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Airway Epithelial Expression of Toll-like Receptor 5 is Down-regulated in Healthy Smokers and Smokers with Chronic Obstructive Pulmonary Disease

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Abstract

The toll-like receptors (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. Based on 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–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 in 50%, 6 of 10 TLRs (1, 2, 3, 4, 5 and 8) were expressed. Compared to nonsmokers, the most striking change was for TLR5, which was down-regulated 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, were highly induced by flagellin in TLR5 high-expressing cells compared to TLR5 low-expressing cells. In the context that TLR5 functions to recognize pathogens and activate innate immune responses, the smoking-induced down-regulation of TLR5 may contribute to smoking-related susceptibility to airway infection, at least for flagellated bacteria.

Introduction

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 toll-like receptors (TLR), are type 1 membrane glycoproteins with extracellular leucine-rich repeat domain, transmembrane domain and cytoplasmic Toll/interleukin-1 receptor (TIR) domain (2,4,5). The TIR domain mediates TLR signaling

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through the TIR-domain-containing adapters myeloid differentiation primary response gene 88 (MyD88), TIR-containing adaptor protein, TIR-containing adaptor-inducing IFN- β and TRIF-related adaptor molecule (4,5).

Ten TLR members have been identified in humans (6,7), expressed on B-cells, NK cells, dendritic cells, macrophages, and non-immune cells, including fibroblasts, epithelial cells and endothelial cells (7,8). TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface, and TLR3, 7, 8 and 9 in endosomes (3,9). Based on 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 shows that airway epithelium TLR5 expression is down-regulated in healthy smokers and smokers with 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 COPD is characterized by bacterial colonization and exacerbations of infection, this data suggests 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.

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. Current smoking status was evaluated based on history, urine analysis for nicotine metabolites, and venous carboxyhemoglobin levels. Healthy nonsmokers were defined as subjects who never smoked and have normal physical exam, lung function and chest x-ray, and their smoking related blood and urine parameters within the nonsmoker range. Healthy smokers were defined as subjects with a current smoking history, normal physical exam, lung function, chest x-ray and their smoking-related urine and blood parameters consistent with that 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 epithelium 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 an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). The concentration was determined using NanoDrop ND-100 spectrophotometer (NanoDrop technologies, Wilmington, DE).

Microarray analysis was performed using Affymetrix (Santa Clara, CA) HG-U133 Plus 2.0 using 1 μ g RNA as previously described (15,16). The quality control criteria included: (1) 3'/5' ratio for GAPDH ≥ 3 ; and (2) scaling factor ≤ 10.0 (19). These data were processed using GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto, CA) 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) in 50% in healthy nonsmokers.

Massive Parallel mRNA Sequencing

The TLR5 expression was additionally studied using massive parallel RNA sequencing (RNA-Seq) in 4 healthy nonsmokers and 6 healthy smokers. 6 µg 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, St Louis, MO) (20–22).

TaqMan Real-time PCR

To further confirm the gene expression of TLR5 in small airway epithelium, TaqMan Real-time PCR was done 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). 18S rRNA was used as the internal control (Life Technologies, Carlsbad, CA). Relative gene expression was determined with CT 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. Antigen recovery was performed using boiling citrate buffer, pH 6.0 (Thermo Fisher Scientific, San Jose, CA) at 100°C for 20 min. Tissue sections were blocked using diluted normal horse blocking serum (Vectastain ABC Elite; Vector Laboratories, Burlingame, CA) for 20 min. Then the sections were incubated with polyclonal rabbit antibody 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 phosphate buffered saline, pH 7.4 (PBS), the slides were incubated with biotinylated secondary antibody 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 4',6-diamidino-2-phenylindole (DAPI) nuclear staining at a concentration of 1:2000 (Life Technologies, Carlsbad, CA) for 5 min. The slides were mounted using slow fade mounting medium (Life Technologies, Carlsbad, CA).

