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Airway Epithelial Expression of TLR5 Is Downregulated in Healthy Smokers and Smokers with Chronic Obstructive Pulmonary Disease

Rui Wang,*† Joumana Ahmed,*,† Guoqing Wang,* Ibrahim Hassan,† Yael Strulovici-Barel,* Jacqueline Salit,* Jason G. Mezey,*‡ and Ronald G. Crystal*

The TLRs are important components of the respiratory epithelium host innate defense, enabling the airway surface to recognize and respond to a variety of insults in inhaled air. On the basis of the knowledge that smokers are more susceptible to pulmonary infection and that the airway epithelium of smokers with chronic obstructive pulmonary disease (COPD) is characterized by bacterial colonization and acute exacerbation of airway infections, we assessed whether smoking alters expression of TLRs in human small airway epithelium, the primary site of smoking-induced disease. Microarrays were used to survey the TLR family gene expression in small airway (10th to 12th order) epithelium from healthy nonsmokers (n = 60), healthy smokers (n = 73), and smokers with COPD (n = 36). Using the criteria of detection call of present (P call) ≥ 50%, 6 of 10 TLRs (TLRs 1-5 and 8) were expressed. Compared with nonsmokers, the most striking change was for TLR5, which was downregulated in healthy smokers (1.4-fold, p < 10^-10) and smokers with COPD (1.6-fold, p < 10^-11). TaqMan RT-PCR confirmed these observations. Bronchial biopsy immunofluorescence studies showed that TLR5 was expressed mainly on the apical side of the epithelium and was decreased in healthy smokers and smokers with COPD. In vitro, the level of TLR5 downstream genes, IL-6 and IL-8, was highly induced by flagellin in TLR5 high-expressing cells compared with TLR5 low-expressing cells. In the context that TLR5 functions to recognize pathogens and activate innate immune responses, the smoking-induced downregulation of TLR5 may contribute to smoking-related susceptibility to airway infection, at least for flagellated bacteria. The Journal of Immunology, 2012, 189: 2217–2225.

The innate immune recognition of pathogens by human airway epithelium and subsequent intracellular signaling is mediated by families of pattern recognition receptors, which recognize the specific molecular structures of pathogens (1–3). Among these pattern receptors, the TLRs are type I membrane glycoproteins with extracellular leucine-rich repeat domain, transmembrane domain, and cytoplasmic Toll/IL-1R (TIR) domain (2, 4, 5). The TIR domain mediates TLR signaling through the TIR domain-containing adapters MyD88, TIR-containing adapter protein, TIR-containing adapter-inducing IFN-β, and TIR-containing adapter-inducing IFN-β–related adapter molecule (4, 5).

Ten TLR members have been identified in humans (6, 7) and are expressed on B cells, NK cells, dendritic cells, macrophages, and nonimmune cells, including fibroblasts, epithelial cells, and endothelial cells (7, 8). TLRs 1, 2, 4, 5, 6, and 10 are expressed on the cell surface and TLRs 3, 7, 8, and 9 in endosomes (3, 9). On the basis of our observation that small airway epithelial TLR5 expression is dramatically suppressed by smoking, we focused this study on TLR5, a TLR known to be expressed on the luminal surface of epithelial cells covering the trachea, bronchi, and alveoli of the respiratory tract (10). TLR5 identifies flagellin, a protein on the surface of motile bacteria that functions to mediate adhesion and invasion at the surface of the epithelial cells (11–14). Using assessment of TLR5 at the mRNA, protein, and functional levels, the data show that airway epithelium TLR5 expression is downregulated in healthy smokers and in smokers with chronic obstructive pulmonary disease (COPD) and the overall levels of expression of TLR5 mediate the levels of airway epithelial response to TLR5 activation. In the context that cigarette smoking is associated with increased susceptibility to infection and that COPD is characterized by bacterial colonization and exacerbations of infection, these data suggest that smoking-mediated suppression of airway epithelial TLR5 may contribute to the increased susceptibility of smokers and smokers with COPD to airway flagellated bacterial infection.

