Toll-Like Receptors in Normal and Cystic Fibrosis Airway Epithelial Cells

Amanda Muir, Grace Soong, Sach Sokol, Bharat Reddy, Marisa I. Gomez, Anna van Heeckeren, and Alice Prince

Departments of Pediatrics and Pharmacology, College of Physicians and Surgeons, Columbia University, New York, New York; and Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, Ohio

Toll-like receptors (TLRs) mediate cellular responses to diverse microbial ligands. The distribution and function of TLRs in airway cells were studied to identify which are available to signal the presence of inhaled pathogens and to establish if differences in TLR expression are associated with the increased proinflammatory responses seen in cystic fibrosis (CF). Isogenic, polarized CF and control bronchial epithelial cell lines, human airway cells in primary culture, and *cftr* null and wild-type mice were compared. TLRs 1–10, MD2, and MyD88 were expressed in CF and normal cells. Only TLR2 transcription was modestly increased in CF as compared with normal epithelial cells following bacterial stimulation. TLR2 was predominantly at the apical surface of airway cells and was mobilized to cell surface in response to bacteria. TLR4 was present in a more basolateral distribution in airway cells, but appeared to have a limited role in epithelial responses. Lipopolysaccharide failed to activate nuclear factor-κB in these cells, and TLR2 dominant negative but not TLR4 dominant negative mutants inhibited activation by both Gram-negative and Gram-positive bacteria. Increased availability of TLR2 at the apical surfaces of CF epithelial cells is consistent with the increased proinflammatory responses seen in CF airways and suggests a selective participation of TLRs in the airway mucosa.

Impaired clearance of inhaled bacteria, especially *Staphylococcus aureus* and *Pseudomonas aeruginosa*, is a major cause of the chronic polymorphonuclear leukocyte (PMN)-dominated airway inflammation characteristic of cystic fibrosis (CF) lung pathology. Bacteria trapped in dehydrated mucus, or their shed products, stimulate adjacent airway epithelial cells as well as alveolar macrophages and PMNs that are recruited to the lungs. Gene microarray studies demonstrate the expected upregulation in transcription of proinflammatory genes in bronchial epithelial cells exposed to *P. aeruginosa* (1). Clinical studies in patients with CF (2–4) as well as numerous *in vitro* studies indicate that cells with CF transmembrane conductance regulator (CFTR) mutations have increased expression of proinflammatory genes, often significantly greater than normal controls (5–8). Absolute amounts of cytokines and chemokines are increased in the airways and, in *cftr* −/− mice, persist for longer than in control animals (9).

The CF pathogens *S. aureus* and *P. aeruginosa* activate a common signaling pathway in airway cells to stimulate epithelial interleukin (IL)-8 expression and initiate the recruitment of PMNs to the airway (10). These organisms, as well as many other pulmonary pathogens, bind to the GalNAcβ1–4Gal moiety exposed on asialylated glycolipids such as asialoGM1 (11). Due to CFTR-associated effects on intracellular pH (12, 13), epithelial cell surface sialylation is decreased in CF, resulting in increased asialoGM1 for bacterial binding (14). A direct correlation between CFTR dysfunction, surface available asialoGM1, and epithelial IL-8 expression has been demonstrated by several laboratories (7, 14, 15). The recognition of asialoGM1 by *P. aeruginosa* pili (14), flagella (16), or staphylococcal surface components activates epithelial IL-8 and mucin (17) expression through a Ca^{2+}-dependent signaling pathway mediated by Src, ERK 1/2 mitogen-activated protein kinases, and nuclear factor (NF)-κB (10).

Toll-like receptors (TLRs) mediate innate immune responses to bacterial components, or pathogen-associated molecular patterns (PAMPs) (18). The 10 mammalian TLR proteins identified thus far share several conserved elements, a leucine-rich external domain, and a cytoplasmic domain with homology to IL-1R (19). Specific TLRs recognize microbial components, such as lipopolysaccharide (LPS) and TLR4 (20, 21), flagella and TLR5 (22), or CpG DNA and TLR9 (23). Of the 10 TLRs identified thus far, TLR2 seems least discriminating. TLR2 forms a heterodimer with TLR1 or TLR6 and can be activated by diverse bacterial products including lipoproteins and peptidoglycan (24). TLR4, which mediates responses to LPS, is part of a receptor/signaling complex that includes LPS binding protein, TLR4, CD14, and MD2 (21).

The distribution and function of TLRs differ substantially in cells of hematopoietic (25), endothelial (26), and epithelial origin, and are variable in epithelial cells at different sites (27–30). The presence of a specific TLR in a given cell type does not guarantee participation in responses to pathogens (31, 32). Coreceptors must be available and the TLRs must be accessible to the corresponding bacterial ligand. The airway epithelium has tight junctions that block the access of inhaled bacteria to basolateral surfaces. Despite chronic infection in the CF lung, these tight junctions are maintained. Superficial bacterial–receptor interactions are sufficient to activate the Ca^{2+} fluxes associated with induction of NF-κB and IL-8 expression in the airway (10). In contrast, many gastrointestinal pathogens or their components must be internalized within mucosal cells to initiate TLR-dependent signaling. The basolateral distribution of major TLRs in gastrointestinal mucosal cells is consistent with their role in signaling the presence of invasive organisms (28, 33–35) and failure to respond to the many bacteria present in the gut lumen. Even in dendritic cells, there is polarization of TLR distribution toward the cell surfaces interacting with bacteria (36).

