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Host polymorphisms in TLR9 and IL10 are associated with the outcomes of experimental Haemophilus ducreyi infection in human volunteers

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ABSTRACT

**Background.** In humans inoculated with *Haemophilus ducreyi*, there are host effects on the possible clinical outcomes - pustule formation vs. spontaneous resolution of infection. However, the immunogenetic factors that influence these outcomes are unknown. Here we examined the role of 14 single nucleotide polymorphisms (SNPs) in seven selected pathogen recognition pathways and cytokine genes on the gradated outcomes of experimental infection.

**Methods.** DNAs from 105 volunteers infected with *H. ducreyi* at 3 sites were genotyped for SNPs using real-time PCR. The participants were classified into 2 cohorts by race and four groups based on whether they formed 0, 1, 2, or 3 pustules. $X^2$ tests for trend and logistic regression analyses were performed on the data.

**Results.** In European Americans, the most significant findings were a protective association of the TLR9 +2848 GG genotype and a risk enhancing association of the TLR9 TA haplotype with pustule formation; logistic regression showed a trend towards protection for the TLR9 +2848 GG genotype. In African Americans, logistic regression showed a protective effect for the IL10 -2849 AA genotype and a risk enhancing effect for the IL10 AAC haplotype.

**Conclusions.** Variations in TLR9 and IL10 are associated with the outcome of *H. ducreyi* infection.
BACKGROUND

*Haemophilus ducreyi* causes chancroid, a sexually transmitted disease that presents as painful genital ulcers and facilitates the transmission and acquisition of the human immunodeficiency virus (HIV) type 1 (1). Due to syndromic management of genital ulcers, the global prevalence of chancroid is currently undefined but has declined in many former areas of high endemicity (2, 3). Recently, *H. ducreyi* was found to be the leading cause of cutaneous ulcers in children in yaws-endemic communities of the South Pacific islands and equatorial Africa (3-7). Thus, *H. ducreyi* is an important threat to global health.

To study the biology of *H. ducreyi*, we developed a model in which healthy adult volunteers are inoculated at 3 sites on an upper arm with identical doses of the genital ulcer isolate, 35000HP (HP, human passaged) (8, 9). Papules develop at infected sites within 24 h and either spontaneously resolve or progress into pustules within 2 to 5 days. Within a person, the outcomes (resolution vs. pustule formation) of infected sites tend to be similar, suggesting a host effect on disease progression (10, 11). When reinfected, volunteers initially classified as “resolvers” or “pustule formers” segregate towards their initial outcomes, confirming a host effect on susceptibility (10).

Experimental pustules and natural ulcers represent a failed immune response. These lesions resemble suppurative granulomas in that they consist of polymorphonuclear leukocytes (PMN) that form an epidermal abscess, a collar of macrophages admixed with regulatory T cells below the abscess, and a deep dermal infiltrate of memory CD4, CD8, and NK cells (12-15). Unlike most bacteria that cause granulomas, *H. ducreyi* is surrounded by PMN and macrophages and is extracellular (16, 17). Thus, evasion of phagocytosis underlies disease progression (18-21). The mechanism of bacterial clearance in resolvers is unknown but likely involves enhanced phagocytic clearance, which may be shaped by the microenvironment at the infected site (10, 22). Comparative transcriptional analysis of skin biopsies obtained after a repeat infection shows that relative to resolvers, the lesional microenvironment of pustule formers is marked by a hyperinflammatory, dysregulated state (22). When infected with *H. ducreyi*, monocyte-derived myeloid dendritic cells (DC) obtained from resolvers have a transcriptional response typical of type 1 DC, while those derived from pustule formers have a mixed response with features of type 1 DC and regulatory DC, marked by upregulation of IL-10 (22). In addition, the preinfection microbiome of resolvers share a similar community structure.
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that significantly differs from the preinfection microbiome of pustule formers, which is more diverse (23). This finding may reflect biases in innate immunity between the two groups that drive different compositions of the microbiome (23). These data led us to hypothesize that there may be an immunogenetic basis for differential innate immune responses to *H. ducreyi* that ultimately determine disease outcome.

Host immunogenetic factors are associated with the outcome of other bacterial sexually transmitted infections (24-27). For instance, single nucleotide polymorphisms (SNP) in the Toll-like receptor (*TLR*) 4 and *TLR9* genes affect the susceptibility to and severity of *Chlamydia trachomatis* infections (24, 25). These polymorphisms affect the ability of the TLRs to detect pathogen associated molecular patterns, impeding the host immune response to infection.

