TLR4 Polymorphisms Mediate Impaired Responses to Respiratory Syncytial Virus and Lipopolysaccharide

Meri K. Tulic, Robert J. Hurrelbrink, Cecilia M. Prêle, Ingrid A. Laing, John W. Upham, Peter Le Souef, Peter D. Sly and Patrick G. Holt

*J Immunol* 2007; 179:132-140; doi: 10.4049/jimmunol.179.1.132
http://www.jimmunol.org/content/179/1/132

References  This article cites 31 articles, 9 of which you can access for free at: http://www.jimmunol.org/content/179/1/132.full#ref-list-1

Why The *JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
TLR4 Polymorphisms Mediate Impaired Responses to Respiratory Syncytial Virus and Lipopolysaccharide

Meri K. Tulic,* Robert J. Hurrelbrink,† Cecilia M. Prèle,‡ Ingrid A. Laing,*¶ John W. Upham,* Peter Le Souef,¶ Peter D. Sly,§ and Patrick G. Holt²*

Severe bronchiolitis following respiratory syncytial virus (RSV) infection occurs in only a small subset of infected infants and the basis for variations in disease severity is not understood. Innate immune responses to RSV are mediated by TLR-4, and the 299Gly and 399Ile alleles of the TLR4 gene have been linked epidemiologically with increased severity of RSV disease in children. We hypothesized that cellular immune responses to RSV mediated by these variant forms of the receptor are defective relative to responses mediated via the common form of the receptor. Human bronchial epithelial cells were transfected with TLR4 constructs encoding the common TLR4 gene sequence (299Asp399Thr), or the 299Gly or 399Ile alleles, and cytokine responses to in vitro RSV challenge were analyzed in the different transfected cells. Follow-up studies compared RSV-induced responses in PBMC from children expressing these same TLR4 genotypes. Human bronchial epithelial expressing 299Gly or 399Ile displayed normal levels of intracellular TLR4 but failed to efficiently translocate the receptor to the cell surface. This was associated with reduced NF-κB signaling post-TLR4 engagement, reduced production of IFNs, IL-8, IL-10, IL-12p35, IL-18, and CCL8, and the absence of acute-phase TNF-α. These findings were mirrored by blunted PBMC responses to RSV in children expressing the same TLR4 variants. Compromised first-line defense against RSV at the airway-epithelial surface of children expressing these TLR4 variants may thus confer increased susceptibility to severe infections with this virus. The Journal of Immunology, 2007, 179: 132–140.

The TLR4 gene which encodes the receptor recognizing bacterial LPS is highly polymorphic. Two cosegregating missense polymorphisms have been identified in the TLR4 gene at minor allele frequencies between 8 and 10% in Caucasian populations (1), which result, respectively, in aspartic acid to glycine substitution at position 299 (Asp299Gly) and threonine to isoleucine substitution at position 399 (Thr399Ile) in the receptor protein. These polymorphisms have been linked with blunted airway (2) and systemic inflammatory responses (3) to inhaled LPS in adults and attenuated LPS-induced responses in primary airway epithelial cells (2). Moreover, they have also been associated with increased risk for severe respiratory syncytial virus (RSV) bronchiolitis (4–6) in previously healthy infants. In this regard, studies in mice have shown that TLR4 recognizes not only bacterial but also viral motifs, including the F protein of RSV (7) and, moreover, TLR4-deficient mice infected with RSV show impaired pulmonary cellular responses and delayed viral clearance (7, 8).

The primary target for RSV in humans is airway respiratory epithelial cells (9) and the first line of defense against the virus involves epithelial production of a plethora of cytokines, chemokines, and immunomodulatory mediators (9, 10). Although RSV infects >50% of infants during their first year of life, only a small percentage (1–3%) of RSV-infected infants develop infections of sufficient severity to require hospitalization (11). It has been reported (12) that the ability of individual infants to increase TLR4 expression on blood monocytes during acute RSV bronchiolitis is inversely proportional to the degree of ensuing hypoxia, suggesting that the ability to up-regulate TLR4 is closely linked to disease severity. In addition, RSV has also been shown to up-regulate TLR4 in airway epithelial cells in vitro (13) and we have previously reported similar TLR4 up-regulation in the upper airways of infected infants (14). It has also recently been reported that these common TLR4 mutations are associated with an increased risk of severe RSV bronchiolitis (6) and increased risk for hospitalization (4) in previously healthy infants. However, the mechanisms by which TLR4 polymorphisms might impair host defense against RSV are currently unknown.