To account for the increased proinflammatory responses typical of CF airway epithelial cells, we postulated that there may be *cftr*-dependent changes in TLR expression or availability, especially at the exposed, apical surfaces of airway epithelial cells. In the studies presented, we examined the transcription and distribution of major TLRs in human CF and control cells as well as in *cftr* null mice. All of the expected components of

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Address correspondence to: Alice Prince, Department of Pediatrics and Pharmacology, College of Physicians and Surgeons, Columbia University, 416 Black Building, 650 W. 168th Street, New York, NY 10032. E-mail: asp7@columbia.edu

Abbreviations: cystic fibrosis, CF; transmembrane conductance regulator, CFTR; dominant-negative, DN; extracellular signal-related kinase, ERK; interleukin-8, IL-8; IL-1 receptor–associated kinase, IRAK; lipopolysaccharide, LPS; mean fluorescence intensity, MFI; myeloid differentiation protein, MyD88; nuclear factor-κB, NF-κB; phosphate-buffered saline, PBS; polymorphonuclear leukocytes, PMNs; reverse transcriptase–polymerase chain reaction, RT-PCR; toll-like receptor, TLR; TNF receptor–associated factor, TRAF.

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the TLR signaling pathway were expressed in CF and control airway epithelial cells. Increased TLR2 expression and enhanced apical mobilization in response to either *P. aeruginosa* or *S. aureus* could be attributed to CFTR dysfunction, consistent with the increased inflammation characteristic of CF lung pathology.

Materials and Methods

Epithelial Cell Culture and Reagents

16HBE cells, a human bronchial epithelial cell line stably expressing episomes encoding CFTR in the sense or antisense orientation, and 9HTEo- cells overexpressing the CFTR R domain that have a CF phenotype (9HTEo-pCep-R) or the pCep vector control (7), were obtained from P. Davis, Case Western Reserve University. 1HAEo- cells were obtained from D. Gruenert, University of Vermont. All have been previously characterized (6, 37). The CF IB-3 and corrected C-38 cell lines were obtained from P. Zeitlin and have been previously characterized (38). The 16HBE cells maintain tight junctions when grown in a polarized fashion at an air–liquid interface. The cells expressing the CFTR antisense construct reflect the expected absence of CFTR function and fail to secrete Cl^- in response reagents that stimulate cAMP production. They were grown in modified Eagle's medium with Earl salts (MEM) supplemented with 10% fetal calf serum and 400 μg/ml G418. Human (CF and normal) airway epithelial cells were isolated from freshly excised nasal polyp tissue using the protease method and grown in a polarized fashion on Transwells (37). Reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA).

Bacterial Strains and Culture Conditions

P. aeruginosa PAO1, as has been previously described (7), was grown in Luria broth overnight and 1–5 $\times$ 10^9 cfu/ml were used to stimulate the monolayers. *S. aureus* RN6390 was grown on CYGP plates overnight, and resuspended in MEM + 0.1% fetal bovine serum to achieve a density of 1–5 $\times$ 10^9 cfu/ml.

Reverse Transcriptase and Real-Time Polymerase Chain Reaction

Epithelial cells grown in 10-cm dishes were grown to 80% confluence and were weaned from serum overnight. The cells were lysed and the RNA was isolated using the Qiagen RNeasy Mini Kit (Valencia, CA). cDNA was made using Omniscript Reverse Transcriptase (Qiagen). For reverse transcriptase (RT)-polymerase chain reaction (PCR), Accuprime Taq (Invitrogen) was used and 35 cycles were run with denaturation at 94° for 45 s and extension at 72° for 1 min with various annealing temperatures as listed in Table 1. For quantitative real-time PCR, cells were stimulated with *P. aeruginosa* PAO1 for 1 h (live bacteria) and 4 h (heat-killed bacteria) or with *S. aur*eus RN6390 for 1 h and 4 h (live bacteria) and 22 h (heat killed bacteria). Amplification was done in a Light Cycler using the DNA Master SYBR Green I kit (Roche, Indianapolis, IN). Thirty-five cycles were run with denaturation at 94° for 8 s and extension at 72° for 12 s. Primers were designed from separate exons for each of the genes. All primers and annealing temperatures are listed in Table 1.