Materials and Methods

Study population

Healthy nonsmokers (n = 60), healthy smokers (n = 73), and smokers with COPD (n = 36) were evaluated using institutional review board-approved protocols at the Department of Genetic Medicine Clinical Research Facility (Weill Cornell Medical College). Current smoking status was evaluated based on history and urine analysis for nicotine metabolites. Healthy nonsmokers were defined as subjects who never smoked, had normal physical examination, lung function, and chest radiograph, and had smoking-related blood and urine parameters within the nonsmoker range. Healthy smokers were defined as subjects with a current smoking history, normal physical examination, lung function, and chest radiograph, and their smoking-
related urine and blood parameters were consistent with those of a current smoker (15,16). The criteria for smokers with established COPD included current smokers who met the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (17,18).

**Sampling airway epithelium**

Small airway epithelium brushes were collected using fiberoptic bronchoscopy (16). Small airway epithelial cells were collected from the 10th to 12th order bronchi. Total cell number was counted, and cell viability was estimated by trypan blue exclusion. Cytology and differential cell count were also carried out.

**RNA extraction and microarray processing**

Total RNA was extracted from epithelial cells using Qiagen RNeasy MinElute kit (Qiagen, Valencia, CA). An aliquot of each RNA sample was run on the Agilent Technologies (Palo Alto, CA). The concentration was determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Microarray analysis was performed using an Affymetrix (Santa Clara, CA) HG-U133 Plus 2.0 using 1 µg RNA as previously described (15,16). The quality control criteria included: 1) 3'5' ratio for GAPDH ≥ 3 and 2) scaling factor ≥10 (19). These data were processed using GeneSpring GX 7.3.1 software (Agilent Technologies) and Partek Genomics Suite v6.5 (Partek Genomics, St. Louis, MO). The data sets were assessed for expression of TLR using criteria of present (P call) ≥50% in healthy nonsmokers.

**Massive parallel mRNA sequencing**

The TLR5 expression was additionally studied using massive parallel RNA sequencing in four healthy nonsmokers and six healthy smokers. Six micrograms of total RNA per subject was processed according to Illumina’s mRNA Sequencing Sample Preparation Guide. Then, the data were analyzed with Partek Genomics Suite v6.5 (Partek Genomics) (20-22).

**TaqMan real-time PCR**

To confirm further the gene expression of TLR5 in small airway epithelium, TaqMan real-time PCR was performed on small airway samples (n = 23 healthy nonsmokers, n = 43 healthy smokers, n = 24 smokers with COPD) that had also been assessed with the HG-U133 Plus 2.0 Microarray. cDNA was synthesized using the TaqMan Reverse Transcriptase Reaction kit (Life Technologies, Carlsbad, CA) with random hexamers as primers (23), and 18S rRNA was used as an internal control (Life Technologies). Relative gene expression was determined with the C_{\text{M}} method.

**Immunofluorescence assessment of TLR5 in airway epithelium**

To determine the effects of smoking on TLR5 expression in airway epithelial cells, bronchial biopsies were obtained by flexible bronchoscopy from the large airway epithelium (3rd to 4th order bronchi) of healthy nonsmokers (n = 8), healthy smokers (n = 12), and smokers with COPD (n = 13). Biopsies were embedded in paraffin, sectioned, cleared with xylene, and rehydrated through graded ethanol series. Ag recovery was performed using boiling citrate buffer, pH 6 (Thermo Fisher Scientific, San Jose, CA) at 100°C for 20 min. Tissue sections were blocked using diluted normal horse blocking serum (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 20 min. Then, the sections were incubated with polyclonal rabbit Ab to TLR5 at 1 µg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Isotype-matched rabbit IgG was used as control. After washing with PBS, pH 7.4, the slides were incubated with biotinylated secondary Ab solution (Vectastain Elite ABC kit) for 30 min. Then, the slides were washed with PBS and incubated with Cy3-conjugated streptavidin at a concentration of 1:900 (Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min, followed by incubation with DAPI nuclear staining at a concentration of 1:2000 (Life Technologies) for 5 min. The slides were mounted using slow fade mounting medium (Life Technologies).