In this present study, we have addressed this issue by comparing the in vitro response to RSV and LPS in bronchial epithelial cells transfected with constructs containing each allele of the TLR4 Asp299Gly (299Asp or 299Gly) and Thr399Ile (399Thr or 399Ile) polymorphisms, measuring markers of cellular immunity. We further extended the studies to investigate the effects of these TLR4 polymorphisms on responses of PBMC to these stimuli.

Materials and Methods

Epithelial cell culture

Human embryonic kidney (HEK293) and human bronchial (16HBE) epithelial (HBE) cells were obtained from American Type Culture Collection. Cells were grown in 75-cm² tissue-culture flasks (Sarstedt) and maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% non-heat-inactivated FCS at 37°C in 5% CO₂, 95% air atmosphere. Cells were negative for Mycoplasma pneumoniae.
Epithelial cell experimental protocol

Epithelial cells were seeded in 24-well plates at 2 × 10^5 cells/well and were left overnight in DMEM 10% FCS. The next day at 80% confluency, cells were transfected with control (LacZ) or TLR4 (299Asp, 299Gly, 399Thr, or 399Ile) constructs. Forty-eight hours after transfection, cells were primed with IFN-γ (10 ng/ml) for 3 h and then stimulated with Escherichia coli LPS (1 μg/ml; Alexis Biochemicals) or human RSV (1 × 10^5 50% tissue culture-infective dose (TCID<sub>50</sub>), strain A-2) or LPS plus RSV and responses were measured 24 h later. Supernatant was harvested to measure innate and adaptive cytokine and chemokine protein production and cell pellets were used for FACs analysis or resuspended in RNAlater (Ambion) and stored at −20°C for RNA extraction and real-time PCR. The LPS used was Lipofectamine 2000 (Invitrogen) in a commercially purified LPS preparation and is free of TLR2 ligand contaminants.

Construction of TLR4<sup>299Gly</sup> or<sup>399Ile</sup> plasmids

Human TLR4<sup>299Asp</sup>/<sup>399Thr</sup> plasmid was a gift from Prof. R. Medzhitov (Yale University School of Medicine, New Haven, CT). The gene was cloned into the pcDNA3 vector between the HindIII and XhoI restriction sites of the multiple cloning region. Site-directed mutagenesis was performed using the GeneTailor kit (Invitrogen Life Technologies) to insert a single point mutation in the TLR4<sup>299Asp</sup>/<sup>399Thr</sup> construct. Mutations were introduced either at nucleotide position 896 (A to G) resulting in the Asp for Gly substitution at amino acid 299 or at nucleotide position 1196 (C to T), producing a Thr for Ile substitution at amino acid 399. Briefly, target DNA was methylated using DNA methylase and then amplified using two overlapping primers, one of which contained the target mutation site. Following mutagenesis, the plasmid was transformed into mrc<sup>B</sup>C wild-type Escherichia coli where the host mrc<sup>B</sup>C endonuclease digested only the methylated template DNA, leaving unmethylated, mutated product behind. Sequence analysis showed no other insertions, deletions, or substitutions anywhere else in the gene.

Transfection

Epithelial cells were transfected in 24-well plates with TLR4 expression constructs using Lipofectamine 2000 reagent (Invitrogen Life Technologies). Briefly, DNA-Lipofectamine 2000 complexes were prepared by combining the diluted Lipofectamine 2000 (2 μl/50 μl medium) with diluted DNA (0.8 μg/50 μl medium) within 30 min. Complexes were added to cells in growth medium, incubated for 24–48 h, and assayed for expression. Transfection efficiency was studied by transfecting the cells with a similar sized plasmid construct expressing lacZ and staining in situ for β-gal expression. Transfected cells appeared blue and transfection efficiency was calculated by counting the number of blue cells and expressing them as a percentage of total number of cells per field of view.

Culture of PBMCs

Cryopreserved PBMC from children heterozygous for the Asp<sup>299</sup> or Thr<sup>399</sup> polymorphisms were compared with the PBMC collected from children homozygous for 299Asp and 399Thr (n = 12). Previous studies in our laboratory have shown that PBMC retain their viability, cellular immune responses, and function after cryopreservation. The PBMC samples were previously collected from two well-characterized community-based cohorts of children. Cells were collected at follow-up from one population when the children were 6.2 ± 0.8 years of age (18) and from the other population when the children were aged 11.8 ± 1.4 years (15). A total of 2 × 10<sup>5</sup> PBMC were plated in 96-well plates and primed with IFN-γ (10 ng/ml) for 3 h before stimulation with LPS (1 ng/ml) or RSV (1 × 10<sup>5</sup> TCID<sub>50</sub>) or a combination of both LPS plus RSV. As with epithelial cells, innate and adaptive immune responses as well as TLR4 receptor expression was measured 24 h after bacterial and/or viral stimulation.

