

# Microarray Analysis Reveals Induction of Lipoprotein Genes in Mucoid *Pseudomonas aeruginosa*: Implications for Inflammation in Cystic Fibrosis

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**The main cause of the high morbidity and mortality of cystic fibrosis (CF) is the progressive lung inflammation associated with *Pseudomonas aeruginosa* colonization. During the course of chronic CF infections, *P. aeruginosa* undergoes a conversion to a mucoid phenotype. The emergence of mucoid *P. aeruginosa* in CF is associated with increased inflammation, respiratory decline, and a poor prognosis. Here we show, by the use of microarray analysis, that upon *P. aeruginosa* conversion to mucoidy, there is a massive and preferential induction of genes encoding bacterial lipoproteins. Bacterial lipoproteins are potent agonists of Toll-like receptor 2 (TLR2) signaling. The expression of TLR2 in human respiratory epithelial cells was ascertained by Western blot analysis. Human respiratory epithelial cells responded in a TLR2-dependent manner to bacterial lipopeptides derived from *Pseudomonas* lipoproteins induced in mucoid strains. The TLR2 proinflammatory response was further augmented in CF cells. Thus, the excessive inflammation in CF is the result of a global induction in mucoid *P. aeruginosa* of lipoproteins that act as proinflammatory toxins (here termed lipotoxins) superimposed on the hyperexcitability of CF cells. Blocking the signaling cascade responding to bacterial lipotoxins may provide therapeutic benefits for CF patients.**

Cystic fibrosis (CF) is the most common lethal inheritable disease affecting Caucasians (19). CF is caused by mutations in the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR), resulting in multiorgan malfunctions, particularly within the respiratory, gastrointestinal, hepatobiliary, and reproductive tracts (29, 44). The lung complications in CF include chronic respiratory infections, which are the main cause of CF remaining an incurable lethal disease (15). The predominant CF pathogen is *Pseudomonas aeruginosa*: the lungs of >90% of all CF patients eventually become colonized with this bacterium (11). A classical feature of *P. aeruginosa* strains infecting CF patients is that they mutate into a mucoid, exopolysaccharide alginate-overproducing form in a process referred to as the conversion to mucoidy (15). The conversion to mucoidy is concomitant with the establishment of chronic bacterial colonization (20, 27). Infections with mucoid *P. aeruginosa* are associated with heightened inflammation, tissue destruction, and pulmonary function decline (3). It has been recognized that the establishment of mucoid *P. aeruginosa* biofilms correlates with a poor prognosis for CF patients (11, 20, 27). Conversion to mucoidy results from mutations that render the *P. aeruginosa* stress response sigma factor AlgU constitutively active (21, 22). This in turn activates genes of the alginate biosynthesis pathway and additional genes that still need to be fully characterized (9, 10).

However, the overproduction of alginate, an immunologically inert exopolysaccharide associated with mucoid conversion, cannot explain the increased inflammation in CF. This

makes it likely that additional, less conspicuous, but potentially more damaging products of *P. aeruginosa* are produced by mucoid *P. aeruginosa* in the CF host. Here we describe the previously unappreciated induction of proinflammatory products in mucoid *P. aeruginosa* and how they affect signaling pathways in host respiratory cells. Using microarray analysis, we found that the most prominently induced genes in mucoid *P. aeruginosa* encode lipoproteins. We show that these *P. aeruginosa* products cause the activation of NF- $\kappa$ B in human lung epithelial cells and that this occurs through Toll-like receptor 2 (TLR2).

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The mucoid *P. aeruginosa* strain PAO578II (*mucA22 sup-2*) and its isogenic nonmucoid PAO6865 (*algU::Tc<sup>r</sup>*) derivative have been described previously (7). For RNA isolation, strains were cultured at 37°C overnight in Luria broth. One milliliter of the overnight culture was used to inoculate 100 ml of Luria broth containing 0.3 M NaCl, and the culture was grown for 4 h at 37°C to mid-log phase (optical density at 600 nm of 0.5).

