Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease

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The immunogenetic factors that influence susceptibility to pneumonia are poorly understood. Recent studies suggest an association of toll-like receptor 4 (TLR4) polymorphisms with increased susceptibility to some infections. Here, we examined whether polymorphisms in TLR4 influence susceptibility to Legionnaires' disease (LD) by using a case-control study to compare the allele frequencies of two SNPs (A896G and C1196T). Cases ($n = 108$) were obtained from a LD outbreak in The Netherlands in 1999. Controls were exposed at the same outbreak, did not develop pneumonia, and were either unmatched ($n = 421$) or matched ($n = 89$) to patients for age, sex, and geographic residence. Allele 896G was associated with LD susceptibility with a frequency of 6.5% in the combined control group (matched and unmatched) vs. 2.5% in patients [odds ratio (OR) of 0.36, 95% confidence interval (C.I.) 0.14–0.91, $P = 0.025$]. In the matched control group comparison, allele 896G also showed a protective association with an OR of 0.27 (95% C.I. 0.09–0.75, $P = 0.008$). An analysis of genotype frequencies (896 AA vs. AG and GG) demonstrated similar protective associations (patient vs. combined control group comparison, OR = 0.35, 95% C.I. 0.14–0.89, $P = 0.02$; matched control group comparison, OR = 0.25, 95% C.I. 0.09–0.71, $P = 0.006$). Allele 1196T cosegregated with allele 896G and, thus, had identical associations. Although previous studies suggest that these TLR4 SNPs are associated with an increased risk of infection, this study demonstrates an association with resistance. This protective association illustrates that an innate immune receptor can mediate either beneficial or deleterious inflammatory responses and that these outcomes vary with different pathogens.

genetic markers | immunity | inflammation

Although lower respiratory tract infections are the most common cause of death due to infectious disease in the United States, the influence of host immunogenetic factors on human susceptibility to pneumonia is poorly understood (1). Toll-like receptors (TLRs) constitute a family of transmembrane proteins that differentially recognize pathogen-associated molecular patterns through an extracellular domain and initiate inflammatory signaling pathways through an intracellular domain (2–5). Because of their central role in regulation of the immune response to pathogens, TLRs are excellent candidate genes for genetic susceptibility studies (6). *Legionella pneumophila*, described in 1976 as the agent of Legionnaires' disease (LD), is a flagellated Gram-negative bacterium that causes from 1% to 30% of cases of community-acquired pneumonia (7–11). *In vitro* studies indicate that *Legionella* is recognized by several TLRs, including TLR2, TLR4, and TLR5 (12–15). We recently demonstrated (13, 16, 17) that TLR5 recognizes bacterial flagellin and that a common dominant TLR5 stop codon variant is associated with susceptibility to LD. It is not known whether polymorphisms in other TLRs influence human susceptibility to LD.

TLR4, the receptor for lipopolysaccharide (LPS), has two common polymorphisms (A896G and C1196T) that are associated with LPS hyporesponsiveness in heterozygous individuals in response to inhaled endotoxin (18). Although several small genetic studies suggest a possible association of these SNPs in heterozygous individuals with an increased risk of some bacterial infections, the influence of these SNPs on human infections remains poorly understood and controversial (19–23). Furthermore, inflammatory responses of heterozygous individuals are not uniformly impaired, and the result appears to depend on the measured phenotype (18, 21, 24, 25). In this study, we use genetic studies to understand the role of TLR4 during human infection with *L. pneumophila*. Herein, we demonstrate that these SNPs are associated with resistance to LD in heterozygous individuals and provide previously undocumented evidence that these variants protect individuals from an infection. These data suggest that clinical outcomes associated with TLR4 polymorphisms can vary substantially for different bacteria and illustrate that the beneficial or deleterious consequences of TLR4-mediated host inflammation can be pathogen-specific.