Western Hybridization

Epithelial cells grown in 10-cm dishes to 80% confluence and weaned from serum overnight, were stimulated with 1 $\times$ 10^9 *P. aeruginosa* PAO1 or *S. aur*eus RN6390 for 1 h, washed with phosphate-buffered saline (PBS), and lysed with buffer containing 1% SDS, 1 mM sodium orthovandate, and 10 mM Tris pH 7.4. Protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Equal amounts of protein were separated by electrophoresis on a 4–12% NUPAGE gel (Invitrogen Life Technologies), transferred to an Immobilon-P, PVDF membrane (Millpore, Bedford, MA), and incubated overnight in 5% skim milk blocking solution. Immunoblotting with anti-TLR2 from Imgenex (San Diego, CA), anti-TLR4, anti-TLR5, or anti–IL-1 receptor–associated kinase (IRAK)-1 from Santa Cruz Biotechnology (Santa Cruz, CA) or anti-actin as a loading control (Sigma, St. Louis, MO) followed by secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology), and detection with Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA) was performed.

Confocal Microscopy and Immunofluorescence Studies

16HBE sense and antisense or primary cells were grown to confluence on Transwell-Clear filters (Corning-Costar, Corning, NY) with an air–liquid interface to form polarized monolayers. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and incubated with 5% normal serum blocking solution for 20 min at room temperature with 0.1% Triton X-100 for permeabilization, if necessary. Primary antibodies (as above) or anti-asialoGM1 (Wako, Richmond, VA) were added for 1 h at room temperature, followed by 3- to 5-min washes. Alexa Fluor 488– and Alexa Fluor 594–conjugated secondary antibodies (Molecular Probes, Eugene, OR) were added for 30 min, washed three times, and cells were mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Flow Cytometry

S. aureus RN6390 (1 $\times$ 10^9 cfu/ml) was added to confluent wells of 16HBE sense and antisense cells after weaning at time points indicated. Nonadherent bacteria were removed with 3–4 PBS washes. After blocking for 15 min with 5% normal serum, primary antibodies were added for 1 h at room temperature and Alexa Fluor 488–conjugated secondary antibodies were added for 30 min, washed, and incubated with Hanks' balanced salt solution + 0.02% EGTA. Detached cells were harvested and transferred to an appropriate tube containing 2% paraformaldehyde to fix. Cells were stored overnight at 4°C and then analyzed with a Becton Dickinson FACSCalibur using CellQuest software.

Animals

Male congenic B6.129P2-*cftr*^{tm1Unc} mice from Jackson Laboratories (Bar Harbor, ME) genotypes *cftr* +/+ (normal) or *cftr* −/− (CF) were caged

TABLE 1. Real-time PCR primers

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)	Annealing Temp. (°C)
TLR1	CTATACACCAAGTTGTCAGC	GTCTCAACTCAGTAAGGTG	57
TLR2	GCCAAAGTCTTGATTGATTGG	TTGAAGTTCTCCAGCTCCTG	57
TLR3	GATCTGTCTCATAATGGCTTG	GACAGATTCCGAATGCTTGTG	57
TLR4	TGGATACGTTTCCTTATAAG	GAAATGGAGGCACCCCTTC	57
TLR5	CTAGCTCCTAATCCTGATG	CCATGTGAAGTCTTTGCTGC	57
TLR6	AGAACTCAACCAGAGGTAAG	GAAGCATATCCTTCGTCATGAG	57
TLR7	GGTATTCCCACGAACACC	GTAAGCTAGGCGGGAG	60
TLR8	TGAGCTGCGCTACCAC	CTTCTGCCTTCGGGTT	55
TLR9	GCAAATACTAGATGTAAGCGC	GCGTTTTGTCGAAGACC	55
TLR10	CCATTCCGGTGTACCT	TGACCTAGCATCCTGAG	52
MD2	GAGGGGCACGGGTAA	ACTTGCATCGGATGAGT	52
MyD88	TGAGCGTTTCGATGCC	GTTGGTGTAGTCGCAG	55
IL-8	TACTCCAAACCTTTCCAACCC	AACTTCTCCACAACCCTCTG	55
β-Actin	GTGGGCCGCTCTAGGCACCA	CGGTTGGCCTTAGGGTTCAGGGGGG	57

in Static Micro-Isolator LPTM cages (Lab Products, Inc., Seaford, DE) and bedded in combination size corncob bedding (The Andersons, Maumee, OH). The normal mice were fed solid chow and the CF mice were fed Peptamen (Nestle Clinical Nutrition, Deerfield, IL). At age 59–74 d, the mice were treated with 20 μl of PBS or PAO1 (2.48×10^8 cfu) intranasally under isoflurane anesthesia. After 24 h, the mice were killed by CO_2 inhalation followed by exsanguination. During dissection, the lungs were inflated with 1 ml of 2% paraformaldehyde in PBS and following removal, immersed in 50 ml of paraformaldehyde in PBS for 48 h, and paraffin embedded.

Immunohistochemisty

Paraffin lung sections were deparaffinzed by successive washes with xylene, 100% ethanol, 95% ethanol, and 70% ethanol. The Vectastain Elite ABC Kit was used to stain the slides in association with Vectastain DAB peroxidase substrate kit (Vector Laboratories). The primary antibodies used were anti-TLR2 (sc-12507), anti-TLR4, (sc-12511), anti-TLR5 (sc-10742), anti-IRAK-1 (sc-7883), anti–tumor necrosis factor-associated receptor (TRAF)6 (sc-7221), and anti-MYD88 (sc-11356). Fluorescent sections were prepared by deparaffinization, followed by treatment with primary antibody anti-asialoGM1 (Wako) and secondary Alexa Fluor 594–conjugated secondary antibody (Molecular Probes). All sections were mounted with Vectashield (Vector Laboratories).