**Microarray analysis.** For microarray analysis and primer extension, RNAs were isolated with Trizol (Invitrogen Life Technologies, Carlsbad, Calif.) and an RNeasy kit (Qiagen, Valencia, Calif.) according to the method described by Lory et al. ([http://cfgenomics.unc.edu/protocols\\_rna\\_prep.htm](http://cfgenomics.unc.edu/protocols_rna_prep.htm)). Labeled cDNAs were generated according to the protocol for the Affymetrix (Santa Clara, Calif.) *Pseudomonas* microarray chip. cDNAs were synthesized by annealing random primers (Invitrogen) to purified RNAs and extending them with SuperScript II (Invitrogen). Transcripts corresponding to *Bacillus subtilis* genes *dap*, *thr*, *phe*, *lys*, and *trp* were spiked into the cDNA synthesis reaction mixtures as a control to monitor cDNA synthesis, labeling, hybridization, and staining efficiency (courtesy of Steve Lory, Harvard Medical School). RNAs were removed by the addition of 1 N NaOH and incubation at 65°C for 30 min. The reaction was neutralized with 1 N HCl, and the cDNAs were purified with a Qiaquick PCR purification kit (Qiagen). The yields were quantified, and cDNAs were fragmented with 0.6 U of DNase I (Amersham Pharmacia Biotech) per  $\mu$ g of cDNA for 10 min at 37°C, followed by heat inactivation. Chips were hybridized overnight at 50°C and then

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washed, stained, and scanned the next day according to the steps of the Affymetrix Microarray Suite software specified for the *Pseudomonas* chip. The results from three independent experiments were merged for each strain. The merged data were used for comparisons, and statistical significance was assessed with Student's *t* test.

**Cell culture.** Primary normal human bronchial epithelial cells (NHBEs) (Cambrex Bio Science, Baltimore, Md.) were cultured in bronchial epithelial medium (BEGM; Cambrex Bio Science). IB3-1 (47) is a CF-affected human airway epithelial cell line. Genotypically, IB3-1 is a compound heterozygote containing the  $\Delta$ F508 mutation and W1282X. The C38 and S9 cell lines, created by correcting IB3-1 cells for chloride conductance by the introduction of functional CFTR (8), were grown in LHC-8 medium (Bio-fluids, Rockville, Md.) supplemented with 10% fetal bovine serum and antibiotics. S9 and C38 cells are both functionally complemented for the major known CFTR effects. They differ in that the CFTR cDNA used to complement IB3-1 cells encodes a complete CFTR molecule in the case of S9 cells, while it lacks the first CFTR extracellular loop in the case of C38 cells (47). NuLi-1 cells, derived from normal human airway epithelial cells by J. Zabner (46), were cultured in collagen-coated plastic dishes (Sigma, St. Louis, Mo.) in serum-free bronchial epithelial cell growth medium with supplements (Clonetics/BioWhittaker, San Diego, Calif.). The 9/HTEo<sup>-</sup> tracheal epithelial cell line (28), obtained from P. Davis (Case Western Reserve University, Cleveland, Ohio), was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2.5 mM L-glutamine (in the presence of 50 U of hygromycin/ml as a marker of the cell line due to its stable transfection with the pCEP2 vector). All cell lines were grown at 37°C in 5% CO<sub>2</sub>. For luciferase and cytokine assays, cells were seeded at  $5 \times 10^5$  or  $1 \times 10^5$  cells/well in 12-well plates, respectively.

**Peptide design and synthesis.** Leader sequences denoting lipopeptide modification (31) were observed for two open reading frames (ORFs) downstream of AlgU-dependent promoters. Two peptides, LPTA(6) (Pam<sub>3</sub>Cys-DKKEE-OH) and LPTB(6) (Pam<sub>3</sub>Cys-DSQTN-OH), consisting of a palmitoylated cysteine (Pam<sub>3</sub>Cys) after the cleavage site plus five amino acids from the amino terminus of each lipoprotein, were synthesized (Bio-Synthesis, Inc., Lewisville, Tex.). The proinflammatory synthetic bacterial lipopeptide Pam<sub>3</sub>Cys-SKKKK-OH was also synthesized (2, 17).