**In vitro human airway epithelial cell differentiation**

Human airway epithelial cells were collected by bronchoscopy from four healthy nonsmokers and cultured in vitro for 7 d (24). Then, the cells were put on air–liquid interface to induce differentiation for 28 d (24). Samples were collected at five time points: days 0, 7, 14, 21, and 28. Affymetrix HG-U133 Plus 2.0 microarrays were used to analyze the gene expression at different time points.

**Airway epithelial cells exposed to cigarette smoke extract**

To prepare aqueous cigarette cell extract (CSE), 1 cigarette (Marlboro Red) was combusted and bubbled through 12.5 ml of culture medium as described previously (15). This medium was defined as “100% CSE.” Different concentrations of CSE diluted with the culture medium were used, ranging from 1 to 3%. In vitro fully differentiated human airway epithelial cells cultured on air–liquid interface for 21 d were exposed to freshly prepared CSE for 48 h. The expression of TLRs 1, 4, and 5 as well as control genes including airway basal cell marker cytokeratin 5, ciliated cell marker DNAI1, and β-actin were assessed by TaqMan real-time PCR with 18S rRNA as the internal control.

**TLR5 function on airway epithelial cells**

Human airway basal cells and differentiated cells on air–liquid interface were exposed to recombinant purified flagellin (100 ng/ml; Invogen, San Diego, CA) and incubated at 37°C, 24 h. Then, cells were collected in TRIzol (Life Technologies), followed by RNA extraction (Qiagen RNeasy MinElute kit; Qiagen). TaqMan real-time PCR reactions were carried out using gene expression assays for the TLR5 target genes, IL-6 and IL-8 (Life Technologies).

To assess the effect of TLR5 inhibition on the ability of airway epithelial cells to respond to flagellin, human airway epithelial cell line BEAS-2B cells (American Type Culture Collection, Manassas, VA) were treated with 5 μg/ml TLR5 neutralizing Ab (PAB-hTLR5; Invivogen, San Diego, CA) or 5 μg/ml purified rat IgG isotype control (eBioscience, San Diego, CA) (12) and TLR5 ligand flagellin (100 ng/ml). Cells were incubated for 24 h, 37°C, and then cells were collected for TaqMan real-time PCR using gene expression assay for IL-6 and IL-8 (Life Technologies). TLR5 small interfering RNA (siRNA) was also used to confirm the effect of TLR5 downregulation on the ability of airway epithelial cells to respond to flagellin. TLR5 and control siRNA (Qiagen) were transfected in BEAS-2B cells for 24 h, then cells were exposed to flagellin (100 ng/ml) for 24 h. Cells were then collected for TaqMan real-time PCR using gene expression assays for IL-6 and IL-8 (Life Technologies).

**Statistical analysis**

GeneSpring and Partek software were used to analyze the HG-U133 Plus 2.0 Microarray data. Average expression values in small airway samples were calculated from normalized expression levels for healthy nonsmokers, healthy smokers, and smokers with COPD. Kruskal-Wallis test was used to assess for differences among groups. For genes where Kruskal–Wallis showed a significant difference (p < 0.05), non-parametric post hoc pairwise comparisons were performed using the mean rank comparison approach of Schach and Hamele (25). For TaqMan RT-PCR analysis, statistical comparisons were calculated using an unpaired, two-tailed t test for two groups using Excel and one-way ANOVA and post hoc pairwise comparisons with Fisher PLSD test for three groups using StatView software (version 5.0). A p value <0.05 was considered significant.

**Web deposition of data**

All data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE30063.

**Results**

**Study population and sampling of the small airway epithelium**

Small airway epithelial samples from a total of 169 individuals, including 60 healthy nonsmokers, 73 healthy smokers, and 36 smokers with COPD (GOLD I, n = 21; GOLD II, n = 13; GOLD III, n = 2; Table I) were analyzed with Affymetrix HG-U133 Plus 2.0 microarrays. There were minor differences in age, ancestral background, and smoking history, but otherwise the groups were comparable, and all fit the established phenotype criteria for each group. The number of airway epithelial cells recovered averaged from 2.3 × 10^6 to 2.0 × 10^7 (Table I). The various categories of airway epithelial cells were as expected from the small airway epithelium (16).