NF-κB Luciferase Reporter Assay

1HAEo- cells grown in 12-well plates to 70–80% confluence were transiently transfected using FuGENE 6.0 (Roche), pNF-κB-luc (Stratagene, La Jolla, CA), pRL-TK (Promega, Madison, WI) to control for transfection efficiency, and either TLR2 DN or TLR4 DN (gifts of Jian Dong Li, House Ear Institute, USC, Los Angeles, CA). After 24 h incubation, cells were stimulated with $Pam_3Cys\text{-}Ser\text{-}Lys_4$ (5 μg/ml), *P. aeruginosa* LPS (10 μg/ml) (Sigma) for 4–24 h, *P. aeruginosa* PAO1 or *S. aureus* RN6390 for 1 h. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Data were plotted as the mean of sextuplicate samples and are representative of at least two independent experiments.

Statistical significance was measured using Bonferoni post-test.

Results

Expression of TLRs 1–10 in CF and Control Cell Lines

The expression of the major TLRs under control, unstimulated conditions were first assessed by RT-PCR using lysates of 16HBE cells (Figure 1A). Each of these TLRs, as well as the adaptor proteins MD2 and MyD88, were present in both the normal and CF cells. Confirmatory studies for TLR2 were done in two other matched CF and control cell lines, 9HTEo-pCep cells (normal physiology) and 9HTEo-pCep-R cells which are "CF-like," as well as the IB-3 and C-38 (CF and corrected) cell lines, which suggested that the CF cells had slightly more abundant TLR2 than the control cells, at least at 24 h after bacterial exposure (Figure 1B). To obtain quantitative data, real-time PCR was performed for TLRs 2 and 4 and the effects of exposure to *S. aureus* or *P. aeruginosa* quantified (Figure 1C). After 4 h of exposure to either organism, there was an increase in TLR2 message in the normal cells ($P < 0.05$), but no increase in TLR4. By 22 h (using heat-killed *S. aureus*) there was a 5-fold increase in TLR2 message in the CF but not the normal cells ($P < 0.05$). Comparable studies could not be done with *P. aeruginosa* due to toxicity to the monolayers. These increases in TLR transcription were quite modest when compared with the induction of IL-8 expression in the same cells, which was increased by 20-fold in the normal cells and 40-fold in the CF cells ($P < 0.001$) following 4 h of bacterial exposure (Figure 1C), as has been previously reported (38).

The corresponding TLR proteins and IRAK-1 in CF and control cell lines were assessed by Western hybridization. Lysates of 16HBE cells, expressing CFTR sense or antisense, obtained following *P. aeruginosa or S. aureus* stimulation, contained TLRs

