Activation of Airway Epithelial Cells by Toll-Like Receptor Agonists

Quan Sha, Ai Q. Truong-Tran, James R. Plitt, Lisa A. Beck, and Robert P. Schleimer

Division of Allergy and Clinical Immunology, Department of Medicine, Johns Hopkins University School of Medicine, Asthma and Allergy Center, Baltimore, Maryland

Toll-like receptors (TLR) play an important role in pathogen recognition and innate immunity. We investigated the presence and function of TLRs in the BEAS-2B airway epithelial cell line and primary bronchial epithelial cells. Standard real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis and Taqman RT-PCR revealed that BEAS-2B cells express mRNA for TLR1–10. Several TLR ligands were tested for their ability to activate gene expression in BEAS-2B cells using limited microarray analyses focusing on genes of the chemokine and chemokine receptor family, cytokines, and signaling pathways. While the TLR3 ligand double-stranded RNA was the most effective epithelial activator, clear responses to flagellin, lipopolysaccharide, CpG, peptidoglycan, and zymosan were also observed. RT-PCR and/or enzyme-linked immunosorbent assay were used to confirm results obtained with microarrays for five of the induced genes: interleukin-8, serum amyloid A, TLR3, macrophage inflammatory protein-3α, and granulocyte-macrophage colony-stimulating factor. Stimulation of epithelial cells with double-stranded RNA induced levels of interleukin-8 exceeding 20 ng/ml and levels of serum amyloid A exceeding 80 ng/ml. Double-stranded RNA, lipopolysaccharide, zymosan A, and flagellin also induced expression of macrophage inflammatory protein-3α and granulocyte-macrophage colony-stimulating factor, which may facilitate immature dendritic cell migration and maturation. These results suggest that airway epithelial cells express several TLRs and that they are functionally active. Epithelial expression of TLRs may be of importance in inflammation and immunity in the airways in response to inhaled pathogens.

The respiratory epithelium is an important interface with the environment, and represents a dynamic system for innate host defense. In addition to providing a barrier to the entry of pathogens, epithelial cells lining the airway have been shown to respond to the presence of microorganisms by producing natural antimicrobial factors and mounting an inflammatory response (1).

Toll-like receptors (TLRs) have been recently recognized to play a major role in pathogen recognition and innate immunity (2, 3). To date, ten TLR family members (TLR1–10) have been identified in the human genome, and different TLRs appear to play crucial roles in the activation of the immune response to distinct pathogen-associated molecular patterns. TLR2 and TLR4 recognize endotoxin, lipoproteins, and some extracellular matrix molecules (3). TLR3 recognizes double-stranded RNA (dsRNA) (4), TLR5 recognizes bacterial flagellin (5). The synthetic imidazoquinolines activate immune cells via TLR7 (6), and TLR9 recognizes CpG-containing DNA (7). The TLR family provides the possibility for a rapid response after exposure to potential pathogens. Signaling through TLR activates the expression of a host of cytokines, chemokines, hematopoietic factors, acute phase proteins, and antimicrobial factors.

Localization of TLR has largely been associated with immune and inflammatory cells (1–4). The purpose of the present studies is to better characterize the expression and function of TLR on human airway epithelial cells. Because airway epithelium comes in contact with numerous potential pathogens, expression of TLR would be of relevance to immunity in the airways.

Respiratory infections are thought to be a leading cause of exacerbation of several airways diseases including asthma, chronic rhinosinusitis, and chronic obstructive pulmonary disease (8–10). The epithelium is the primary target for respiratory viruses, and thus these cells are likely to play a pivotal role in viral-induced lung inflammation. In this regard it is interesting to note that *in vitro* studies have demonstrated that viral infection of lung epithelial cells leads to the production of a variety of proinflammatory cytokines and chemokines (11–13).

We demonstrate here that epithelial cells express mRNA for all TLR and that several known TLR ligands activate epithelial cells to express chemokines, cytokines, and host defense molecules, including acute phase proteins and complement proteins. Among the induced genes were macrophage inflammatory protein (MIP)-3α and granulocyte macrophage–colony-stimulating factor (GM-CSF), which would be expected to recruit and activate immature dendritic cells that might be important in early triggering of adaptive immune responses. Although several different TLR ligands were able to stimulate epithelial cells, the most effective was Poly (I)•Poly (C), a synthetic dsRNA copolymer of inosinic and cytidilic acids, which induces cell activation in part via TLR3 (4) and is thought to mimic the effects of dsRNA intermediates produced during viral infection of cells (14). Expression of functional Toll-like receptors by airway epithelial cells has considerable implications with regard to innate immune responses and disease pathogenesis.

Materials and Methods

The following reagents were purchased from the indicated sources: Dulbecco's modified Eagle's medium/F-12, heat-inactivated fetal calf serum, L-glutamine, penicillin/streptomycin (Invitrogen, Grand Island, NY), bronchial epithelial basal medium (Clonetics, San Diego, CA), collagen (Vitrogen; Collagen Biomaterials, Palo Alto, CA), double-stranded Poly (I)•Poly (C) (dsRNA; Amersham, Piscataway, NJ), zymosan A from *Saccharomyces cerevisiae*, lipopolysaccharide (LPS) from *Escherichia coli*, serotype 0111:B4 (Sigma, St. Louis, MO), peptidoglycan (PGN) from Staphylococcus aureus (Fluka), flagellin isolated from *Salmonella typhimurium* strain 14,028 (Apotech), CpG (2,216), a synthetic oligodeoxynucleotide that contains CpG motifs mimicking bacterial DNA (5′-ggGGGA**CG**AT**CG**TCggggG-3′; the small letters represent phosphorothioate linkages, the capital letters represent phosphodiester

