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Association of TLR4 Polymorphisms with Symptomatic Respiratory Syncytial Virus Infection in High-Risk Infants and Young Children

Agnes A. Awomoyi,* Prasad Rallabhandi,† Toni I. Pollin,† Eva Lorenz,‡ Marcelo B. Sztein,§ Marina S. Boukhvalova,‖ Val G. Hemming,‖# Jorge C. G. Blanco,‖ and Stefanie N. Vogel2*

Respiratory syncytial virus (RSV) is a leading cause of infant mortality worldwide. Although anti-RSV Ab prophylaxis has greatly reduced infant mortality in the United States, there is currently no vaccine or effective antiviral therapy. RSV fusion (F) protein activates cells through TLR4. Two single nucleotide polymorphisms (SNPs) encoding Asp299Gly and Thr399Ile substitutions in the TLR4 ectodomain were previously associated with TLR4 hyporesponsiveness and increased susceptibility to bacterial infection. Prevalence of these SNPs was analyzed in a case series of 105 DNA samples extracted from archived nasal lavage samples from high-risk infants/young children with confirmed RSV disease who participated in two seminal clinical trials for anti-RSV prophylaxis. Frequencies of TLR4 SNPs in the case series were compared with those of literature controls, healthy adults, infants, and young children who presented with symptoms of respiratory infections (but not preselected for high risk for RSV). Both SNPs were highly associated with symptomatic RSV disease in this largely premature population (p < 0.0001), with 89.5% and 87.6% of cases being heterozygous for Asp299Gly and Thr399Ile polymorphisms versus published control frequencies of 10.5% and 6.5%, respectively. The other two control groups had similarly low frequencies. Our data suggest that heterozygosity of these two extracellular TLR4 polymorphisms is highly associated with symptomatic RSV disease in high-risk infants and support a dual role for TLR4 SNPs in prematurity and increased susceptibility to RSV not revealed by analysis of either alone. The Journal of Immunology, 2007, 179: 3171–3177.

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The Journal of Immunology

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lead to severe lower respiratory tract disease. Two passively administered Abs, RespiGam and Synagis (MedImmune), provide significant prophylactic protection to high-risk infants (7, 8). The idea that the immune response also plays an adverse role in RSV-induced disease is based largely on results of failed clinical trials in which infants vaccinated with formalin-inactivated RSV exhibited greatly enhanced incidence of severe disease or died upon natural infection (reviewed in Ref. 9).

TLR4 is the primary signaling receptor for Gram-negative LPS (reviewed in Ref. 10). Although structurally unrelated to LPS, RSV fusion (F) protein also triggers TLR4 and, like LPS, uses CD14 as a coreceptor (11, 12). The ability to clear RSV has been reported to be significantly impaired in mice with tlr4 mutations (11, 12), consistent with the inability of such mice to contain many bacterial pathogens (reviewed in Ref. 13). In contrast to these findings, Ehl et al. (14) failed to observe an effect of TLR4 deletion on RSV clearance, yet stated that the reason for this discrepancy was unclear. In contrast, Haebeler et al. (15) showed that the early lung NF-κB response to RSV is both alveolar macrophage and TLR4 dependent, suggesting that increased susceptibility to RSV may be, in part, secondary to a failure to elicit an appropriate inflammatory response through TLR4.

Arbour et al. (16) identified two human TLR4 single nucleotide polymorphisms (SNPs) that encode single amino acid substitutions, Asp299Gly (rs4986790) and Thr399Ile (rs4986791), in the ectodomain of TLR4. Both SNPs were associated with an LPS-hyporesponsive phenotype in human airway epithelial cells and alveolar macrophages and a blunted response to inhaled LPS. Kiechl et al. (17) reported that subjects carrying the Asp299Gly polymorphism were more susceptible to bacterial infections but had a lower risk of atherosclerosis. Lorenz et al. (18) reported that the Asp299Gly variant was found at a higher
rate in septic shock patients, and septic shock patients with either SNP exhibited a higher incidence of Gram-negative infection. Collectively, an overwhelming body of functional and genetic data indicates an important role for TLR4 in control of infection and supports the hypothesis that polymorphisms in the TLR4 extracellular domain may reduce the capacity of the host to respond optimally to infection.

