TLR4 mutations are associated with endotoxin hyporesponsiveness in humans

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There is much variability between individuals in the response to inhaled toxins, but it is not known why certain people develop disease when challenged with environmental agents and others remain healthy. To address this, we investigated whether *TLR4* (encoding the toll-like receptor-4), which has been shown to affect lipopolysaccharide (LPS) responsiveness in mice[1,2], underlies the variability in airway responsiveness to inhaled LPS in humans[3]. Here we show that common, co-segregating missense mutations (Asp299Gly and Thr399Ile) affecting the extracellular domain of the TLR4 receptor are associated with a blunted response to inhaled LPS in humans. Transfection of THP-1 cells demonstrates that the Asp299Gly mutation (but not the Thr399Ile mutation) interrupts TLR4-mediated LPS signalling. Moreover, the wild-type allele of TLR4 rescues the LPS hyporesponsive phenotype in either primary airway epithelial cells or alveolar macrophages obtained from individuals with the *TLR4* mutations. Our findings provide the first genetic evidence that common mutations in *TLR4* are associated with differences in LPS responsiveness in humans, and demonstrate that gene-sequence changes can alter the ability of the host to respond to environmental stress.

We investigated the genetic basis for the physiologic response to inhaled endotoxin or LPS for several reasons. First, endotoxin is associated with the development and progression of asthma and other forms of airway disease. In the domestic setting, the concentration of endotoxin is associated with the clinical severity of asthma[4], and, among exposed workers, endotoxin is the most significant component of the bioaerosol that is associated with the development[5] and progression[6] of airway disease. Endotoxin may also have a role in the pathophysiological consequences of air pollution[7]. Second, the ability of the host to respond to endotoxin is highly variable. Differences between individuals have been reported in the release and synthesis of cytokines by human monocytes stimulated with LPS *in vitro*[8], and a patient with recurrent bacterial infections has been reported to be refractory to the *in vivo* and *in vitro* effects of LPS (ref. 9). We have recently found that normal, healthy, non-asthmatic subjects demonstrate a reproducible airway response to an incremental LPS inhalation challenge test; some subjects developed airflow obstruction when challenged with low concentrations of LPS and others were unaffected by high concentrations of inhaled LPS (ref. 3). Third, the molecular events leading to cell recognition and response to LPS are becoming more clearly defined. Recent attention has focused on the toll receptor family, specifically TLR4. LPS initiates signal transduction through the TLR4 receptor, and this pathway is enhanced by CD-14 (ref. 10) and MD-2 (ref. 11), and is activated through NF-κB and AP-1 (ref. 12). Mutations in *Tlr4* (ref. 1) have been reported in mouse strains that are defective in their response to LPS, and disruption of *Tlr4* results in an LPS hyporesponsive phenotype[2]. Based on these observations, we hypothesized that mutations in *TLR4* may be associated with diminished airway responsiveness to inhaled LPS in humans.

Using SSCP, we screened the entire coding region of *TLR4* in all 83 subjects in our study population; 10 (12%) subjects had a band variant detected by SSCP (Fig. 1*a*). Direct sequencing detected an A→G substitution at nt 896 from the start codon of the *TLR4* cDNA (ref. 12). To confirm our findings, we sequenced the 83 unrelated probands in the forward and reverse directions with primers designed to amplify nt 896. We found the same ten individuals to have the A896G substitution and the remaining individuals to have the more common *TLR4* allele. One of the ten individuals with the A896G substitution was homozygous for this mutation; the remaining nine had a single, mutant allele. The allelic frequency of the 896 guanine substitution was 6.6% in our study population, 7.9% in a control population from Iowa[13] and 3.3% in the parental chromosomes of the Centre d'Etude du