**Expression of TLRs in small airway epithelium of healthy nonsmokers, healthy smokers, and smokers with COPD**

Microarrays were used to analyze the gene expression of the TLR family at the mRNA level in the small airway epithelium of healthy nonsmokers, healthy smokers, and smokers with COPD. Using the criteria of detection call of present (P call) ≥50%, 6 of 10 TLRs (TLRs 1–5 and 8) were expressed in the small airway epithelium.
that in healthy nonsmokers (4.4-fold, p = 0.0001, fold-change 4.4; Fig. 1A). Of the 6 TLRs expressed, only TLRs 4 and 5 had any significant differences among the groups (Fig. 1B, 1C). Expression of TLR4 in healthy smokers was downregulated compared with that in healthy nonsmokers (p < 0.001, fold-change 1.4; Fig. 1B), consistent with previous studies that TLR4 expression is reduced in nasal epithelium of smokers compared with that in nonsmoker controls (26). However, there was no significant change of TLR4 expression level between healthy smokers and COPD smokers (26). The COPD smoker group included: GOLD I, n = 1; GOLD II, n = 1; and GOLD III, n = 2. The numbers of these individuals on COPD-related medications are indicated in the next row.

Two separate methods were used to validate the microarray data. First, TaqMan RT-PCR was used to validate the TLR5 expression in 23 healthy nonsmokers, 43 healthy smokers, and 24 smokers with COPD. Consistent with the microarray data, the TaqMan RT-PCR analysis showed that TLR5 was downregulated in healthy smokers (1.9-fold, p < 0.0001) and in smokers with COPD (1.4-fold, p < 0.04; Fig. 2A). In addition, massive parallel mRNA sequencing was carried out on a subgroup of four healthy nonsmokers and six healthy smokers, which was part of the microarray data. Consistent with the microarray and TaqMan RT-PCR data, TLR5 expression was 1.7-fold lower in healthy smokers compared with that in healthy nonsmokers (p < 0.002; Fig. 2B, 2C).

On the basis of these data, we focused on TLR5 expression. Two separate methods were used to validate the microarray data. First, TaqMan RT-PCR was used to validate the TLR5 expression in 23 healthy nonsmokers, 43 healthy smokers, and 24 smokers with COPD. Consistent with the microarray data, the TaqMan RT-PCR analysis showed that TLR5 was downregulated in healthy smokers (1.9-fold, p < 0.0001) and in smokers with COPD (1.4-fold, p < 0.04; Fig. 2A). In addition, massive parallel mRNA sequencing was carried out on a subgroup of four healthy nonsmokers and six healthy smokers, which was part of the microarray data. Consistent with the microarray and TaqMan RT-PCR data, TLR5 expression was 1.7-fold lower in healthy smokers compared with that in healthy nonsmokers (p < 0.002; Fig. 2B, 2C).

**TLR5 localization in human airway epithelium**

Bronchial biopsies obtained by flexible bronchoscopy from the airway epithelium of healthy nonsmokers, healthy smokers, and smokers with COPD were stained with an anti-TLR5 Ab to assess the distribution of TLR5 protein expression in airway epithelium cells. In healthy nonsmokers, healthy smokers, and smokers with COPD, the TLR5 protein was expressed mainly on the apical side of the human airway epithelium (Fig. 3). The immunofluorescence of TLR5 in healthy smokers (Fig. 3E–H) and smokers with COPD (Fig. 3I–L) was decreased compared with that in healthy nonsmokers (Fig. 3A–D).

Human airway epithelial cells differentiated in vitro were used to assess further the expression pattern of TLR5. The gradual appearance of ciliated cell-specific marker β-tubulin IV during in vitro differentiation showed the successful differentiation of human airway epithelial cells (Fig. 4A–E). Consistent with this, the expression pattern of ciliated cell-specific gene FOXJ1 showed upregulation with differentiation (Fig. 4F), and the basal cell-specific gene KRT14 was downregulated with differentiation (Fig. 4G). Consistent with the immunofluorescence expression pattern from the biopsies, TLR5 gene expression was upregulated as the basal cells differentiated to a ciliated epithelium (Fig. 4H).

