Variants in Toll-like Receptor 1 and 4 Genes Are Associated With Chlamydia trachomatis Among Women With Pelvic Inflammatory Disease

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Background. Toll-like receptors (TLRs) are involved in the innate immune response. We examined whether TLR variants are associated with Chlamydia trachomatis infection among women with pelvic inflammatory disease (PID).

Methods. We tested whether 18 tagging single nucleotide polymorphisms (tagSNPs) assayed in 4 TLR genes (TLR1, TLR2, TLR4, TLR6) and 2 adaptor molecules (TIRAP, MyD88) were associated with C. trachomatis among 205 African American women with clinically suspected PID from the PID Evaluation and Clinical Health Study. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs). An empirical P value of <.004 was considered significant.

Results. Women with PID who carried the TLR4 rs1927911 CC genotype had significantly increased odds of C. trachomatis (OR, 3.7; 95% CI, 1.6–8.8; P = .002). The TLR1 rs5743618TT genotype was also associated with C. trachomatis (OR, 2.8; 95% CI, 1.3–6.2; P = .008).

Conclusions. Among African American women with PID, variants in the TLR1 and TLR4 genes, which may increase signaling, were associated with increased C. trachomatis infection.

Chlamydia trachomatis is the most common bacterial sexually transmitted infection in the United States [1] and can lead to serious complications such as pelvic inflammatory disease (PID) and its subsequent sequelae, including ectopic pregnancy, infertility, and chronic pelvic pain [2, 3]. However, some women may develop PID and reproductive sequelae following chlamydial infection, whereas others may not [1, 4–6]. As the mechanisms underlying the pathogenesis of C. trachomatis have yet to be completely elucidated, the reasons for this variability in outcome are unknown. It is known that Chlamydia spp. can infect epithelial cells, causing secretion of proinflammatory cytokines [7–9]. Researchers have suggested that this inflammatory response may be responsible for disease progression [9], especially among those with chronic infections. Thus, it is possible that variations in host innate immune receptor genes may contribute to the variability in outcomes following chlamydial genital tract infection.

The innate immune system serves as the first line of defense after exposure to pathogens and depends on pattern recognition receptors (PRRs) for microbial recognition [10–12]. A conserved family of PRRs called the Toll-like receptor (TLR) family [11] is responsible for induction of inflammatory cytokine and chemokine genes and priming of the adaptive immune system that leads to microbial elimination. Ten different TLRs (TLRs 1–10) have been identified in humans, and the overlap between them allows identification of a diverse range of pathogens through ligand binding [10–12]. Adaptor molecules, including myeloid differentiation primary response protein 88 (MyD88) and Toll/interleukin 1 receptor domain–containing adaptor protein (TIRAP), help to mediate TLR
signaling [10]. However, as important as TLRs and their adaptor molecules are for a healthy immune response, variations in these genes may lead to an overexuberant or inadequate response, possibly influencing disease progression.

Because TLRs are expressed in the reproductive tract [12] and can stimulate proinflammatory genes after binding to a bacterial ligand, it is possible that they may play a role in chlamydial infection. Studies have examined TLR pathways in chlamydial infections and have suggested a role for TLR2 in cytokine production [13, 14]. O’Connell et al [14] found that TLR2 was required for interleukin 8 (IL-8) expression, whereas TLR4/MD-2 had minimal effects on cytokine production in vitro. Darville et al [15], using a murine model of genital tract infection, found that mice genetically deficient for TLR2 exhibited reduced ovudipt pathology and production of select proinflammatory cytokines, whereas TLR4-deficient mice had outcomes similar to wild-type mice. However, very few studies have examined TLRs in Chlamydia trachomatis pathogenesis among human subjects. Studies among Dutch white populations have been unable to show any significant associations between functional polymorphisms in the TLR4 and TLR2 genes and Chlamydia trachomatis [15–17]. Our objective was to explore the role of TLR2, TLR4, TLR6, MyD88, and TIRAP gene polymorphisms in Chlamydia trachomatis infection among women with clinically suspected PID.