Methods

Human Subjects and Data Collection. Approval for human study protocols was obtained from the human subjects review boards at the University of Amsterdam Medical Center, the University of Washington Medical Center, and the Western Institutional Review Board. All participants gave written, informed consent. Genomic DNA was purified from peripheral blood leukocytes from 10 ml of blood. Enrollment of the patients and controls from the LD outbreak after a flower show in the Netherlands has been described in ref. 13. Of the 188 patients identified in the original investigation of the outbreak, 141 consented for the study. There were 18 individuals who died, and no DNA was available for genotyping; 108 cases were available (93 definite LD, 15 probable LD) with both DNA and epidemiologic data for analysis. Controls were drawn from the exhibitors who had worked at the flower show and were at high risk for exposure to *Legionella*. We contacted 1,616 controls by letter to be in the study. The first 508 who completed the questionnaire had blood drawn for genetic analysis. All of the patients and controls were from The Netherlands, and >95% of both groups were Caucasian–Dutch.

Molecular Biology. SNP discovery and genotyping were performed by PCR amplification of TLR4 from genomic DNA followed by sequencing. The cloned variants of TLR4 were sequenced with Big Dye Terminator version 3.0 and analyzed on an ABI PRISM 3700 capillary sequencer (Applied Biosystems). The sequence was aligned and analyzed with the programs PHRED/PHRAP and CONSED (26). Genotyping was carried out

Abbreviations: TLR, toll-like receptor; LD, Legionnaires' disease; LPS, lipopolysaccharide; OR, odds ratio.

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Table 1. TLR4 SNP allele and genotype frequencies in patients and controls

Base pair	Amino acids	Patients (n = 108), no. (freq.)	All control (n = 508)				Matched control (n = 89)			
			No. (freq.)	OR	95% C.I.	P	No. (freq.)	OR	95% C.I.	P
Allele/haplotype										
896A·1196C	299D, 399T	199 (0.975)	926 (0.935)				159 (0.914)			
896G·1196T	299G, 399I	5 (0.025)	64 (0.065)	0.36	0.14–0.91	0.025	15 (0.086)	0.27	0.09–0.75	0.008
Genotype/diplotype										
896AA·1196CC	299DD, 399TT	97 (0.951)	431 (0.871)				72 (0.828)			
896AG·1196CT	299DG, 399TI	5 (0.049)	64 (0.129)	0.35	0.14–0.89	0.025	15 (0.172)	0.25	0.09–0.71	0.006
896GG·1196TT	299GG, 399II	0 (0.0)	0 (0.0)				0 (0.0)			

TLR4 genotypes were determined in patients and controls. OR and 95% C.I. represent a comparison of the case group with the respective control group with an unadjusted analysis. Numbers in parentheses indicate frequencies (freq.).

with a MassARRAY technique (Sequenom, San Diego), a chip-based MALDI–TOF mass spectrometer-based method (27). Multiplex SNP assays were designed by using SPECTRODESIGNER software (Sequenom); 384-well plates containing 5 ng of DNA in each well were amplified by PCR following Sequenom's specifications. After PCR, arctic shrimp alkaline phosphatase (Sequenom) was added to samples to prevent future incorporation of unused dNTPs that could interfere with the primer extension assay. Allele-discrimination reactions were conducted by adding the extension primer(s), DNA polymerase, and a mixture of dNTPs and dideoxy NTPs to each well. MassEXTEND clean resin (Sequenom) was added to the mixture to remove extraneous salts that could interfere with MALDI–TOF analysis. Genotypes were determined by spotting 15 nl of each sample onto a 384 SpectroCHIP (Sequenom), which was subsequently read by the MALDI–TOF mass spectrometer.

Statistics. Univariate analysis was performed for categorical variables with a χ^2 test and for continuous variables by using a t test. Multivariate logistic regression analysis was performed with SPSS 11.5.0 and HPLUS as described in refs. 13, 28, and 29. Two-tailed testing was used to evaluate statistical significance.