**Transfection, luciferase reporter, and cytokine assays.** To monitor transient NF- $\kappa$ B activation, we seeded the primary NHBE, IB3-1, C38, and S9 cell lines at  $5 \times 10^5$  cells per well in a 12-well plate and transfected them by using the Effectene transfection reagent (Qiagen) with 0.05  $\mu$ g of a human TLR2 (hTLR2) or dominant-negative TLR2 (DN-TLR2) expression plasmid, 0.15  $\mu$ g of an NF- $\kappa$ B-responsive luciferase reporter construct, 0.15  $\mu$ g of a control *Renilla* luciferase construct, and 0.05  $\mu$ g of a control  $\beta$ -galactosidase reporter construct for normalization. Eighteen hours after transfection, the cells were incubated with a stimulus (tumor necrosis factor alpha [TNF- $\alpha$ ], 20 ng/ml; lipopolysaccharide [LPS], 1  $\mu$ g/ml; Pam<sub>3</sub>Cys, 5  $\mu$ g/ml; or lipopeptides, 5  $\mu$ g/ml) for 6 h and assayed for luciferase activity by use of a luciferase assay reagent (Promega) or for  $\beta$ -galactosidase activity by use of the Galacto-Star luminescence system (Tropix, Bedford, Mass.). The transfection efficiency was controlled by standardizing the luciferase activity to constitutive  $\beta$ -galactosidase production. For the cytokine assay, NHBE confluent monolayers were incubated with LPS (1  $\mu$ g/ml), bacterial lipopeptides (1 or 10  $\mu$ g/ml), or no stimulation for 24 h. Cell culture supernatants were assayed at a 100-fold dilution for secreted interleukin-8 (IL-8) according to the manufacturer's instructions (R&D Systems, Minneapolis, Minn.). All experiments were repeated at least three times, and all errors shown represent experimental errors for at least three independent cultures.

**Western blot analysis and antibodies.** Protein samples were prepared by homogenizing lung epithelial cells in lysis buffer (8.5% sucrose and protease inhibitors [10 mg of leupeptin/ml, 1  $\mu$ M pepstatin, 1.8 mg of E64/ml, and 5 mg of N $\alpha$ -p-tosyl-L-lysine chloromethyl ketone/ml]) (all chemicals were from Sigma Chemical Co.) on ice. Protein concentrations were determined by the use of a micro BCA protein assay reagent kit (Pierce, Rockford, Ill.). An antibody against TLR2 for Western blotting was obtained from Imgenex (San Diego, Calif.). Immune complexes were detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad, Hercules, Calif.). The signal was visualized by incubating the complexes with Super Signal chemiluminescent substrate (Pierce). Densitometric analyses of Western blots were performed with NIH Image software (<http://rsb.info.nih.gov/nih-image>). Western blot quantification was performed as previously described (13, 14, 43). A blocking antibody against TLR2 was obtained from Douglas Golenbock.

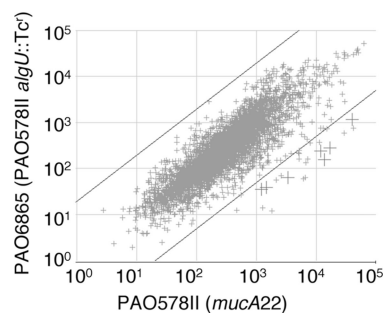


FIG. 1. Global gene expression analysis by use of microarrays reveals massive and selective lipoprotein gene induction in mucoid *P. aeruginosa*. The experimental points represent merged expression data from three independent cultures run on three separate chips for each strain. Mucoid strain PAO578II carries the typical CF mutation *mucA22*, which causes mucoidy in *P. aeruginosa*. Gene expression in PAO578II was compared to that in the isogenic, nonmucoid, *algU* knockout strain PAO6865. Large crosses, lipoprotein genes induced in mucoid *P. aeruginosa* ( $P < 0.001$ ). Diagonal lines delimit regions of  $\geq 30$ -fold induction.

