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A *TLR*6 polymorphism is associated with increased risk of Legionnaires' Disease

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Abstract

Legionella pneumophila (Lp), the etiologic agent of Legionnaires' Disease (LD), is an important cause of community-acquired and nosocomial pneumonia. However, the host immune and genetic determinants of human susceptibility to Lp are poorly understood. Here we show that both TLR6 and TLR1 cooperate with TLR2 to recognize Lp in transfected HEK293 cells. We also perform a human genetic association study of 14 candidate single nucleotide polymorphisms in Toll-like receptors (*TLR*s) 1, 2, and 6 in 98 LD cases and 268 controls from the Netherlands. No polymorphisms in *TLR*1 or *TLR*2 were associated with LD. A *TLR*6 polymorphism, 359T>C (rs5743808), was associated with an elevated risk of LD in genotypic and dominant (OR 5.83, $p=7.9\times10^{-5}$) models. The increased risk in persons with 359 TC or CC genotypes was further enhanced among smokers. In a multivariate model, 359T>C was associated with a higher risk of LD (OR 4.24, p=0.04), than any other variable, including age and smoking. Together, these data suggest that the human TLR6 variant, 359T>C, is an independent risk factor for LD.

Keywords

*TLR*6; polymorphism; Legionnaires' Disease; *Legionella pneumophila*

Introduction

Legionella pneumophila (Lp) is a Gram-negative, facultative intracellular bacterium that opportunistically infects human alveolar macrophages and causes a pneumonic illness known as Legionnaires' Disease (LD) in normal and immunocompromised hosts. LD is

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Conflicts of Interest

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estimated to account for up to 15% of all hospital admissions for community-acquired pneumonia in North America and Europe[1–5] and may also be an underreported cause of nosocomial pneumonia.[6–8] Despite its epidemiologic importance, the immunologic and genetic factors that underlie human susceptibility to Lp infection remain poorly understood.

The innate immune system is critical to host defenses against both extracellular and intracellular pathogens. Toll-like receptors (TLRs) are central to the repertoire of innate immune receptors that recognize bacterial pathogens, including Lp. TLR2 recognizes a number of molecular motifs on bacteria, including di-acylated and tri-acylated lipopeptides, peptidoglycan, lipotechoic acid, and GPI (glycosylphosphatidyl-inositol)-linked proteins.[9–11] TLR2 signaling in response to bacterial ligands requires heterodimerization with one of two co-receptors, TLR1 or TLR6, which mediate recognition of tri-acylated or di-acylated lipoproteins, respectively.[12–15] TLR2 recognizes Lp and regulates cytokine production in infected macrophages and after aerosolized *in vivo* infection of mice.[16–19] However, the contributions of TLR2 co-receptors TLR1 and TLR6 to Lp immune recognition have not yet been defined.

Our current understanding of the immunopathogenesis of *Legionella* infection relies heavily on *in vivo* studies in mice and *in vitro* macrophage studies. Human genetic studies permit study of immunologic mechanisms during natural infection, especially when coupled to functional immune studies. Our group and others have described common human polymorphisms that are associated with clinical susceptibility to infection and also regulate pathogen-induced immune responses by primary immune cells *in vitro*. [20–27] Given the central role of TLR2 in *Legionella* pathogenesis, we hypothesized that common genetic variants in *TLR*1, 2, or 6 modulate susceptibility to LD in humans. In this study, we investigated whether TLR1 or TLR6 cooperate with TLR2 to recognize Lp and if polymorphisms in *TLR*s 1, 2, or 6 are associated with susceptibility to LD.

Results

Legionella pneumophila is recognized by TLR1 and TLR6

To determine whether TLR2 responses to Lp are mediated through heterodimerization with TLRs 1 or 6, we measured NF-κB activity in HEK293 cells transfected with a murine *TLR*2 construct with or without constructs for murine *TLR*1 or *TLR*6. HEK293 cells transfected with *TLR*2 plus *TLR*1 and stimulated with 10^6, 10^7, or 10^8 cfu/ml of Lp had significantly greater NF-κB activity than HEK293 cells transfected with *TLR*2 (T2) alone ($p \leq 0.001$, $p < 0.005$, and $p < 0.005$, respectively) (Fig. 1). Cells transfected with *TLR*2 plus*TLR*6 also had greater activity in response to 10^6, 10^7, or 10^8 cfu/ml of Lp in comparison to cells expressing TLR2 alone ($p < 0.01$, $p < 0.005$, and $p < 0.005$, respectively). As a control, responses to IL-1β, which stimulates NF-κB activity through the IL-1 receptor, were equally robust among cells transfected with different combinations of TLR constructs or empty vector. As an additional positive control, responses to the lipopeptide PAM3 were present in cells transfected with TLR2 alone as well as TLR2/1 and TLR2/6 heterodimers. Responses to LPS, a TLR4 ligand, were not detected, as expected. These results suggest that either TLR1 or TLR6 are required for full recognition of Lp by TLR2.