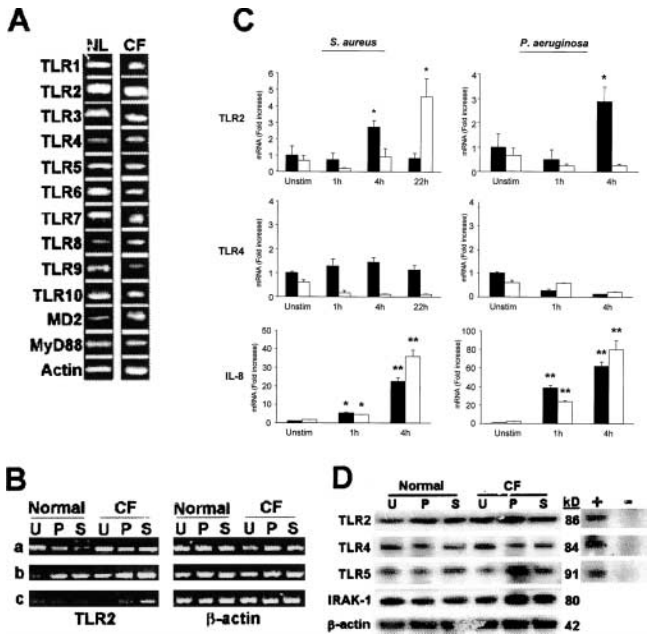

Figure 1. Expression of TLRs 1–10, MD2, and MyD88 in normal and CF airway epithelial cells. (*A*) 16HBE cells expressing CFTR in the sense (Normal) or antisense (CF) were analyzed by RT PCR under control unstimulated conditions and (*B*) following 24 h bacterial stimulation with *P. aeruginosa* PAO1 (P) or *S. aureus* RN6390 (S) compared with unstimulated (U) in (a) 16HBEsense (Normal) and antisense (CF), (b) C-38 (Normal) and IB-3 (CF), (c) 9HTEo-pCep (Normal) and pCepR (CF) cells, or (*C*) real-time quantitative PCR under control unstimulated (Unstim) conditions or following stimulation with *S. aureus* RN6390 or *P. aeruginosa* PAO1. Values for real-time PCR were normalized to β-actin and are shown as the fold change in expression relative to the endogenous level in unstimulated normal cells. Each bar represents the mean of triplicate samples. *Filled bars*, normal; *open bars*, CF. IL-8 is included as a control for CFTR-associated changes in transcription (note *y* axis), $*P < 0.05$, $**P < 0.01$. (*D*) Western immunoblot showing amounts of TLRs 2, 4, 5, and IRAK-1 in whole cells lysates of normal and CF unstimulated (U) cells or following bacterial stimulation with *P. aeruginosa* PAO1 (P) or *S. aureus* RN6390 (S) and compared with β-actin. Cell lysates for controls were (+) Jurkat for TLR2 and TLR4, A431+EGF for TLR5, and (−) Mouse macrophage for TLR2 and TLR4, SW480 for TLR5. One representative experiment of three independent studies is shown.

2, 4, and 5, the TLRs that respond to the major surface components of bacteria, lipoproteins and peptidoglycan, LPS and flagella, respectively, as well as IRAK-1 (Figure 1D).

Distribution of TLRs in Polarized Airway Cells

To mediate responses to inhaled pathogens, TLRs should be available at the apical surface of cells. Using confocal microscopy, CF and normal airway epithelial cell lines were imaged to establish and compare the distribution of TLRs in these polarized cells (Figure 2). In CF cells in primary culture, TLR2 was particularly abundant at the apical surface, even in the absence of exogenous bacterial stimulation (Figure 2A). TLR4 and TLR5 were readily visualized along the basolateral aspects of the cells. Airway cells in primary culture from normal control cells appeared similar to the CF cells (data not shown). We then compared the amount and distribution of major TLRs and kinases in the CF and control cell lines, demonstrating that the patterns of TLR distribution are similar in the transformed cells as well as the cells in primary culture (Figure 2B). No major differences

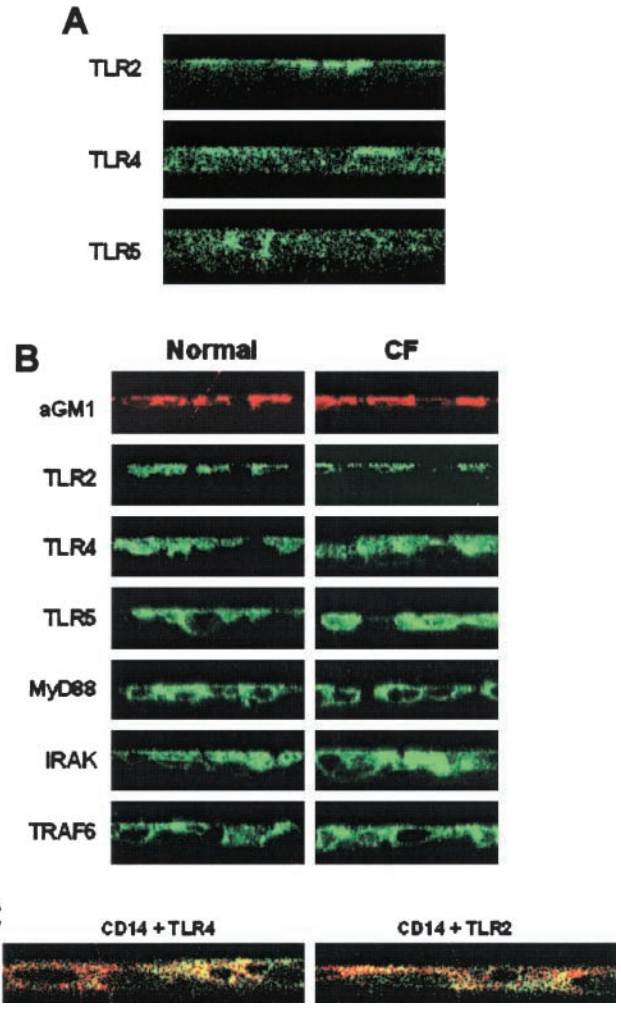

Figure 2. TLR and CD14 distribution in airway cells in primary culture and cell lines. (*A*) Primary airway epithelial cells isolated from human CF nasal polyps were grown with an air–liquid interface and analyzed by confocal microscropy for TLRs 2, 4, 5. TLR2, as compared with TLRs 4 or 5, was primarily apical. (*B*) 16HBE sense (Normal) and antisense (CF) cell lines grown in a polarized fashion with asialoGM1 (aGM1) shown as a control for apical staining. (*C*) Colocalization of CD14 (*green*) and TLRs 2 and 4 (*red*) in polarized primary airway cells following *P. aeruginosa* PAO1 stimulation.

in the unstimulated CF and control cells were apparent. Because CD14 is a coreceptor for LPS, the cells were also imaged to document the presence of CD14 and any colocalization with TLRs in these cells (Figure 2C).