## RESULTS

**The most highly induced genes in mucoid *P. aeruginosa* encode lipoproteins.** It has been shown that the conversion to mucoidy in *P. aeruginosa* CF isolates occurs via mutations in the *mucA* gene (22) that activate the alternative sigma factor AlgU (21, 22). We tested how the activation of AlgU in a mucoid *P. aeruginosa* strain carrying the most common *mucA* mutation found in CF isolates affected global gene expression. PAO578II carries the *mucA22* mutation and an additional *sup-2* mutation, also common among CF isolates, that renders it responsive to growth conditions for the maximal production of alginate (35). The strain PAO6865 is an *algU* knockout (*algU::Tc<sup>r</sup>*) derivative of PAO578II (4). When the microarray expression data (Fig. 1) for PAO578II and PAO6865 were compared, the analysis revealed massive and selective lipoprotein induction in mucoid *P. aeruginosa*, with high expression ratios (Fig. 1, crosses, and Table 1). We found that 70% of the genes showing induction above 30-fold encoded uncharacterized lipoproteins (Table 1). The highest levels of AlgU-dependent expression were observed with the lipoprotein genes *lptE* and *lptG*. Two of the lipoprotein-encoding genes, *lptA* and *osmE*, have known AlgU-dependent promoters (10). The majority of the other highly induced genes encoding products with lipoprotein leader sequences contained an AlgU promoter consensus motif (Table 2). Since lipoproteins have been implicated in inflammatory processes and the pathogenesis of several important bacterial infections, including *Mycobacterium tuberculosis* (5), *Treponema pallidum* (40), *Listeria monocytogenes* (12), and *Borrelia burgdorferi* (32), this observation warranted further analysis.

***P. aeruginosa* lipopeptides stimulate NF- $\kappa$ B activation in human lung epithelial cells.** Considering the preponderance of lipoprotein genes induced in mucoid *P. aeruginosa*, we next investigated whether lipoproteins and/or lipopeptides could induce inflammation in clinically relevant host cells. *P. aeruginosa* colonization is largely limited to the lower airways, and the bronchioles of the CF lung are where respiratory epithelial cells are exposed to *Pseudomonas* products. To determine

TABLE 1. Activation of lipoprotein gene expression in mucoid *P. aeruginosa*<sup>a</sup>

Gene no. <sup>b</sup>	Gene name	Description <sup>c</sup>	Fold activation	<i>P</i> value
PA1323		Unknown	103	$9.8 \times 10^{-6}$
PA3691	<i>lptE</i>	Lipoprotein LptE	87	$4.9 \times 10^{-6}$
PA5526	<i>lptG</i>	Lipoprotein LptG	61	0.0005
PA3819	<i>slyB</i>	Possible porin	56	0.0002
PA3692 <sup>d</sup>	<i>lptF</i>	Lipoprotein LptF	53	0.004
PA4876	<i>osmE</i>	Lipoprotein OsmE	49	$6.0 \times 10^{-5}$
PA0737	<i>lptD</i>	Lipoprotein LptD	49	0.0002
PA1592	<i>lptA</i>	Lipoprotein LptA	35	$4.8 \times 10^{-6}$
PA0062	<i>lptC</i>	Lipoprotein LptC	34	$8.8 \times 10^{-5}$

<sup>a</sup> The data shown are for all *P. aeruginosa* genes with activation exceeding 30-fold in mucoid cells and with *P* values of <0.001.

<sup>b</sup> Genes are ordered by decreasing levels of induction.

<sup>c</sup> A Lpt designation indicates the presence of a typical lipoprotein signal sequence.

<sup>d</sup> PA03692 *lptF* was included based on a genetic linkage to PA03691 *lptE* and a statistically significant induction (*P* < 0.005).

whether *P. aeruginosa* lipopeptides activate NF- $\kappa$ B in human respiratory epithelial cells, we first measured NF- $\kappa$ B-dependent promoter activity by use of a luciferase reporter plasmid in primary human bronchial epithelial cells (NHBEs). We exposed NHBEs to the lipopeptides LPTA(6) and LPTB(6) (9), derived from *Pseudomonas* lipoproteins induced in mucoid cells. Responses to LPTA(6) and LPTB(6) were detected (Fig. 2A) that were comparable to the response to the standard bacterial lipoprotein (sBLP) (2, 17). NHBEs did not respond to *P. aeruginosa* LPS. As a negative control, we used a palmitylated cysteine residue (Pam<sub>3</sub>Cys) that does not stimulate TLR2 (2, 17). Pam<sub>3</sub>Cys did not cause NF- $\kappa$ B activation, consistent with a requirement for both the acyl groups and a peptide moiety for lipopeptide recognition by TLRs (2). These results show that lipopeptides derived from lipoproteins induced in mucoid *P. aeruginosa* activate NF- $\kappa$ B in human respiratory epithelial cells.