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Address correspondence to: Dr. Robert P. Schleimer, Division of Clinical Immunology, Department of Medicine, Asthma and Allergy Center, Baltimore, MD 21224. E-mail address: rschleim@jhmi.edu

Abbreviations: third component of complement, C3; threshold cycle, Ct; double-stranded RNA, dsRNA; glyceraldehyde phosphate dehydrogenase, GAPDH; granulocyte macrophage–colony-stimulating factor, GM-CSF; interleukin, IL; lipopolysaccharide, LPS; macrophage inflammatory protein, MIP; nuclear factor-κB, NF-κB; primary bronchial epithelial cells, PBEC; peptidoglycan, PGN; real-time polymerase chain reaction, RT-PCR; serum amyloid A, SAA; Toll-like receptor, TLR.

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linkages 3′ of the base, and the bold letters represent CpG dinucleotides. (Sigma Genosys, Woodlands, TX).

Cell Culture

The BEAS-2B cell line, derived from human bronchial epithelium transformed by an adenovirus 12-SV40 hybrid virus (15) was a gift from Dr. Curtis Harris. The BEAS-2B cells were grown as monolayers in Dulbecco's modified Eagle's medium/F-12 supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2 in humidified air. Cells that had reached ~ 80–90% confluence were challenged for 18 h with dsRNA (25 μg/ml), LPS (1 μg/ml), PGN (10 μg/ml), CpG DNA (3 μg/ml), zymosan A (100 μg/ml), or flagellin (10 ng/ml).

Selected results were confirmed using human primary bronchial epithelial cells (PBEC). PBEC were obtained as described (16). Cells were cultured in bronchial epithelial basal medium containing bovine pituitary extract, hydrocortisone, human recombinant epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, bovine serum albumin-fatty acid free, gentamicin, and amphotericin-B at 37°C with 5% CO_2. PBEC were plated in 6-well culture plates coated with collagen and used only at their first passage.

RNA Extraction, Reverse Transcriptase–Polymerase Chain Reaction, and Real-Time Polymerase Chain Reaction

Total RNA was isolated using the RNeasy Mini kit (QIAGEN, Valencia, CA). RNA (0.5 μg) was reverse transcribed using a $p(dT)_{15}$ primer (Roche, Indianapolis, IN) and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems) using conditions provided by the manufacturer. One microliter of each cDNA sample, corresponding to 25 ng total RNA, was used for standard polymerase chain reaction (PCR) and real-time PCR. The primers and probes for TLR 1–10, MIP-3α, GM-CSF, and glyceraldehydes phosphate dehydrogenase (GAPDH) are listed in Table 1. Standard PCR was performed in a total volume of 25 μl with the following components: 1 × PCR buffer (Applied Biosystems), 0.25 mM dNTPs (Invitrogen), 3 mM $MgCl_2$, 0.6 μM of each primer, and 1.25 U of Ampli Taq Gold (Applied Biosystems). PCR conditions were 94°C for 15 s, 65°C for 60 s for 40 cycles. Samples were visualized using agarose gel electrophoresis.

Real-time PCR was performed in an ABI PRISM 7,700 Sequence Detection System thermal cycler (PE Applied Biosystems, Foster City, CA) to quantify TLR1–10, MIP-3α, GM-CSF, and GAPDH mRNA. Real-time PCR was performed in a total volume of 25 μl with the following components: 1 × TaqMan PCR buffer, 5.5 mM $MgCl_2$, 0.25 mM of dATP, dCTP and dGTP, 0.5 mM dUTP, 0.25 U AmpErase UNG, 0.75 U Ampli Taq Gold, 0.4 μM of each primer, and 0.2 μM of the Taqman probe. Cycle parameters were 50°C for 2 min to activate UNG, 95°C for 10 min to activate *Taq*, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fold change of test gene mRNA was expressed as $2^{-\Delta\Delta Ct}$ (ΔCt = the difference in threshold cycles for the test gene and GAPDH, ΔΔCt = the difference of ΔCt between stimulated and non-stimulated control).

Gene Expression Arrays

Human nuclear factor (NF)-κB signaling pathway GEArray Q series and Human chemokine and receptor GEArray Q series (SuperArray, Bethesda, MD) microarrays were used to evaluate the gene expression profile of BEAS-2B cells. An aliquot of 5 μg total RNA was used as the template to produce ^{32}P labeled cDNA probes. Microarray analysis was performed as described by the manufacturer. The image of the array was recorded using a storage phosphor screen (Molecular Dynamics, Amersham Pharmacia). The digital image was converted to digital data using ImageQuant software. The raw data were saved for further analysis using the GEArrayAnalyzer software. The relative amount of a given gene transcript was estimated by comparing its signal intensity with the signal derived from β-actin.

Enzyme-Linked Immunosorbent Assay

Supernatants from untreated and stimulated cell cultures were collected and stored at −80°C for enzyme-linked immunosorbent assay (ELISA) of interleukin (IL)-8, MIP-3α, GM-CSF (R&D Systems, Minneapolis, MN), and serum amyloid A (SAA) (PHASE RANGE; Tridelta, Morris Plains, NJ) according to the manufacturer's instructions. The detection limits for assay of IL-8, MIP-3α, GM-CSF, and SAA are 10 pg/ml, 0.47 pg/ml, 3 pg/ml, and 0.3 ng/ml, respectively.