The nonsynonymous *TLR*6 polymorphism 359T>C is associated with LD

To determine whether human *TLR*1, 2, or 6 polymorphisms are associated with susceptibility to legionellosis, we used a case-control study of an epidemic outbreak in The Netherlands. A description of this outbreak has previously been published[28] and the clinical features of genotyped cases and controls are summarized in Table 1. We analyzed 14 candidate polymorphisms, 5 in *TLR*1, 3 in *TLR*2, and 6 in *TLR*6 in 98 cases and 268 controls. Two *TLR*6 SNPs (rs3821985 and rs3775073) had HWE p values ≤ 0.001 and were not analyzed further. Since *TLR*1 and *TLR*6 are contiguous genes on chromosome 4p14, we evaluated the linkage disequilibrium pattern for the 5 *TLR*1 and 4 *TLR*6 SNPs in the control population (Fig. 2). The majority of R^2 values were <0.65, indicating a low to moderate degree of linkage. We found no associations with LD for any of the *TLR*1 variants, including rs5743618, a non-synonymous SNP at base pair 1805 in the transmembrane domain of *TLR*1 that regulates signaling (Table 2).[26, 27] Similarly, no *TLR*2 variant was associated with LD. The frequency of a single *TLR*6 SNP, rs5743808 (359T>C), a nonsynonymous variant in the extracellular leucine rich repeat domain of the protein (encoding an isoleucine-to-threonine transition at amino acid residue 120), was greater in cases compared to controls (genotypic analysis: $p = 7.9 \times 10^{-5}$) (Table 2). This association remained significant after a conservative Bonferroni adjustment for multiple comparisons ($p = 9.5 \times 10^{-4}$). The association best fit a dominant model (comparing TT genotypes to TC/CC) with an Odds Ratio (OR) of 5.83 for LD ($p = 7.9 \times 10^{-5}$) in cases compared to controls (Table 3). Among cases, 15% carried the C allele (had TC or CC genotype) compared to 3% of controls. Odds ratios for LD were similar whether cases were compared to all controls (n=263) or controls without Pontiac fever (n=234) (OR of 5.83 vs. 5.17, respectively), a clinical marker of *Legionella* exposure (Table 3).

We then compared cases to controls with serological evidence of exposure to Lp and found that the risk of LD associated with TC/CC genotypes was further enhanced when cases were compared to seropositive controls: 15% of cases had a TC or CC genotype compared to 0% of exposed, seropositive controls and 3% of all controls (Table 3). To ensure that our results were not due to population admixture, we also examined genotype frequencies for 359T>C in 86 controls and 97 cases matched for age, sex and place of residence within 25 km. The increased risk of LD in cases as compared to matched controls was still seen, although the OR was lower than for the general control group (OR of 3.75, p=0.03) (Table 3).

We previously identified a *TLR*5 polymorphism (1174C>T, or 392R>STOP) associated with susceptibility and two *TLR*4 polymorphisms (896A>G (299D>G) and 1196C>T (399T>I)) associated with resistance to LD.[23, 24] We found no evidence of interaction between any of these previously defined risk alleles and *TLR*6 359T>C (data not shown), suggesting that 359T>C is an independent susceptibility locus for LD.

*TLR*6 359T>C confers a higher risk of LD than other genetic and non-genetic risk factors

Previously reported risk factors for LD include older age, gender, smoking, diabetes, alcohol use, and chronic respiratory illness.[29–33] In our cohort, older age, diabetes mellitus, and chronic respiratory disease were each significantly associated with an increased risk of LD (Table 4) in a univariate analysis. Smoking was also associated with increased LD risk that

did not reach significance in the univariate analysis (p=0.2). Conversely, alcohol use was significantly associated with protection from LD (Table 4). We performed a multivariate analysis using all variables with a p≤0.2 in the univariate analysis as well as the TLR5 1174C>T and TLR4 896A>G and 1196C>T variants previously associated with LD. In the multivariate analysis, the 359T>C variant retained a significant association with LD (OR 4.24, *p*=0.04, dominant analysis) (Table 4).

Since smoking was also associated with a greatly increased risk of LD (OR 3.51, *p*=0.002) in our multivariate analysis, we stratified our analysis by smoking status. Among smokers, individuals with TC or CC genotypes had a significantly elevated risk of LD compared to controls (OR 8.75, *p*=0.005, unadjusted analysis) (Table 5). Among nonsmokers, TC/CC genotypes were associated with somewhat less increased risk (OR 4.07, p=0.03, unadjusted analysis) (Table 5). After adjustment for age and alcohol, both the associations among smokers and nonsmokers became nonsignificant (p=0.11 and p=0.06, respectively), likely due to small numbers. These results suggest that the association of 359T>C with LD may be more pronounced in smokers than non-smokers.

We next examined NF-κB signalling in response to heat-killed Lp in HEK 293 cells transfected with constructs containing the 359T (wild type) or 359C (SNP) variant of human TLR6, but found no differences in Lp- or $Pam2CSK_4$-mediated responses (data not shown).