In Vitro Human Airway Epithelial Cell Differentiation

Human airway epithelial cells were collected by bronchoscopy from 4 healthy nonsmokers and cultured *in vitro* for 7 days (24). Then the cells were put on air-liquid interface to induce differentiation for 28 days (24). Samples were collected at 5 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 days were exposed to freshly prepared

CSE for 48 hr. The expression of TLR1, 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, Imgenex, San Diego CA) and incubated at 37°C, 24 hr. Then cells were collected in Trizol (Life Technologies), followed by RNA extraction (Qiagen RNeasy MinElute kit, Qiagen, Valencia, CA). 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 (ATCC, Manassas, VA) were treated with 5 μ g/ml TLR5 neutralizing antibody (PAB-hTLR5, Invivogen, San Diego, CA) or 5 μ g/ml purified rat IgG isotype control (eBioscience, San Diego, CA) (12), and TLR5 ligand flagellin (100 ng/ml). Cells were incubated for 24 hr, 37°C, and then cells were collected for TaqMan Real-time PCR using gene expression assays for IL-6 and IL-8 (Life Technologies). TLR5 siRNA was also used to confirm the effect of TLR5 down-regulation on the ability of airway epithelial cells to respond to flagellin. TLR5 and control siRNA (Qiagen, Valencia, CA) were transfected in BEAS-2B cells for 24 hr, then cells were exposed to flagellin (100 ng/ml) for 24 hr. 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 was used to assess for differences among groups. For genes that Kruskal-Wallis showed a significant difference ($p < 0.05$), non-parametric post-hoc pairwise comparisons were done using the mean rank comparison approach of Schaich and Hamerle (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 (GEO) site (<http://www.ncbi.nlm.nih.gov/geo>). Accession number is GSE30063.

Results

Study Population and Sampling of the Small Airway Epithelium

Small airway epithelium 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, $n=13$; and 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, smokers and smokers with COPD. Using the criteria of detection call of present (P call) in 50%, 6 of 10 TLRs (TLR 1, 2, 3, 4, 5 and 8) were expressed in the small airway epithelium (Figure 1A). Of the 6 TLR expressed, only TLR4 and 5 had any significant differences among the groups (Figure 1B, C). Expression of TLR4 in healthy smokers was down regulated compared to healthy nonsmokers ($p < 0.001$, fold-change 1.4, Figure 1B), consistent with previous studies that TLR4 expression is reduced in nasal epithelium of smokers compared to nonsmoker controls (26). However, there was no significant change of TLR4 expression level between healthy smokers and COPD smokers ($p > 0.05$; Figure 1B) and healthy nonsmokers and COPD smokers ($p > 0.05$; Figure 1B). Expression of TLR5 in both smokers and smokers with COPD showed down-regulation with 1.4-fold decrease ($p < 10^{-4}$) and 1.6-fold decrease ($p < 10^{-4}$), respectively, compared to TLR5 expression in healthy nonsmokers (Figure 1C). There was no significant difference between healthy smokers and COPD smokers ($p > 0.05$, Figure 1C).

Based on this 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 down-regulated in healthy smokers (1.9-fold, $p < 0.0001$) and in smokers with COPD (1.4-fold, $p < 0.04$; Figure 2A). In addition, massive parallel mRNA sequencing was carried out on a subgroup of 4 healthy nonsmokers and 6 healthy smokers, which were 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 to healthy nonsmokers ($p < 0.002$; Figure 2B, C).

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 antibody 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 (Figure 3). The immunofluorescence of TLR5 in healthy smokers (Figure 3E–H) and smokers with COPD (Figure 3I–L) was decreased compared to healthy nonsmokers (Figure 3A–D).

Human airway epithelial cells differentiated *in vitro* were used to further assess 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 (Figure 4A–E). Consistent with this, the expression pattern of ciliated cell-specific gene FOXJ1 showed up-regulation with differentiation (Figure 4F) and the basal cell-specific gene KRT14 was down-regulated with differentiation (Figure 4G). Consistent with the immunofluorescence expression pattern from the biopsies, TLR5 gene expression was up-regulated as the basal cells differentiated to a ciliated epithelium (Figure 4H).