In this study, we examined whether SNPs in genes that encode pathogen recognition receptors (PRR), control innate immune responses, or encode cytokines correlated with the outcomes of experimental infection in two cohorts of experimentally infected European Americans (EA) and African Americans (AA). As innate immune responses appear to be important in determining outcome, we analyzed SNPs in TLRs, nucleotide oligomerization domain (NOD)-like receptors, single immunoglobulin interleukin (IL) 1 receptor (SIGIRR) and IL-10.

**METHODS**

**Sample collection**

Between March 2000 and June 2014, we collected blood from 144 healthy adult volunteers, who had no history of previous *H. ducreyi* infection (Figure 1). Each volunteer was inoculated with strain 35000HP on one arm at 3 sites vertically spaced 3 cm apart on the skin overlying the upper deltoid via 1.9 mm puncture wounds made with an allergy testing device, which delivers the bacteria to the epidermis and dermis. Each site received identical doses of 35000HP, which was prepared from dedicated freezer lots according to US Food and Drug Administration guidelines. Most participants were enrolled in mutant versus parent comparison trials and were also infected on the opposite arm with isogenic mutants derived from 35000HP, which can be attenuated or fully virulent for pustule formation (9). Resolvers who formed pustules at sites inoculated with virulent mutants were considered capable of pustule formation; 3 such participants were excluded from the analysis.
In the model, we attempt to deliver a standard dose of ~ 90 colony forming units (CFU) of 35000HP. However, *H. ducreyi* has a tendency to clump, which causes variation in actual dose. Data based on infection of 299 participants show a significant effect of dose on pustule formation, which increases by 0.7% per CFU ($P = 0.001$). To adjust for potential differences in doses between the resolvers and pustule formers, we excluded 15 participants who had been inoculated with 35000HP doses < 34 CFU and ≥ 130 CFU.

From the remaining 126 persons, 19 samples were lost and 2 samples were not amplifiable; thus, we recovered amplifiable DNA from 105 participants. The participants were divided into EA and AA cohorts by self-report. Each cohort was divided into 4 groups, who formed 0 (resolvers), or 1, 2, or 3 pustules (pustule formers) at 35000HP-inoculated sites. The participants included 59 EA (33 males and 26 females; age range 21 – 59 years; mean age ± standard deviation (SD), 36.3 ± 11.8 years) and 46 AA (29 males and 17 females; age range 21 – 64 years; mean age ± SD, 42.3 ± 10.6 years) (Figure 1).

**Figure 1:** Participant and sample selection flow chart for the European American (EA) and the African American (AA) cohorts. Data are no. of participants or samples.
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Ethics Statement

Study protocols and informed consent statements were approved by the Division of Microbiology and Infectious Diseases of the National Institutes of Allergy and Infectious Diseases and by the Institutional Review Board of Indiana University.

DNA isolation

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using the Accuspin™ System–Histopaque®-1077 kit (Sigma-Aldrich). DNA was isolated from PBMC using the High Pure PCR Template Preparation Kit (Roche Applied Science).

SNP determination

The isolated DNA was genotyped for 14 SNPs in seven genes (Table I) using Real Time PCR assays on the LightCycler 480 (Roche Molecular Diagnostics, Almere, The Netherlands). The PCR conditions were: initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 60 s, and elongation at 72 °C for 1 s. For the SNP IL10 -1082 A>G, annealing was done at 55 °C for 1 minute each cycle, using primer sequences as described in Supplementary file 1, which can be found in the online version of this article.