TLR Signaling Components in *cftr* −/− Mice

The distribution of the TLRs in CF and control animals and the effects of *P. aeruginosa* infection were examined (Figure 3). In contrast to the studies performed *in vitro*, these experiments take into account *in vivo* expression of cytokines, such as interferon-γ and tumor necrosis factor-α, which can influence TLR expression (32). TLR2, TLR4, and TLR5 were abundant in airway mucosal cells with no major differences in the distribution of these receptors following *P. aeruginosa* infection in the mice (Figure 3A). IRAK-1, MyD88, and TRAF6 were also present in the airway cells, all key elements in downstream TLR-mediated signaling (39, 40). To establish whether CF-dependent differences in the surface expression of bacterial receptors could be detected, we also used fluorescence-labeled antibodies. It was possible to visualize the previously established differences in surface asialoGM1 expression in uninfected control and CF lungs using TRITC-labeled anti-asialoGM1 (Figure 3B). However, the distribution and amount of FITC-labeled TLRs appeared equivalent in the CF and control cells (data not shown), and the horseradish peroxidase–labeled sections shown were more informative.

Mobilization of Signaling Components to the Cell Surface in Response to Bacterial Ligands

The localization of TLRs within cells is dependent upon the degree of differentiation (27), as well as exposure to bacterial components (31). Flow cytometry was used to quantify the number of cells with surface exposed receptors and to compare the numbers of receptors/cell as indicated by mean fluorescence intensity (Figure 4). There was a small but consistent increase in surface TLR2 in the CF cells and increased mobilization of TLR2 in response to *S. aureus* in both the CF cells as well as the normal cells (Figure 4A). The ΔMFI of asialoGM1 also increased by 7.7-fold in the CF compared with control cells in response to *S. aureus*. The kinetics of receptor mobilization was also compared and found to be similar (Figure 4B). As a control, asialoGM1, known to be increased in CF, was also found to be mobilized to a greater extent in the CF cells following bacterial exposure. There was no increase in surface TLR4 in either the CF or control cells in response to bacterial ligands including LPS in the presence of serum to provide LPS-binding protein (data not shown).

TLR2 and TLR4 Responses to Bacteria

The participation of airway TLRs in response to bacteria were examined. We were particularly interested in the role of TLR4 and epithelial responses to LPS and Gram-negative bacteria. The availability of the co-receptor CD14 was confirmed and its likely association with TLR4 visualized by confocal imaging (Figure 2C). The relative participation of TLR2 and TLR4 in signaling were compared by stimulating 1HAEo- cells (known to express both receptors) transfected with either a TLR2 DN mutant or a TLR4 DN mutant or vector control, and measuring NF-κB activation following stimulation with a TLR2 agonist, $Pam_3Cys\text{-}Ser\text{-}Lys_4$, with *P. aeruginosa* LPS in the presence of serum to provide LPS binding protein, or with intact bacteria (*P. aeruginosa* or *S. aureus*) (Figure 5). These experiments were done with normal airway cells because the endogenous activation of NF-κB activity in the CF cells cannot be negated by transfection of dominant-negative forms of the involved receptors. Despite incubation periods of up to 24 h, LPS did not activate NF-κB expression, and the TLR4 DN had no effect on endogenous luciferase activation. In contrast, the TLR2 agonist, as well as both *S. aureus* and *P. aeruginosa*, stimulated NF-κB and were significantly inhibited by the TLR2 DN mutant ($P < 0.01$ for each), but not by the TLR4 DN. To verify the function of the TLR4 DN mutant, we transfected RAW cells with the TLR4 DN construct and demonstrated a 50% inhibition of luciferase activity as compared with cells transfected with the vector control, in response to LPS (data not shown).

Discussion

TLRs, coreceptors, associated adaptor proteins, and signaling kinases are critical in mediating cellular responses to microbial components. In this report, we compared the distribution of several TLRs and associated kinases in CF and normal respiratory epithelial cells to determine if increased availability is associated with the increased inflammatory responses to inhaled bacteria,