Discussion

Our data show that TLR6 mediates recognition of Lp and that a common polymorphism is associated with susceptibility to LD. *In vitro*, the presence of TLR1 or TLR6 was required for maximal TLR2-mediated responses to heat-killed Lp in transfected HEK cells. *In vivo*, a natural variant of human TLR6, 359T>C, was associated with elevated risk of LD. Previously published work has implicated TLR2 in the mammalian innate immune response to *Legionella*.[16–19, 35, 36] Although the majority of Gram-negative organisms have an LPS that signals through TLR4, the LPS structure of *Legionella* is atypical and appears to predominantly signal through TLR2.[11, 16, 19, 37] In addition, the *Legionella* structural protein, peptidoglyan-associated lipoprotein (PAL), signals via TLR2.[38] Studies of *Legionella* pulmonary infection in *Tlr2*$^{-/-}$mice have shown 10 to 100-fold higher CFUs in the lung compared to wildtype counterparts[17] and enhanced intracellular growth of *Legionella* has been shown in *Tlr2*-deficient murine macrophages.[16] Our functional data suggests that TLR2/6 and TLR2/1 cooperate to recognize Lp. Our genetic association results further suggest that *TLR*6 is a genetic locus of LD susceptibility. Although we did not detect an association between *TLR*2 polymorphisms and LD, these results do not rule out a contribution of *TLR*2 variants to LD susceptibility since only three candidate SNPs in *TLR*2 (597T>C, 1350T>C, and 2258G>A) were evaluated in our study.

Our study has several potential limitations. Although our results suggest an association of *TLR*6 with LD, we cannot exclude the possibility that the 359T>C SNP is in linkage with a nearby causative variant and serves as a marker for this alternative risk locus. *TLR*6 is located in continuity with *TLR*s 1 and 10 along a 54 kb segment of chromosome 4p14 and the potential role of *TLR*10 variants in LD susceptibility was not evaluated in this study. The

similar NF-κB responses of the TLR6 359T and 359C variants in response to heat-killed Lp could be explained if the 359T>C polymorphism primarily modulates initial uptake or intracellular trafficking of live bacteria by the macrophage rather than NF-κB-driven cytokine responses in response to heat-killed Lp. However, we have not evaluated this possibility. As with other genetic association studies, genotyping error can occur or confounding may exist due to the unaccounted-for effects of population stratification or ethnic admixture. We judged ethnic admixture to be unlikely to cause confounding, since >95% of both cases and controls were of Caucasian Dutch background. We also included place of residence (along with age and sex) as one of the matching criteria in the original study design to control for possible population stratification and found that the association of the 359T>C SNP with LD was similar whether we used the general control group (OR 5.83, $p=7.9\times10^{-5}$) or the smaller, matched control (OR 3.75, $p=0.03$) group as the comparator. Another potential weakness of this study is the relatively small number of LD cases (98 analyzed out of 188 identified in the original outbreak). Nonetheless, our case sample size is larger than most outbreaks reported in the medical literature. Ideally, these findings will be investigated in a future cohort of different ethnicity.

Few genetic association studies have addressed the role of *TLR*6 in infectious diseases. One study reported an association of the 359T>C and 745C>T, among other polymorphisms, with tuberculosis in an African population.[39] The *TLR*6 variant 745C>T has a reported association with increased risk of invasive aspergillosis after stem cell transplantation.[40] The mechanisms responsible for the association of *TLR*6 variants with these diverse pathogens -- from an intracellular bacterium to an extracellular mould-- remain unknown. Several studies have investigated the role of *TLR*6 variants in mediating altered cytokine signaling to pathogens or pathogen motifs. In functional studies, 745T (249S) and the synonymous SNP 1083C have been reported to be associated with decreased whole blood IL-6 responses to bacterial lipopeptides, and SNP 1083C was additionally associated with decreased IL-6 responses to *M. tuberculosis* lysate and BCG.[41] Other authors have demonstrated a link between TLR6, phagocytosis, and autophagy. For example, TLR6 and 2 cooperate to recognize zymosan, a TLR2 ligand from yeast, and recruit the autophagy marker, LC3, to zymosan-containing phagosomes.[42, 43]

The magnitude of LD risk posed by *TLR*6 359T>C variant (OR 5.83, dominant analysis) is greater than that posed by other polymorphisms previously associated with LD, specifically, *TLR5* 1174C>T, *TLR*4 896A>G, and *TLR*4 1196C>T (ORs 0.40–2.24, Table 4).[23, 24] Furthermore, we found no evidence of an interaction between the *TLR*6 359C allele and any of the *TLR*5 or *TLR*4 risk alleles. These results support a role for human TLR6 in the immunopathogenesis of LD. If validated in other genetic association studies of LD, one could imagine the usefulness of this SNP in outbreak settings, where individuals at highest risk could be targeted for pre-emptive therapy. Similarly, the *TLR*6 359T>C polymorphism could be used prospectively to identify immunocompromised individuals with enhanced genetic risk for legionellosis.