Table 1: Genes, SNPs, alleles, rs numbers, and haplotype configurations analyzed in this study. Abbreviations: SNP, single nucleotide polymorphisms; rs number, reference SNP identification number.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Allele</th>
<th>rs number</th>
<th>Haplotype configurations at selected loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>-16934</td>
<td>T&gt;A</td>
<td>rs4696480</td>
<td>TG/TA/AG</td>
</tr>
<tr>
<td></td>
<td>+2477</td>
<td>G&gt;A</td>
<td>rs5743708</td>
<td>TG/TA/AG</td>
</tr>
<tr>
<td>TLR4</td>
<td>+896</td>
<td>A&gt;G</td>
<td>rs4986790</td>
<td>...</td>
</tr>
<tr>
<td>TLR9</td>
<td>-1237</td>
<td>T&gt;C</td>
<td>rs5743836</td>
<td>TA/TG/CA/CG</td>
</tr>
<tr>
<td></td>
<td>+2848</td>
<td>A&gt;G</td>
<td>rs352140</td>
<td>TA/TG/CA/CG</td>
</tr>
<tr>
<td>NOD1</td>
<td>+32656</td>
<td>T&gt;G</td>
<td>rs6958571</td>
<td>...</td>
</tr>
<tr>
<td>NOD2</td>
<td>+2104</td>
<td>C&gt;T</td>
<td>rs2066844</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>+2722</td>
<td>C insertion</td>
<td>rs2066847</td>
<td>...</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>-146</td>
<td>G&gt;T</td>
<td>rs7396562</td>
<td>GCA/GGC/TGG/TTA</td>
</tr>
<tr>
<td></td>
<td>+53</td>
<td>C&gt;T</td>
<td>rs4074794</td>
<td>GCA/GGC/TGG/TTA</td>
</tr>
<tr>
<td></td>
<td>+935</td>
<td>G&gt;A</td>
<td>rs3210908</td>
<td>GCA/GGC/TGG/TTA</td>
</tr>
<tr>
<td>IL10</td>
<td>-2849</td>
<td>A&gt;G</td>
<td>rs6703630</td>
<td>AAC/AAT/AGC/GAC/GGC/AGT/GGT</td>
</tr>
<tr>
<td></td>
<td>-1082</td>
<td>A&gt;G</td>
<td>rs1800896</td>
<td>AAC/AAT/AGC/GAC/GGC/AGT/GGT</td>
</tr>
<tr>
<td></td>
<td>-819</td>
<td>C&gt;T</td>
<td>rs1800871</td>
<td>AAC/AAT/AGC/GAC/GGC/AGT/GGT</td>
</tr>
</tbody>
</table>
**Statistical Analyses**

Statistical analyses were performed using Graphpad Instat 3. Results between sample groups were examined for Hardy Weinberg equilibrium. \( \chi^2 \) tests for trends were performed where appropriate to assess differences in genotype distributions between the groups (0, 1, 2, or 3 pustules). Haplotype distribution (Table 1) was inferred using PHASE software and analysed using \( \chi^2 \) tests for trends. Carrier trait analyses were performed to examine synergy in protective or risk enhancing associations of different SNPs and haplotypes. To reduce data complexity, binary logistic regression was performed using SPSS v20.

**Analysis of H. ducreyi CpG motifs**

To determine the potential immunostimulatory activity of 35000HP DNA, we calculated the CpG index for \( H. ducreyi \) exactly as described previously (28, 29). The results for \( H. ducreyi \) were compared to those calculated previously for \( Streptococcus pneumoniae, Neisseria meningitidis, \) and \( Haemophilus influenzae \).

**RESULTS**

**Genotyping results**

Amplifiable DNA was recovered from 105 persons who were infected with \( H. ducreyi \) and met inclusion criteria. The participants included 59 EA and 46 AA who formed 0, 1, 2, or 3 pustules (Figure 1). In each cohort, there were no significant differences in the doses of \( H. ducreyi \) among the four outcome groups (data not shown). Table 2 shows the overall frequency of the genotypes in each cohort.

We assessed potential links between SNPs and haplotypes and the outcome of infection by using \( \chi^2 \) tests. Within each ethnicity, \( \chi^2 \) tests for trend on the SNPs and haplotypes showed multiple significant results (Figure 2 and Figure 3). There were significant protective associations against pustule formation for the \( TLR9 +2848 \) GG (\( P = 0.004 \)) and *G genotypes (\( P = 0.041 \)) and for the \( IL10 \) AGC haplotype (\( P = 0.009 \)) in the EA cohort. A significant risk enhancing association for pustule formation was found for the haplotype \( TLR9 \) TA in the EA cohort (\( P = 0.005 \)); a borderline risk enhancing association was found for the haplotype \( IL10 \) AAC (\( P = 0.058 \)) in the AA cohort. No significant results were found for the other analysed SNPs or haplotypes.
### Table 2: Genotype frequencies and percentages of the SNPs in the cohorts. Data represent the number of persons and (their percentage) in each cohort. Abbreviations: SNP, single nucleotide polymorphism; EA, European American; AA, African American; WT, wild type; HZ, Heterozygous; MT, Mutant allele