**Acute exposure to CSE decreases TLR5 gene expression in differentiated human airway epithelial cells**

On the basis of immunofluorescence staining, the TLR5 protein was expressed mainly on the apical side of the human airway epithelium. To assess whether cigarette extract could directly

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**Table I. Demographics of the study population and biologic samples**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Nonsmokers</th>
<th>Healthy Smokers</th>
<th>Smokers with COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>60</td>
<td>73</td>
<td>36</td>
</tr>
<tr>
<td>Sex (n, M/F)</td>
<td>38/22</td>
<td>52/21</td>
<td>28/8</td>
</tr>
<tr>
<td>Age (y)</td>
<td>41 ± 12</td>
<td>43 ± 7</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>Race (n, black/white/other)</td>
<td>26/23/11</td>
<td>45/17/11</td>
<td>13/13/10</td>
</tr>
<tr>
<td>Smoking history (pack-years)</td>
<td>26 ± 16</td>
<td>38 ± 24</td>
<td></td>
</tr>
<tr>
<td>Urine nicotine (ng/ml)</td>
<td>—</td>
<td>1,327 ± 1,627</td>
<td>1,160 ± 1,548</td>
</tr>
<tr>
<td>Urine cotinine (ng/ml)</td>
<td>—</td>
<td>1,285 ± 1,040</td>
<td>1,469 ± 625</td>
</tr>
<tr>
<td>Blood carboxyhemoglobin (%)</td>
<td>0.4 ± 0.8</td>
<td>1.8 ± 1.8</td>
<td>2.8 ± 1.8</td>
</tr>
<tr>
<td>Pulmonary function parameters†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC</td>
<td>106 ± 13</td>
<td>109 ± 13</td>
<td>104 ± 21</td>
</tr>
<tr>
<td>FEV1</td>
<td>105 ± 14</td>
<td>107 ± 14</td>
<td>82 ± 22</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>82 ± 6</td>
<td>80 ± 5</td>
<td>63 ± 7</td>
</tr>
<tr>
<td>TLC</td>
<td>99 ± 13</td>
<td>100 ± 12</td>
<td>105 ± 19</td>
</tr>
<tr>
<td>DLCO</td>
<td>98 ± 14</td>
<td>94 ± 11</td>
<td>76 ± 16</td>
</tr>
<tr>
<td>GOLD stage (I/II/III)†</td>
<td>—</td>
<td>—</td>
<td>21/13/2</td>
</tr>
<tr>
<td>Medication use (number of patients)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-agonist</td>
<td>—</td>
<td>—</td>
<td>6</td>
</tr>
<tr>
<td>Anticholinergic</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Inhaled corticosteroid</td>
<td>—</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>Epithelial cells‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number recovered ×10⁶</td>
<td>6.4 ± 2.9</td>
<td>7.5 ± 3.3</td>
<td>6.3 ± 2.7</td>
</tr>
<tr>
<td>Percentage epithelial cells (%)</td>
<td>99.1 ± 1.2</td>
<td>99.0 ± 1.3</td>
<td>98.4 ± 1.5</td>
</tr>
<tr>
<td>Percentage inflammatory cells (%)</td>
<td>0.9 ± 1.2</td>
<td>1.0 ± 1.3</td>
<td>1.6 ± 1.5</td>
</tr>
<tr>
<td>Differential cell count§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciliated (%)</td>
<td>72.0 ± 8.9</td>
<td>64.1 ± 12.0</td>
<td>60.3 ± 10.6</td>
</tr>
<tr>
<td>Secretory (%)</td>
<td>6.7 ± 3.6</td>
<td>8.4 ± 4.1</td>
<td>11.4 ± 5.6</td>
</tr>
<tr>
<td>Basal (%)</td>
<td>12.6 ± 6.6</td>
<td>14.5 ± 8.0</td>
<td>15.3 ± 8.5</td>
</tr>
<tr>
<td>Undifferentiated columnar (%)</td>
<td>7.8 ± 3.4</td>
<td>12.2 ± 6.8</td>
<td>11.4 ± 4.1</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.  
‡Smokers with “established COPD” defined by the GOLD criteria (13). The COPD smoker group included: GOLD I, n = 21; GOLD II, n = 13; and GOLD III, n = 2. The numbers of these individuals on COPD-related medications are indicated in the next row.  
†Pulmonary function testing parameters are given as percentage of predicted value with the exception of FEV1/FVC, which is reported as percentage observed. For individuals with COPD, FVC, FEV1, and FEV1/FVC are postbronchodilator values.  
§Small airway epithelium.  
¶As a percentage of small airway epithelium recovered.  
— Not applicable; DLCO, diffusing capacity; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; TLC, total lung capacity.
downregulate TLR5 expression in human airway epithelial cells, fully differentiated human airway epithelial cells cultured on air–liquid interface for 21 d were exposed to freshly made CSE for 48 h. Three different CSE concentrations were used (1, 2, and 3%); none of these levels was toxic to the cells (data not shown).