Methods and methods

Reagents, Bacteria and Cells

Ultrapure lipopolysaccharide (LPS) was from *E. coli* 0111:B4 (InvivoGen). Lipopeptide PAM3Cys-SKKKK (triacylated, PAM3) was from InvivoGen. IL-1β was from Pierce Endogen. *Legionella pneumophila* (Lp) Philadelphia serogroup 1 strain (ATCC 33152) was heat killed at 65°C for 30 minutes at concentrations of 10^6, 10^7, or 10^8 CFU/ml (corresponding to MOIs of ~2.5:1, ~25:1 and ~ 250:1, respectively) for stimulation assays. HEK293 cells (obtained from A. Hajjar) were cultured in a 96 well flat-bottomed tissue culture plate at ~5×10^4 cells/well in DMEM with high glucose (Mediatech) plus 10% heat-inactivated FBS (Hyclone). Cells were transiently transfected with 5 μL of transfection reagent comprised of a 1:1 mix of 0.25 M $CaCl_2$ containing 2 × BBS (50 mM BES, 280 mM NaCl, and 1.5 mM NaH_2PO_4) and DNA expression vectors for *Renilla* luciferase (driven by constitutively active β-actin promoter (control for transfection efficiency)), ELAM-1 firefly luciferase (driven by NF-κB), murine MD2, and murine CD14, along with the following HA-tagged constructs: murine *TLR*2 alone; murine *TLR*2 with murine*TLR*6; or murine *TLR*2 with murine TLR1.[44, 45] The total amount of DNA added per well was adjusted to 0.05 μg by the addition of empty vector. Transfected cells were washed once after 4h and stimulated the following day with TLR ligands, or heat-killed Lp for 4 hours, then lysed and processed for luciferase readings per the manufacturer's instructions for the Dual Luciferase Reporter Assay System (Promega, Madison, WI).

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 and the University of Washington. All procedures for human subjects were consistent with ethical standards set by the 1964 Helsinki Declaration. Each participant gave written informed consent. Enrollment of cases and controls from an LD outbreak in Bovenkarspel, has been described previously.[28, 31, 46] Of the 188 cases (133 confirmed, 55 probable) identified in the original investigation of the outbreak, DNA and epidemiologic data were available from 98 cases (84 confirmed LD, 14 probable LD) and 268 controls (Table 1) for this study. A confirmed case of LD was defined as radiographic finding of pneumonia and laboratory evidence (positive urine antigen, sputum culture, or serology) of Lp infection. A probable case was defined as a radiographic finding of pneumonia in a person attending the flower show during the epidemic period and no evidence of alternate pathogens.[31] Individuals recruited as controls were exhibitioners who worked at the flower show, completed a questionnaire, and had blood drawn for genetic analysis. A subset of the controls had definitive evidence of exposure consisting of a positive serology for Lp (n=57) or clinical diagnosis of Pontiac Fever (n=29), a manifestation of Lp infection distinguishable from LD by its short incubation time and absence of respiratory symptoms. Data on incubation times was available since both the time of exposure (coinciding with flower show attendance) and date of clinical presentation were known. All of the cases and controls were from the Netherlands and >95% were of Caucasian Dutch ancestry.

SNP selection

For the LD genetic association study, we investigated SNPs in *TLR*s 1, 2 and 6 previously reported to have associations with infectious disease or altered immune responses (Table 2). We also investigated a single nonsynonymous SNP in *TLR*6 (rs5743808) as well as two SNPs in *TLR*1 or 6 flanking regions (rs17616434 and rs3924112) with no prior reports of associations. SNP annotation and mapping was confirmed using the online NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp).

Genomic Techniques

Genomic DNA was purified from peripheral blood leukocytes from 10 ml of blood. Genotyping was performed using a chip-based matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF) Mass Array technique (Sequenom), as described previously.[47] Cluster plots were visually inspected to ensure accurate genotyping.