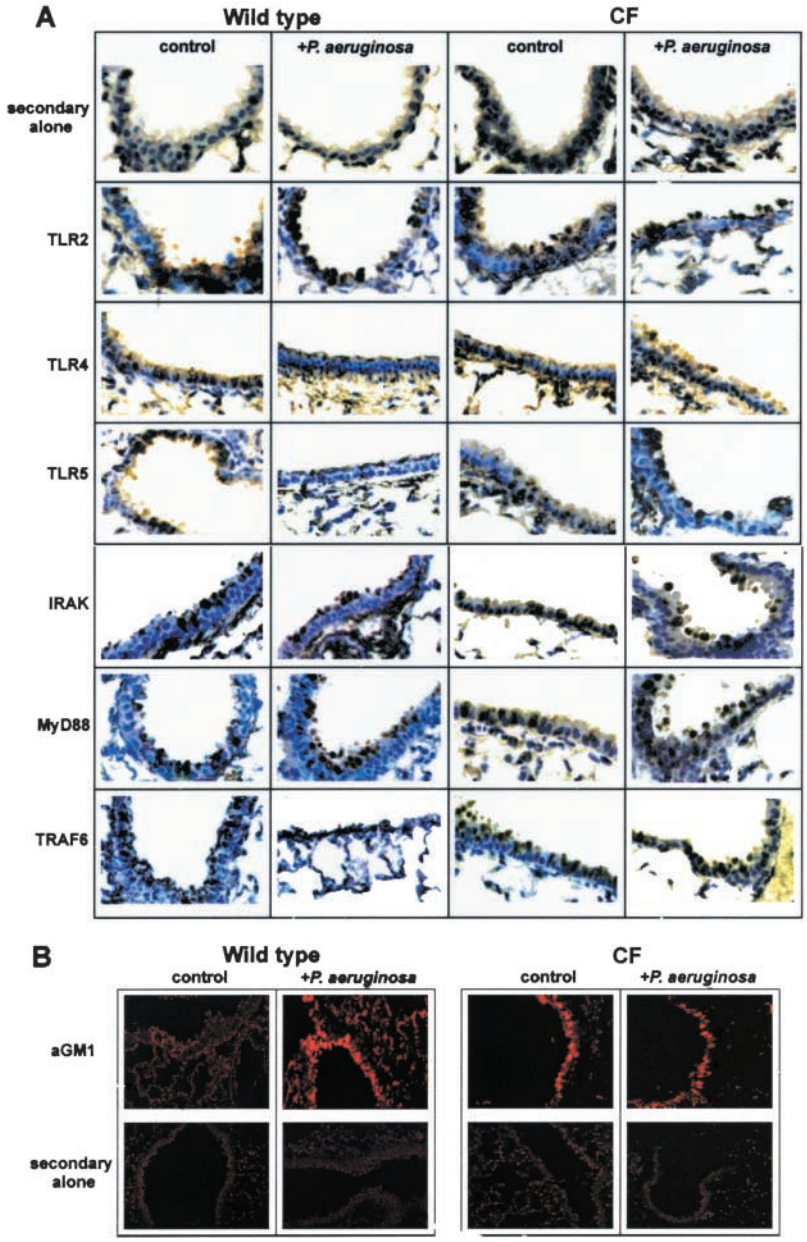

Figure 3. In vivo distribution of TLRs and asialoGM1 in *cftr*−/− and wild-type mice in response to *P. aeruginosa* infection. (*A*) Horseradish peroxidase–labeled antibodies were used to detect the TLRs 2, 4, 5, IRAK-1, MyD88, and TRAF6 in paraffin-embedded sections of lung tissue (×40). (*B*) TRITC-labeled anti-asialoGM1 detects increased labeling in unstimulated CF cells.

characteristic of CF pulmonary pathology. Excessive PMN-dominated inflammation in the CF lung has been attributed to exogenous factors: exposure to bacterial products (41), increased availability of asialoGM1 receptors (14), and correspondingly increased IL-8 expression (38). In addition, clinical as well as experimental data support the hypothesis that mistrafficked CFTR causes endogenous activation of NF-κB and IL-8 expression, as a consequence of increased $[Ca^{2+}]_i$ and cell stress (5, 8). Either pathway could potentially affect the availability of TLRs. Using several different experimental techniques, our data suggest that the expression of TLRs is not directly affected by CFTR dysfunction. There was a small but consistent increase in TLR2 availability on the surface of CF cells exposed to *P. aeruginosa* or *S. aureus*, although this was not as great as the increase in asialoGM1.

Different types of airway epithelial cells were used in these studies, because no single cell line or animal model is ideal for all of the experiments. Arguably, human epithelial cells in primary culture might be optimal to study. However, there are difficulties in controlling for prior exposure to bacteria that could affect TLR expression, by either upregulation or by the induction of tolerance. No normal mucosal tissues are exposed to the amount of bacterial contamination as are CF cells. To establish if lack of CFTR function, by itself, affects TLR expression, 16HBE cells expressing CFTR in the sense or antisense orientation are a useful model in that they are isogenic, polarized, and retain the tight junctions critical to epithelial function, as well as the altered surface sialylation and increased IL-8 expression, characteristic of defective CFTR activity (7, 37). Thus, we focused primarily on these cells to determine the distribution and effects of CFTR on TLR expression.

In vivo studies using the *cftr*−/− mouse were consistent with the *in vitro* experiments with cell lines; TLR2, 4, and 5 expression were constitutive and the major TLRs and associated kinases were available in the airway cells. The *in vivo* studies also demonstrate that in the presence of chemokines and cytokines produced during an infection, there were still no major differences in TLR expression in the CF and control lung. The *cftr* −/− mouse is not an entirely satisfactory model, as these mice do not develop