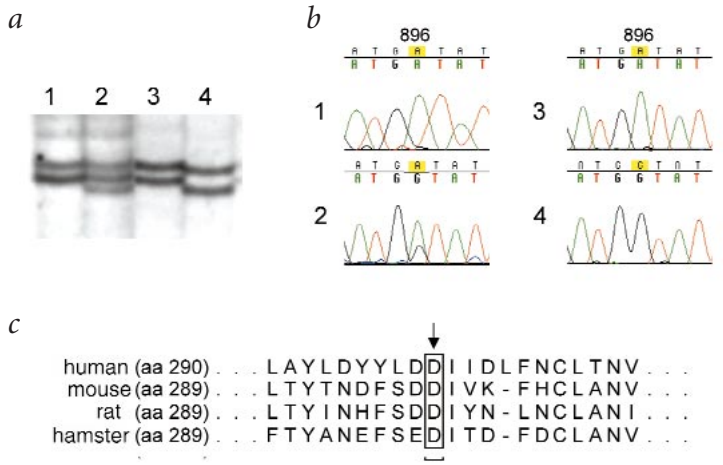

Fig. 1 A common missense mutation in human *TLR4*. ***a***, The altered electrophoretic mobility of band variants identified by single-stranded conformation polymorphism (SSCP) gel. ***b***, The sequencing results for the samples in lanes 1–4. Lanes 2 and 4 are samples in which a band shift was observed. Upon sequencing, the samples in lanes 1 and 3 were homozygous for alanine at position 896, whereas the sample in lane 2 was identified as heterozygous with both an alanine and a guanine at position 896, and the sample in lane 4 was homozygous for a guanine at position 896. ***c***, The aspartic acid residue at position 299 is conserved between species. The sequence surrounding the amino acid altered by the *TLR4* mutation was aligned for human[12], mouse[1], rat and hamster (D. Golenbock, pers. comm.). The aspartic acid at position 299 is indicated (arrow).

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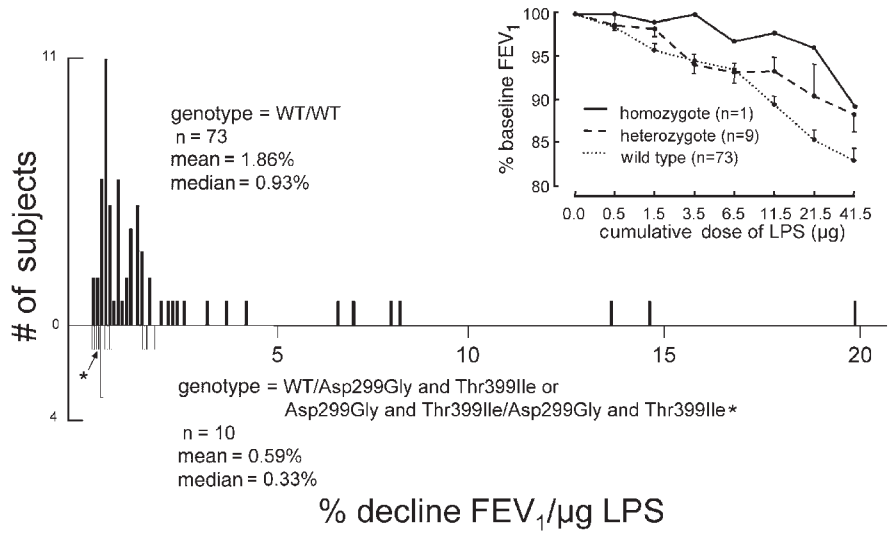
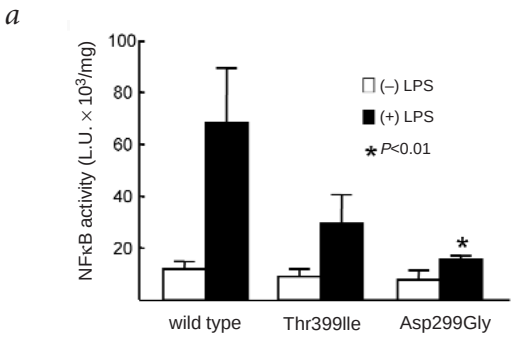