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Allele</th>
<th>EA cohort WT</th>
<th>EA cohort HZ</th>
<th>EA cohort MT</th>
<th>AA cohort WT</th>
<th>AA cohort HZ</th>
<th>AA cohort MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>-16934</td>
<td>T&gt;A</td>
<td>6 (10)</td>
<td>27 (48)</td>
<td>26 (44)</td>
<td>23 (50)</td>
<td>20 (43)</td>
<td>3 (7)</td>
</tr>
<tr>
<td>TLR2</td>
<td>+2477</td>
<td>A&gt;G</td>
<td>54 (92)</td>
<td>5 (8)</td>
<td>0 (0)</td>
<td>45 (98)</td>
<td>1 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>TLR4</td>
<td>+896</td>
<td>A&gt;G</td>
<td>52 (88)</td>
<td>7 (12)</td>
<td>0 (0)</td>
<td>39 (85)</td>
<td>7 (15)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>TLR9</td>
<td>-1237</td>
<td>T&gt;C</td>
<td>46 (78)</td>
<td>11 (19)</td>
<td>2 (3)</td>
<td>13 (28)</td>
<td>24 (52)</td>
<td>9 (19)</td>
</tr>
<tr>
<td>TLR9</td>
<td>+2848</td>
<td>A&gt;G</td>
<td>19 (32)</td>
<td>28 (47)</td>
<td>12 (20)</td>
<td>2 (4)</td>
<td>27 (59)</td>
<td>17 (37)</td>
</tr>
<tr>
<td>NOD1</td>
<td>+32656</td>
<td>T&gt;GG</td>
<td>35 (59)</td>
<td>22 (37)</td>
<td>2 (3)</td>
<td>24 (52)</td>
<td>17 (37)</td>
<td>5 (11)</td>
</tr>
<tr>
<td>NOD2</td>
<td>+2104</td>
<td>C&gt;T</td>
<td>52 (88)</td>
<td>7 (12)</td>
<td>0 (0)</td>
<td>46 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NOD2</td>
<td>+3020</td>
<td>C insertion</td>
<td>54 (92)</td>
<td>5 (8)</td>
<td>0 (0)</td>
<td>46 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>-146</td>
<td>G&gt;T</td>
<td>47 (80)</td>
<td>10 (17)</td>
<td>2 (3)</td>
<td>21 (46)</td>
<td>22 (48)</td>
<td>3 (7)</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>+53</td>
<td>C&gt;T</td>
<td>47 (80)</td>
<td>10 (17)</td>
<td>2 (3)</td>
<td>21 (46)</td>
<td>22 (48)</td>
<td>3 (7)</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>+935</td>
<td>G&gt;A</td>
<td>27 (46)</td>
<td>25 (42)</td>
<td>7 (12)</td>
<td>41 (89)</td>
<td>4 (9)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>IL10</td>
<td>-2849</td>
<td>A&gt;G</td>
<td>36 (61)</td>
<td>20 (34)</td>
<td>3 (5)</td>
<td>25 (54)</td>
<td>16 (35)</td>
<td>5 (11)</td>
</tr>
<tr>
<td>IL10</td>
<td>-1082</td>
<td>A&gt;G</td>
<td>20 (34)</td>
<td>25 (42)</td>
<td>14 (24)</td>
<td>15 (33)</td>
<td>23 (50)</td>
<td>8 (17)</td>
</tr>
<tr>
<td>IL10</td>
<td>-819</td>
<td>C&gt;T</td>
<td>27 (48)</td>
<td>26 (44)</td>
<td>6 (9)</td>
<td>18 (39)</td>
<td>22 (48)</td>
<td>6 (13)</td>
</tr>
</tbody>
</table>
Figure 2: Bar plots and trend lines for SNPs and haplotypes found to have significant effects on the outcome of experimental infection in European American using the X² tests for trends. The data shows the % of volunteers who carried a particular SNP or haplotype in the four outcome groups. Analyses are shown for A, TLR9 +2848 *G genotype; B, TLR9 +2848 GG genotype; C, TLR9 haplotype TA; and D, IL-10 haplotype AGC. The data in panels A, B and D show protective effects against pustule formation, while the data in panel C shows a risk enhancing effect.
Figure 3: Bar plot and trend line for the IL10 AAC haplotype, which had a significant risk enhancing effect on the outcome of experimental infection in African Americans using the $X^2$ tests for trends. The data shows the % of volunteers who carried this haplotype in the four outcome groups.