Flow Cytometry

BEAS-2B cells were grown to 80% confluence in 6-well culture plates. Cells were washed three times with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution and removed from culture plates by treatment with Versene (Invitrogen Corporation, Carlsbad, CA) for 10 min. For each analysis, 10^6 cells were incubated in 30 μl of phosphate-buffered saline/0.2% bovine serum albumin containing 30 μg/ml monoclonal mouse IgG_1 phycoerythrin (PE) anti-human Toll-like receptor 3 (TLR3.7 clone; eBioscience, San Diego, CA) antibody and 4 mg/ml of human IgG (Sigma Chemicals), to reduce nonspecific binding, at 4°C for 30 min. Cells were washed and resuspended in phosphate-buffered saline/0.2% bovine serum albumin buffer and immediately analyzed with a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software. Fluorescence was determined on all cells for each sample after debris, dead cells, and aggregates were excluded by forward angle and side scatter gating. Mean fluorescence intensity was compared with control staining using an irrelevant isotype-matched mouse monoclonal antibody. For each sample, at least 20,000 events were collected, and histograms were generated. Data are expressed as means ± SEM.

TABLE 1. Oligonucleotide primers and probes used for RT-PCR and real-time PCR

cDNA	Forward	Reverse	Probe
TLR1	CAGTGTCTGGTACACGCATGGT	TTTCAAAAACCGTGTCTGTTAAGAGA	TGCAACATCATGACCAAAGACAAAGAACCT
TLR2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACCT	CCATCCCATGTGCGTGG
TLR3	CCTGGTTTGTTAATTGGATTAACGA	TGAGGTGGAGTGTTGCAAAGG	CATACCAACATCCCTGAGCT
TLR4	GCAGTGAGGATGATGCCAGGAT	GCCATGGCTGGGATCAGAGT	TGTCTGCCTCGCGCC
TLR5	TGCCTTGAAGCCTTCAGTTATG	CCAACCACCACCATGATGAG	CCAGGGCAGGTGCTTATCTGACCTTAACA
TLR6	GAAGAAGAACAACCCTTTAGGATAGC	AGGCAAACAAAATGGAAGCTT	TGCAACATCATGACCAAAGACAAAGAACCT
TLR7	TTACCTGGATGGAAACCAGCTAC	TCAAGGCTGAGAAGCTGTAAGCTAG	AGATACCGCAGGGCCTCCCGC
TLR8	AGCGGATCTGTAAGAGCTCCATC	CCGTGAATCATTTTCAGTCAAGAC	CCTGACAACCCGAAGGCAGAAGGC
TLR9	GCAGTCAATGGCTCCCAGTTC	GCGGTAGCTCCGTGAATGAGTG	CCCGCAATAAGCTG
TLR10	TTATGACAGCAGAGGGTGATGC	CTGGAGTTGAAAAAGGAGGTTATAGG	TTGACCCCAGCCACAACGACACTG
MIP-3α	TCCTGGCTGCTTTGATGTCA	GAAGAATACGGTCTGTGTATCCAAGAC	TGCTGCTACTCCACC
GM-CSF	AGGGCCCCTTGACCATGA	CAAAGGTGATAGTCTGGGTTGCA	CAGCACTGCCCTCCAACCCCG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC

The software package Primer Express (Applied Biosystems) was used to identify appropriate primer sets. The 5′ end of the probes of TLR1–10, MIP-3α, and GM-CSF were labeled with 6-carboxyfluorescein (FAM) as a reporter, the 3′ end of the probes of TLR2, 3, 4, 9 were labeled with the minor groove binder/nonfluorescent quencher (MGBNFQ) and the 3′ end of the probes of TLR1, 5, 6, 7, 8, 10, MIP-3α, and GM-CSF were labeled with 6-carboxytetramethylrhodamine (TAMRA) as the quenching dye. The GAPDH probe was labeled on the 5′ end with 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE) and on the 3′ end with TAMRA as the quenching dye. All sequences are presented in the 5′→3′ direction.

Definition of abbreviations: GAPDH, glyceraldehydes phosphate dehydrogenase; GM-CSF, granulocyte macrophage–colony-stimulating factor; MIP, macrophage inflammatory protein; TLR, Toll-like receptor.

Statistical Analysis

Data are expressed as mean values ± SEM. Statistical significance of differences was determined with a Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

We first set out to determine the expression of mRNA for Toll-like receptors in BEAS-2B cells and PBEC and perform microarray analysis of epithelial gene expression in response to TLR agonists. Assessment of TLR mRNA expression in BEAS-2B cells and PBEC by RT-PCR is shown in Figure 1A. All TLR with the exception of TLR8 were found to be expressed in BEAS-2B cells, and all ten TLRs were found to be expressed in PBEC. Data in Figure 1B show the levels of expression of TLR mRNA in BEAS-2B cells determined using Taqman real-time PCR. It is clear from data in the figure that levels of TLR8 and TLR10 mRNA were present but quite low. Based upon the threshold cycle (Ct) values, the most highly expressed TLRs were TLR3, TLR2, TLR4, and TLR5, whereas the lowest expression was detected for TLR10, TLR8, and TLR7 (Figure 1B).

We next screened the TLR ligands LPS, PGN, zymosan A, CpG DNA, and dsRNA to study their effects on epithelial cells. The putative receptors of these stimuli are indicated in Figure 1B. BEAS-2B cells were treated with the agonists for 18 h, total RNA was isolated and gene expression was monitored by microarray. Although several stimuli induced genes detected by the microarrays (data not shown), by far the most effective stimulus among those tested was dsRNA. Inspection of representative microarrays shown in Figure 2 reveals that a number of genes were upregulated by dsRNA. A few of the many induced genes are labeled in the figure. Data shown in this figure are from the NF-κB signaling pathway GEArray, which includes numerous cytokines, chemokines, and signaling molecules. We also used the chemokine and receptor array and found that dsRNA was the most effective among the TLR agonists. Data in Table 2 present genes that were statistically significantly induced by dsRNA by 2 fold or more in cells stimulated with dsRNA. These genes include cytokines, chemokines, acute phase proteins, and signaling factors.