Fig. 2 Airway responsiveness to inhaled LPS and *TLR4* genotype. The percentage decline in FEV_1/µg LPS was calculated following administration of the cumulative LPS dose. Subjects above the *x* axis (filled bars) are homozygous for the wild-type allele (WT/WT); subjects below the *x* axis (open bars) are either heterozygous or homozygous (*) for the missense Asp299Gly and Thr399Ile alleles. *P* values are presented for the comparison of the percentage decline in FEV_1/µg LPS between subjects with the wild-type genotype (n=73) and those with the Asp299Gly and Thr399Ile alleles (n=10). Inset, the dose-response curves for all study subjects grouped by genotype. As the LPS inhalation challenge was terminated if a study subject decreased his/her FEV_1 by 20%, the number of subjects decreased as the cumulative dose of LPS increased. For example, at a cumulative dose of 41.5-µg inhaled LPS, only 35 wild-type, 6 heterozygous and 1 homozygous subjects contribute to the calculation of the FEV_1.

Polymorphisme Humain (CEPH) population[14]. The study population and the Iowa control population were in Hardy-Weinberg equilibrium for distribution of the *TLR4* mutation ($P>0.20$), but the CEPH population was not ($P=0.002$). The A896G substitution results in replacement of a conserved aspartic acid residue with glycine at amino acid 299 (Fig. 1*b*). This missense mutation (Asp299Gly) is in the fourth exon of *TLR4*, and alters the extracellular domain of this receptor. We found an additional missense mutation (replacing a non-conserved threonine with an isoleucine at amino acid 399 (Thr399Ile) in the extracellar domain of the TLR4 receptor) co-segregating with the Asp299Gly substitution. We directly sequenced the region of exon 3 encoding the intracellular domain, which corresponds to the location of the missense mutation in *Tlr4* reported in C3H/HeJ mice[1], and found no sequence variants in any of our study subjects.

Based on our experience[3] and a standard approach to the definition of airway hyperreactivity, we categorized our study subjects as 'LPS responsive' if subjects decreased their FEV_1 by at least 20% at any point during the LPS inhalation challenge, or 'hyporesponsive' if they had a less than 20% decline in their FEV_1 after inhaling a cumulative dose of 41.5 µg LPS. Of the 83 unrelated study subjects who completed the LPS inhalation challenge test, 52 (63%) were responsive to inhaled LPS and 31 (37%) were hyporesponsive to inhaled LPS. The co-segregating mutant (Asp299Gly and Thr399Ile) sequence variants occurred in three LPS-responsive (5.8%) and seven LPS-hyporesponsive (22.6%) study subjects ($P=0.029$). Among the 73 subjects with the common *TLR4* allele, the dose-response slope (percentage decline FEV_1/cumulative dose of inhaled LPS) averaged 1.86% (range 0.01–19.78%), whereas the dose-response slope for the 10 subjects with the mutant alleles (Asp299Gly and Thr399Ile) was significantly less ($P=0.037$), averaging 0.59% (range 0.00–1.59%; Fig. 2). The subject homozygous for both mutant alleles (Asp299Gly and Thr399Ile) was hyporesponsive to inhaled LPS