Although TLR5 expression did not change significantly when treated with 1 and 2% CSE, TLR5 expression was significantly downregulated when exposed to 3% CSE (fold-change 1.4, \( p < 0.0001 \); Fig. 5C). There was a trend of decreased TLR4 expression in the 3% CSE treatment group, but it was not statis-
tically significant \((p > 0.05; \text{Fig.~5B})\). As a control, the expression of TLR1, which did not have significant change in vivo, also did not change significantly in vitro (Fig. 5A). The expression level for other control genes, including ciliated cell marker DNAI1, basal cell marker cytokeratin 5 (KRT5), and an internal control \(\beta\)-actin, were not significantly changed by CSE (Fig. 5D–F).

**In vitro TLR5 function**

To explore whether the expression of TLR5 was related to its function, flagellin, a specific activator of TLR5, was used to activate TLR5 in two types of human airway epithelial cells. Human airway basal cells had lower TLR5 expression levels compared with those of basal cells differentiated on air–liquid interface to a ciliated epithelium \((p < 0.01; \text{Fig.~6A})\). After flagellin activation for 24 h, the human airway basal cells (TLR5 low-expressing cells) showed lower expression levels of downstream genes of the flagellin–TLR5 pathway, IL-6 and IL-8, compared with those of differentiated cells (Fig. 6B, 6C).

A specific neutralizing polyclonal Ab of TLR5 was further used to assess the specificity of flagellin–TLR5 activation. Compared with mock controls, the addition of flagellin and rat IgG control to the human BEAS-2B airway epithelial cell line increased IL-6 expression (10-fold increase, \(p < 0.0001\)) and IL-8 (34-fold increase, \(p < 0.001; \text{Supplemental Fig.~1A, 1B}\)). The TLR5 inhibitor decreased the expression of both IL-6 (1.5-fold decrease, \(p < 0.02\)) and IL-8 (2.3-fold decrease, \(p < 0.01; \text{Supplemental Fig.~1A, 1B}\)). TLR5 siRNA was also used to confirm the specificity of flagellin–TLR5 activation in the human BEAS-2B airway epithelial cell line. After activation with flagellin for 24 h, the cells transfected with control siRNA showed significant upregulation of IL-6 (29-fold increase, \(p < 0.0001\)) and IL-8 expression (124-fold increase, \(p < 0.0001; \text{Fig. ~7}\)). The TLR5 siRNA decreased the expression of both IL-6 (1.8-fold decrease, \(p < 0.003\)) and IL-8 (1.4-fold decrease, \(p < 0.01; \text{Fig.~7}\)).
Discussion

The TLR family belongs to the pattern recognition receptors, which play a fundamental role in the activation of innate immune responses against pathogens (2, 4). After recognizing the specific pathogen-associated molecular patterns, TLRs induce NF-κB signaling and the MAPK pathway to trigger the secretion of proinflammatory cytokines and chemokines (27, 28). To explore the expression pattern of TLRs in human airway epithelium and the relationship to smoking and COPD, the expression of TLRs in human airway epithelium of healthy nonsmokers, healthy smokers, and smokers with COPD was assessed. The data demonstrate that 6 of 10 TLRs are expressed in human small airway epithelium, with TLR5 mRNA expression significantly reduced in healthy smokers and smokers with COPD compared with that in healthy nonsmokers. Consistent with the mRNA data, immunofluorescence Ab assessment of biopsies from lung epithelium showed that TLR5 protein is expressed mainly on the apical side of the human airway epithelium and is decreased in healthy smokers and smokers with COPD compared with that in healthy nonsmokers. Addition of the TLR5-specific activator flagellin to differentiated airway epithelial cells resulted in marked upregulation of the TLR5 downstream genes, IL-6 and IL-8, compared with minimal IL-6 and IL-8 upregulation in TLR5 low-expressing airway basal stem/progenitor cells. Finally, when TLR5 was blocked with a specific Ab, IL-6 and IL-8 expression levels were significantly decreased, confirming the specific interaction of flagellin and TLR5 in inducing the downstream IL-6 and IL-8 expression in airway epithelial cells.