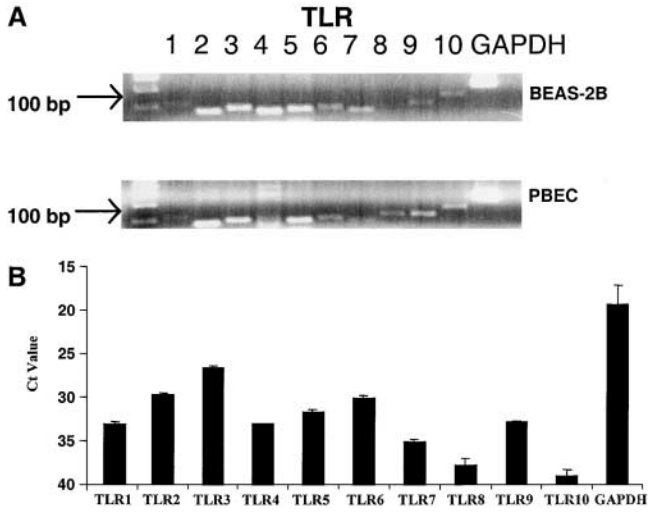

Figure 1. RT-PCR and quantitative real-time PCR analysis of the expression of mRNA for TLRs in BEAS-2B cells and PBEC. (*A*) Total cellular RNA was extracted and subjected to RT-PCR using specific primers to human TLR 1–10 and GAPDH. The amplified products were resolved on a 3% agarose gel and the bands were visualized by ethidium bromide staining (image shown is representative of $n = 3$). (*B*) Taqman real-time PCR assessment of TLR expression in BEAS-2B cells. Values shown are mean ± SEM Ct from three separate experiments, calculated as described in Materials and Methods. The scale is inverted, so that the *higher histogram bars* represent higher levels of mRNA. TLR ligands used to stimulate epithelial cells are indicated in the figure.

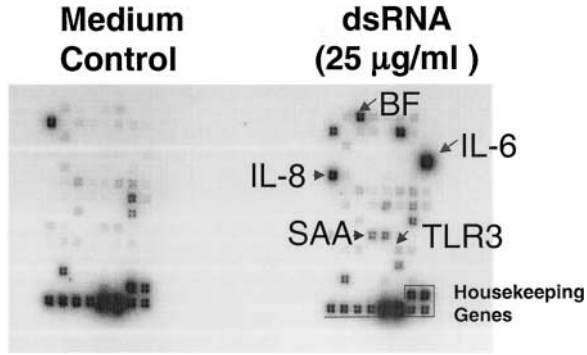

Figure 2. Assessment of gene expression after stimulation of BEAS-2B cells with dsRNA. BEAS-2B cells were left untreated or stimulated with dsRNA (25 μg/ml) for 18 h. A number of genes were upregulated by dsRNA (*right*); only a few of them are labeled. Data shown in this image are from the NF-κB signaling pathway GEArray and represent three separate experiments.

Interestingly, among the genes found to be modestly induced (i.e., 2-fold or less but $P < 0.05$ for $n = 3$) by dsRNA in the array studies were several of TLR genes themselves, including TLR3, the putative receptor for dsRNA (Figure 3A). We therefore attempted to confirm expression and induction of TLR3 mRNA by quantitative real-time PCR. Data in Figure 3B confirm the expression and induction of TLR3 mRNA by dsRNA in BEAS-2B (*open bars*) and PBEC (*solid bars*) using real-time PCR. Expression of TLR3 mRNA was increased 13- and 14-fold ($P < 0.05$), respectively, in BEAS-2B and PBEC by dsRNA. LPS induced TLR3 mRNA expression by 2-fold ($P < 0.05$) in BEAS-2B, but had no effect in PBEC. CpG, PGN, and zymosan had no effect on levels of mRNA for TLR3 in either BEAS-2B or PBEC.

TABLE 2. Selected genes induced by dsRNA (25 μg/ml, 18 h) in BEAS-2B cells

Gene	Fold Induction
IL-6	7.9*†
IL-8	6.9‡
Properdin B-factor	5.4‡
ICAM-1	4.6†
IL-2	3.3†
Serum amyloid A	3.3†
MAP/ERK kinase kinase 1	3.2†
IKKE	3.2†
Complement C3	3.1†
I-TAC	2.8†
GM-CSF	2.7†
RANTES	2.6†
TNF-α IP1	2.6†
IL-12A	2.5‡
MyD88	2.0†

Definition of abbreviations: GM-CSF, granulocyte macrophage–colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MAP/ERK, mitogen-activated protein/extracellular signal–regulated protein kinase; RANTES, regulated on activation, normal T cells expressed and secreted; TNF-α, tumor necrosis factor-α.

* Mean of three separate experiments.

† $P < 0.05$.

‡ $P < 0.01$.