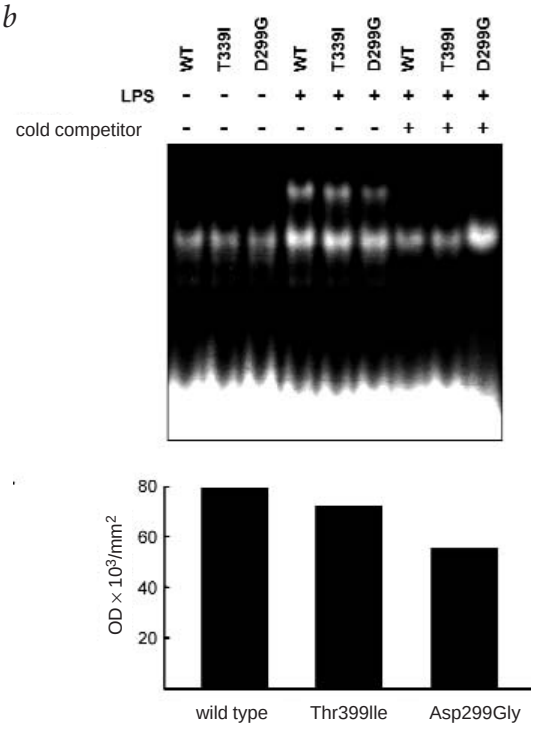


Fig. 3 Functional significance of *TLR4* mutations in THP-1 cells. THP-1 cells were transfected with *TLR4* (ref. 14) expression plasmids (wild type, Asp299Gly or Thr399Ile) and the cells stimulated with LPS. ***a***, NFκB activity following LPS stimulation is significantly ($P<0.01$) less for the THP-1 cells transfected with the Asp299Gly plasmid compared with cells transfected with the wild-type *TLR4* plasmid. ***b***, A gel shift showing that LPS induces nuclear translocation and DNA binding to the NFκB sequence. A diminished gel shift was observed for the mutant *TLR4* allele encoding the Asp299Gly amino acid substitution. The higher molecular weight band is eliminated by an excess of unlabelled NFκB probe.

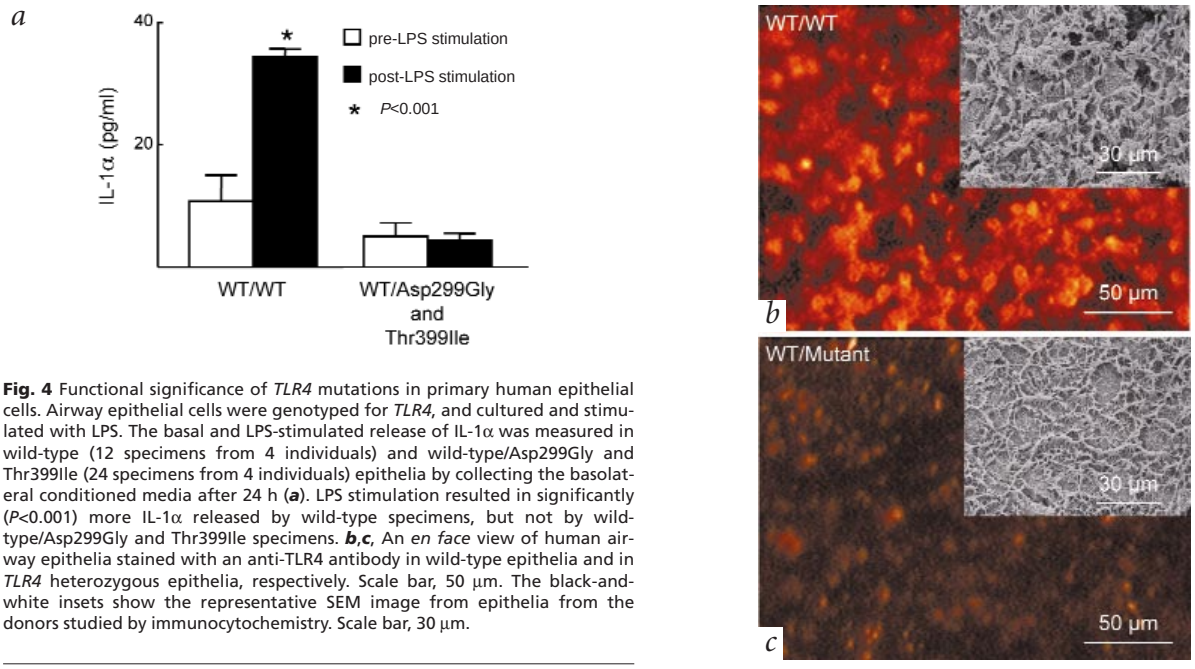


Fig. 4 Functional significance of *TLR4* mutations in primary human epithelial cells. Airway epithelial cells were genotyped for *TLR4*, and cultured and stimulated with LPS. The basal and LPS-stimulated release of IL-1α was measured in wild-type (12 specimens from 4 individuals) and wild-type/Asp299Gly and Thr399Ile (24 specimens from 4 individuals) epithelia by collecting the basolateral conditioned media after 24 h (***a***). LPS stimulation resulted in significantly ($P<0.001$) more IL-1α released by wild-type specimens, but not by wild-type/Asp299Gly and Thr399Ile specimens. ***b***,***c***, An *en face* view of human airway epithelia stained with an anti-TLR4 antibody in wild-type epithelia and in *TLR4* heterozygous epithelia, respectively. Scale bar, 50 μm. The black-and-white insets show the representative SEM image from epithelia from the donors studied by immunocytochemistry. Scale bar, 30 μm.