Viral propagation and plaque assay

RSV was propagated in HeLa cells. Briefly, cells were infected with RSV at a multiplicity of infection of 0.1. Six days after incubation at 37°C in 5% CO<sub>2</sub>, 95% air, cell supernatants were harvested, cells were disrupted by freezing and thawing, and debris was pelleted by low-speed centrifugation. The initial stock (1 × 10<sup>5</sup> TCID<sub>50</sub>) was aliquoted and kept frozen at −135°C. A fresh aliquot was thawed for each experiment and leftover virus was never refrozen. In some experiments, UV radiation was used to inactivate RSV. To inactivate the virus, a UV lamp was held 10 cm over the RSV in an open petri dish on ice for 30 min. After irradiation, no evidence of viable RSV could be detected by plaque-forming assay.

Quantitative RT-PCR

Total RNA was isolated using an RNeasy Mini kit (Qiagen) according to the manufacturer’s directions. Reverse transcription was performed using the Omniscript RT kit (Qiagen) according to the manufacturer’s protocol with oligo(dT) (Promega) and Superasin (Geneworks). Intron-spanning primers were designed in-house using Primer Express Software (Applied Biosystems). Reverse-transcribed RNA samples were diluted 1/5 and quantitated by real-time PCR using Quantitect SYBR Green Master Mix (Qiagen) on the ABI PRISM 7900HT (Applied Biosystems). Melting curve analysis was used to assess the specificity of the assay. Copy numbers were determined by 10-fold serial dilutions of plasmid standards and normalized to the reference gene ubiquitin-conjugating enzyme E2D2 (UBC) (19). Quantitative PCR was used to measure CCL8 (or MCP-2), IL-5, IL-6, IL-12p35, IL-12p40, IL-18, IFN-β, IFN-γ, myxovirus protein A (MxA), and TLR4 mRNA expression.

Surface and intracellular TLR4 expression

Surface and intracellular TLR4 expression of PBMC and epithelial cells was studied using a biotin-conjugated anti-human TLR4 (clone H1TA25, dilution 1/25) (eBioscience) as the primary Ab and streptavidin conjugated to PE (1/400 dilution; BD Pharmingen) as the secondary Ab. All samples were incubated with Abs for 30 min at 4°C. For intracellular TLR4 staining, cells were washed and then fixed and permeabilized in Cytofix/Cytoperm buffer (BD Pharmingen) for 30 min at 4°C before staining for TLR4. Ten thousand events were collected and analyzed using a FACSCalibur (BD Biosciences) and CellQuest software (BD Biosciences). For LPS-binding experiments, LPS-Alexa 488 from E. coli (Invitrogen Life Technologies/Molecular Probes) (5 μg/ml) was used to stimulate epithelial cells transfected with TLR4 expression constructs for an hour before harvesting and the cells stained for surface TLR4 expression. The number of LPS plus TLR4<sup>+</sup> cells was analyzed by flow cytometry.

Cytokine and chemokine measurements

IL-5, IL-6, IL-10, IL-13, IFN-β, and TNF-α protein in supernatants was measured using in-house time-resolved fluorometry. BD Pharmingen Abs were used for capture and detection and standard curves were generated by using serial dilutions of Abs. Europium-labeled streptavidin and enhancement solution (Delfia Wallac) were used for detection. IL-8 protein was measured using a commercially available ELISA kit (BD BioSciences/BD Pharmingen). The limit of detection for time-resolved fluorometry assays was 5 pg/ml and for IL-8 ELISA it was 6 pg/ml.