Tal et al. (19) provided evidence that full-term babies were at higher risk for RSV infection if they carried either TLR4 SNP. However, full-term babies are at relatively low risk for complicated RSV infections when compared with babies born prematurely, without or with BPD, or with congenital heart defects (6). We therefore postulated that inheritance of these TLR4 polymorphisms would increase susceptibility to RSV infection in a high-risk (e.g., largely premature) population. Our hypothesis was that TLR4 polymorphisms would be overrepresented in a cohort of premature infants/children with documented RSV infection. Herein, we provide compelling evidence derived from archival samples that these two TLR4 SNPs are highly associated with RSV disease in high-risk infants. Our results indicate that a fully functional immune response to natural RSV infection, particularly in high-risk infants, and we speculate that inheritance of TLR4 SNPs may underlie prematurity or other risk factors for RSV infection.

Materials and Methods

DNA samples

DNA was extracted (Puregene DNA purification system; Gentra) from 105 of 165 archived nasal lavage samples of participants of early clinical trials with documented RSV infection (8, 20) (i.e., 54 of 64 and 51 of 101 samples from the two studies, respectively) (Table I). Samples from children who were RSV-negative were not archived. As required by our Institutional Review Boards, no linkage exists between sample codes and demographic data. Nasal lavage samples were concentrated by centrifugation and 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly concentrated by centrifugation. Thereafter, the manufacturer’s instructions were followed with minor modification: 3.3 mg/ml glycogen was added to 100% isopropanol during DNA precipitation. DNA was similarly

Table I. Summary of prophylactic Ab trials for RSV from which case specimens were derived

<table>
<thead>
<tr>
<th>Study</th>
<th>Groothuis et al. (RespiGam) (20)</th>
<th>Impact-RSV (Palivizumab; Synagis) (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Randomized placebo-controlled trial</td>
<td>Randomized double-blind placebo-controlled trial</td>
</tr>
<tr>
<td>Prophylaxis Centers</td>
<td>Multicenter: 5 centers, all within United States</td>
<td>Multicenter 139 centers: United States, 119; Canada, 9; United Kingdom, 11</td>
</tr>
<tr>
<td>Trial size</td>
<td>249 (high dose (n=81); low dose (n = 79)); control (n = 89)</td>
<td>1502 (Palivizumab (n = 1002); placebo (n = 500))</td>
</tr>
<tr>
<td>Male Gender %</td>
<td>56.3</td>
<td>56.9</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White %</td>
<td>63</td>
<td>57.9</td>
</tr>
<tr>
<td>Black %</td>
<td>26</td>
<td>24.2</td>
</tr>
<tr>
<td>Hispanic %</td>
<td>10</td>
<td>10.9</td>
</tr>
<tr>
<td>Asian %</td>
<td>2</td>
<td>2.25</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>4.75</td>
</tr>
<tr>
<td>Study groups</td>
<td>Prematurity (no BPD); gestational age ≤35 weeks (n = 60)</td>
<td>Prematurity (no BPD); gestational age ≤35 weeks (n = 740); Prematurity and BPD (n = 762)</td>
</tr>
<tr>
<td>Confirmed RSV episodes</td>
<td>64 (high dose, 19; low dose, 16; control, 29)</td>
<td>101 (Palivizumab, 48; placebo, 53)</td>
</tr>
</tbody>
</table>