METHODS

This study utilized data from the PID Evaluation and Clinical Health (PEACH) study. This was the first randomized clinical trial to compare inpatient and outpatient treatment in preventing long-term complications among 831 women with mild to moderate PID. The methods of subject recruitment, data collection, and follow-up have been reported elsewhere [18]. Briefly, between March 1996 and February 1999, women aged 14–37 years were recruited from emergency departments, obstetrics and gynecology clinics, sexually transmitted disease clinics, and private practices throughout the eastern, southern, and central regions of the United States. The women who had clinically suspected PID and gave informed consent were eligible for the PEACH study. Eligible women had a history of pelvic discomfort for less than 30 days, findings of pelvic organ tenderness (uterine or adnexal) on bimanual examination, and leukorrhea and/or mucopurulent cervicitis and/or untreated but documented gonococcal or chlamydial cervicitis. The University of Pittsburgh Institutional Review Board approved the study.

A total of 290 women (205 with self-reported non-Hispanic African ancestry, 51 with non-Hispanic European ancestry, and 34 with “other” ancestry) with previously stored buffy coats (n = 237) or serum samples (n = 50) were genotyped for TLR and adaptor molecule single-nucleotide polymorphisms (SNPs) in this study. There were no significant differences in the genotype frequencies between women genotyped with buffy coats or serum samples. Because the sample size for European (n = 51) and “other” (n = 34) ancestry groups were small, they were excluded from the association analyses.

Gynecological examinations were performed at baseline and 5 and 30 days posttreatment. Endometrial biopsy specimens were obtained for histological assessment of endometritis, chlamydial polymerase chain reaction (PCR), and gonococcal culture. Upper genital tract infection was defined as a positive PCR for Chlamydia trachomatis or a positive culture for Neisseria gonorrhoeae performed on the endometrial biopsy. PCR and cultures were performed at a central reference laboratory. For the patients with endometrial biopsies, 2 reference pathologists separately evaluated at least 1 section stained with hematoxylin and eosin and at least 1 stained with methyl green–pyronin. Disagreements about the presence or absences of neutrophils and plasma cells were settled by both pathologists reading the slides together and coming to an agreement. Histological endometritis was based on a modification of the criteria proposed by Kiviat et al [19]. Self-reported pregnancy, based on a positive urine/blood test or physician’s diagnosis, was assessed over a mean of 84 months.

Buffy coat and serum samples were genotyped for TLR and TLR adaptor-molecule SNPs. For this study, 18 tagging SNPs (tagSNPs), including 3 from TLR1 (rs5743618, rs5743817, rs4833095), 3 from TLR2 (rs3804099, rs11938228, rs1898830), 3 from TLR6 (rs1039559, rs5743810, rs3775073), 4 from TLR4 (rs4986790, rs1927911, rs11536887, rs5030728), 2 from MyD88 (rs4988457, rs7744), and 3 from TIRAP (rs3802813, rs8171374, rs7932976), were chosen from HapMap (http://hapmap.ncbi.nlm.nih.gov/). For each TLR gene, HapMap was used to identify SNPs with a minor allele frequency ≥0.05. Blocks of linkage disequilibrium (LD) were then identified, and a subset of tagSNPs was chosen to account for the variation across the gene.

All SNPs were genotyped by fluorescence polarization [20]. PCR reaction included 2.5 μL of 1× PCR buffer (Invitrogen), 1.0 μL MgCl₂, 4 μL dNTPs, 1.5 μL of each primer, 0.1 μL Taq polymerase (Invitrogen), and 13.4 μL H₂O, for a total volume of 25.0 μL. Amplification was performed using a Peltier Thermal Cycler (MJ Research). Thermal cycling conditions were 95°C for 3 minutes, then 35 cycles of 95°C for 30 seconds for denaturing, 55°C for 30 seconds for annealing, 72°C for 30 seconds for extension, and a final extension step of 72°C for 1 minute. PCR products were resolved by electrophoresis in a 3% agar gel and visualized under UV light after ethidium bromide staining. Genotypes were assigned by direct comparison to controls of sequence-confirmed genotypes, and a 5% random resample was included for consistency of the genotyping. To check for possible genotyping errors, all SNPs in the control group were tested for deviations from Hardy–Weinberg equilibrium. Furthermore, allele frequencies in our cohort were compared to those reported on HapMap.org.

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null distribution and determined that an observed $P$ value <.004 would be significant (at empirical $\alpha \leq .05$). All analyses were performed using SAS/Genetics version 9.1.3 (Cary, NC).