Western blot analysis

PBMC from individuals homozygous for 299Asp and 399Thr or heterozygous for Asp<sup>299</sup>Gly or Thr<sup>399</sup>Ile were stimulated with 1 ng/ml LPS for 0, 30, 60, 120 min and harvested by centrifugation. PBMC were lysed in protein lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100 (pH 7.6)) supplemented with 5 mM sodium fluoride, 10 mM sodium molybdate, 1 mM sodium pyrophosphate, 2 mM sodium orthovanadate and 1× protease inhibitors (Complete Mini, protease inhibitor mixture tablets; Roche). Approximately, 25 μg of protein lysate was resolved per lane of a 12% SDS-PAGE gel and transferred to nitrocellulose membrane (Pall Scientific). Membranes were blocked for at least 1 h in 5% skim milk in TBS/0.05% Tween 20 (block buffer) followed by a 2-h incubation with primary Abs diluted in block buffer or 5% BSA in TBSO.05% Tween 20 according to the manufacturer’s guidelines. Following four sequential 5-min washes in TBS/0.05% Tween 20, membranes were incubated with HRP-conjugated anti-rabbit IgG. Bound secondary Ab was detected using chemiluminescence (Roche Diagnostics) and visualized using CL-XPosure film (Pierce). Anti-phospho-IXββ (Ser<sup>32</sup>) Ab was purchased from Cell Signaling Technology, anti-IXββ (C-21) from Santa Cruz Biotechnology, and anti-β-tubulin from Abcam.

Statistical methods

All results are expressed as mean ± SEM. Differences between individual groups were analyzed initially using the nonparametric Friedman test followed by a Wilcoxon-signed ranks test for paired responses and the Mann-Whitney U test for unpaired responses. SPSS statistical package was used for all analysis. Significance was set at p < 0.05.
Results

Epithelial cells respond to LPS and/or RSV following transfection with a TLR4 expression construct

Using flow cytometry, we found unstimulated human kidney (HEK293) and HBE (16HBE) cell lines had low constitutive expression of TLR4 (3.6 ± 0.4% and 4.6 ± 0.8% positive, respectively) and as a consequence produced low levels of cytokines when stimulated by LPS and/or RSV. These responses were significantly higher than those seen in control, unstimulated cells (Fig. 1). Transfection of 16HBE or HEK293 epithelial cells with LacZ or TLR4 construct alone had no effect on cytokine production in unstimulated cells (Fig. 1). We were routinely able to transfect at least 90% of all HEK293 or 16HBE cells with TLR4 constructs (299Asp/399Thr (common allele), 299Gly (mutant 1), or 399Ile (mutant 2)) as demonstrated by in situ β-gal staining of transfected cells (data not shown).

To characterize the cytokine profile produced by TLR4 299Asp and 399Thr-transfected 16HBE or HEK293 cells following bacterial and viral exposure, cells were exposed to LPS and/or RSV and cytokines measured in the supernatant 24 h later. IL-8 was the most abundant cytokine produced by TLR4-transfected 16HBE (Fig. 1) and HEK293 (Fig. 2) epithelial cells, followed by IL-6 and TNF-α. Both RSV and LPS also induced a small but statistically significant production of Th2 cytokines IL-10, IL-5, and IL-13 in TLR4-transfected 16HBE (Fig. 1) and to a lesser extent in HEK293 epithelial cells (Fig. 2) when compared with LacZ-transfected cells (Fig. 1). Production of IL-8 and TNF-α in response to LPS plus RSV in 16HBE cells was additive (Fig. 1). This was not the case for HEK293 epithelial cells (Fig. 2). Priming with IFN-γ for 3 h before LPS or RSV exposure had no significant effect on cytokine or chemokine production (mRNA and protein levels) in either 16HBE or HEK293 epithelial cells (data not shown).

As RSV is a potent inducer of type I IFNs, we measured MxA mRNA expression levels following TLR4 299Asp/399Thr transfection, as this has been shown to be an accurate and reliable marker of IFN-β bioactivity (20). MxA mRNA was significantly increased at 6 h after RSV or LPS exposure in both HEK293 and 16HBE TLR4-transfected epithelial cells compared with control LacZ-transfected cells (Fig. 3). The magnitude of the MxA mRNA response was significantly larger in 16HBE after RSV than after LPS stimulation and 16HBE production was much more sensitive than HEK293. Subsequent experiments therefore focused upon 16HBE cells.

FIGURE 1. Characterization of bronchial epithelial cell response to LPS and RSV following TLR4 transfection. Th1 and Th2 cytokine responses in HBE cells (16HBE) transfected with control LacZ ( ), n = 6) or TLR4 299Asp/399Thr ( , n = 6) expressing constructs. Responses were measured under control, nonstimulated conditions or 24 h after RSV, LPS, or LPS plus RSV exposure. †, p < 0.05 vs LacZ control in unstimulated cells; *, p < 0.05 vs respective LacZ-transfected cells.