with a 0.28% decline in FEV_1/μg of inhaled LPS. This subject is one of a pair of monozygotic twins; the other twin was subsequently phenotyped and was also found to be hyporesponsive to inhaled LPS, with a 0.34% decline in FEV_1/μg of inhaled LPS. To be certain that other mutations in *TLR4* were not responsible for the hyporesponsive phenotype, we sequenced the entire coding region and splice sites of *TLR4* in the ten individuals who were least responsive to inhaled LPS ($FEV_1 \geq 90\%$ after inhaling a cumulative dose of 41.5 μg LPS). One of these individuals was found to have another missense mutation in *TLR4* (A→G substitution at nt 137, resulting in replacement of a tyrosine with cysteine at aa 46), and also had the co-segregating mutations. Consequently we sequenced this region in all remaining study subjects and found the Tyr46Cys substitution in only one additional individual, who was responsive to inhaled LPS. Thus the Tyr46Cys substitution does not appear to have a role in responsiveness to inhaled LPS.

As LPS-induced airflow obstruction is associated with activation of macrophages and airway epithelia, release of proinflammatory cytokines and recruitment of PMNs to the airspace[3,15], we investigated the biological significance of the Asp299Gly and Thr399Ile mutations. First, transfection of THP-1 cells (which contain wild-type *TLR4*) with either wild-type or mutant alleles of *TLR4* demonstrated that the cells transfected with the Asp299Gly allele did not respond normally to LPS stimulation, whereas those transfected with the Thr399Ile allele had an intermediate response to LPS (Fig. 3*a*,*b*). Second, airway epithelia obtained from heterozygous individuals with both mutations (Asp299Gly and Thr399Ile) did not respond to LPS stimulation (Fig. 4*a*) and had less TLR4 receptor expression on their apical surface (Fig. 4*b*). Third, overexpression of the wild-type allele of *TLR4* restored LPS responsiveness in either primary airway epithelial cells (Fig. 5*a*) or alveolar macrophages (Fig. 5*b*) obtained from individuals with the *TLR4* mutations.

Our results provide the first direct evidence that a sequence polymorphism in *TLR4* is associated with an endotoxin hyporesponsive phenotype in humans. An understanding of the role of TLR4 in LPS signalling in humans will alter our approach to Gram-negative sepsis, as well as other diseases thought to be mediated by endotoxin (such as the systemic inflammatory response syndrome, the acute respiratory distress syndrome[16] and airway disease[4–6]). Although humans with the TLR4 substitutions may be more resistant to localized forms of endotoxin-induced inflammation, these individuals may be more susceptible to a systemic inflammatory