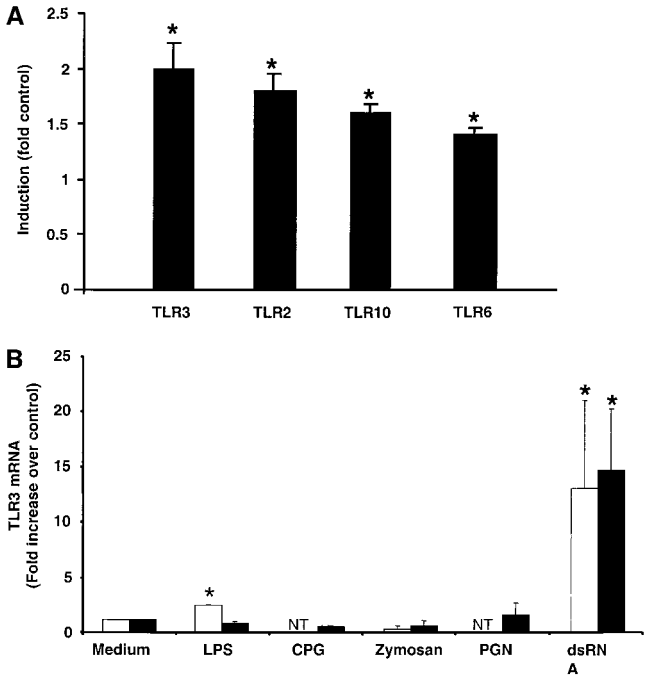

Figure 3. (*A*) Analysis of induction of mRNA for TLR genes by dsRNA using the NF-κB signaling pathway GEArray. The data were normalized to the housekeeping gene β-actin and are expressed as the mean ± SD ($n = 3$) fold of control. $^*P < 0.05$, significantly different from unstimulated cells. (*B*) Confirmation of induction of TLR3 mRNA by dsRNA in BEAS-2B (*open bars*) and PBEC (*closed bars*) using real-time PCR. Cells were treated with LPS (1 μg/ml), zymosan A (100 μg/ml), or dsRNA (25 μg/ml) for 18 h. In addition, PBEC were also treated with CpG (3 μg/ml) and PGN (10 μg/ml). Total cellular RNA was isolated and reverse transcribed to cDNA and the cDNA were used for real-time PCR as described in MATERIALS AND METHODS ($n = 3$–8). $^*P < 0.05$, compared with unstimulated cells.

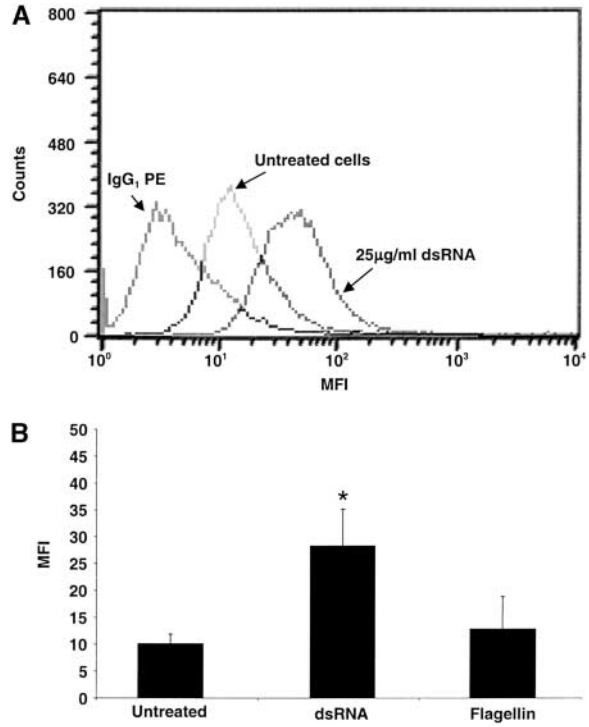

Figure 4. (*A*) Flow cytometric analysis of TLR3 expression on epithelial cells. PBE cells were treated with 25 μg/ml dsRNA for 18 h and then stained with PE conjugated anti-TLR3 mAb (TLR3.7). *Histograms* display fluorescence intensity of cells labeled with human IgG isotype-matched control Ab (*far left*), untreated cells labeled with PE anti-TLR3 mAb (*middle*) and dsRNA-treated cells labeled with TLR3 antibody (*far right*). (*B*) Mean fluorescence intensity (MFI) data from separate experiments show TLR3 protein levels were increased by 2.8-fold in BEAS-2B cells stimulated with dsRNA ($^*P < 0.05$). $n = 4$, $^*P < 0.05$ compared with unstimulated cells.

To confirm the mRNA results, we assessed the level of TLR3 in resting and dsRNA (25 μg/ml, 18 h) stimulated cells using flow cytometry. Results in Figure 4 show clear basal expression of TLR3 and induction in dsRNA-stimulated BEAS-2B cells (2.8-fold, $P < 0.05$, $n = 4$). Stimulation of BEAS-2B with 10 ng/ml of flagellin did not produce a significant effect on expression of TLR3 (Figure 4B).

To confirm the microarray results, we selected a few induced genes for ELISA assay in BEAS-2B cell culture supernatants. Data in Figure 5A show that dsRNA dramatically increased IL-8 secretion, producing levels of IL-8 in excess of 20 ng/ml ($P < 0.05$, $n = 3$). LPS and zymosan A were considerably weaker stimuli which induced levels of IL-8 of 1.6 and 0.6 ng/ml, respectively ($P > 0.05$, $n = 3$). CpG DNA failed to induce IL-8 production (data not shown). Although IL-2 and IL-12 mRNA were found to be induced in the microarrays (Table 2), none of the stimuli tested induce either IL-2 or IL-12 protein as tested by ELISA (data not shown). Another gene induced by dsRNA was the acute phase protein SAA (Table 2). Recent studies suggest that SAA plays a role in atherosclerosis and rheumatoid arthritis (17, 18). This acute phase protein induces leukocyte chemotaxis, probably via a newly described receptor, formyl peptide receptor-like 1 (19). ELISA experiments demonstrated that dsRNA increased levels of SAA from ~ 5 ng/ml to ~ 80 ng/ml, whereas the other stimuli failed to induce SAA production (Figure 5B).