FIGURE 2. Characterization of kidney epithelial cell response to LPS and RSV following TLR4 transfection. Th1 and Th2 cytokine responses in human kidney epithelial cells (HEK293) transfected with control LacZ ( , n = 6) or TLR4 299Asp/399Thr ( , n = 6) expression constructs. Responses were measured under control, nonstimulated conditions or 24 h after RSV, LPS, or LPS plus RSV exposure in cells. †, p < 0.05 vs LacZ control in unstimulated cells; *, p < 0.05 vs respective LacZ-transfected cells.
Epithelial cell responses to LPS and/or RSV are blunted following transfection with TLR4299Asp/399Thr, 299Gly, or 399Ile expression constructs

Single amino acid substitutions were introduced at position 299 or 399, respectively, in the TLR4299Asp/399Thr construct. Sequence analysis confirmed that no other variations were introduced anywhere else in the TLR4 gene (data not shown). Epithelial cells transfected with either the TLR4299Gly (Fig. 4A) or 399Ile (Fig. 4B) produced significantly lower levels of IL-8 and IL-6 protein, as well as MxA mRNA following LPS or RSV stimulation when compared with TLR4299Asp/399Thr-transfected cells. In addition, when both LPS and RSV stimuli were simultaneously added to TLR4299Gly- or 399Ile-transfected cells, IL-6 protein and MxA mRNA production was attenuated toward baseline but production of IL-8 protein remained unaffected (Fig. 4, A and B). TLR4 mRNA levels were similar in cells expressing any of the three different TLR4 sequences and stimulation with LPS and/or RSV had no effect on TLR4 mRNA expression (Fig. 4C).

Epithelial cell response to UV-treated RSV in epithelial cells transfected with TLR4299Gly or 399Ile expression constructs

To determine whether viral replication was essential for RSV-induced proinflammatory responses in TLR4299Asp/399Thr-transfected cells and whether attenuation of this response was still seen in cells transfected with the TLR4299Gly or 399Ile constructs, the virus was UV inactivated before addition to 16HBE cultures. UV inactivation had no significant effect on the magnitude of RSV-induced inflammatory response in TLR4299Asp/399Thr-transfected 16HBE cells when compared with the noninactivated RSV (Fig. 5). In addition, UV inactivation of the virus had no effect on the relative IL-8 response levels of epithelial cells transfected with 299Asp/399Thr compared with 299Gly or 399Ile constructs (Fig. 5).
To elucidate the potential mechanism(s) involved in attenuated responses to RSV in cells transfected with the TLR4 299Asp/399Thr, 299Gly, or 399Ile constructs, we compared surface and intracellular expression of the receptor, on their surface (Fig. 6). This high level of surface expression was maintained at 72 h posttransfection. However, in cells transfected with TLR4 299Gly or 399Ile constructs, there was a lack of surface expression of TLR4 at all time points, despite high intracellular expression as early as 24 h posttransfection that was maintained at 48 and 72 h (Fig. 6). Similar levels of intracellular TLR4 protein expression were seen in epithelial cells transfected with the three different TLR4 sequences (Fig. 4C). Together, these results suggest that in epithelial cells transfected with the 299Gly or 399Ile forms of the receptor, the receptor fails to translocate to the cell surface and is trapped within the cell. This interpretation was supported by experiments with fluorochrome-labeled LPS, where there was a dose-dependent increase in the number of LPS plus TLR4+ cells in TLR4 399Thr-transfected cells following LPS exposure (Fig. 7A), increasing from 0.38% in unstimulated epithelial cells to 50.3% LPS plus TLR4+ cells following 5 μg/ml LPS stimulation. In contrast, in TLR4 399Ile-transfected cells, the number of LPS plus TLR4+ cells remained unchanged following LPS stimulation, irrespective of the dose of LPS used (Fig. 7B).

Reduced PBMC response to LPS or RSV in individuals heterozygous for the Asp299Gly or Thr399Ile polymorphisms in TLR4

Bone marrow-derived cells exemplified by PBMC are intrinsically more sensitive to TLR4 stimulation than epithelial cells and display up to a 4 log-fold lower LPS stimulation threshold (data not shown). We next sought to determine whether the relative attenuation of TLR4-mediated responses to LPS and RSV in epithelial cells transfected with 299Gly or 399Ile constructs was supported by data from bone marrow-derived cells from children with TLR4 Asp299Gly and Thr399Ile polymorphisms. To address this question, we studied PBMC from children homozygous for the 299Asp and 399Thr or heterozygous at position 299 or 399, by comparing their responses to viral and bacterial stimuli. The genotype frequencies in our population were similar to those previously described in the literature (2). All children homozygous for 299Asp were also homozygous for 399Thr. Furthermore, 11 of the 12 children who were identified as heterozygous for the 299Gly polymorphism were also heterozygous for the Thr399Ile polymorphism. This confirms the earlier observation that these polymorphisms usually segregate together (21).