**TLR2 is expressed in human lung epithelial cells and responds to *P. aeruginosa* lipopeptides.** In contrast to its relatively high expression in lymphoid tissues, TLR2 is believed to be expressed only at a low level in epithelial cells, although TLR2 has been detected in human epithelial HeLa cells (36). For this study, we extended these investigations to verify whether TLR2 is expressed in human respiratory epithelial cells. TLR2 protein expression was detected by Western blots in the bronchial epithelial cell line IB3-1, derived from a CF

patient, by using a monoclonal antibody against human TLR2 (Fig. 2B). Equal amounts of TLR2 were observed in IB3-1 cells and in their CFTR-corrected, genetically matched derivatives C38 and S9 cells (Fig. 2B). Similar results were obtained with primary NHBEs. Since TLR2 is known to be the receptor for bacterial lipoproteins, we next tested whether TLR2 is involved in *P. aeruginosa* lipopeptide-stimulated NF- $\kappa$ B activation in NHBEs (37, 41, 45). In cotransfection experiments with hTLR2 and DN-TLR2 cDNAs, a dependence on TLR2 for LPTA(6) stimulation was observed, as detected by an NF- $\kappa$ B-dependent luciferase reporter assay (Fig. 2C). In addition to NHBEs, two human respiratory epithelial cell lines, 9HTEo<sup>-</sup> (28) and NuLi-1 (46), were tested. Both the dependence on TLR for stimulation with LPTA(6) (Fig. 3A and B) and the presence of TLR2 (Fig. 3C) were demonstrated in experiments with 9HTEo<sup>-</sup> and NuLi-1 cells.

**TLR2 is involved in *Pseudomonas* lipopeptide-induced NF- $\kappa$ B activation in CF cells.** We next assessed the role of *Pseudomonas* lipoproteins and TLR2 in the stimulation of NF- $\kappa$ B in IB3-1 cells, derived from a compound heterozygote CF patient carrying the  $\Delta$ F508 CFTR and W1282X CFTR alleles. In addition to the CFTR mutant cell line IB3-1, its CFTR-corrected derivatives C38 and S9 were included in the study. IB3-1, C38, and S9 cells were transfected with TLR2 or DN-TLR2, exposed to the LPTA lipopeptide, and tested for the ability to activate the NF- $\kappa$ B reporter gene. The CF

TABLE 2. *P. aeruginosa* AlgU ( $\sigma^E$ ) promoter consensus sequence in front of the *lpt* genes

Gene category and no.	Gene name	AlgU consensus <sup>a</sup>	Distance (bp) from initiation codon
Lpt-encoding genes with AlgU promoter consensus sequence			
PA0737	<i>lptD</i>	TC <b>GAACT</b> TGGATCCGTTTCGACGATGGCTACTACGGCT	155
PA1323	Unknown	CT <b>GAACT</b> TTTTCACTGCGGCGCCTAT <b>CAA</b> CTCCTTT	30
PA5526	<i>lptG</i>	A <b>GAAT</b> TCCCTCGATTGCGACGGT <b>CACA</b> AGGGCAA	55
PA1592	<i>lptA</i>	T <b>GAACT</b> TCACGCCAGCGCAAATGTT <b>CAA</b> AGGGCTA	35
PA3262	<i>lptB</i>	TT <b>GAACT</b> TATCCGCGCGCACCTGTT <b>TCCT</b> ATTGCCCA	82
PA3819	<i>slyB</i>	T <b>GAACT</b> TGGTGGTTTTTGGCCAG <b>TCCT</b> AGGCAAGG	60
PA4876	<i>osmE</i>	G <b>GAACT</b> CTGCGCGAGGGCCTGCCT <b>TCCAA</b> TGTTCC	37
Lpt-encoding genes with no discernible AlgU promoter consensus sequence			
PA0062	<i>lptC</i>		
PA3691	<i>lptE</i>		

<sup>a</sup> The AlgU consensus sequence was GAACTT-(16 or 17 bp)-TCCAA-(5 or 6 bp). The consensus matches are shown in bold; underscored residues map mRNA 5' ends.