**RESULTS**

Compared with chlamydial-negative women, chlamydial-positive women were more likely to be under the age of 25 ($P < .001$) and have elevated CRP ($>5$ mg/dL; $P = .0003$) and endometritis ($P = .006$), but were less likely to have bilateral adnexal tenderness ($P = .02$) or an elevated temperature ($P = .02$) (Table 1). All other demographic variables were similar between groups.

Logistic regression revealed that among women with PID, the TLR4 rs1927911 CC genotype increased the odds of chlamydial infection (adjusted odds ratio [AOR], 2.8; 95% CI, 1.3-6.2), although this association was not statistically significant after permutations (observed $P = .008$). No significant associations were found between any other TLR or adaptor-molecule SNP and chlamydia. A subanalysis among 164 women who had data on upper genital tract infection revealed that the TLR1 rs5743618 TT genotype (AOR, 2.8; 95% CI, 1.2-8.1; $P = .02$) and the TLR4 rs1927911 CC genotype (AOR 3.5; 95% CI, 1.6-8.8; $P = .002$) were both associated with upper genital tract infection (Table 3). We ran an exploratory analysis to
examine the association between the TLR1 and TLR4 SNPs in a subset of 51 white women. We found that women who carried the TLR1 rs5743618 TT genotype (OR, 2.1; 95% CI, .5–9.8) and the TLR4 rs1927911 CC genotype (OR, 1.1; 95% CI, .3–3.7) were more likely to test positive for C. trachomatis infection. These results were nonsignificant.

Although our power was limited, in a subset of 94 women with C. trachomatis, we found that the TLR1 rs5743618 TT genotype was more frequent in women who did not become pregnant over follow-up compared with women who did (93% vs 79%; Table 4). However, because of small cell size, we were unable to perform logistic regression. Women who carried the GG genotype for TLR1 rs4833095 had decreased pregnancy rates (adjusted hazard ratio [AHR], 0.5; 95% CI, .3–.9; P = .04).

**DISCUSSION**

In this cohort of African American women with mild to moderate PID, TLR1 and TLR4 variants were associated with C. trachomatis infection. These variants also displayed trends toward increased odds of upper genital tract infection. TLR1 variants were also associated with decreased pregnancy rates among a subset of women with C. trachomatis infection. This study provides novel insight into the pathogenesis of C. trachomatis infection.

Toll-like receptors initiate microbial elimination through the production of inflammatory cytokines and chemokines via activation of nuclear factor κ-B (NF-κB) [10–12]. Although this initially results in a healthy immune response, variations in these genes may alter TLR signaling, possibly playing a role in disease progression. Variations in these genes may also explain the variability seen in the course and outcome of C. trachomatis infection. Our results show that TLR1 and TLR4 variants may play a role in the development of chlamydial PID. Chlamydia spp. infect epithelial cells, leading to the secretion of proinflammatory cytokines [7–9]. This inflammatory response may be responsible for long-term damage of the reproductive tract following C. trachomatis infection [21, 22]. We found that compared with chlamydial-negative women, chlamydial-positive women were more likely to have elevated CRP. C-reactive protein is an acute-phase protein and is an indicator of inflammation, suggesting that the women with PID due to chlamydial infection had an increased systemic inflammatory response compared with women with PID without chlamydial infection. Chlamydial-positive women were also more likely to have evidence of histologic endometritis. It is possible that chlamydial infection more frequently causes detectable endometrial inflammation in women with PID symptoms. However, the diagnosis of PID was likely more specific in women with PID symptoms and documented chlamydial infection.

We found that among women with PID, the CC genotype of TLR4 SNP rs1927911 was associated with increased odds of C. trachomatis infection. Studies among Dutch white populations have not found significant associations between TLR4 variants and C. trachomatis [15, 17]. However, our patient populations differed, and the authors did not include rs1927911 in

### Table 3. Associations Between Selected Single-Nucleotide Polymorphisms (SNPs) and Upper Genital Tract Infection (UGTI)