Responses to LPS (1 ng/ml) or RSV (3 × 10^4 PFU) in PBMC from the children who were homozygous for 299Asp and 399Thr were characterized by increased production of MxA, IFN-β, IL-12p35, CCL8 (or MCP-2), and IL-18 mRNA as determined at 2 h after stimulation (Fig. 8A), and IL-8, TNF-α, IL-10, and IL-6 protein production. The protein production was seen as early as 2 h after stimulation and reached maximal response by 24 h (Fig. 8B). IL-12p40 mRNA was not detected after RSV stimulation, however, we measured small but significant increases in IL-12p40 mRNA after LPS exposure (Fig. 8A). In general, LPS induced stronger cytokine responses from PBMC than RSV (Fig. 8B), however, RSV induced stronger MxA and IFN-β mRNA signals than LPS (Fig. 8A).

PBMC from individuals heterozygous for Asp299Gly or Thr399Ile (n = 12) had significantly reduced MxA, IL-12p35, CCL8, and IL-18 mRNA as determined at 2 h after stimulation (Fig. 8A), and IL-8, TNF-α, IL-10, and IL-6 protein production. The protein production was seen as early as 2 h after stimulation and reached maximal response by 24 h (Fig. 8B). IL-12p40 mRNA was not detected after RSV stimulation, however, we measured small but significant increases in IL-12p40 mRNA after LPS exposure (Fig. 8A). In general, LPS induced stronger cytokine responses from PBMC than RSV (Fig. 8B), however, RSV induced stronger MxA and IFN-β mRNA signals than LPS (Fig. 8A).
FIGURE 7. LPS binding to TLR4 in cell lines transfected with TLR4 constructs. The number of TLR4 plus LPS cells in 16HBE epithelial cells transfected with the TLR4 299Asp/399Thr (A) or TLR4 399Ile (B) expression construct and stimulated with exogenous fluorescently tagged LPS (1–5 μg/ml) for 1 h. Results are representative of three separate experiments.

FIGURE 8. PBMC responses to LPS and RSV in individuals with different TLR4 genotypes. Cytokine mRNA (A) and protein (B) responses to LPS or RSV in PBMC from individuals homozygous for 299Asp and 399Thr (■, n = 12) or heterozygous for Asp299Gly or Thr399Ile (□, n = 12). Cytokine mRNA was measured at 2 h after RSV or LPS stimulation. B, Cytokine protein responses were measured at both 2 h (left) and 24 h (right) after stimulation. Results are expressed as mean ± SE of mean (SEM). *p < 0.05 and **p < 0.01 vs homozygous subjects.
Reduced expression of surface TLR4 on the surface of PBMC from heterozygote individuals. A, Intensity of TLR4 expression on the surface of CD14+ monocytes in individuals heterozygous for 299Asp and 399Thr (n = 4) or heterozygous for 299Gly or Thr399Ile (n = 4) at 24 h after LPS or RSV stimulation in PBMC. Results are expressed as mean ± SE of mean (SEM). †, p < 0.05 vs homozygote control response; *, p < 0.05 vs responses in respective homozygous subjects. B, Western blot analysis of whole cell lysates in PBMC from individuals heterozygous for 299Asp and 399Thr (left) or heterozygous for 299Gly or Thr399Ile (right) at 0, 30, 60, and 120 min post-LPS stimulation, using an Ab to the phosphorylated form of IκBα (P-IκBα). Anti-β-tubulin served as a loading control. These data are representative of blots performed on four individuals.

Reduced expression of TLR4 on the surface of PBMC from children heterozygous for Asp299Gly or Thr399Ile polymorphisms in TLR4

Using FACS analysis and Abs directed against TLR4, we showed significantly reduced expression of TLR4 on the surface of stimulated or nonstimulated monocytes in PBMC from patients heterozygous for Asp299Gly or Thr399Ile when compared with surface TLR4 expression in PBMC from patients homozygous for 299Asp and 399Thr (Fig. 9A). Although LPS or RSV treatment had no effect on expression of the TLR4 receptor in monocytes from heterozygous subjects, both LPS and RSV significantly reduced the number of surface receptors in homozygous cells (Fig. 9A).