DNA samples were first genotyped using a fluorogenic 5’-nucleic-TaqMan assay in the ABI PRISM 7900HT sequence detection system (Applied Biosystems). Two primers for each polymorphism and an oligonucleotide probe (labeled at the 5’ end with fluorescent dye, VIC or 6FAM, and at the 3’ end with quencher, MGBNFQ) for each allele were synthesized based on TLR4 GenBank accession no. AF177765: Asp299Gly: forward, 5’-TGAGAATTCCATGATATGATTAG-3; reverse 5’-TGGTGAGAACCTGCACCCATTAC-3; reverse 5’-AGGAGGTTGCTGTCTC-3; reverse 5’-GAAACTGATGCCATTACGGTTC-3; Thr399Ile: forward, 6FAM-ACCTCGATGTAGATATGATATTAT-MGBNFQ; polymorphic allele, VIC-ACCTCGATGTAGTTAT-MGBNFQ; polymorphic allele, VIC-TAGGCTGATTGTTGTTCC-MGBNFQ. Amplification was performed in 25 μl containing 10 ng of DNA, 900 nM of each primer, 200 nM of each probe, and 1× TaqMan Universal PCR master mix (Applied Biosystems) in 96-well plates. PCR conditions were as follows: initial denaturation at 95°C, 10 min, followed by 60 cycles of denaturation at 95°C, 15 s, annealing and extension at 60°C, 60 s.

Each DNA sample was also genotyped by sequencing (Biopolymer Core Facility, University of Maryland, Baltimore) after labeling DNA with Big Dye Terminator cycle sequencing kit, v.3.1 (Applied Biosystems) as described (21). Fifty nanograms of genomic DNA was amplified in a 50-μl volume using 10 ng of the forward 5’-CTCTTAGAGGG CCTGTG CAAT-3' and reverse 5’-GAAAGATGTTGACCTGCAAAAC-3' primers encompassing 576 bp of the DNA region containing both TLR4 polymorphisms.

Haplotype phase was determined by cloning PCR products derived from 13 randomly selected, doubly heterozygous genomic DNAs as described (21).

Statistical analysis

Allele and genotype frequencies were estimated by gene counting in 105 samples from high-risk, RSV-infected subjects. Control data was derived from 25 published studies (16–18, 22–43) (totaling 7092 healthy subjects of various ethnicities for the Asp299Gly polymorphism; data from 9 of the 25 published studies [17, 18, 25, 27, 31, 34, 35, 37, 41], totaling 2213 subjects, served as controls for the Thr399Ile polymorphism). DNA samples from 52 healthy adult volunteers from the Greater Baltimore area and 45 pediatric samples from infants and young children with symptomatic, non-RSV respiratory infection were also analyzed (see above). Fisher’s exact tests were used to assess statistical significance of differences in carrier frequencies (proportion of individuals with at least one copy of the
minor allele) and allele frequencies between cases and controls, both separately for each published control sample and for all control samples pooled together. The 95% confidence intervals (CI) for carrier frequencies were calculated using the exact method as implemented in SAS version 9 (SAS).

Results
Case series DNA samples were derived from archived nasal lavages of children who participated in two early clinical trials (8, 20) of Ab-mediated prophylaxis. Table I summarizes these studies and shows that all subjects recruited were at high risk for RSV: the majority were premature (≤35 wk gestation, without or with BPD), but the earlier trial (20) included some subjects with congenital heart disease. In both multicenter trials, subjects were ethnically diverse, with the two trials being very closely matched for ethnicity and gender (Table I). In both studies, subjects were treated with placebo or Ab, and caregivers instructed to return if the child showed signs of respiratory infection. Nasal lavages of symptomatic children were collected and first tested locally. All RSV-positive samples from the multiple trial sites were sent to a single independent laboratory for confirmation. A total of 165 samples from both studies (64 and 101, respectively) were confirmed to be RSV positive, and of these, 105 nasal lavage samples (54 and 51, respectively) contained sufficient material for DNA extraction. Genotype analysis of each DNA sample derived from these RSV-infected subjects by two distinct methods revealed that both TLR4 SNPs were highly over-represented in our case series compared with literature controls (p < 0.0001; Table II and Table III). Specifically, 94 of 105 samples from high-risk infants and children with RSV (89.5%; includes 48 of 54, or 88.9%, and 46 of 51, or 90.2%, in the two separate studies) were heterozygous for the Asp299Gly polymorphism, in contrast to 742 of 7092 control healthy individuals (10.5%) derived from data pooled from 25 published studies (see Fig. 1 legend). Similarly, for the Thr399Ile polymorphism, 92 of 105 (87.6%; includes 46 of 54, or 85.2% and 46 of 51, or 90.2%, in the two separate studies) of our case samples were heterozygous, versus published data of 144 of 2213 healthy controls (6.5%). Ninety-two of 105 samples carried both SNPs heterozygously, whereas no individuals were found to be homozygous for either polymorphism. Fig. 1 illustrates the carrier frequencies of the published control populations versus that of our RSV subjects. For both variants, both carrier and allele frequencies of the minor allele (Gly299 and Ile399) in the cases were significantly higher than in each individual control sample as well as the respective pooled control sample (p < 0.0001 for all comparisons; Tables II and III). Frequencies of the two polymorphisms were virtually identical in the two separate trials (see Fig. 1 legend). The odds ratios (OR) for the association of RSV case status with Asp299Gly and Thr399Ile carriers were 69.3 (95% CI: 36.9, 129.9) and 99.4 (95% CI: 54.3, 182.0), respectively.