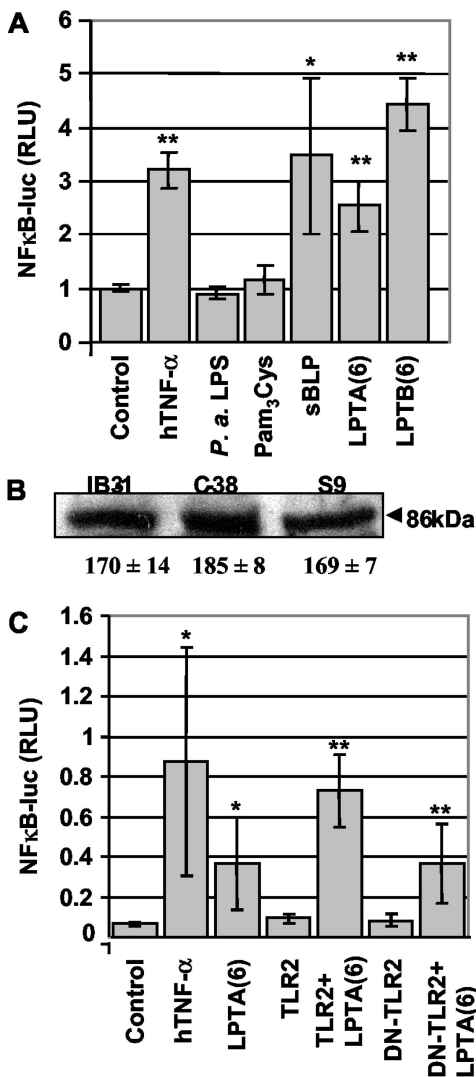


FIG. 2. Lipopeptides induce inflammatory response in human respiratory epithelial cells. (A) Lipopeptides induce NF-κB activation in primary human bronchial epithelial cells. NHBEs were transfected with an NF-κB-responsive luciferase construct and incubated for 6 h in the presence of 1 μg of LPS/ml, 20 ng of TNF-α/ml, or 5 μg of lipopeptide/ml. The data were normalized to a cotransfected β-galactosidase construct. sBLP, synthetic bacterial lipopeptide (Pam<sub>3</sub>Cys-SKXXX-OH); LPTA(6), N terminus of *lptA* gene product (Pam<sub>3</sub>Cys-DKKEE-OH); LPTB(6), N terminus of *lptB* gene product (Pam<sub>3</sub>Cys-DSQTN-OH). (B) Western blot analysis of TLR2 protein expression in CF (IB3-1) and CFTR-corrected (C38) cells with an anti-hTLR2 monoclonal antibody (IMG-319), detected as an 86-kDa band. Equal amounts of proteins were loaded. A densitometry analysis of the TLR2 protein was performed for three independent experiments with NIH Image 1.62 software. The numbers underneath the gel represent the means ± standard errors (arbitrary density units). (C) NF-κB luciferase assay with primary NHBEs showing that TLR2 activates the LPTA(6)-induced response in epithelial cells by employing DN-TLR2. \*, *P* < 0.05; \*\*, *P* < 0.01.

(IB3-1) cells showed a higher reactivity to *P. aeruginosa* lipopeptides than the CFTR-corrected, genetically matched C38 and S9 cells, in a TLR2-dependent manner (Fig. 4A). Transfection with TLR2 further enhanced LPTA-induced NF-κB activation in the mutant IB3-1 cells. Taken together, these data indicate that the TLR2-dependent activation of NF-κB in re-

sponse to *Pseudomonas* lipopeptides is augmented in CFTR mutant epithelial cells.