Acute Exposure to CSE Decreases TLR5 Gene Expression in Differentiated Human Airway Epithelial Cells

Based on immunofluorescence staining, the TLR5 protein was expressed mainly on the apical side of the human airway epithelium. In order to assess whether cigarette extract could directly down-regulate TLR5 expression in human airway epithelial cells, fully

differentiated human airway epithelial cells cultured on air-liquid interface for 21 days were exposed to freshly made CSE for 48 hr. Three different CSE concentrations were used (1%, 2%, and 3%); none of these levels were toxic to the cells (not shown). Although TLR5 expression did not change significantly when treated with 1% and 2% CSE, TLR5 expression was significantly down-regulated when exposed to 3% CSE (fold-change 1.4, $p < 0.0001$, Figure 5C). There was a trend of decreased TLR4 expression in 3% CSE treatment group, but it was not statistically significant ($p > 0.05$, Figure 5B). As a control, the expression of TLR1, which did not have significant change *in vivo*, also did not change significantly *in vitro* (Figure 5A). The expression level for other control genes, including ciliated cell marker DNAI1, basal cell marker cytokeratin 5 (KRT5) and an internal control β -actin, were not significantly changed by CSE (Figure 5D–F).

***In Vitro* TLR5 Function**

In order 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 to basal cells differentiated on air-liquid interface to a ciliated epithelium ($p < 0.01$; Figure 6A). After flagellin activation for 24 hr, the human airway basal cells (TLR5 low-expressing cells) showed lower expression levels of down-stream genes of flagellin-TLR5 pathway, IL-6 and IL-8, compared to differentiated cells (Figure 6B, C).

A specific neutralizing polyclonal antibody of TLR5 was further used to assess the specificity of flagellin-TLR5 activation. Compared to 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, $p < 0.001$; Supplemental Figure 1A, B). The TLR5 inhibitor decreased the expression of both IL-6 (1.5 fold decrease, $p < 0.02$) and IL-8 (2.3 fold decrease, $p < 0.01$; Supplemental Figure 1A, B). TLR5 siRNA was also used to confirm the specificity of flagellin-TLR5 activation in human BEAS-2B airway epithelial cell line. After activation with flagellin for 24 hr, the cells transfected with control siRNA showed significant up-regulation of IL-6 (29-fold increase, $p < 0.0001$) and IL-8 expression (124-fold increase, $p < 0.0001$; Figure 7A, 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, $p < 0.01$; Figure 7A, B).

Discussion

The toll-like receptor family belongs to the pattern recognition receptors that 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- κ B signaling and the MAP kinase pathway to trigger the secretion of pro-inflammatory 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 demonstrates that 6 out of 10 TLRs are expressed in human small airway epithelium, with TLR5 mRNA expression significantly reduced in healthy smokers and smokers with COPD compared to healthy nonsmokers. Consistent with the mRNA data, immunofluorescence antibody 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 to healthy nonsmokers. Addition of the TLR5 specific activator flagellin to differentiated airway epithelial cells resulted in marked up-regulation of the TLR5 downstream genes, IL-6 and IL-8, compared to minimal IL-6 and IL-8 up-regulation in TLR5 low-expressing airway basal stem/progenitor cells. Finally, when TLR5 was blocked with a specific antibody, 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. Together, the data demonstrates 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 suggests smoking-related down-regulated expression of TLR5 in the airway epithelium may contribute to smoking induced suppression of airway innate defenses to flagellated bacteria (29–31).

Toll-like Receptors 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 non-immune 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 lipopolysaccharide (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 induce the expression of genes involved in innate immunity including pro-inflammatory cytokines, chemokines and other effector molecules (41–43). Decreased expression/function of the TLRs has significant consequences to host defenses. For example, (44) and 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).