To insure the validity of our genotyping methods, DNA constructs encoding WT TLR4, Asp299Gly and/or Thr399Ile polymorphisms (44) were included as positive controls in the TaqMan assay. Each of the 105 DNA samples derived from the case series was also sequenced and confirmed TaqMan results. To preclude the possibility that these samples were contaminated, all of these DNA preparations were subjected to independent analysis by deCODE Genetics Ltd. (Reykjavik, Iceland; www.decode.com) for analysis of other polymorphic markers. After genotyping for TLR4 SNPs, sufficient DNA remained in 53 of 105 original samples to permit further analysis after whole genome amplification (using 59 distinct markers, including 27 highly polymorphic microsatellite markers). The independent analysis of these 53 samples (derived from both studies) revealed no evidence of relatedness among samples and that there were no more than two alleles per locus detected for any individual, indicating that the samples were not contaminated.

Nasal lavages from RSV-negative children from the original trials were not similarly archived and, therefore, were not available for analysis. However, DNA from two additional control groups was similarly analyzed for prevalence of the two TLR4 SNPs. First, DNA derived from 52 healthy, ethnically diverse adults, recruited from the Greater Baltimore area, were also genotyped (see adult controls on Fig. 1). Similar to published values, 7 of 52 individuals were heterozygous for the Asp299Gly polymorphism (13.5%; OR = 54.9 [95% CI: 20.0, 151.1] and p < 0.0001 for RSV subjects versus these controls) and 4 of 52 individuals were heterozygous for the Thr399Ile polymorphism (7.7%; OR = 84.9 [95% CI: 26.3, 274.6] and p < 0.0001 for RSV subjects versus these controls). Three of the seven polymorphic individuals carried both polymorphisms heterozygously.

A pediatric control group included 45 nasal lavage specimens from infants and children who were not selected as being at high risk for RSV, but who presented with respiratory symptoms consistent with viral infection (Fig. 1), none of whom were diagnosed as RSV-positive. Similar to the literature control values and those of the healthy adult subjects, the frequencies of the Asp299Gly (Table IV) and Thr399Ile (Table V) polymorphisms were quite low, i.e., 4.4% (OR = 183.7 [95% CI: 39.0, 865.0] and p < 0.0001 versus RSV subjects) and 2.2% (OR = 311.4 [95% CI: 39.5, 2456.4] and p < 0.0001 for RSV subjects versus these pediatric controls), respectively.

Table II. Analysis of carrier and allele frequencies of Asp299Gly TLR4 polymorphism in a case series of high-risk infants with symptomatic RSV