![FIGURE 5. TLR5 gene expression in differentiated human airway epithelial cells by acute CSE exposure.](image)

![FIGURE 6. TLR5 expression level is related to its function in mediating flagellin-induced airway epithelium expression of inflammatory mediators.](image)
such as asthma and COPD (26, 51, 52). Harmful to the host and leads to chronic inflammatory conditions, over- or under-TLR signaling activation can be fundamental for pathogenic clearance, over- or under-TLR signaling activation can be harmful to the host and leads to chronic inflammatory conditions such as asthma and COPD (26, 51, 52).

TLRs and innate immunity

The mechanism for innate immune recognition of pathogens and signaling evolved rapidly after the discovery of Toll protein in *Drosophila* (32). To date, 10 TLR members have been identified in humans, expressed on various immune and nonimmune cells such as B cells, NK cells, dendritic cells, macrophages, fibroblasts, epithelial cells, and endothelial cells. Different TLRs activate the immune response to specific pathogen-associated molecular patterns (5, 33, 34). For example, TLR2 and TLR4 recognize endotoxin, lipoteichoic acid, and LPS (35, 36); TLR3 recognizes dsRNA (37); TLR5 recognizes bacterial flagellin (11, 12); both TLR7 and TLR8 recognize synthetic imidazoquinolines and ssRNA (38, 39); and TLR9 recognizes unmethylated bacterial CpG DNA (40). Interaction of the agonist with the TLRs facilitates receptor dimerization, triggering activation of either MyD88-dependent or -independent pathways and finally induces the expression of genes involved in innate immunity including proinflammatory cytokines, chemokines, and other effector molecules (41–43). Decreased expression/function of the TLRs has significant consequences to host defenses (44). For example, TLR4-deficient mice have persistent infection with *Haemophilus* (45). TLR5-deficient mice develop spontaneous colitis (46) and exhibit impaired CD4 T cell response to a flagellated pathogen (47).

**TLRs in airway epithelium**

There is considerable evidence that activation of airway epithelial TLRs amplify the airway immune response by production and secretion of chemotactic factors and cytokines, upregulation of cell surface adhesion molecules, and increased expression of antimicrobial peptides (8, 48). Some of these chemokines include TNF-α, IL-6, IL-8, and MIP-3α, which are essential for phagocyte recruitment, granuloma formation, and clearance of bacterial infection in the lung (49, 50). Although fundamental for pathogen clearance, over- or under-TLR signaling activation can be harmful to the host and leads to chronic inflammatory conditions such as asthma and COPD (26, 51, 52).

There has been considerable interest in the function of TLR4 in the lung (26, 51–53). Hongjia et al. (51) demonstrated that TLR4 expression in the airway epithelium is significantly increased in both mRNA and protein levels in mice treated with house dust mite, and TLR4 expression on airway epithelium plays an essential role in house dust mite-induced activation of alveolar macrophages. In the human nasal epithelium, TLR4 gene expression of smokers is decreased compared with that of non-smokers, and individuals with severe COPD have decreased TLR4 expression compared with that of those with less severe disease (26). In vitro CSE treatment of an airway epithelial cell line results in a dose-dependent downregulation of TLR4 expression (26).