Toll-like Receptors 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, up-regulation of cell surface adhesion molecules and increased expression of antimicrobial peptides (8,48). Some of these chemokines include tumor necrosis factor α (TNF- α), IL-6, IL-8 and macrophage inflammatory protein-3 α (MIP-3 α), which are essential for phagocytes 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 to nonsmokers and individuals with severe COPD have decreased TLR4 expression compared to those with less severe disease (26). *In vitro* cigarette smoke extract treatment of an airway epithelial cell line results in a dose-dependent down-regulation of TLR4 expressions (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 *P. aeruginosa* and plays an important role in the initiation of

the host in inflammatory reaction to clear the invading pathogen. There are some reports of *Pseudomonas aeruginosa* isolated from the sputum of COPD patients (54–58) and the acquisition of *Pseudomonas 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 yr mortality (54). In the present study, we found that the TLR5 expression in human small airway epithelium is down-regulated in healthy smokers and smokers with COPD compared to healthy nonsmokers. *In vitro* activation of airway epithelial cells with 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 hr, showing that cigarette smoke down-regulated 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 to recognize pathogens and activate innate immune responses, the smoking-induced down-regulation of TLR5 may contribute to smoking-related susceptibility to airway infection with flagellated bacteria in the healthy smokers and smokers with COPD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expression of the genes in the TLR family in human small airway epithelium. **A.** HG-U133 Plus 2.0 microarrays were used to analyze the gene expression of the TLR family at the mRNA level in the small airway epithelium of healthy nonsmokers. Using the criteria of detection call of present (P call) in 50%, 6 out of 10 TLRs (TLR 1, 2, 3, 4, 5 and 8) were expressed in the small airway epithelium of healthy nonsmokers. **B–C.** TLR4 and 5 expression pattern in the small airway epithelium of healthy nonsmokers, healthy smokers, and smokers with COPD; **B.** TLR4; **C.** TLR5.

Figure 2.

Validation of TLR5 expression in the human small airway epithelium. TaqMan RT-PCR and massive parallel mRNA sequencing were used to validate the TLR5 expression in human small airway epithelium. **A.** TaqMan RT-PCR assessment of small airway epithelium of 23 healthy nonsmokers, 43 healthy smokers and 24 smokers with COPD. Compared to nonsmokers, TLR5 was down-regulated in healthy smokers (1.9-fold, $p < 0.0001$) and in smokers with COPD (1.4-fold, $p < 0.04$). **B.** **C.** Massive parallel mRNA sequencing of the small airway epithelium of 4 healthy nonsmokers and 6 healthy smokers. TLR5 expression was 1.7-fold lower in healthy smokers compared to healthy nonsmokers ($p < 0.002$).

Figure 3.

Immunofluorescence TLR5 localization in human airway epithelium. Bronchial biopsies of healthy nonsmokers, healthy smokers and smokers with COPD from the large airway epithelium were stained with anti-TLR5 antibody. **A–D.** Healthy nonsmokers; **A.** IgG control; **B.** anti-TLR5; **C.** IgG control; and **D.** anti-TLR5. **E–H.** Healthy smokers; **E.** IgG control; **F.** anti-TLR5; **G.** IgG control; **H.** anti-TLR5. **I–L.** Smokers with COPD; **I.** IgG control; **J.** anti-TLR5; **K.** IgG control; and **L.** anti-TLR5.

Figure 4.

TLR5 expression during airway epithelial cell differentiation *in vitro*. Human airway epithelial cells were induced to differentiate *in vitro* on air-liquid interface. **A–E.** Ciliated cell-specific marker β -tubulin IV staining during *in vitro* differentiation; **A.** Day 0; **B.** Day 7; **C.** Day 14; **D.** Day 21; and **E.** Day 28. **F–H.** Gene expression during basal cell to ciliated epithelium differentiation; **F.** Ciliated cell-specific gene FOXJ1 expression; **G.** Basal cell-specific gene KRT14 expression; **H.** TLR5 expression.

Figure 5.