<table>
<thead>
<tr>
<th>Asp299Gly</th>
<th>Literature Controls</th>
<th>RSV Subjects</th>
<th>( \chi^2 ) p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total subjects</td>
<td>7092</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>AA (Asp/Asp)</td>
<td>6313 (89.0%)</td>
<td>11 (10.5%)</td>
<td></td>
</tr>
<tr>
<td>AG (Asp/Gly)</td>
<td>742 (10.5%)</td>
<td>94 (89.5%)</td>
<td></td>
</tr>
<tr>
<td>GG (Gly/Gly)</td>
<td>37 (0.5%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
<tr>
<td>Carrier frequency</td>
<td>0.11</td>
<td>0.90</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.10,10.12)</td>
<td>(0.82,0.95)</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.00–0.21</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Allele (G) Frequency</td>
<td>0.06</td>
<td>0.45</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.05,0.06)</td>
<td>(0.38,0.52)</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.00–0.11</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*The case series of 105 infants and children with symptomatic RSV comprises two separate study samples composed of 54 and 51 subjects (of 64 and 101 subjects in the original studies, respectively). Frequencies of the two polymorphisms were virtually identical in the two studies (Asp299Gly, 90.2% vs 88.9%, \( \chi^2 p = 0.83, \) Fisher’s exact \( p = 1.00)\).
Several previous studies have suggested, but did not formally demonstrate, that Asp299Gly and Thr399Ile polymorphisms cosegregate (16, 18, 37, 41). Of the 92 case specimens that were genotyped as being heterozygous for both polymorphisms, 13 were randomly chosen for haplotype phase analysis. In 12 of 13 samples, both polymorphisms were found within the same chromosomal homologue (independent assortment rejected at $p < 0.0001$).

Taken collectively, these data indicate that high-risk infants and children who contracted symptomatic RSV infection have a much higher than normal frequency of Asp299Gly and Thr399Ile polymorphisms and the majority of cases have both risk alleles on the same chromosomal homologue.

**Discussion**

The remarkable degree of association of two cosegregating polymorphisms that encode structural modifications to the ectodomain of TLR4 (16, 44, 45) with symptomatic RSV in a largely premature population (8, 20) provides compelling evidence for a role for this key innate immune signaling molecule in response to viral infection. Arbour et al. (16) originally reported that these two SNPs resulted in diminished LPS responsiveness in vitro and in vivo. We recently confirmed and extended this finding in vitro by demonstrating that these same SNPs resulted in diminished responsiveness to LPS as well as purified RSV F protein and another structurally unrelated TLR4 agonist, chlamydial heat shock protein 60 (44). Specifically, HEK293T cells transiently transfected with an expression vector that encodes both TLR4 polymorphisms on a single allele, as seen in 87.6% of the case specimens analyzed herein, exhibited a significantly diminished response to RSV F protein at all concentrations tested (44).

Modeling studies (44, 45) predict that these two amino acids lie in the same “face” of the TLR4 protein and that inheritance of both SNPs would introduce conformational changes that could potentially alter interaction of TLR4 with other molecules required for signaling. A TLR4 deletion mutant, Δ300–637, fails to bind MD-2, an extracellular protein required for LPS-induced TLR4 signaling (46). We recently observed that the in vitro response to purified RSV F protein is entirely MD-2-dependent in the HEK293T transfection system (data not shown). Thus, it is possible that the polymorphic amino acids diminish the response to all three agonists by interfering with the ability of TLR4 to associate with MD-2. Clearly, development of a strong proinflammatory response is required to clear RSV, and severe RSV has been associated with a more dominant Th2 response (reviewed in Ref. 9). Thus, diminished signaling in cells that express polymorphic TLRs, particularly when both mutations are expressed on the same protein, may significantly mitigate the ability of the host to control infection and thereby increase susceptibility to multiple pathogens that are sensed by TLR4, including RSV. In addition, a diminished capacity to respond to RSV F protein through TLR4 could potentially impact the efficacy of future RSV vaccines.

However, the remarkably high degree of association of the two TLR4 SNPs with RSV infection in our case series (~90% prevalence) suggests that the phenotype may be more complex. Notably, the genotype frequencies in the cases (largely premature, RSV-positive subjects) are inconsistent with those expected under Hardy-Weinberg equilibrium. For example, for the Asp299Gly polymorphism, the allele frequency for the Gly allele is 45% in the cases (Table II). Based on this observation, the expected proportions for the Asp/Gly, Asp/Gly and Gly/Gly genotypes in the case series would be 30, 50, and 20%, respectively, whereas actual frequencies are 10, 90, and 0%. Although this is clearly a significant departure ($p < 0.0001$) from Hardy-Weinberg proportions, it is consistent with selecting for an associated phenotype from a general population in which 1) the minor (Gly) allele is relatively rare (i.e., 6%) and, therefore, the minor allele homozygotes (Gly/Gly) are very rare (<1%), and 2) the Gly/Gly heterozygote, but not the Gly/Gly genotype, is a necessary prerequisite in most cases to manifest the phenotype.