In the gene array studies, dsRNA upregulated GM-CSF and MIP-3α by 2.7- and 1.6-fold, respectively. Because these genes are of theoretical importance in the recruitment and activation of immature dendritic cells, we confirmed expression of mRNA for these two genes using real-time quantitative PCR and measured the protein in cell culture supernatant by ELISA for BEAS-2B. Data in Figure 6A show that LPS (1 μg/ml), zymosan (100 μg/ml), dsRNA (25 μg/ml), and flagellin (10 ng/ml) all induced the expression of GM-CSF mRNA, whereas neither CpG or PGN induced GM-CSF mRNA. A similar pattern of response was observed when we analyzed MIP-3α (Figure 6B). Namely, MIP-3α mRNA was induced by LPS, dsRNA, zymosan, and flagellin, but not by PGN or CpG (Figure 6B). Double-stranded RNA, LPS, and flagellin significantly induced secretion of GM-CSF into the cell culture supernatant (Figure 6C). Among the TLR agonists, only dsRNA induced detectable MIP-3α in cell culture supernatant (Figure 6D).

The observations in BEAS-2B cells were confirmed in PBEC. Data in Figure 7A show that GM-CSF mRNA was induced by LPS, zymosan, PGN, and dsRNA, though the response was only statistically significant ($P < 0.05$) for PGN and dsRNA. Induction of MIP-3α mRNA was also observed in cells stimulated by LPS, PGN, zymosan, and dsRNA (for dsRNA only, $P < 0.05$), whereas CpG had no effect (Figure 7B).

ELISA assays indicated that GM-CSF and MIP-3α protein were produced by PBEC spontaneously (Figures 7C and 7D). GM-CSF was increased by all stimuli tested; however, only dsRNA and zymosan produced statistically significant responses ($P < 0.05$). Similarly, high levels of MIP-3α were observed after

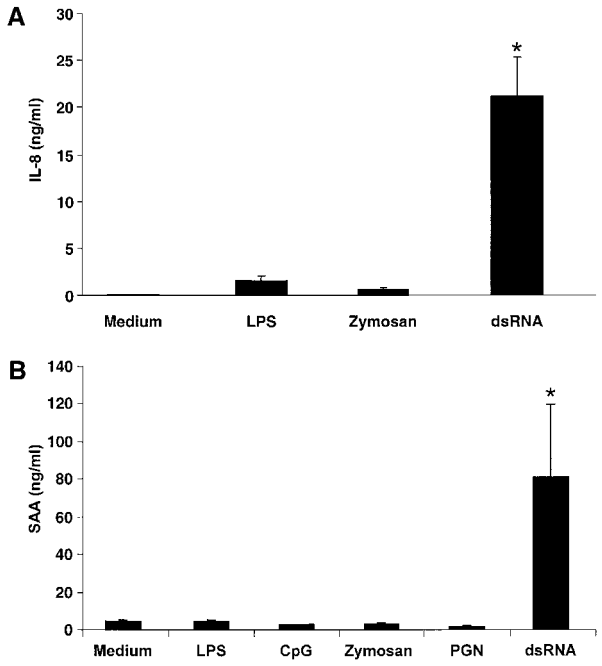

Figure 5. Assessment of the induction of IL-8 and SAA by TLR agonists in BEAS-2B cell supernatant. BEAS-2B were stimulated with dsRNA (25 μg/ml), LPS (1 μg/ml), PGN (10 μg/ml), CpG DNA (3 μg/ml), or zymosan A (100 μg/ml) for 18 h, the supernatants were collected and were tested in an ELISA specific for IL-8 (*A*) or SAA (*B*). n = 3–10, $^*P < 0.05$ compared with unstimulated cells.

stimulation with several TLR agonists; dsRNA was the most effective agonist tested (19.2-fold, $P < 0.005$), followed by PGN (6.7-fold, $P < 0.05$) and zymosan (2.9-fold, $P < 0.05$).

Discussion

The lung is the target of a substantial number of infectious agents that produce agonists for TLRs, including gram-negative and -positive bacteria, mycobacteria, fungi, viruses, and numerous helminths. Despite this fact, relatively little is known about the expression of Toll-like receptors on bronchial epithelial cells. Cario and coworkers (20) demonstrated the presence of TLR2, TLR3, TLR4, and TLR5 on intestinal epithelial cells, and several laboratories have demonstrated that epithelial cells from various sources can respond to bacterial endotoxins (1, 21, 22).