Statistics

Fourteen candidate polymorphisms, 5 in *TLR*1, 3 in *TLR*2 and 6 in *TLR*6, were genotyped in cases and controls. We examined Hardy Weinberg Equilibirum *p* values and SNP genotypic frequencies in the cases and controls using Stata 11.1 software (StataCorp) and the user-written package "GENASS."[48] All SNPs analyzed for association with LD were in Hardy Weinberg equilibrium using a cutoff *p* value of ≥ 0.001 (χ^2 goodness-of-fit test) in the control group to ensure that there were no genotyping errors or major effects of population heterogeneity. Two SNPs in TLR6 (rs3821985 and rs3775073) showed significant departure from Hardy-Weinberg equilibrium (HWE) among control subjects ($p<0.001$) and were not further evaluated. The remaining twelve SNPs passed the HWE p value test ($p\geq0.001$) and were assessed for association with LD, using a genotypic model in the first-pass analysis; those that had a significant association ($p<0.05$) were then investigated under dominant and recessive genetic models. In the dominant model, carriers of the less common allele (01 and 11 genotypes) were compared with homozygous subjects for the major allele (00 genotype). In the recessive model, individuals homozygous for the rare allele (11 genotypes) were compared to heterozygotes and major allele homozygotes (01 and 11 genotypes). SNP associations with LD were also analyzed within subgroups of smokers and nonsmokers and in cases compared to specific control groups (controls without Pontiac fever, seropositive controls, or matched controls). For the *TLR*6 359T>C polymorphism, we used univariate logistic regression to assess the relative magnitude of risk conferred by this genetic variant compared to traditional risk factors for LD and the previously described TLR4 and TLR5 risk alleles. We then performed multivariate logistic regression to determine whether nongenetic risk factors modified the genetic association at the 359T>C locus. Age, diabetes, chronic respiratory illness, smoking status, and alcohol use were included as variables in multivariate logistic regression. Genetic interactions between *TLR*6 359T>C (rs5743808) and *TLR*5 1174C>T (rs144418928), *TLR*4 1196C>T (rs4986791), or TLR4 896A>G (rs4986790), respectively, were investigated using an expectation–maximization algorithm implemented by the "hapipf" function in Stata. The Pearson χ^2 test and Student's *t* test were used to assess categorical and continuous clinical variables, respectively. Two-sided testing

was used for all comparisons to evaluate statistical significance, with a p value of <0.05 considered as significant.

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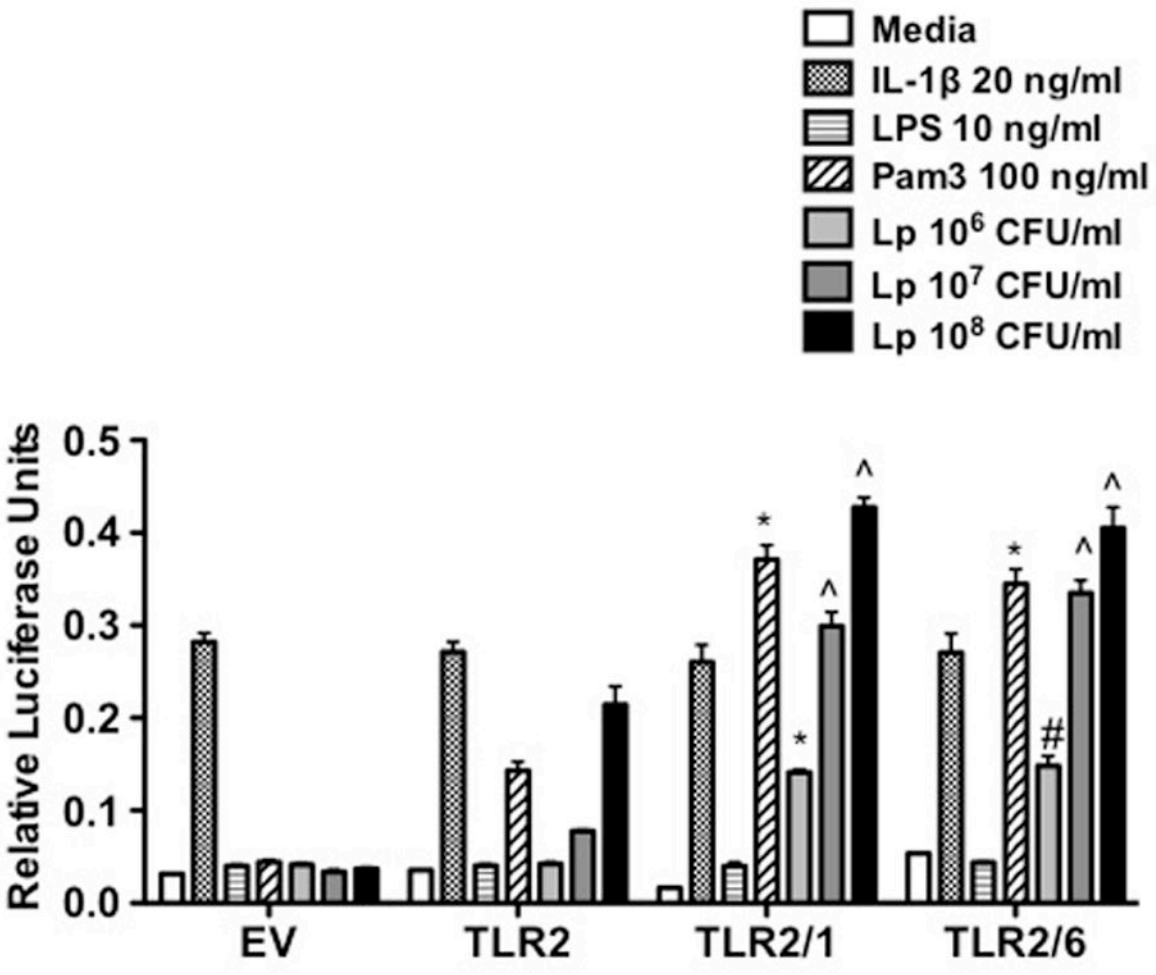