<table>
<thead>
<tr>
<th>SNPs and Genotypes</th>
<th>UGTI Negativea (N = 101), No. (%)</th>
<th>UGTI Positivea (N = 64), No. (%)</th>
<th>AORb (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs5743618</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG + GT</td>
<td>29 (30.9)</td>
<td>6 (12.2)</td>
<td>Referent</td>
<td>...</td>
</tr>
<tr>
<td>TT</td>
<td>65 (69.2)</td>
<td>43 (87.8)</td>
<td>3.1 (1.2–8.1)</td>
<td>.0214</td>
</tr>
<tr>
<td>rs4833095</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA + AG</td>
<td>41 (44.1)</td>
<td>17 (31.5)</td>
<td>Referent</td>
<td>...</td>
</tr>
<tr>
<td>GG</td>
<td>52 (55.9)</td>
<td>37 (68.5)</td>
<td>1.7 (0.8–3.5)</td>
<td>.1377</td>
</tr>
<tr>
<td>TLR 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1927911</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>43 (43.4)</td>
<td>18 (30.0)</td>
<td>Referent</td>
<td>...</td>
</tr>
<tr>
<td>CT</td>
<td>40 (40.4)</td>
<td>19 (31.7)</td>
<td>1.2 (0.5–2.6)</td>
<td>.6898</td>
</tr>
<tr>
<td>CC</td>
<td>16 (16.2)</td>
<td>23 (38.3)</td>
<td>3.5 (1.6–8.8)</td>
<td>.0021</td>
</tr>
</tbody>
</table>

Logistic regression was used to derive odds ratios, 95% CIs, and p values. Abbreviations: AOR, adjusted odds ratio; CI, confidence interval.

a Adjusted for age and history of infertility.

b Cell count too small for analysis.

### Table 4. Associations Between Selected Single-Nucleotide Polymorphisms (SNPs) and Pregnancy Among a Subset of Women With Chlamydia trachomatis Infection

<table>
<thead>
<tr>
<th>SNPs and Genotypes</th>
<th>Pregnant (N = 62), No. (%)</th>
<th>Not Pregnant (N = 32), No. (%)</th>
<th>AORb (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs5743618</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG + GT</td>
<td>10 (21.2)</td>
<td>2 (7.1)</td>
<td>NAb</td>
</tr>
<tr>
<td>TT</td>
<td>37 (78.7)</td>
<td>26 (92.9)</td>
<td>...</td>
</tr>
<tr>
<td>rs4833095</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA + AG</td>
<td>19 (41.3)</td>
<td>9 (29.0)</td>
<td>Referent</td>
</tr>
<tr>
<td>GG</td>
<td>27 (58.7)</td>
<td>22 (71.0)</td>
<td>0.5 (3–9)</td>
</tr>
<tr>
<td>TLR 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1927911</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>18 (31.6)</td>
<td>12 (37.5)</td>
<td>Referent</td>
</tr>
<tr>
<td>CT</td>
<td>17 (29.8)</td>
<td>10 (31.3)</td>
<td>1.2 (0.4–3.9)</td>
</tr>
<tr>
<td>CC</td>
<td>22 (38.6)</td>
<td>10 (31.3)</td>
<td>1.0 (0.6–2.1)</td>
</tr>
</tbody>
</table>

Logistic regression was used to derive odds ratios and 95% CIs. Abbreviation: AOR, adjusted odds ratio; CI, confidence interval; NA, not applicable.

a Adjusted for age and history of infertility.

b Cell count too small for analysis.
their study. It is possible that TLR4 plays a role in \textit{C. trachomatis} pathogenesis. TLR4 is expressed in the female reproductive tract and has been reported to be present in the endocervix, endometrium, and Fallopian tubes [12, 23–25]. In addition, TLR4 can recognize both chlamydial LPS and cHSP60 [26]. Several retrospective studies have found cHSP60 to be linked with chlamydia-associated tubal infertility and PID [27–30], whereas others have determined immune responses to cHSP60 correlate with protection from infection or disease [31, 32]. However, little is known about the functional or clinical relevance of TLR4 rs1927911. In a white population consisting of 110 lung transplant recipients and 422 healthy controls, the TT genotype was found to increase the odds of bronchiolitis obliterans (OR, 4.20; 95% CI, 1.43–12.35; \( P = .005 \)) [33]. Functional analyses are needed to determine the relevance of rs1927911. Because this SNP is located in the intron, it may be in LD with another functional SNP. However, using Haploview, we were unable to find any reported SNPs in strong LD with this variant in African American populations.