Results and Discussion

We first examined *Legionella* cases for previously undescribed, nonsynonymous TLR4 polymorphisms that might be associated with susceptibility to LD. We sequenced the coding region of TLR4 in 99 *Legionella* cases and found 5 individuals with the previously characterized A896G (D299G) and C1196T (T399I) SNPs and 4 different individuals with a previously described A2081G SNP (K694R, 4 of 96 individuals, National Center for Biotechnology Information dbSNP rs5030722) (18). We detected several rare, nonsynonymous SNPs: G26A (G9E, 1 of 99 individuals), A137G (Y46C, 1 of 99), G842A (C281Y, 2 of 96), A986G (N239S, 1 of 96), G2360A (R787H, 1 of 97), and G2288A (R763H, 1 of 96). We also detected several synonymous SNPs: A267G (E89E, 1 of 95), T315C (S105S, 1 of 99), C435A (P145P, 2 of 96), A1062G (K354K, 1 of 76), C1329T (F443F, 1 of 95), G1959A (K653K, 4 of 96), and T2004G (G668G, 4 of 96). These data indicate that there are no novel high-frequency nonsynonymous SNPs in the patient population.

To determine whether TLR4 is associated with susceptibility to LD, we examined two SNPs (A896G and C1196T) that have previously been shown (18) to be associated with LPS hyporesponsiveness. We used a case-control study design with individuals from the Bovenkarspel epidemic described in refs. 13, 30, and 31). Patients included individuals with radiologically confirmed pneumonia occurring during the epidemic time period around the West Friese flower show in the town of Bovenkarspel in The Netherlands. Controls were drawn from a pool of exhibitors who were likely to have been exposed to the contaminated water product that caused the epidemic. To provide an additional control for population admixture, 89 of the 508 controls were matched to patients for their place of residence ±25 km, as well as for age and sex.

We determined the genotype and allele frequencies of individuals for the two nonsynonymous TLR4 SNPs (Table 1). There was no significant departure from Hardy–Weinberg equilibrium of the observed and expected frequencies of cases or controls for these SNPs. The allele frequency of 896G was 2.5% in the patients compared with 6.5% in the entire control group (includes matched and unmatched controls) and 8.6% in the matched control group. In an unadjusted analysis, the odds ratio (OR) for the association of allele 896G with developing LD was 0.36 (95% C.I. 0.14–0.91, P = 0.025) when compared with the entire control group. The OR for the comparison with the matched control group showed an even stronger association, with an OR of 0.27 (95% C.I. 0.09–0.75, P = 0.008). The 896G and 1196T alleles cosegregated, and both were associated with resistance to LD.

A previous study (18) suggests that the 896G allele acts in a dominant fashion with respect to the wild-type 896A allele. Based on these findings, we compared the genotype frequency of 896AG heterozygotes in both patients and controls. Similar to the allelic analysis, genotype 896AG was associated with resistance to LD. The frequency of 896AG was 4.9% in the patients compared with 12.9% in the entire control group [OR 0.35 (95% C.I. 0.14–0.89, P = 0.025)] and 17.2% in the matched control group [OR 0.25 (95% C.I. 0.09–0.71, P = 0.006)].

We next considered whether any variables might be confounders in the analysis, an unlikely possibility, given that there is no overt biologic relationship of TLR4 to any of the baseline characteristics. The baseline variables of the patient and control groups have been analyzed previously (13), and it was demonstrated that smoking status and age had a significant association with LD. We repeated the TLR4 analysis with an adjustment for age (stratified into age 0–60 and >60) and smoking status. We chose conditional logistic regression for analysis of both the combined control group and matched control group comparisons because of our interest in a consistent analysis. For the patient vs. matched control group comparison, we found a similar magnitude of association of TLR4 SNPs with LD susceptibility when we adjusted for age and smoking status [SNP 896G, OR 0.337 (95% C.I. 0.09–1.15, P = 0.083)]; age >60, OR 9.60 (95% C.I. 4.19–21.97, P = 0.000); smoking history, OR 3.21 (95% C.I. 1.41–7.33, P = 0.006). With the patient vs. combined control group comparison, we also found a similar magnitude of association of TLR4 SNPs with LD susceptibility when we adjusted for age and smoking status [SNP 896G, OR 0.412 (95% C.I. 0.13–1.27, P = 0.123)]; age >60, OR 24.14 (95% C.I. 13.02–44.73, P = 0.000); smoking history, OR 2.83 (95% C.I. 1.52–5.26, P = 0.001). Although the P for the association of