Tal et al. (19) previously reported the association of severe RSV infection with inheritance of Asp299Gly and Thr399Ile polymorphisms in a cohort of Israeli infants, all of whom were full-term: ~16.2% and 17.2% of subjects possessed the polymorphic alleles for these SNPs, respectively, compared with 4.4% for both SNPs in the uninfected control group, indicating an association of these TLR4 SNPs with severe RSV infection. The frequencies of the TLR4 risk alleles in our pediatric control group (infants/children with non-RSV viral infections/not selected for high risk for RSV) were similar to those in the Israeli controls, supporting a role for these SNPs in high-risk RSV cases, suggesting that selection for RSV infection alone is unlikely to fully account for our observations.

However, in contrast to the study of Tal et al., all of the subjects from the two anti-RSV trials were enrolled because they were at high risk for RSV infection (Table I), with the majority of enrollees being premature (95% <35 wk gestational age) of whom 52% had BPD. Previous studies identified an association of the Asp299Gly polymorphism and prematurity in a Finnish population (28); however, differences between carrier rates in preterm (10.6%) versus full-term (8.3%) singletons also fail to account for...
the prevalence of polymorphisms observed in our case series. Har
tel et al. (47) failed to confirm an association between inheritance of
these two TLR4 polymorphisms and prematurity. Possibly, a
lack of stratification by degree of prematurity masked detection of
a true association of extreme prematurity with TLR4 polymor-
phisms if the SNPs were inherited by only the most premature
subjects. Therefore, it is possible that diminished TLR4 signaling,
as a consequence of inheritance of TLR4 hyporesponsive, poly-
morphic variants, underlies prematurity by rendering the fetus
more susceptible to infection in utero. This hypothesis is consistent
with the observation that chorioamnionitis is the most commonly
identifiable cause of prematurity (reviewed in Ref. 48).

Most premature infants have pulmonary dysfunction and often
BPD as a result of oxidant stress, volutrauma, and exposure to
infection of the developing lung. In a survey of commonly used
mouse strains, Soucie et al. (49) found that adult, TLR4-deficient
C3H/HeJ mice (reviewed in Ref. 13) possessed the largest alveoli,
suggesting that defective TLR4 signaling in utero alters the normal
course of lung maturation. Similarly, Sampath et al. (50) reported
a 50% increase in alveolar air space area in 2-wk-old neonatal
mouse lungs from C3H/HeJ mice, associated with lung bacterial
colonization and chronic inflammation. Recently, Zhang et al. (51)
observed that TLR4−/− mice develop emphysematous changes in
the lung due to up-regulation of endogenous NOX3, a NADPH
oxidase that results in increased reactive oxygen generation, elastin
degradation, and morphologic changes in the absence of inflam-
mation. These data indicate that TLR4 is required for maintaining
lung homeostasis by limiting bacterial colonization and chronic
inflammation as well as modulating oxidant/antioxidant balance.
This may be particularly relevant to the preterm lung exposed an-
tenatally and perinatally to Gram-negative bacteria and oxidant
stress. Given that Drosophila Toll was first identified as being
required for dorsal-ventral patterning in fly embryos (52), it is
tempting to speculate that TLR4 signaling in utero may provide an
important developmental signal to the developing lung, and that a
failure to signal at WT levels through TLR4 results in infection in
utero (leading to premature birth) and/or morphological changes
that could predispose a premature infant to development of BPD.
In this regard, Prince et al. (48) demonstrated that intraamniotic
administration of LPS to fetal mice resulted in an increase in the
number of alveolar type II cells through the activation of TLR4 and
NF-κB signaling. Surfactant protein A, a key protein in lung
development, has been identified as an endogenous TLR4 agonist
(53). Thus, a diminished capacity of fetuses with TLR4 polymor-
phisms to respond appropriately in utero to infectious stimuli or
developmental signals like surfactant protein A might result in
impaired lung development and, later, exhibit increased suscepti-
bility to RSV infection. However, Prince et al. (54) later reported
that intraamniotic LPS administration to embryonic E15 mouse
fetuses resulted in increased luminal volume density of fetal mouse
lungs at E17 and E18 and decreased distal lung branching, con-
sistent with BPD. One potentially unifying concept that would help
to explain these apparently conflicting observations is that the tim-
ing and strength of the TLR4 signal during developmentally re-
sponsive periods ultimately dictates the physiological outcome in
both murine and human lung.