In the present study, we have demonstrated that mRNA for all known TLRs were expressed in the BEAS-2B airway epithelial cell line as well as primary bronchial epithelial cells. The TLR that were most highly expressed were TLR2, TLR3, TLR5, and TLR6. Among the known TLR agonists, LPS (agonist for TLR4), PGN and zymosan A (agonists for TLR2), one of the CpG DNA (oligodeoxynucleotide 2216, agonist for TLR9), and dsRNA (agonist for TLR3) were tested for the ability to activate epithelial cells. Our gene array data showed that dsRNA was the most effective of these stimuli. We did observe epithelial cell activation with agonists for TLR2 (zymosan A and PGN), TLR4 (LPS), and TLR5 (flagellin), suggesting that epithelial cells express functional TLR2, TLR3, TLR4, and TLR5. Interestingly, there was some agreement between responses to agonists and relative TLR expression based on Taqman real-time PCR analysis, because TLR3 had the lowest Ct (highest relative mRNA), followed by TLR2 and then TLR5. Conversely, TLR9 was one of the least highly expressed TLRs, and CpG had no detectable ability to activate the epithelial cells. Although levels of TLR4 were low, the LPS preparation used was able to activate epithelial cells. Double-stranded RNA induced mRNA for a number of genes including chemokines, cytokines, signaling factors, acute phase proteins, and TLRs including TLR3 in both BEAS-2B and PBE cells. The induction of TLR3 levels by dsRNA was confirmed at the protein level by flow cytometric analysis of stimulated cells. TLR3 is an interesting Toll-like receptor, and TLR3 mRNA has been previously shown to be expressed in mouse lung (4). Monocytes/macrophages express mRNA for most TLRs except TLR3. TLR3 is expressed primarily in mature dentritic cells (23) and NKT cells (24). TLR3 has a unique structure among the TLRs in that it has five exons and the protein is encoded by exons 2 through 5. This is in contrast to all of the other TLRs, which are encoded by only one or two exons (3). A reduced response to the dsRNA analog Poly (I)•Poly (C) was observed in TLR3-deficient mice (4). Epithelial cell expression of functional TLR3 is of interest, because airway epithelial cells are the target of infection by several viruses. TLRs can influence the outcome of viral infections, and TLR3 is presumably involved in immunity to respiratory viruses that produce dsRNA. A recent study showed that activation of antiviral response in primary mouse macrophages is stronger and more prolonged with TLR3 stimulation as compared with stimulation via TLR4 (25). Thus, TLR3 may allow epithelial cells to respond

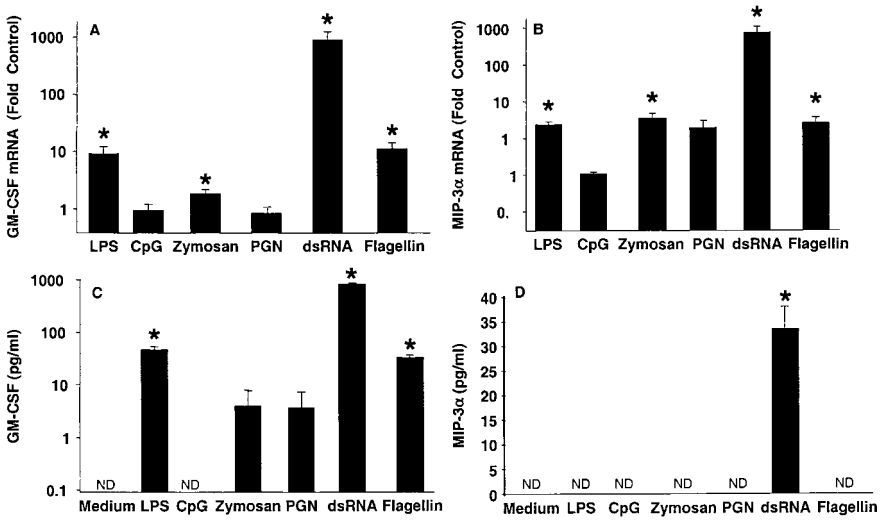

Figure 6. (*A*, *B*) Assessment of mRNA for GM-CSF (*A*) and MIP-3α (*B*) by real-time PCR. BEAS-2 B cells were treated with LPS, CpG DNA, zymosan, PGN, dsRNA, or flagellin for 18 h, and total RNA were isolated and subjected to RT and quantitative real-time PCR by using specific primers and probes for GM-CSF and MIP-3α. (*C*, *D*) Assessment of levels of GM-CSF protein (*C*) or MIP-3α protein (*D*) in cell-free supernatant. ND; not detected. n = 5–6, $^*P < 0.05$ compared with unstimulated cells.

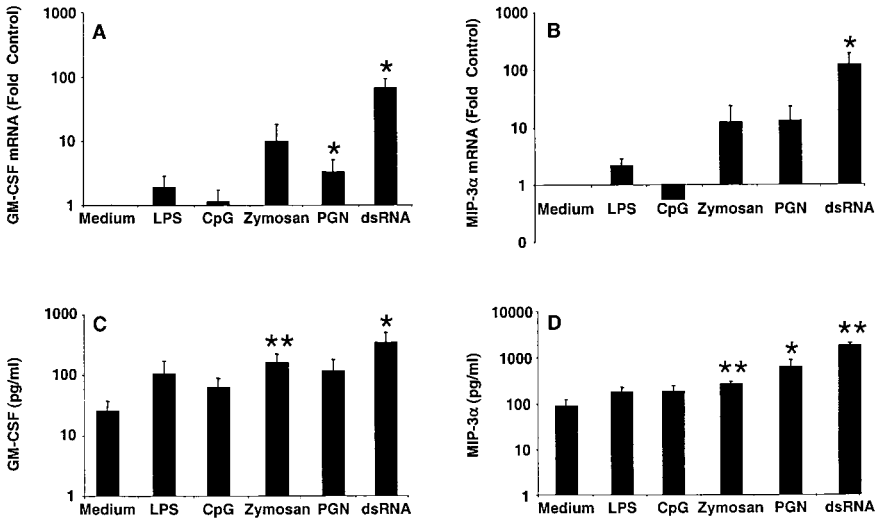

Figure 7. (*A*, *B*) Assessment of mRNA for GM-CSF (*A*) and MIP-3α (*B*) by real-time PCR in PBEC. Primary cells were treated with LPS, CpG DNA, zymosan, PGN, and dsRNA for 18 h, and total RNA were isolated and subjected to RT and quantitative real-time PCR by using specific primers and probes for GM-CSF and MIP-3α. (*C*, *D*) Assessment of levels of GM-CSF protein (*C*) or MIP-3α protein (*D*) in cell-free supernatants. $n = 5$–6, $^*P < 0.05$, $^{**}P < 0.005$ compared with unstimulated cells.

to pathogenic airway viruses such as rhinovirus and respiratory syncytial virus.