TLR5 gene expression in differentiated human airway epithelial cells by acute CSE exposure. Fully differentiated human airway epithelial cells cultured on air-liquid interface for 21 days were exposed to freshly made CSE for 48 hr. **A.** TLR1 expression; **B.** TLR4 expression; **C.** TLR5 expression; **D.** ciliated cell marker DNAI1 expression; **E.** basal cell marker KRT5 expression; **F.** β-actin expression.

Figure 6.

TLR5 expression level is related to its function in mediating flagellin-induced airway epithelium expression of inflammatory mediators. **A.** TLR5 expression levels in human airway epithelial basal cells and human airway differentiated cells assessed by TaqMan RT-PCR. **B.** IL-6 expression after human airway epithelial basal cells and human airway differentiated cells were exposed to flagellin. **C.** IL-8 expression after human airway epithelial basal cells and human airway differentiated cells were exposed to flagellin.

Figure 7.

Down-regulation 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 hours, then cells were exposed to flagellin for 24 hr. Expression of TLR5 downstream genes IL-6 and IL-8 was assessed by TaqMan RT-PCR. **A.** IL-6 expression. **B.** IL-8 expression. The cells transfected with control siRNA showed significantly up-regulation of IL-6 (29-fold increase, $p<0.0001$) and IL-8 expression (124-fold increase, $p<0.0001$; Figure 7A, 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, $p<0.01$; Figure 7A, B).

Table I**Demographics of the Study Population and Biologic Samples¹**

Parameter	Healthy nonsmokers	Healthy smokers	Smokers with COPD ²
n	60	73	36
Sex (male/female)	38/22	52/21	28/8
Age (yr)	41 ± 12	43 ± 7	51 ± 7
Race (B/W/O) ³	26/23/11	45/17/11	13/13/10
Smoking history (pack-yr)	-	26 ± 16	38 ± 24
Urine nicotine (ng/ml)	-	1327 ± 1627	1660 ± 1548
Urine cotinine (ng/ml)	-	1285 ± 1040	1469 ± 625
Blood carboxyhemoglobin (%)	0.4 ± 0.8	1.8 ± 1.8	2.8 ± 1.8
Pulmonary function parameters ⁴			
FVC	106 ± 13	109 ± 13	104 ± 21
FEV1	106 ± 14	107 ± 14	82 ± 22
FEV1/FVC	82 ± 6	80 ± 5	63 ± 7
TLC	99 ± 13	100 ± 12	105 ± 19
DLCO	98 ± 14	94 ± 11	76 ± 16
Gold stage (I/II/III) ²	-	-	21/13/2
Medication use			
β-agonist	-	-	6
Anticholinergic	-	-	2
Inhaled corticosteroid	-	-	3
Epithelial cells ⁵			
Number recovered ×10 ⁶	6.4 ± 2.9	7.5 ± 3.3	6.3 ± 2.7
% epithelial cells	99.1 ± 1.2	99.0 ± 1.3	98.4 ± 1.5
% inflammatory cells	0.9 ± 1.2	1.0 ± 1.3	1.6 ± 1.5
Differential cell count ⁶			
Ciliated (%)	72.0 ± 8.9	64.1 ± 12.0	60.3 ± 10.6
Secretory (%)	6.7 ± 3.6	8.4 ± 4.1	11.4 ± 5.6
Basal (%)	12.6 ± 6.6	14.5 ± 8.0	15.3 ± 8.5
Undifferentiated columnar (%)	7.8 ± 3.4	12.2 ± 6.8	11.4 ± 4.1

¹Data are presented as mean ± standard deviation.

²Smokers 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.

³B = black, W = white, O = other.

⁴Pulmonary function testing parameters are given as % of predicted value with the exception of FEV1/FVC, which is reported as % observed; FVC - forced vital capacity, FEV1 - forced expiratory volume in 1 sec, TLC - total lung capacity, DLCO - diffusing capacity. For individuals with COPD, FVC, FEV1, and FEV1/FVC are post-bronchodilator values.

⁵Small airway epithelium.

⁶As a % of small airway epithelium recovered.