**Mucoid *P. aeruginosa* lipopeptides induce IL-8 production.** IL-8 is a potent chemoattractant for neutrophils that has been implicated in a neutrophil infiltration and inflammatory cascade in CF (26). Elevated levels of the chemokine IL-8 represent one of the hallmarks of excessive inflammation in CF (3, 15, 19). Thus, we tested whether lipopeptides corresponding to the highly induced lipoproteins in mucoid *P. aeruginosa* could induce IL-8 production in human lung epithelial cells. To measure IL-8 production, we stimulated confluent NHBE monolayers for 24 h with *P. aeruginosa* LPS, with 1 or 10 μg of lipopeptide/ml, or a palmitylated cysteine control or left the cells unstimulated. Both lipopeptides, LPTA(6) and LPTB(6), induced detectable IL-8 levels in culture supernatants (Fig. 4B). Furthermore, the induction of IL-8 production was at least partially suppressed with TLR2-blocking antibodies (Fig. 4C). IL-8 was not induced in response to either LPS or palmitylated cysteine. Thus, primary human respiratory epithelial cells have the machinery to recognize and respond to bacterial lipoproteins and produce IL-8 upon stimulation with *Pseudomonas* lipopeptides.

**DISCUSSION**

The results of this study show that *P. aeruginosa* causes excessive inflammation in CF due to a massive and selective induction of lipoprotein genes upon conversion to mucoidy. The lipopeptides derived from such proteins show a strong proinflammatory potential in primary lung epithelial cells. In our view, these products are toxic to the CF host and thus should be considered bacterial toxins. Due to their significance in CF and their implications in pathogenesis in other infections (1, 24, 33), lipoproteins should be viewed as a double-edged sword in host-pathogen interactions: they can serve both as signals recognized by the host to activate its defenses and as agents causing excessive host damage by the pathogen in some situations, such as in CF. We have consequently designated the highest expressing lipoprotein genes in mucoid *P. aeruginosa* as genes encoding lipotoxins (LPTs): *lptA*, *lptC*, *lptD*, *lptE*, *lptF*, and *lptG* (Table 1).

Increased levels of IL-8 have been noted in the sputum and bronchoalveolar lavage of patients with CF (3, 34). IL-8 is known to play a major role in inflammatory pathogenesis in the airways of CF patients (3, 23, 34, 39). Synthetic lipopeptides corresponding to the N termini of the mature, processed LPTs caused IL-8 production in primary human epithelial cells, as shown here, and in human macrophages derived from peripheral blood monocytes (9). Previous studies have shown IL-8 secretion in response to *P. aeruginosa* in human bronchial epithelial cells (6, 7, 42), although the factors responsible have not been identified. Our results show that the primary human respiratory epithelial cells have the machinery to recognize and respond to bacterial lipoproteins and that the production of IL-8 in CF may be due to stimulation with *Pseudomonas* lipopeptides.

CF cells showed increased responses to lipopeptide stimulation relative to genetically matched CFTR-corrected cell lines. This indicates that CF cells can be primed for TLR2 signaling to levels that are higher than normal. Thus, both the