Figure 1. TLRs 1 and 6 are required for TLR2-mediated recognition of *Legionella pneumophila*

HEK293 cells were transiently transfected with the indicated murine constructs plus CD14, MD2, ELAM luciferase, and *Renilla* luciferase. The total amount of DNA added per well was adjusted to 0.05 μg by the addition of empty vector. Transfected cells were washed once after 4h and stimulated the following day with the indicated ligands (LPS, negative control; Pam3 and IL-1β, positive controls) or *Legionella pneumophila*. After 4h the cells were lysed and NF-κB activation was measured as the ratio of firefly to *Renilla* luciferase activity (Relative Luciferase Units). Data are mean ± SEM of triplicate wells, and represent one of four independently performed experiments. EV, empty vector. Pam3, Pam3CysSK4. P values calculated using Student's t test. $^{\#}p<0.01$ relative to TLR2 alone; ^p<0.005 relative to TLR2 alone; *p≤0.001 relative to TLR2 alone.

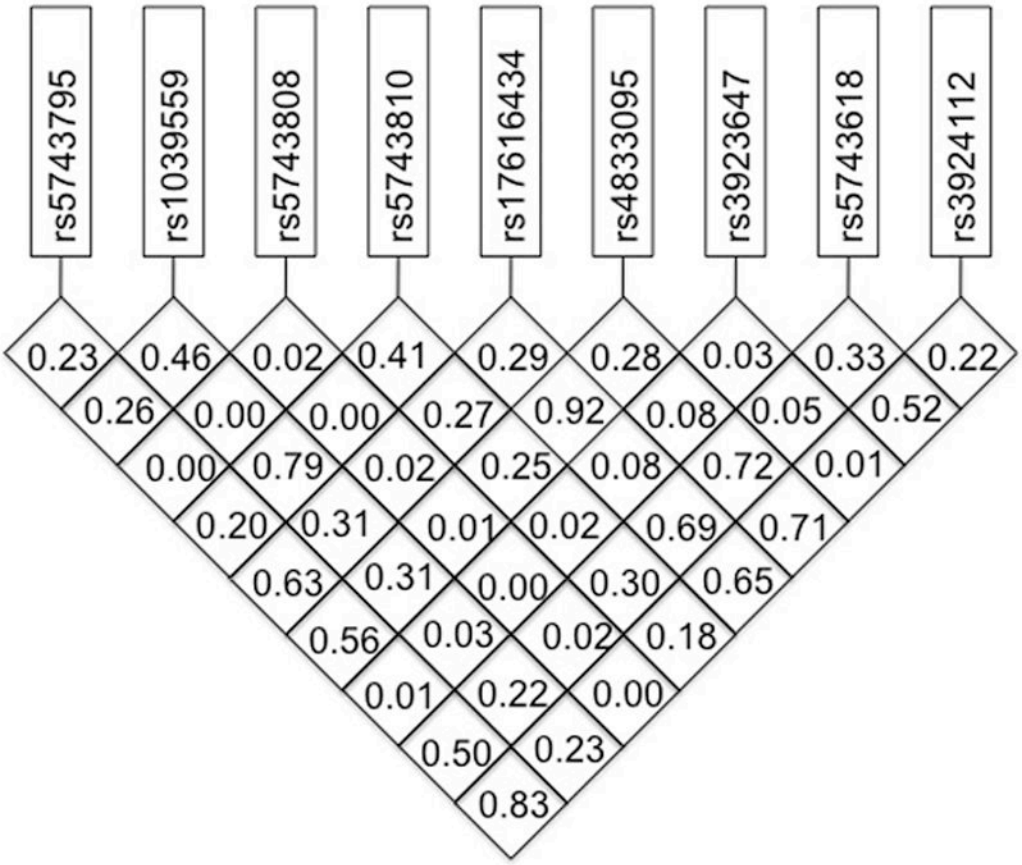

Figure 2. Linkage disequilibrium among *TLR*1 and *TLR*6 polymorphisms

The first row of numbers represents the frequency of the minor allele of each polymorphism. All other numbers represent R^2 values for 5 *TLR*1 and 4 *TLR*6 polymorphisms on chromosome 4p14. R^2 values were calculated for the control group using the "pwld" function in STATA. A value of 1 indicates full linkage (complete co- inheritance of the two alleles); a value of 0 indicates no linkage.

Table 1

Case and Control Characteristics

Characteristics	Controls	Cases[1]
Total individuals	268	98
Age (mean±SD)*	46.9(±14.2)	63.7(±10.2)
Smoking status‡		
Nonsmokers (%)	135(0.59)	45 (0.51)
Smokers (%)	95 (0.41)	43 (0.49)
Urine Antigen[2] (%)		
absent	—	36 (0.38)
present	—	59 (0.62)
Fever[3](%)		
absent	—	26 (0.30)
present	—	61 (0.70)
ICU (%)		
no	—	80 (0.82)
yes	—	18 (0.18)
Pontiac Fever (%)		
no	239 (0.89)	—
yes	29 (0.11)	—
Seropositive (%)	57 (0.21)	—
mean incubation time (days ±SD)[4]	—	7.5 (±3.4)
mean length of stay (days ±SD)[5]	—	19.1 (±21.6)

[1] Cases includes individuals with definite and probable LD.