By identifying flagellin, TLR5 induces early signaling dedicated to protective innate immune responses against microorganisms (11, 12). Zhang et al. (10) demonstrated that airway epithelium TLR5 senses *Pseudomonas aeruginosa* and plays an important role in the initiation of the host inflammatory reaction to clear the invading pathogen. There are some reports of *P. aeruginosa* isolated from the sputum of COPD patients (54–58), and the acquisition of *P. aeruginosa* occasionally is associated with a COPD exacerbation (57). *P. aeruginosa* isolation in sputum in patients hospitalized for acute exacerbation of COPD has been reported to be a prognostic marker of 3-y mortality (54). In the current study, we found that the TLR5 expression in human small airway epithelium is downregulated in healthy smokers and smokers with COPD compared with that in healthy nonsmokers. In vitro activation of airway epithelial cells with the TLR5 agonist flagellin induced the increased expression of the TLR5 downstream chemokines and cytokines, and this activation was attenuated after blocking the TLR5 function. Consistent with these observations, Maunders et al. (59) evaluated gene expression profiling with air/whole mainstream cigarette smoke treatment of the differentiated tracheobronchial epithelium in vitro for 24 h, showing that cigarette smoke downregulated the expression of TLR5. However, the mechanism of why cigarette smoking should repress selective TLRs in vitro and in vivo is unknown due to the complicated regulating pathways and complex tobacco components.

In the context that TLR5 functions on the airway epithelium, but the levels of expression are suppressed by smoking, both in healthy smokers and COPD smokers. In the context that airway cells with low expression of TLR5 respond less vigorously with downstream mediators when exposed to a TLR5 agonist than do airway cells with high TLR5 expression, the data suggest smoking-related downregulated expression of TLR5 in the airway epithelium may contribute to smoking-induced suppression of innate defenses to flagellated bacteria (29–31).

**FIGURE 7.** Downregulation of TLR5 expression in human airway epithelial cells decreases TLR5 downstream gene expression. The human airway epithelial cell line BEAS-2B was transfected with TLR5 siRNA and control siRNA for 24 h, then cells were exposed to flagellin for 24 h. Expression of TLR5 downstream genes IL-6 and IL-8 was assessed by TaqMan RT-PCR. (A) IL-6 expression. The cells transfected with control siRNA showed significant upregulation of IL-6 (29-fold increase, p < 0.0001) and IL-8 (124-fold increase, p < 0.0001) expression (A, B). The TLR5 siRNA decreased the expression of both IL-6 (1.8-fold decrease, p < 0.003) and IL-8 (1.4-fold decrease, p < 0.01) (A, B).

Together, the data demonstrate that TLR5 functions on the airway epithelium, but the levels of expression are suppressed by smoking, both in healthy smokers and COPD smokers. In the context that airway cells with low expression of TLR5 respond less vigorously with downstream mediators when exposed to a TLR5 agonist than do airway cells with high TLR5 expression, the data suggest smoking-related downregulated expression of TLR5 in the airway epithelium may contribute to smoking-induced suppression of innate defenses to flagellated bacteria (29–31).

**TLRs and innate immunity**

The mechanism for innate immune recognition of pathogens and signaling evolved rapidly after the discovery of Toll protein in *Drosophila* (32). To date, 10 TLR members have been identified in humans, expressed on various immune and nonimmune cells such as B cells, NK cells, dendritic cells, macrophages, fibroblasts, epithelial cells, and endothelial cells. Different TLRs activate the immune response to specific pathogen-associated molecular patterns (5, 33, 34). For example, TLR2 and TLR4 recognize endotoxin, lipoteichoic acid, and LPS (35, 36); TLR3 recognizes dsRNA (37); TLR5 recognizes bacterial flagellin (11, 12); both TLR7 and TLR8 recognize synthetic imidazoquinolines and ssRNA (38, 39); and TLR9 recognizes unmethylated bacterial CpG DNA (40). Interaction of the agonist with the TLRs facilitates receptor dimerization, triggering activation of either MyD88-dependent or -independent pathways and finally induces the expression of genes involved in innate immunity including proinflammatory cytokines, chemokines, and other effector molecules (41–43). Decreased expression/function of the TLRs has significant consequences to host defenses (44). For example, TLR4-deficient mice have persistent infection with *Haemophilus* (45). TLR5-deficient mice develop spontaneous colitis (46) and exhibit impaired CD4 T cell response to a flagellated pathogen (47).
Disclosures

The authors have no financial conflicts of interest.

References