**Carrier trait analyses**

We assessed the synergy in protective or risk enhancing associations between combined SNPs or haplotypes and the outcome of infection by $X^2$ tests for trends. Two combinations of variables showed a significant association with the severity of *H. ducreyi* infection. In the EA cohort, only the TLR9 +2848 *G* genotype combined with the IL10 AGC haplotype had an increased significance of a protective effect compared to any of the single SNPs ($P = 0.012$). In the AA cohort, the IL10 -2849 *G* genotype combined with the SIGIRR TTG haplotype had an increased significance of a protective effect compared to single SNPs or haplotypes ($P = 0.02$).
**Logistic regression**

We used forward stepwise binary logistic regression with dichotomized groups of the formed pustules as the dependent variable to produce models for each cohort. Only variables with \( P < 0.2 \) in the \( X^2 \) tests for trend were included in the models. In the EA cohort, the model included SNPs at \( TLR2 \) -16934, \( TLR9 +2848 \), and \( SIGIRR +935 \), the \( TLR9 \) haplotype TG and the \( IL10 \) haplotypes AGC and GGC. In the AA cohort, the model included SNPs at \( IL10 -819 \) and \( IL10 -2849 \), the \( SIGIRR \) haplotype TTG, and the \( IL10 \) haplotype AAC. The major results are shown in Table 3. In the EA cohort, there was a trend towards a protective association with the \( TLR9 +2848 \) GG genotype (\( P = 0.052 \)); in the AA cohort, the \( IL10 -2849 \) AA genotype showed a significant protective association (\( P = 0.032 \)) and the \( IL10 \) AAC haplotype a significant risk enhancing association (\( P = 0.024 \)). In general, these results were consistent with the trends analysis shown in Figure 2 and Figure 3. No significant results were found for the other analysed SNPs and haplotypes.

**Table 3**: Results of logistic regression on probable association models. Abbreviations: EA, European American; AA, African American; OR, odds ratio; CI, confidence interval.

<table>
<thead>
<tr>
<th>Genotype / Haplotype</th>
<th>Outcome</th>
<th>Cohort</th>
<th>( P ) value</th>
<th>ORa</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>( TLR9 +2848 ) GG</td>
<td>1,2,3 vs. 0</td>
<td>EA</td>
<td>0.052</td>
<td>0.42</td>
<td>0.17-1.01</td>
</tr>
<tr>
<td>( IL10 -2849 ) AA</td>
<td>1,2,3 vs. 0</td>
<td>AA</td>
<td>0.032</td>
<td>0.18</td>
<td>0.04-0.86</td>
</tr>
<tr>
<td>( IL10 ) AAC Haplotype</td>
<td>1,2,3 vs. 0</td>
<td>AA</td>
<td>0.024</td>
<td>3.08</td>
<td>1.16-8.13</td>
</tr>
</tbody>
</table>

a Group dichotomization by outcome (0, 1, 2 or 3 pustules).

**Calculated CpG index**

Since TLR9 is activated by CpG motifs in bacterial DNA, we calculated a CpG index for 35000 HP DNA and compared it to results previously described for several other bacterial pathogens (28). While a CpG index < 1 is considered immunoinhibitory, a CpG index >1 is regarded as immunostimulatory. The calculated CpG index for \( H. ducreyi \) was 6.6, which was similar to the indices calculated for \( S. pneumoniae \) and \( H. influenzae \) (Table 4).
DISCUSSION

Here we sought to find contributions of host immunogenetic factors on the outcome of experimental *H. ducreyi* infection. Because the Hardy-Weinberg equilibrium showed differences in the genotypes of the AA and EA, these cohorts were analysed separately. Our cohorts were unique in that the participants had clearly distinguishable phenotypes and could be placed into defined groups (0, 1, 2 or 3 pustules), which allowed us to do a trend analysis. The fact that all our participants were infected with *H. ducreyi* likely permitted us to find significant genetic associations with disease outcomes despite our small sample size.