[2] Presence or absence of *L. pneumophila* antigen in urine.

[3] Fever defined as temperature above 38.5 degrees Celsius.

[4] Incubation time defined as days between exposure and onset of symptoms.

[5] Length of hospital stay in days.

* p value < 0.0001.

‡ P=0.22 for distribution of smokers vs. nonsmokers among cases and controls

Table 2

*TLR*1, *TLR*2 and *TLR*6 Polymorphism Genotype Frequencies in Legionnaires' Disease[1]

SNP base pair (aa)[2]		Genotype frequency (%)[3] 00	01	11	*Chi*^{2}	*p*	HWE *p*[4]
***TLR*6**							
rs5743795	controls	159 (0.61)	84 (0.32)	17 (0.07)	—	—	0.22
−1,401G>A	cases	62 (0.68)	28 (0.31)	1 (0.01)	4.46	0.09	—
rs1039559	controls	81 (0.31)	122 (0.46)	61 (0.23)	—	—	0.25
−502T>C	cases	27 (0.30)	32 (0.36)	30 (0.34)	4.51	0.10	—
rs5743808	controls	255 (0.97)	8 (0.03)	0 (0.00)	—	—	1.00
+359T>C (120I>T)	cases	82 (0.85)	14 (0.14)	1 (0.01)	18.93	7.9×10^{-5}	—
rs5743810	controls	95 (0.36)	120 (0.46)	47 (0.18)	—	—	0.40
+745C>T (249P>S)	cases	34 (0.38)	35 (0.39)	20 (0.22)	1.41	0.49	—
rs3821985	controls	138 (0.53)	85 (0.33)	37 (0.14)	—	—	2.1×10^{-4}
+1083C>G (T361T)	cases	45 (0.53)	30 (0.36)	9 (0.11)	—	—	
rs3775073	controls	137 (0.54)	82 (0.32)	37 (0.14)	—	—	9.4×10^{-5}
+1263A>G (K421K)	cases	47 (0.51)	32 (0.34)	14 (0.15)	—	—	
***TLR*1**							
rs17616434	controls	133 (0.52)	102 (0.39)	23 (0.09)	—	—	0.59
−12,424T>C	cases	50 (0.55)	34 (0.38)	6 (0.07)	0.66	0.72	—
rs4833095	controls	134 (0.53)	98 (0.38)	23 (0.09)	—	—	0.41
+743T>C (248N>S)	cases	43 (0.47)	41 (0.45)	7 (0.08)	1.24	0.54	—
rs3923647	controls	249 (0.94)	17 (0.06)	0 (0.00)	—	—	1.00
+914A>T (305H>L)	cases	82 (0.90)	9 (0.10)	0 (0.00)	1.23	0.35	—
rs5743618	controls	114(0.44)	110 (0.43)	33 (0.13)	—	—	0.43
+1805G>T (602S>I)	cases	40 (0.43)	44 (0.48)	8 (0.09)	1.39	0.50	—
rs3924112	controls	164 (0.62)	82 (0.31)	17 (0.06)	—	—	0.15
+4,642C>T	cases	63 (0.68)	29 (0.31)	1 (0.01)	4.26	0.11	—
***TLR*2**							
rs3804099	controls	80 (0.30)	132 (0.50)	51 (0.19)	—	—	0.79
+597T>C (199N>N)	cases	26(0.29)	37 (0.41)	27 (0.30)	4.62	0.10	—

SNP		Genotype frequency (%)[3]					
base pair (aa)[2]		00	01	11	*Chi²*	*p*	HWE *p*[4]
rs3804100	controls	211(0.85)	36 (0.15)	0 (0.00)	—	—	0.62
+1350T>C (450S>S)	cases	85(0.91)	8 (0.09)	0 (0.00)	2.14	0.20	—
rs5743708	controls	243(0.93)	19 (0.07)	0 (0.00)	—	—	1.00
+2258G>A (753R>Q)	cases	88(0.94)	6 (0.06)	0 (0.00)	0.08	1.00	—

[1] Total numbers of cases and controls may not sum to 268 and 98, respectively, due to failed genotyping for small numbers of individuals. Controls include 29 individuals with Pontiac Fever.

[2] Base pair position given relative to ATG start site along with corresponding amino acid (aa) within parentheses.

[3] **00** denotes homozygosity for major (common) allele, **01** denotes hetereozygosity, and 11 denotes homozygosity for minor allele in the Netherlands population.

[4] HWE, p value for Hardy-Weinberg equilibrium test performed in control population. Two *TLR*6 SNPs (rs3821985 and rs3775073) with HWE p values ≤ 0.001 are presented in this table but were not analyzed further.