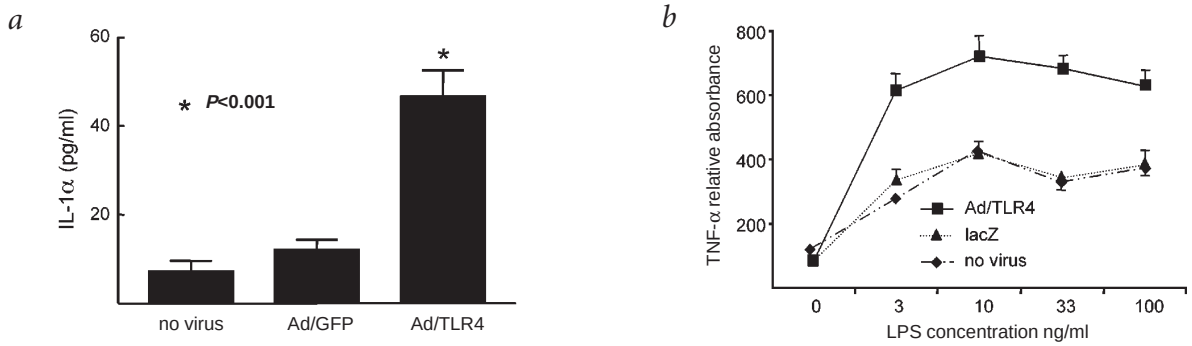


Fig. 5 Rescue of the LPS hyporesponsive phenotype. We infected heterozygote (WT/Asp299Gly and Thr399Ile) airway epithelia (***a***) or homozygote (Asp299Gly and Thr399Ile/Asp299Gly and Thr399Ile) alveolar macrophages (***b***) with a recombinant adenovirus vector expressing TLR4 (ref. 22). After collecting the basal specimen, the epithelia were exposed to LPS on the apical side for 6 h, and the medium was collected after 24 h. Heterozygous airway epithelia produced significantly ($P<0.001$) more IL-1α after infection with the adenovirus vector expressing TLR4 than before transfection.

response initiated or exacerbated by endotoxin, as has been found with the C3H/HeJ mouse, which is more susceptible to *Salmonella typhimurium*[17]. The extracellular domain of TLR4 and the specific amino acid changes that we have identified may be important in receptor function and may provide key therapeutic targets to modulate LPS signalling. It is possible that a missense mutation affecting the extracellular domain of TLR4 either disrupts the transport of this receptor to the cell membrane or impairs ligand binding or protein interactions. Although our data support the former possibility, replacement of the conserved aspartic acid with glycine at position 299 theoretically causes disruption of the α-helical protein structure, resulting in an extended β-strand[18]. Although our findings demonstrate that changes in gene sequence alter the ability of the host to respond to environmental stress, not all of the subjects who were hyporesponsive to LPS had mutations in *TLR4*, and not everyone with the *TLR4* mutation was hyporesponsive to inhaled LPS. This suggests that these mutations in *TLR4* act in concert with other genetic changes or acquired factors to influence the complex physiological response to inhaled LPS.

Methods

Study subjects. Our study population consisted of 83 healthy, adult volunteers (31 men, 52 women) aged 18–50 y who were specifically recruited for this investigation. Exclusion criteria and screening studies have been described[3]. To assess the allelic frequency of *TLR4* sequence variants in individuals of European disease, we screened a well-characterized Iowan population[13] and the CEPH population[14] for specific sequence variants identified in our 83 study subjects.

Endotoxin. We prepared solutions of endotoxin for inhalation according to a standard protocol using lyophilized *Escherichia coli* (serotype 0111:B4, Sigma) LPS as described[3].

Inhalation challenge protocol. All subjects were exposed by inhalation challenge to buffered sterile saline (HBSS) followed by increasing concentrations of LPS. The solutions were delivered via a DeVilbiss 646 nebulizer powered by compressed air at 30 psi (DeVilbiss) and a Rosenthal dosimeter (Laboratory for Applied Immunology). After the HBSS, subsequent inhalations delivered increasing doses of LPS as follows: 0.5 μg, 1.0 μg, 2.0 μg, 3.0 μg, 5.0 μg, 10 μg and 20 μg. The entire protocol delivered a total of 41.5 μg of LPS, a dose that could be inhaled on a daily basis by an agricultural worker[19].

Physiological measurements. We used a Spirotech S600 spirometer to assess pulmonary function as described[3]. Baseline spirometry was recorded after inhalation of saline, then at 1, 10, 20 and 30 min following inhalation of each dose of LPS and compared with the post-saline baseline spirometry. If the study subject's FEV_1 was greater than 80% of the baseline measurement at the final assessment (30 min post-saline), we continued the inhalation challenge and administered the next dose of LPS. The challenge test was terminated when any of the following criteria were met: (i) the subject did not wish to continue for any reason; (ii) the subject's FEV_1 decreased 20% or greater from baseline; or (iii) a cumulative dose of 41.5 μg had been achieved. Of 84 subjects enrolled in the study, 1 subject withdrew before completion of the LPS inhalation challenge test, 52 subjects had at least a 20% decline in the FEV_1 during the LPS inhalation challenge test, and 31 subjects inhaled a cumulative dose of 41.5 μg LPS and did not decrease their FEV_1 by 20%.