TLR2 has been recognized to play a role in chlamydial infections [13, 14]. However, we did not find any significant associations between TLR2 variants and \textit{C. trachomatis}, although we only had 35% gene coverage for TLR2. Other TLR2 variants that were not tagged by our SNPs should be further examined. TLR2 can form a dimer with TLR1 to recognize a wide range of pathogens, and TLR1 can affect the level of signaling through TLR2 [34]. We found an association between the TLR1 rs5743618 TT genotype and increased \textit{C. trachomatis} infection. TLR1 variants were also associated with decreased pregnancy rates among women with \textit{C. trachomatis} infection. In addition, rs5743618 was associated with upper genital tract infection after adjusting for age. The association between the TT genotype and chlamydia did not reach statistical significance after permutations. However, our sample size limited our power. TLR1 rs5743618 is a nonsynonymous mutation and results in an amino acid change. The G allele has been reported to be associated with deficient TLR signaling in comparison with the T allele [35–38] and has been reported to reduce leprosy [35, 36, 39]. Hawn et al [35] reported that the T allele expressed significantly greater NF-\( \kappa B \) signaling in transfected HEK293 cells compared with the G allele. Because rs5743618 has possible functional relevance, it should be further explored in \textit{C. trachomatis} pathogenesis.

Interactions between the responses elicited by chlamydial stimulation of multiple TLRs and other innate immune receptors may also be involved in determining the balance of cytokine production and thus the ultimate outcome [40]. Functional interactions between TLR2 and TLR1 or TLR6 have been demonstrated in vitro, with TLR6 and TLR1 enhancing or impeding, respectively, the TLR2-dependent response to phenol-soluble modulin, a factor secreted by \textit{Staphylococcus epidermidis} [34]. Thus, the ratio of different TLRs within a cell may modify the response to a given ligand. It is clear that interactions between TLR2 and other TLRs following \textit{C. trachomatis} infection need to be further explored. Due to our sample size, we did not examine any interactions, but we acknowledge that our results may be masked by gene–gene or gene–environment interactions.

Our study has several strengths. First, data were obtained from a large, multicenter, prospective, randomized clinical trial with comprehensive demographic, clinical, and obstetric measurements. These findings are generalizable to patients treated for clinically suspected PID. Not all women in the PEACH study had blood samples available for analyses. However, important demographic and clinical characteristics between women with and without blood samples did not differ. This is the first study to examine the role of several TLR and adaptor-molecule SNPs in chlamydial PID. However, our sample size limited our power. We also had low SNP coverage for our genes. Other TLR variants and variants in other PRRs that recognize Chlamydiae [40–42] should be explored in chlamydial pathogenesis. We also relied on an internal control group for comparison. Therefore, women in the control groups all had clinically suspected PID. This may have biased comparisons between chlamydial-positive and chlamydial-negative women toward the null. For comparisons of postchlamydial PID sequelae, this was not a limitation.

Studies in the mouse model of chlamydial genital tract infection have revealed TLR2 activation is critical for the development of oviduct pathology [13, 14, 43]. TLR2 activation is the result of engagement of TLR1/2 or TLR2/6 heterodimers [34]. The association of the TLR1 rs5743618 TT genotype with increased \textit{C. trachomatis} as a cause of PID and a trend for increased upper genital tract infection and decreased pregnancy raises the possibility that we have discovered a genetic risk predictor of enhanced disease due to chlamydia. This is supported by studies documenting that the T allele leads to significantly greater NF-\( \kappa B \) signaling and inflammatory cytokine production [35]. Although the CC genotype of TLR4 SNP rs1927911 was associated with increased odds for chlamydial infection as a cause of PID, this allele was not associated with decreased pregnancy. This may not be surprising because TLR4-deficient mice demonstrate levels of pathology similar to wild-type mice after chlamydial infection [13].

Further exploration of the role of innate immune receptors in the course and outcome of \textit{C. trachomatis} is needed to delineate its pathogenesis. This should include comparisons between women with chlamydial PID and women with uncomplicated lower genital \textit{C. trachomatis} infection. Such research may lead to better management and control of \textit{C. trachomatis}, possibly by identification of biomarkers that predict patients with increased risk that require increased screening and would benefit most from a vaccine.
Notes

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References