Reduced IκBα phosphorylation in PBMC from patients heterozygous for TLR4 Asp299Gly or Thr399Ile

The large variety of cytokines and chemokines that are produced in LPS- or RSV-infected epithelial cells result from direct activation of TLR4. Engagement of TLR4 initiates an intracellular signaling cascade leading to activation of transcription factors responsible for initiation of transcription of proinflammatory genes. NF-κB is central to expression of most of these genes (22, 23). In resting cells, NF-κB is sequestered in the cytoplasm by interaction with the IκB (inhibitor of NF-κB) family of inhibitory proteins. Following activation, IκB complex is phosphorylated resulting in its degradation via ubiquitin-mediated pathway. This allows the NF-κB complex to be translocated to the nucleus to initiate transcription. Measuring the degree of IκBα phosphorylation on Ser27 as a surrogate marker of NF-κB activation, we demonstrated in Fig. 9B that in PBMC from patients homozygous for the 299Asp and 399Thr, IκBα phosphorylation occurred 30 min after LPS stimulation, reached maximal activation by 60 min, and decreased thereafter. In patients heterozygous for Asp299Gly or Thr399Ile, IκBα phosphorylation also reached its maximal activation by 60 min poststimulation and then decreased. Although the kinetics of phosphorylation remained the same, the level of IκBα activation was lower in PBMC from heterozygous compared with homozygous patients (Fig. 9B).

Discussion

Compelling evidence from animal model systems suggests a central role for TLR4 in activation of the innate immune system in response to microbial stimuli (6 – 8). However, in humans, the situation is less well-understood. In particular, there is little information available relating to the role of TLR4 in microbial immunity in early childhood, when infection risk is maximal. Of particular interest in this context is RSV, which is the most common cause of infant hospitalization in developed countries (24). The TLR4 mutations Asp299Gly and Thr399Ile have been associated with severity of RSV infection in infants (4 – 6), but the underlying mechanism(s) have not been defined. In this study, we have addressed the hypothesis that these mutations may be associated with delayed and/or attenuated triggering of the innate immune response to RSV and to the bacterial stimulus LPS.

Airway epithelial cells are the primary target for RSV (25) and an initial cycle of infection and replication in the upper airways is usually required before upper airway infections can progress deeper into the respiratory tree. This very early phase of the respiratory infection cycle provides a window of opportunity for pathogen eradication via innate defense mechanisms, provided they can be mobilized rapidly and at a sufficient level of intensity. As demonstrated in Figs. 1 and 4, epithelial cells are capable of mounting this first line of defense themselves via direct TLR4-mediated recognition of both viral and bacterial motifs, triggering rapid production of a broad array of cytotoxic defense mediators (such as type I IFN) as well as mediators (such as chemokines/TNF-α) which are required for recruitment of effector cells. However, despite the presence of TLR4 mRNA, resting epithelial cells are normally not responsive to LPS or RSV due to their low constitutive surface expression of TLR4 protein (26, 27). An induction signal is required to up-regulate TLR4 translocation to the epithelial surface and this can include infection with live virus including RSV (13). As demonstrated in our experiments, surface expression of TLR4 and ensuing responsiveness to LPS or RSV can also be achieved by transfection of epithelial cells with the common form of TLR4 (TLR4 299Asp and 399Thr), resulting in potent cytokine and ensuing chemokine production dominated by high levels of neutrophil chemoattractant IL-8 protein, TNF-α which plays a key role in neutrophil recruitment via endothelial cell priming (28) and antiviral MxA/IFN-β mRNA. Neutrophils are the predominant inflammatory cells in the lungs during human RSV infection. The production of these effector molecules was similar in epithelial cells with or without IFN-γ priming suggesting that they do not require IFN-γ for efficient cytokine production. Optimal IL-8 and...
TNF-α production in 16HBE cells was achieved with simultaneous exposure to both LPS and RSV. In addition, transfected bronchial respiratory epithelial cells (16HBE) induced a much stronger IFN response 24 h after RSV exposure than transfected kidney epithelial cells (HEK293); the additive effects of LPS plus RSV on MxA mRNA signal were only seen in 16HBE. Because respiratory epithelial cells are the primary target for RSV, this may explain their increased sensitivity to the pathogen.

Bronchial epithelial cells transfected with TLR4 299Gly or 399Ile displayed a markedly reduced innate response to both bacterial and viral stimuli relative to cells transfected with the TLR4 299Asp/399Thr construct (Fig. 4). Both 299Gly and 399Ile constructs were equally effective at abolishing IL-8 and IL-6 responses in epithelial cells and for IL-6 in particular, responses were similar to those elicited by control cells transfected with a LacZ-expressing construct (Fig. 4). Although expression of the 399Ile construct completely inhibited MxA responses, expression of the 299Gly construct caused only a partial block. Swedish children heterozygous for the Asp299Gly polymorphism displayed reduced LPS-induced IL-12 (p70) responses (29). Systemic inflammatory hypersensitivity to inhaled LPS has also been reported in healthy adults with this genotype (2, 3).