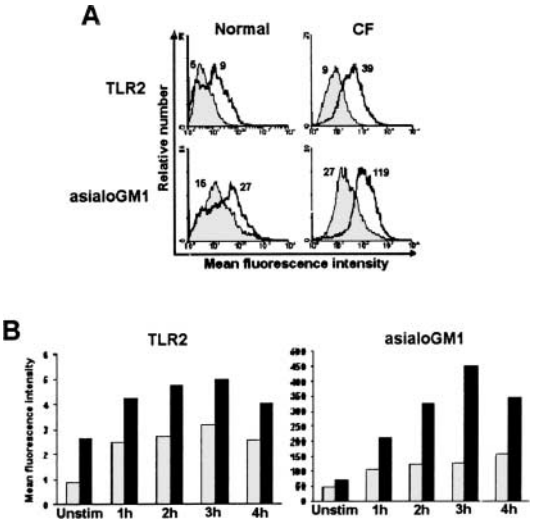

Figure 4. Surface expression of TLR2 and asialoGM1 was quantified by flow cytometry on the surface of 16HBE sense (Normal) and antisense (CF) cells. (*A*) Histograms show endogenous (*shaded section*) and *S. aureus*–induced (*bold line*) TLR2 and asialoGM1 in normal and CF cell lines. (*B*) Kinetics of TLR2 and asialoGM1 mobilization to the cell surface. The mean fluorescence intensity of normal (*lightly shaded bars*) and CF (*solid bars*) cells is shown for the 4 h following exposure to *S. aureus* RN6390. The experiment was performed three times and representative data is shown.

spontaneous lung infection, although they do have increased and prolonged airway inflammation similar to that seen in human disease (6). Despite reported differences in the regulation of the TLR2 promoter in mice and humans (25), the patterns of TLR2 expression in murine airways were similar to those observed in human cells. Thus, TLR expression in the *cftr* −/− mouse lung appears to be similar to that in humans, and is unlikely to account for the failure of the *cftr* null mice to more accurately mimic human disease.

Although we failed to detect a global effect of CFTR dysfunction on TLR expression in the lung, we did appreciate the participation of TLR2 in airway cells responses to bacteria. Both *S. aureus* and *P. aeruginosa* stimulation appeared to be signaled through TLR2, despite the presence of TLR4 in these cells. The availability of TLR2 on the apical surface of respiratory

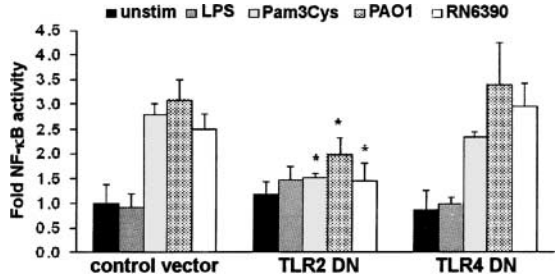

Figure 5. Activation of NF-κB is inhibited by dominant-negative mutation in TLR2 but not TLR4. NF-κB promoter activity was measured by luciferase assay using 1HAEo- cells transfected with an empty vector control, TLR2 DN, or TLR4 DN mutant following stimulation with LPS from *P. aeruginosa*, $Pam_3Cys\text{-}Ser\text{-}Lys_4$ (Pam3Cys), a TLR2 agonist, *P. aeruginosa* PAO1 or *S. aureus* RN6390. Luciferase units for each sample were normalized to the unstimulated empty vector control and fold NF-κB activity plotted. The TLR2 DN significantly inhibited activity stimulated by $Pam_3Cys\text{-}Ser\text{-}Lys_4$, *P. aeruginosa* and *S. aureus* ($P < 0.001$).

epithelial cells suggests a role for this TLR in airway responses to Gram-negative as well as Gram-positive pathogens. The participation of specific TLRs in airway cells clearly differs from what is observed in cells of hematopoietic origin. TLR4, which is critical in signaling systemic responses to LPS, was substantially less involved in epithelial signaling, although clearly present in the epithelial cells. Airway epithelial cells, like other mucosal epithelia, are not particularly responsive to LPS as compared with myeloid cells (41). Our findings are similar to a recent report documenting the presence of intracellular TLR4 in BEAS cells (42). However, in the 1HAEo- cells that we studied, unlike the BEAS cells, there was no detectable activation of NF-κB in response to LPS, which may be due to as yet undefined differences in these cell lines. A lack of TLR4 expression was deemed responsible for similar observations in A549 cell lines (43). However, our data indicate that both murine and human airway epithelial cells express the requisite components of the TLR-signaling cascade. Perhaps superficial presentation of LPS is insufficient to engage TLR4 signaling, as at other mucosal sites only invasive Gram-negative pathogens initiate inflammation (35). The lack of TLR4 involvement in epithelial responses to LPS does not suggest that the lung itself is unresponsive. LPS introduced intravenously is a potent stimulus for neutrophil accumulation in the lung, a response mediated by endothelial TLR4 expression (44). Inhaled LPS in rodents activates inflammation, although this may be mediated by alveolar macrophages or alveolar endothelial cells (45). Although epithelial TLR4 expression may not be critical in signaling lumenal LPS, TLR4 in other cell types in the lung may assume this function.

Airway epithelial cells, as participants in the mucosal immune system express all the requisite components of the TLR signaling pathway. Consistent with their function in protecting the respiratory tract from inhaled pathogens, there is a selective mobilization of TLR2 to the apical surface of the airway cells in response to bacteria, and specifically in response to CF pathogens. However, there is no evidence that TLR-associated signaling is directly affected by *cftr* dysfunction.

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