ± 10.7                          | 0.887                     |             |
| No. (%) below age 14 yr       | 65 (61.9)                            | 118 (53.2)                           | 0.153                     | 0.69 (0.43–1.12) |
| No. (%) above age 14 yr       | 40 (38.1)                            | 104 (46.8)                           |                           |             |
| Sex (no. male/no. female)     | 55/50                                | 118/104                              | 0.906                     | 0.97 (0.61–1.54) |

<sup>a</sup> Comparisons of genotypes were carried out between wild-type and the sum of mutated alleles (\( \chi^2 \) two-sided test). Abbreviations: wt, wild type; het, heterozygous; hom, homozygous.

<sup>b</sup> Boldface values indicate statistically significant differences.
indicated that the TLR4-D299G SNP might have a greater functional impact than the TLR4-T399I SNP (2). Moreover, the transfection of THP-1 cells with either the wild-type or the mutant alleles of TLR4 demonstrated that the cells transfected with the TLR4-D299G allele do not respond normally to LPS stimulation, while those transfected with the TLR4-T399I allele have an intermediate response, causing a more mild phenotype (25). In this context, the replacement of the conserved aspartic acid with glycine at position 299 theoretically causes disruption of the α-helical protein structure, resulting in an extended β strand, while substitution of isoleucine for threonine at position 399 should not alter the structure of the extracellular domain of the receptor (6, 25). However, this might not be the case in vivo, since recent gene association studies have shown that the above-mentioned SNPs could affect disease development in a different way. For example, Kiechl et al. observed that the TLR4-D299G SNP has a more profound protective effect against atherosclerosis risk than TLR4-T399I (12), while only the TLR4-T399I SNP seems to affect the susceptibility to ulcerative colitis (33) or the development of chronic pulmonary obstructive disease in smokers (28) and protects Caucasian patients with chronic hepatitis caused by hepatitis C virus from fibrosis progression (8). Similarly, in this study we demonstrated that the presence of the TLR4-T399I SNP confers a more profound effect than TLR4-D299G, predisposing individuals to GAS tonsillar infection and protecting individuals from Haemophilus influenzae tonsillar infection.

In conclusion, our findings indicate that TLR4 polymorphisms predispose individuals to GAS infections and protect them against Haemophilus influenzae infections in the tonsils. This result further elucidates the role that host immune genetic variations might play in susceptibility to common infections and tonsillar disease.

ACKNOWLEDGMENTS

This work was supported by grants of the Research Committee of the University of Thessaly, Thessaly, Greece.

We thank V. Karanikas for critical appraisal of the manuscript.