Mutation detection. We isolated genomic DNA from whole blood obtained from the study subjects using a rapid salt isolation procedure. Overlapping primer sets were designed across the *TLR4* coding sequence such that products did not exceed 200 bp. We derived primers from flanking intronic sequences to include all splice sites. Standard PCR reactions were prepared, except that genomic DNA (10–20 ng) was used as template. Amplification products were separated on non-denaturing, fan-cooled gels containing 5% acrylamide/bis (19:1), 0.5×TBE and 2.5% glycerol for 3 h at 20 W and 15 h at 5 W using standard procedures[20]. A subset of PCR products were also run on MDE gels. The gels were subjected to silver staining and aberrant bands extracted from the gel, reamplified and sequenced in both directions. To verify the sequence of the aberrant band, we used the same primers to amplify and sequence genomic DNA from each subject. At least one individual without the aberrant band was also sequenced for comparison. The DNA sequence was determined with a Model 377 automated DNA sequencer (Perkin Elmer). The SSCP and sequence analyses were performed by investigators 'blinded' to the LPS response phenotype of the study subjects.

Transfection of THP-1 cells. We maintained THP-1 cells in RPMI media supplemented with 10% fetal calf serum (Gibco), L-glutamine (2 mM) and penicillin/streptomycin (10,000 U). We transfected cells with a mixture of DNA (1 μg) and Effectene (25 μl; Qiagen) in a 60-mm^2 dish. The DNA mix consisted of NFκB reporter plasmid encoding the luciferase gene (0.5 μg; Clontech), MD-2 Flag plasmid (0.25 μg) and 0.25 μg of each *TLR4* (ref. 12) expression plasmid (wild type, Asp299Gly, or Thr399Ile). After 24 h, the cells were stimulated with LPS (100 ng/ml) for 6 h. We measured total luciferase activity using a commercially available method (Tropix). Briefly, cells were centrifuged for 5 min in a clinical centrifuge and pellets resuspended in 1 ml lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, 1% Triton X-100). Light emission was quantified in a luminometer (Analytical Luminescence Laboratory).

Isolation of nuclear extracts and electrophoretic mobility shift assays. The nuclear pellets were prepared by resuspending THP-1 cells in 0.4 ml lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA), placing them on ice for 15 min and vigorous mixing after the addition of 10% Nonidet P-40 (25 μl). After a 30-s centrifugation (16,000*g*, 4 °C), the pelleted nuclei were resuspended in 50 μl extraction buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) and incubated on ice for 20 min. The insoluble fraction was removed by a 14,000-r.p.m. spin for 10 min and nuclear extracts stored at 70 °C. We carried out the DNA-binding reaction (EMSA) at RT in a mixture containing nuclear proteins (5 μg), poly (d(I-C)) (1 μg) and ^{32}P-labelled double-stranded oligo-nucleotide probe (15,000 c.p.m.) for 30 min. The samples were fractionated through a 5% polyacrylamide gel in 1×TBE (6.05 g/l Tris base, 3.06 g/l boric acid, 0.37 g/l EDTA-Na_2H_2O).

Isolation and culture of primary airway epithelial cells. We obtained airway epithelial cells from surgical polypectomies of non-CF patients or from trachea and bronchi of lungs removed for organ donation as described[21]. Freshly isolated cells were seeded at a density of 5×10^5 cells/cm^2 onto collagen-coated, 0.6-cm^2 diameter millicell polycarbonate filters (Millipore). The culture media consisted of a 1:1 mix of DMEM/Ham's F12, 5% Ultraser G (Biosepra SA), penicillin (100 U/ml), streptomycin (100 μg/ml), 1% nonessential amino acids and insulin (0.12 U/ml). Epithelia were tested for transepithelial resistance, and for morphology by scanning electron microscopy. Fourteen days after seeding, the basal release of IL-1α was measured in homozygous wild-type (12 specimens from 4 individuals), WT/Asp299Gly and Thr399Ile (24 specimens from 4 individuals) epithelia by collecting the basolateral conditioned media after 24 h. The epithelia were then exposed to LPS (100 ng/ml) on the apical side for 6 h, and the basolateral media collected after 24 h. We measured IL-1α using a commercially available ELISA (R&D).