Table 4: Calculated CpG indices.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Size (Mb)</th>
<th>G+C (%)</th>
<th>CpG/ kb(^d)</th>
<th>Total CpG(^d)</th>
<th>Stimulatory(^e)</th>
<th>Inhibitory(^f)</th>
<th>CpG index(^g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemophilus ducreyi</em></td>
<td>1.7</td>
<td>38.2</td>
<td>40.9</td>
<td>112.2</td>
<td>124.8</td>
<td>110.4</td>
<td>6.6</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>1.91</td>
<td>38.2</td>
<td>72.8</td>
<td>109.1</td>
<td>105.5</td>
<td>96.4</td>
<td>7.2</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>2.22</td>
<td>39.5</td>
<td>78.0</td>
<td>69.5</td>
<td>82.4</td>
<td>66.5</td>
<td>8.6</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>2.27</td>
<td>51.5</td>
<td>132.7</td>
<td>130.6</td>
<td>78.4</td>
<td>140.0</td>
<td>-106.8</td>
</tr>
</tbody>
</table>

\(^a\) Deviations in specified motif occurrences compared to those expected based on genomic G+C content.  
\(^b\) Consensus stimulatory and inhibitory CpG hexamer motifs  
\(^c\) Number of CpG hexamer motifs (NNCGNN) in each genome normalized to 100 kb of DNA  
\(^d\) Total frequency of CpG hexamer motifs (NNCGNN)  
\(^e\) Frequency of stimulatory CpG hexamers (RRCGYY)  
\(^f\) Frequency of inhibitory CpG hexamers (NCCGNN and NNCGRN)  
\(^g\) Index calculated from the difference between stimulatory and inhibitory hexamers, multiplied by the total CpG hexamers, normalized to 1 kb.  
\(^h\) Data are taken from Table 3 of reference (28)

In the EA cohort, we found that the tendency to resolve experimental infection was associated with the TLR9 +2848 *G* and GG genotypes, but the TA haplotype of this gene showed a risk enhancing effect for pustule formation. In contrast, Sanders et al. showed a protective association for TLR9 +2848 GA or AA alleles in control children vs. those with bacterial meningitis in the Netherlands; the protective effect is against *N. meningitidis* but not against *S. pneumoniae* or *H. influenzae* (28). The TLR9 +2848 AA genotype is also associated with
a decreased incidence of positive blood cultures in children who have meningococcal meningitis, again suggesting that some degree of protection against Neisseria meningitidis is conferred by this genotype (30).

One explanation of the different effects of these TLR9 alleles on susceptibility to bacterial infection could be that the activation of TLR9 is triggered by binding of unmethylated bacterial CpG DNA motifs, which lead to the production of inflammatory cytokines (31). The amount and structure of CpG motifs in bacterial DNA affect its ability to activate TLR9; calculated CpG indices > 1 are pro-inflammatory while those < 1 are anti-inflammatory (28, 29). The calculated CpG index for N. meningitidis is very low (-106.8) relative to S. pneumoniae (8.6) and H. influenzae (7.2). This data led to the hypothesis that the TLR9 +2848 GA or AA alleles might compensate for the anti-inflammatory potential of meningococcal DNA and protect the host against disease (28). The CpG index of H. ducreyi 35000HP DNA calculated by the same methodology (28, 29) was 6.6. Since pustule formation is marked by hyperinflammatory responses in tissue and dendritic cells (22), perhaps the TLR9 +2848 *G and GG alleles counter hyperinflammatory responses to H. ducreyi that lead to tissue damage. Similarly, in Ghanaian children with malaria, the TLR9 +2848 GG genotype is not associated with protection against parasitemia (i.e., infection) but is associated with protection from symptomatic disease (i.e., inflammation) (32). In our cohort, the contrasting result found for the TLR9 TA haplotype may be due to the fact that this haplotype lacks the protective TLR9 +2848 *G and GG genotypes. Since TLR9 +2848 G is a synonymous coding SNP, how this SNP affects TLR9 expression and subsequent activity is unclear.