TLR4 with LD in the adjusted analysis was not statistically significant, the magnitude of the association was similar to the unadjusted analysis. The *P* was higher because of the low frequency of the polymorphism and the loss of degrees of freedom from multiple adjustments during the logistic regression. Taken together, the magnitudes of the association in the adjusted analyses confirmed our findings from the unadjusted analyses. We previously demonstrated (13) that the TLR5 stop codon was associated with increased susceptibility to LD in nonsmokers but not in smokers. In light of these findings, we investigated whether the TLR4 association depended on smoking status. We stratified the analysis by smoking status (with an adjustment for age) and found that TLR4 SNP 896G was not associated with resistance to LD in nonsmokers [OR 0.79 (C.I. 0.14–4.54, $P = 0.79$)]. There was a nonsignificant trend toward an association in smokers [OR 0.40 (95% C.I. 0.10–1.64, $P = 0.20$). Because of small numbers, this subanalysis did not have adequate statistical power to fully address this question.

In this article, we show that TLR4 SNPs are associated with resistance to LD. This evidence indicates that SNPs A896G and C1196T can be associated with protection from an infection and suggests that variation in TLR4 responses can have beneficial or deleterious consequences that are pathogen-dependent. The association of TLR4 with protection from LD was found in individuals with heterozygous genotypes. Although there is evidence in airway epithelial cells that SNP 896G (299D) is LPS-hyporesponsive and acts in a dominant fashion with respect to the wild-type allele 896A (299G), there is conflicting evidence (18, 24, 25) that monocytes and whole blood from heterozygous individuals show no deficit in LPS signaling. This apparent contradiction may be partially explained by the use of different cell types in these studies. Our genetic findings in heterozygous individuals support a model in which alleles 896G and 1196T act in a dominant fashion with respect to the wild-type alleles in their association with resistance to LD. Similarly, previous genetic studies (19–23, 32–35) with these TLR4 SNPs suggest that altered susceptibility to infection is found in heterozygous individuals.

Previous association studies (19–23, 32–35) with SNPs A896G and C1196T have shown either no effect or an association with increased risk from infection. The conditions that showed an association with increased infectious risk include mortality from systemic inflammatory response syndrome, severe acute infections (e.g., pneumonia, pyelonephritis, peritonitis, diverticulitis, and sepsis), septic shock, Gram-negative bacteremia, and respiratory syncytial virus bronchiolitis (19–23). Even though recognition of pathogens is required to initiate an appropriate immune response, the ensuing inflammatory cascade can also cause pathology and adverse outcomes. It is not known why these TLR4 SNPs are associated with different susceptibility to *Legionella* in comparison with other pathogens. *Legionella* is an intracellular Gram-negative bacterium with an unusual LPS structure that is primarily recognized by TLR2 rather than TLR4 (12). We and others (13, 15) have shown that TLR4 does not mediate recognition of *Legionella* in mouse bone marrow macrophages stimulated *in vitro*, a result that contrasts with many other Gram-negative bacteria that are recognized by TLR4. We also found that TLR4 does not affect bacterial growth during *in vivo Legionella* infections in mice inoculated via an intranasal route (36). In contrast, Kikuchi *et al.* (14) found that TLR4 mediates IL-12 production *in vitro* in murine dendritic cells stimulated with *Legionella*. By influencing dendritic cell function, TLR4 may alter the adaptive immune response to *Legionella* that subsequently affects susceptibility to human infection. Together, these studies indicate that *Legionella* stimulates TLR4 in an unusual fashion that differs from other Gram-negative bacteria and may be cell-specific. These results illustrate the importance of using genetic studies to understand the complexity of *in vivo* human infections because of the limitations of *in vitro* and murine *in vivo* systems.

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