The attenuated responses of cells transfected with 299Gly or 399Ile cannot be attributed to reductions in the amount of TLR4 message transcribed. However, the lack of surface TLR4 expression at the time of stimulation (48 h posttransfection) in TLR4 299Gly- or 399Ile-transfected epithelial cells when compared with TLR4 299Asp/399Thr-transfected cells (Fig. 6) is a potential explanation. Low surface TLR4 expression would confer poor capacity to bind ligand following exposure (Fig. 7). Despite the lack of surface TLR4 expression in 299Gly- and 399Ile-transfected epithelial cells, these cells do express TLR4 protein intracellularly as early as 24 h after transfection, and the receptor is maintained there for at least 72 h. We have also demonstrated, at least for IL-8 and type I IFN production, that there is residual cytokine production in TLR4 299Gly- and 399Ile-transfected cells, albeit markedly dampened. Although these cells lack detectable levels of surface receptor, activation of intracellular TLR4 by LPS could be sufficient to initiate signal transduction (30). Alternatively, it is possible that this cytokine production may occur via a TLR4-independent mechanism. A TLR4-independent pathway has recently been reported by Kato et al. (31) who described a novel cytoplasmic protein, retinoic-acid-inducible gene 1 (RIG-1), involved in recognition of viral components and triggering antiviral responses in fibroblasts and dendritic cells. A similar protein may also be present in epithelial cells and may contribute to the innate immune response to RSV. Second, complete inhibition of LPS- or RSV-induced responses may require both 299Gly and 399Ile to occur together. Although we did not directly test this in our model by transfecting the epithelial cells with double constructs, the fact that both of these missense polymorphisms are found in the extracellular domain of the receptor, are known to occur together, and we have shown them individually to have similar functional effect, collectively suggest that they may interact in an additive fashion.

The second part of this study sought to establish the relative function of these TLR4 variants in bone marrow-derived cells. Our results using PBMC from children suggest comparable response pattern to those seen with epithelial cells. Impaired cytokine responses (at protein level) can be demonstrated from as early as 2 h after LPS and/or RSV exposure in PBMC heterozygous for the Asp299Gly or Thr399Ile polymorphisms (Fig. 8B). Attenuated cytokine responses were associated with reduced mRNA expression for neutrophil (IL-8) and monocyte (CCL8) chemotaxtractants as well as reduced IL-18 and IL-12 (Fig. 8A). These functional changes were supported by reduced expression of TLR4 on the surface of monocytes heterozygous for Asp299Gly or Thr399Ile (Fig. 9A) and significantly down-regulated levels of 1β,25-phosphorylation signal inside these cells (Fig. 9B). Evidence that this phenomenon may be applicable to many cell types, as well as to immunity to viruses other than RSV, is supported by Machida et al. (32) who have demonstrated that hepatitis C virus induces TLR4 expression in B cells of infected individuals compared with healthy controls and also enhances their production of proinflammatory cytokines IFN-β and IL-6.

The effect of the TLR4 299 and 399 amino acid changes on receptor conformation, activity of downstream signaling molecules, and/or chaperone proteins remains to be explored. In this study, however, we present evidence that TLR4 299Gly and 399Ile alleles result in reduced function of the receptor in both transfected epithelial cell lines and in monocytes from individuals with these TLR4 alleles, following exposure to bacterial and/or viral stimuli. This impaired function may be attributed to reduced surface expression of TLR4 and therefore attenuated ligand binding, consequently resulting in reduced activation of the intracellular NF-κB-signaling pathway. The resultant weak innate immune response may contribute to enhanced susceptibility to infection in these individuals.

Acknowledgments
We thank Prof. Paul Young (University of Queensland, Brisbane, Queensland, Australia) for initial stock of RSV, Prof. R. Medzhitov (Yale University School of Medicine, New Haven, CT) for the TLR4 299Asp/399Thr plasmid, Assoc. Prof. Prue Hart (Division of Molecular Biotechnology, Institute for Child Health Research, Perth, Western Australia) for the HEK293 cell line, Matthew Wikstrom (Division of Cell Biology, Institute for Child Health Research) for his expert FACS analysis, and Stephanie Yerkovich (Division of Cell Biology, Institute for Child Health Research, Perth, Western Australia) for her assistance with molecular techniques.

Disclosures
The authors have no financial conflict of interest.

References