REFERENCES

1. Anagnostou, G., et al. 1992. Nasopharyngeal colonization during the first year of life. J. Infect. Dis. 165(Suppl.)S58–S62.
2. Arbour, N. C., et al. 2000. TLR4 mutations are associated with endotoxin hyposresponsiveness in humans. Nat. Genet. 25:187–191.
3. Bisno, A. L., M. O. Brito, and C. M. Collins. 2003. Molecular basis of group A streptococcal virulence. Lancet Infect. Dis. 3:191–200.
4. Chatwal, G. S., and D. J. McMillan. 2005. Uncovering the mysteries of invasive streptococcal diseases. Trends Mol. Med. 11:152–155.
5. Cortés, G., and M. R. Wessels. 2009. Inhibition of dendritic cell maturation by group A Streptococcus. J. Infect. Dis. 200:1152–1161.
6. Gibat, J. F., J. Garmier, and B. Robson. 1987. Further developments of protein secondary structure prediction using information theory. J. Mol. Biol. 198:425–443.
7. Hawn, T. R., et al. 2009. Toll-like receptor polymorphisms and susceptibility to urinary tract infections in adult women. PLoS One 4e:9900.
8. Huang, H., et al. 2007. A gene signature identifies the risk of developing cirrhosis in patients with chronic hepatitis C. Hepatology 46:297–306.
9. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. Nat. Immunol. 5:987–995.
10. Jeong, J. H., et al. 2007. Bacteriologic comparison of tonsillar core in recurrent tonsillitis and tonsillar hypertrophy. Laryngoscope 117:2146–2151.
11. Kerakawuchi, H., Y. Kurono, and G. Mogi. 1997. Immune responses against Streptococcus pyogenes in human palatine tonsils. Laryngoscope 107:634–639.
12. Kiechl, S., E. Lorenz, M. Reindl, C. J. Wiedermann, F. Oberhollenzer, E. Bonora, J. Willeit, and D. A. Schwartz. 2002. Toll-like receptor 4 polymorphisms and atherogenesis. N. Engl. J. Med. 347:185–192.
13. Kurono, Y., T. Fujiyoshi, and G. Mogi. 1989. Secretory IgA and bacterial adherence to nasal mucosal cells. Ann. Otol. Rhinol. Laryngol. 98:273–277.
14. Kvestad, E., et al. 2005. Heritability of recurrent tonsillitis. Arch. Otolar- yngol. Head Neck Surg. 131:E33–E36.
15. Lindroos, R. 2000. Bacteriology of the tonsil core in recurrent tonsillitis and tonsillar hyperplasia—a short review. Acta Otolaryngol. 54(Suppl.):206–208.
16. Lorenz, E. K. L. Frees, and D. A. Schwartz. 2001. Determination of the TLR4 genotype using allele-specific PCR. Biotechniques 31:22–24.
17. Lysenko, E. S., A. J. Ratner, A. L. Nelson, and J. N. Weiser. 2005. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. PLoS Pathog. 1:e1.
18. Mogens, K. 2003. Haemophilus, p. 623–635. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. ASM Press, Washington, DC.
19. Moon, E. R. 1986. The carrier state: Haemophilus influenzae. J. Anti- biotics 39:737–746.
20. Park, J. M., V. H. Ng, S. Maeda, R. F. Rest, and M. Karin. 2004. Anthracyclins O and other gram-positive cytolsins are Toll-like receptor 4 agonists. J. Exp. Med. 200:1647–1655.
21. Poltorak, A., et al. 1998. Defective LPS signaling in CH3HeJ and C3BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085–2088.
22. Ruoff, K. L., R. A. Whitey, and D. Brighton. 2003. Streptococcus, p. 405–421. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. ASM Press, Washington, DC.
23. Schröder, N. W., and R. R. Schumann. 2005. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect. Dis. 5:156–164.
24. Schwandner, R., R. Dziarski, H. Wescbe, M. Rothe, and C. J. Kirschning, 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. J. Biol. Chem. 274:17406–17409.
25. Schwartz, D. A. 2002. The genetics of innate immunity. Chest 121 (3 Suppl.): 62S–68S.
26. Skoulakis, C., et al. 2008. Level of Streptococci pyogenes in patients with recurrent tonsillitis and tonsillar hypertrophy. Scand. J. Infect. Dis. 40:899–903.
27. Speletas, M., et al. 2009. TLR2 and TLR4 polymorphisms in familial Mediterranean fever. Hum. Immunol. 70:750–753.
28. Speletas, M., et al. 2009. Association of TLR4-T399I polymorphism with chronic obstructive pulmonary disease in smokers. Clin. Dev. Immunol. 2009:652086.
29. Speletas, M., et al. 2008. Pneumonia caused by Candida krusei and Candida glabrata in a patient with chronic myeloid leukemia receiving imatinib mesylate treatment. Med. Mycol. 46:259–263.
30. Stjernquist-Desatnik, A., K. Pfaller, and C. Schalen. 1991. High recovery of Haemophilus influenzae and group A streptococci in recurrent tonsillar infection or hypertrophy as compared with normal tonsils. J. Laryngol. Otol. 105:439–441.
31. Takeuchi, O., et al. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11:443–451.
32. Toner, G. J., T. J. Stewart, J. B. Campbell, and J. Hunter. 1986. Tonsil flora in the very young tonsillolactomy patient. Clin. Otolaryngol. Allied Sci. 11:171–174.
33. Torok, H. P., J. Glas, L. Tonench, T. Maccass, and C. Fowbacy, 2004. Polymorphisms of the lipopolysaccharide-signaling complex in inflammatory bowel disease: association of a mutation in the Toll-like receptor 4 gene with ulcerative colitis. Clin. Immunol. 112:85–91.
34. Van Stau, B. K., et al. 2003. Does the tonsillar surface flora differ in children with and without tonsillar disease? Acta Otolaryngol. 123:873–878.
35. Voelcker, V., et al. 2008. Hyaluronan fragments induce cytokine and metalloproteinase upregulation in human melanoma cells in part by signalling via TLR4. Exp. Dermatol. 17:100–107.
36. Yuan, F. F., et al. 2008. Clinical relevance of TLR2, TLR4, CD14 and FagammaRIIA gene polymorphisms in Streptococcus pneumoniae infection. Immunol. Cell Biol. 86:268–270.