Variation in IL10 polymorphisms and IL-10 production are linked to various immunosuppressive or inflammatory conditions. In our study, we found the IL10 -2849 AA genotype in the AA cohort had a statistically significant protective effect against pustule formation. Two studies reported an association between IL10 -2849 AA and low IL-10 production by endotoxin-stimulated whole blood (33, 34). The finding that IL10 -2849 AA is associated with resolution is consistent with our previous report showing that DC cells derived from resolvers have less IL-10 transcription and secretion than pustule formers in response to H. ducreyi (22). IL-10 is an anti-inflammatory cytokine that inhibits the activation and function of T cells, NK cells and macrophages (35). Production of high levels of IL-10 by DC during H. ducreyi infection could promote Th2 as well as regulatory T cell responses and inhibit the activation of Th1 cells and macrophages, leading to impaired clearance of H. ducreyi (22).
The *IL10* AGC haplotype had a protective effect on *H. ducreyi* infection in the EA cohort, while the *IL10* AAC haplotype showed a risk enhancing effect in the AA cohort. Several studies suggest that protection against infection is linked to low IL-10 producing haplotypes while risk enhancement is linked to high IL-10 producing haplotypes (36, 37). The AAC haplotype has been shown and the ACG haplotype is assumed to be low IL-10 producers due to the inclusion of the genotype *IL10* -2849 A (36). If this is the case, one would expect both haplotypes to be protective against *H. ducreyi*. However, the levels of IL-10 expression could be influenced by *IL10* -1082 genotypes; PBMC from European cohorts with the *IL10* -1082 GG genotype secrete more IL-10 than those with the *IL10* -1082 AA genotype in response to *C. trachomatis* (38). Similarly, *Helicobacter pylori* infected patients with the *IL10* -1082 GG genotype express more IL-10 in mucosal biopsies than those with the AA genotype (37). Additionally, the general genetic background of the EA and AA cohorts might affect IL-10 expression. As no plasma or peripheral blood samples were available from the *H. ducreyi*-infected cohorts, we were unable to correlate their IL-10 secretion capacity with the two IL-10 haplotypes.

In the EA cohort, the TLR9 +2848 *G* genotype combined with the *IL10* AGC haplotype had an increased significance of a protective effect compared to the single SNPs, which may be due to the potential anti-inflammatory effects of both SNPs discussed above. In the AA cohort, the *IL10* -2849 *G* genotype combined with the SIGIRR TTG haplotype also had an increased significance of a protective effect. The *IL10* -2849 *G* genotype is associated with high production of IL-10 (33, 34). SIGIRR is a negative regulator of the TLR pathways and SIGIRR deficiency in mice leads to hyperinflammatory response and tissue damage in microbial infections (39). Currently, there are no other reports on associations of the SIGIRR TTG haplotype with any inflammatory conditions. The SIGIRR -146TT genotype, which is contained in the TTG haplotype, is significantly associated with the susceptibility to systemic lupus erythematosus (40). Perhaps hyperinflammatory responses potentially conferred by the SIGIRR TTG haplotype are offset by potentially higher levels of IL-10 induced by the *IL10* -2849 *G* genotype, leading to a balanced inflammatory response against *H. ducreyi* and effective clearance of the pathogen.

In the human challenge model, there are no effects of race or age on pustule formation, but men form pustules at rates approximately 1.7 fold higher than women, consistent with the high male to female ratio seen in natural chancroid (1). Men and women were included in this study. Analysis for potential gender influences on the results using Mantel-Haenszel
tests in conjunction with Tarone’s tests showed no significant differences between results related to gender.

Since differences in innate immune responses are associated with the outcome of *H. ducreyi* infection, we chose to include genes only from innate immune pathways in this study. One effect of this targeted approach was a reduced need for corrections for multiple comparisons. In addition, the statistical tests utilized in this study provide a clear picture through both univariate and multivariate testing, while the logistic regression model already accounts for multiple comparisons in its design.

Although we found associations between TLR9 and IL-10 SNPs with outcome, no significant links were found for other SNPs in several other genes encoding PRRs. Compared to most immunogenetic studies, which usually compare large groups of infected patients to healthy controls, our cohorts were small; it is possible that the lack of finding other associations was due to our small sample size.

In summary, this is the first study to shed light on the immunogenetic factors affecting the outcome of *H. ducreyi* infection. Our results could be used to predict the risk of susceptibility to *H. ducreyi* infection in future studies. Studies on the effects of the TLR9, IL10, and SIGIRR SNPs on immune responses to *H. ducreyi* are also needed to gain better understanding of differential host susceptibility to the pathogen.

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