In our study, we found that dsRNA increased expression of its putative receptor TLR3 and strongly induced many proinflammatory cytokines and chemokines, as well as complement and acute phase proteins, all of which may exert some antiviral effects in airway epithelial cells. We have not determined whether dsRNA is activating cells through expressed receptors or an intracellular receptor or by internalization. TLR3 has been shown to be a major receptor for dsRNA (4) and can exist in both intracellular and extracellular forms (26). Based on our flow cytometric evaluations TLR3 is expressed, at least in part, on the plasma membrane of epithelial cells. Future studies will be required to determine the relevant localization of TLR3. Whether TLR3 is the main receptor responsible for the actions of dsRNA in our studies (as opposed to DNA PKR or other receptors) or via another mechanism is yet to be determined.

dsRNA induced more than 20 ng/ml IL-8 in the BEAS-2B cell culture supernatant, whereas the other TLR stimuli tested were considerably weaker. This finding confirms the IL-8 gene upregulation in the gene array analysis and has implications in airway innate immune responses to pathogens that may generate dsRNA. Infection of the upper airway with rhinovirus is known to induce a profound neutrophil response, as may be expected if IL-8 is produced (27). We were surprised that among the genes induced by dsRNA were several acute phase proteins and complement proteins, including SAA, the third component of complement (C3) and properdin factor B. In recent studies, we have detected mRNA for several proteins of the alternative complement pathway, including factors B, H, I, and C3 in human sinonasal surgical samples (VanderMeer and colleagues, unpublished observations). The present results suggest that epithelial cells may rapidly generate complement proteins for purposes of host defense when exposed to pathogens that express agonists for TLRs. The liver is generally regarded as the major source of complement proteins and acute phase proteins such as SAA. We have used ELISA to comfirm that dsRNA indeed increased the secretion of SAA in BEAS-2B cells. SAA induces chemotaxis of polymorphonuclear cells (PMNs), monocytes, and T lymphocytes, and causes the release of tumor necrosis factor-α, IL-1β, and IL-8 from PMNs (28). In both clinical and experimental studies, SAA has also been reported to be a very early marker of inflammation and tissue injury in a variety of conditions. In general, changes in SAA mirror those of C-reactive protein but in some circumstances may be greater (29). The expression of SAA in histologically normal human tissues suggests that it may play a local role, either by serving as a first line of defense or in maintaining the function of human tissues. The low affinity receptor for f-met-leu-phe, formyl peptide receptor-like 1, which is also a receptor for lipoxin A4, has recently been shown to act as a receptor for SAA (19). Although the biologic importance of these findings awaits further investigation, they raise the possibility that SAA may be locally produced and present in the airways. One of the functions of SAA as a first line of defense in human tissues may be the induction of adhesion, migration, and tissue infiltration of monocytes, neutrophils, lymphocytes, and mast cells (30, 31), thus helping to recruit them to inflamed areas. Local expression of SAA by epithelial cells might serve to protect the tissues from invading microorganisms.

We also found that TLR ligand stimulation led to expression of mRNA and protein for GM-CSF and the CC chemokine, MIP-3α in both BEAS-2B and PBEC. MIP-3α mRNA is mainly expressed in lung, appendix, liver, and some lymphoid organs (32, 33) and by many cell types both of hematopoietic and nonhematopoietic origins (e.g., fibroblasts, keratinocytes, endothelial cells) (32, 33). DCs reside in close proximity to airway epithelial cells. Immature DCs derived from CD34+ hematopoietic progenitor cells migrate most vigorously in response to MIP-3α, but also to MIP-1α and RANTES. MIP-3α/CCL20 is a unique functional ligand for the recruitment of a distinct population of CCR6-expressing immature DCs to the airway for subsequent antigen presentation (34). Reibman and coworkers recently found that when human bronchial epithelial cells were stimulated with the cytokines tumor necrosis factor-α, IL-1β, IL-4, and IL-13, MIP-3α was induced (34). Levels of mRNA and protein for GM-CSF correlated among the various TLR agonists used. Interestingly, there was an apparent dissociation between expression of mRNA and expression of protein in the case of MIP-3α. Although several agonists induced mRNA (dsRNA, LPS, zymosan, and flagellin), only dsRNA induced significant quantities of MIP-3α protein. This may reflect differences in production levels and assay sensitivities for the ELISAs used to detect GM-CSF and MIP-3α or it may reflect a more fundamental heterogeneity in TLR signaling.

Taken together, our studies demonstrate that airway epithelial cells express a host of functional TLRs and that expression of these receptors may be increased by cell activation. Activation of epithelial cells with TLR agonists, especially dsRNA, triggers the increased expression of a number of chemokines, cytokines, signaling molecules and complement proteins. We speculate that

activation of TLRs on airway epithelial cells may facilitate the migration into the airways and local maturation of DCs as a result of epithelial production of MIP-3α and GM-CSF. The role of these epithelial responses in host immunity and in the etiology of airways disease is under active investigation.

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