Immunohistochemistry. Ciliated airway epithelia were fixed with 4% paraformaldehyde at 4 °C for 10 min and rinsed three times with PBS. The unpermeabilized epithelia were then incubated for 30 min with a 1:100 dilution of donkey serum (Jackson ImmunoResearch Laboratories) followed by a 3-h incubation with an anti-TLR4 antibody (gift from Tularik) applied to the apical side at 4 °C. After careful rinsing, we incubated epithelia with Cy2-labelled antirabbit donkey IgG (1:200; Jackson ImmunoResearch Laboratories) for 1 h. We used non-rabbit serum and secondary antibody alone as negative controls (data not shown).

Electron microscopy. Airway epithelia grown on permeable supports were fixed 14 d after seeding in 2.5% gluteraldehyde and processed for scanning electron microscopy using standard procedures.

Preparation and infection of Ad/TLR4. Briefly, TLR4 plasmid[22] was blunt-ended and cloned into the shuttle vector pAd5/CMVk-NpA using the *Eco*RV site at titres of ~10^{10} infectious units (I.U.)/ml, as described[23]. Four-

teen days after seeding of the airway epithelia (20 specimens from 4 individuals), we added 50 MOI (multiplicity of infection) of the recombinant viruses (Ad/TLR4 and Ad/eGFP in phosphate buffered saline) to the basolateral surface of the epithelia for 30 min. After infection, we incubated epithelia at 37 °C for an additional 48 h before the LPS stimulation assay. To assay for basal LPS response, the media was changed with fresh Ultroser G (500 μl), and collected after 24 h to measure the basal IL-1α secretion to the basolateral side. After collecting the basal specimen, we exposed epithelia to LPS (100 ng/ml) on the apical side for 6 h and collected basolateral media after 24 h. To assay for gene-transfer efficiency, we dissociated the epithelia with 0.05% trypsin and EDTA (0.53 mM). Fluorescence from 50,000 individual cells was analysed using fluorescence-activated cell analysis (FACScan, Lysys II software, Becton Dickinson). The percentage of GFP-positive cells ranged between 52% and 76%. We collected human alveolar macrophages by bronchoalveolar lavage (BAL) from our homozygous (Asp299Gly and Thr399Ile/Asp299Gly and Thr399Ile) study subject as described[15]. Macrophages were seeded onto a 96-well plate at a density of 10^5 cells per well. The cells were infected after 4 h with Ad/TLR4 in a CaPi coprecipitate[24] at an MOI of 50, and a Ca^{++} concentration of 5.6 mM. After 16 h, the cells were exposed to LPS (100 ng/ml) in 1% serum for 6 h, and the media was collected. We measured TNF-α using a commercially available ELISA (R&D).

Statistical analysis. The statistical analysis was designed to determine whether specific mutations in *TLR4* were associated with the airway hyporesponsiveness to inhaled LPS. A one-tailed test was chosen to match the one-sided hypothesis of LPS hyporesponsiveness in the mutant allele group[25]. In a 2×2 analysis, we used a Fisher's one-tailed exact test to determine whether specific mutations of *TLR4* occurred more frequently in study subjects considered LPS hyporesponsive compared with those with a normal airway response to inhaled LPS. As the distribution of the dose-response slope (% decline FEV_1/cumulative dose of inhaled LPS) was highly skewed, the parametric *t*-test was not appropriate for comparing the common *TLR4* allele group with the mutant allele group. Moreover, the standard deviation of the dose-response slope was six times larger in the common allele group. The preferred test in this case is a two-sample permutation *t*-test. On account of the sample sizes, we used a Monte-Carlo permutation *t*-test based on 10,000 permutations[25].

GenBank accession numbers. *TLR4*, U93091, U88880 and AF057025.

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