Table 3

Frequency of *TLR6* polymorphism rs5743808 in cases and different control groups

	Genotype frequency (%)					Dominant Analysis			
	TT	***TC***	***CC***	***Chi²***	***p***	**TT (%)**	**TC/CC (%)**	**OR (95% CI)**	***p***
Pontiac fever included									
Controls[1]	255 (0.97)	8 (0.03)	0 (0.00)			255 (0.97)	8 (0.03)	1.00	
Cases	82 (0.85)	14 (0.14)	1 (0.01)	18.93	7.9×10^{-5}	82 (0.85)	15 (0.15)	5.83 (2.21–16.39)	7.9×10^{-5}
Pontiac fever excluded									
Controls[2]	226 (0.97)	8 (0.03)	0 (0.00)			226 (0.97)	8 (0.03)	1.00	
Cases	82 (0.85)	14 (0.14)	1 (0.01)	16.00	3.4×10^{-4}	82 (0.85)	15 (0.15)	5.17 (1.96–14.54)	2.3×10^{-4}
Seropositive									
Controls[3]	56 (1.00)	0 (0.00)	0 (0.00)			56 (1.00)	0 (0.00)		
Cases	82 (0.85)	14 (0.14)	1 (0.01)	9.60	0.002	82 (0.85)	15 (0.15)	—	0.001
Matched									
Controls[4]	82 (0.95)	4 (0.05)	0 (0.00)			82 (0.95)	4 (0.05)	1.00	
Cases	82 (0.85)	14 (0.14)	1 (0.01)	5.92	0.04	82 (0.85)	15 (0.15)	3.75 (1.12–16.08)	0.03

[1] Controls include individuals with Pontiac Fever, as in Table 1.

[2] Controls exclude individuals with Pontiac fever.

[3] Controls with positive serology for Lp.

[4] Controls matched to cases by age, gender, and place of residence within 25 km.

Table 4

Predictors of Risk of Legionnaires' Disease in a Dutch population

	univariate analysis				multivariate analysis			
Characteristics	**N**	**OR**	**95% CI**	***p***	**N**	**OR**	**95% CI**	***p***
TLR6 359 TC/CC[1]	360	5.83	2.39–14.25	<0.001	274	4.24	1.05–17.17	0.04
Diabetes mellitus	357	4.57	1.68–12.39	0.003	274	1.48	0.31–7.16	0.49
TLR5 1174 CT/TT[2]	366	2.24	1.14–4.39	0.02	274	1.91	0.67–5.46	0.23
Chronic respiratory illness	364	1.54	1.02–2.35	0.04	274	0.66	0.17–2.51	0.54
Smoking	318	1.36	0.83–2.22	0.22	274	3.51	1.61–7.63	0.002
Older age[3]	362	1.12	1.09–1.15	<0.001	274	1.14	1.10–1.19	<0.001
Female gender	361	0.82	0.51–1.30	0.39	274	—	—	—
TLR4 896 GA/AA[4]	363	0.45	0.19–1.03	0.06	274	—	—	—
TLR4 1196 CT/TT[5]	360	0.40	0.16–0.97	0.04	274	0.59	0.16–2.11	0.42[6]
Alcohol use	316	0.45	0.26–0.77	0.004	274	0.42	0.20–0.88	0.02

[1] TC and CC genotypes compared to TT genotype for TLR6 rs5743808 (359T>C) polymorphism (dominant analysis).

[2] CT and TT genotypes compared to CC genotype for TLR5 (1174C>T).

[3] Age analyzed as continuous variable.

[4] GA and GG genotypes compared to AA genotype for TLR4 (896A>G).

[5] CT and TT genotypes compared to CC genotype for TLR4 (1196C>T).

[6] These data pertain to combined genotype category of TLR4 896 GA/AA or TLR4 1196 CT/TT since SNPs TLR4 896A>G and 1196C>T are in complete linkage disequilibrium in this population (all 896AA genotypes co-segregate with 1196CC and all 896GA genotypes co-segregate with 1196 CT).

Table 5

Frequency of *TLR6* polymorphism rs5743808 by smoking status

	Genotype frequency (%)					Dominant Analysis			
	TT	***TC***	***CC***	***Chi²***	***p***	**TT (%)**	**TC/CC (%)**	**OR (95% CI)**	***p***
Smokers									
Controls	90 (0.98)	2 (0.02)	0 (0.00)			90 (0.98)	2 (0.02)	1.00	
Cases	36 (0.84)	6 (0.14)	1 (0.02)	9.63	0.005	36 (0.84)	7 (0.16)	8.75 (1.54–88.61)	0.005
Nonsmokers									
Controls	129 (0.96)	5 (0.04)	0 (0.00)			129 (0.96)	5 (0.04)	1.00	
Cases	38 (0.86)	6 (0.14)	0 (0.00)	5.61	0.03	38 (0.86)	6 (0.14)	4.07 (0.97–17.70)	0.03