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Analysis of carrier and allele frequencies of Asp299Gly TLR4 polymorphism in a pediatric population with symptoms of respiratory infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UMMC Study Patients</td>
</tr>
<tr>
<td>Total subjects</td>
<td>45</td>
</tr>
<tr>
<td>AA (Asp/Asp)</td>
<td>43 (95.6%)</td>
</tr>
<tr>
<td>AG (Asp/Gly)</td>
<td>2 (4.4%)</td>
</tr>
<tr>
<td>GG (Gly/Gly)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Carrier frequency</td>
<td>0.04a</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.01, 0.15)</td>
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<tr>
<td>Allele (G) frequency</td>
<td>0.02a</td>
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<tr>
<td>(95% CI)</td>
<td>(0.01, 0.08)</td>
</tr>
</tbody>
</table>

*p < 0.0001 vs RSV patients.

<table>
<thead>
<tr>
<th>Table V</th>
<th>Analysis of carrier and allele frequencies of Thr399Ile TLR4 polymorphism in a pediatric population with symptoms of respiratory infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UMMC Study Patients</td>
</tr>
<tr>
<td>Total subjects</td>
<td>45</td>
</tr>
<tr>
<td>CC (Thr/Thr)</td>
<td>44 (97.8%)</td>
</tr>
<tr>
<td>CT (Thr/Ile)</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>TT (Ile/Ile)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Carrier frequency</td>
<td>0.02a</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.00, 0.12)</td>
</tr>
<tr>
<td>Allele (T) frequency</td>
<td>0.01a</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.00, 0.06)</td>
</tr>
</tbody>
</table>

*p < 0.0001 vs RSV patients.
Last, we considered the possibility that such a high degree of inheritance of TLR4 polymorphisms in our case series was attributable to a racial/ethnic bias. The racial/ethnic compositions of subjects recruited into the two trials were highly similar (Table I) and quite similar to that of the adult control group (Materials and Methods). Due to the de-identification of samples required to study these unique archival specimens, we were precluded from ascertaining the race/ethnicity (or any other additional data) of the subjects who were genotyped. Nonetheless, the highest reported frequency for the Asp299Gly polymorphism that has been associated with a particular ancestry/ethnicity is 19.6% in a control group of 882 Western Africans (55). Thus, even if every single subject in our case series were of this particular descent, the anticipated frequency would be 19.6%, rather than the observed ~90%. Furthermore, it is important to note that race/ethnicity was not identified as a risk factor in the analyses of the original anti-RSV prophylaxis trial results (8, 20) nor was it identified as a risk factor for RSV infection by the American Academy of Pediatrics in their recent Revised Policy Statement for the use of Ab prophylaxis in high-risk infants (56).

In summary, the unique opportunity to analyze archival samples derived from the original clinical trials of Ab prophylaxis for RSV infection has provided important insights into the role of TLR4 signaling competence not revealed by analysis of premature children or RSV-infected children alone. Experiments to delineate fully the contribution of TLR4 signaling to lung development and how this impacts later responsiveness to an organism that is sensed by innate immune system through TLR4, are in progress.

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Disclosures
Dr. Heming is one of the founding partners of Virion Systems Inc. (VSI). Drs. Blanco and Boukhvalova are full-time employees of VSI. VSI performs contracted research for MedImmune and receives royalty income from the sales of the drug Synagis. Neither Dr. Blanco nor Dr. Boukhvalova derives financial benefit from MedImmune. No other potential conflicts of interest relevant to this article were reported.

References