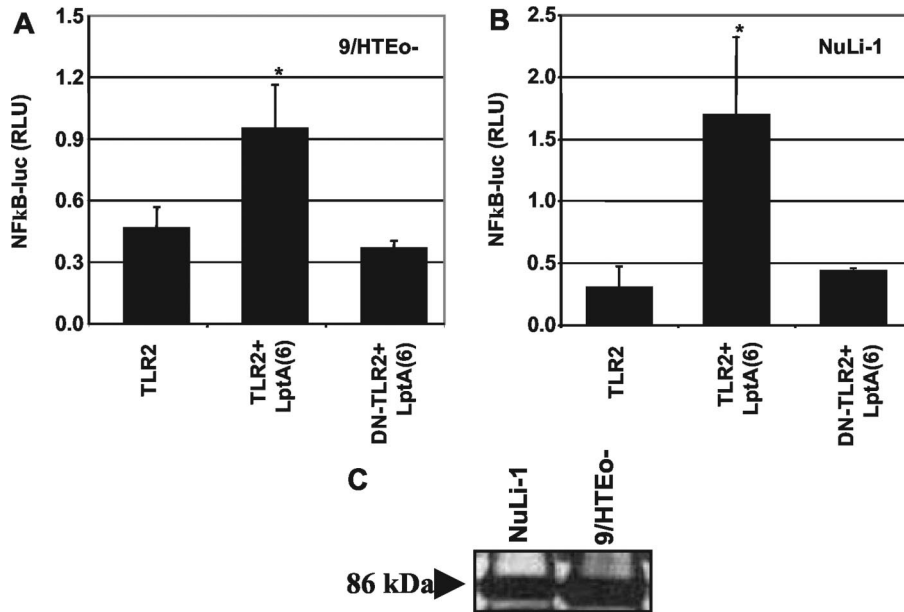


FIG. 3. TLR-dependent induction of NF- $\kappa$ B-mediated transcription in bronchial epithelial cells stimulated with *P. aeruginosa* lipopeptide. 9/HTEo<sup>-</sup> (A) and NuLi-1 (B) cells (both derived from normal human lung cells) were transiently cotransfected with an NF- $\kappa$ B-responsive luciferase reporter plasmid and TLR2 or DN-TLR2 and then incubated for 6 h in the presence of 10  $\mu$ g of lipopeptide (LptA)/ml. The data were normalized to a cotransfected  $\beta$ -galactosidase construct and are means  $\pm$  standard deviations. (C) Western blot analysis of TLR2 protein expression in NuLi and 9/HTEo<sup>-</sup> cells with an anti-hTLR2 monoclonal antibody (IMG-319). Equal amounts of proteins were loaded. \*,  $P < 0.05$ .

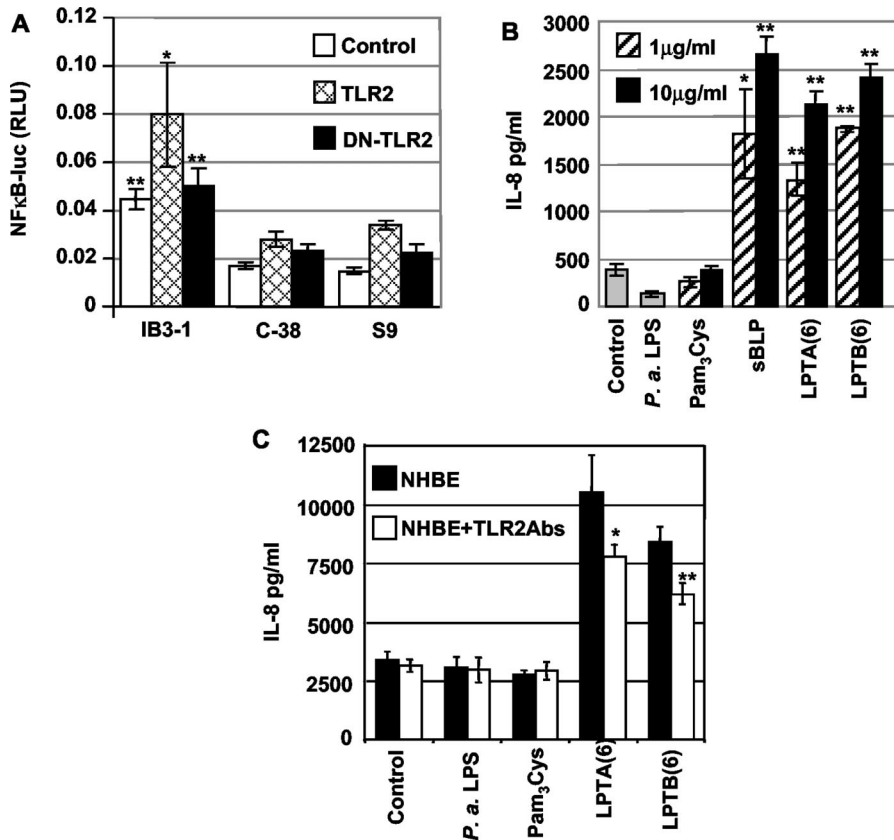


FIG. 4. Proinflammatory action of LptA lipopeptide in the CF bronchial epithelial cell line IB3-1 and its CFTR-corrected derivatives C38 and S9 and TLR2 dependence of the response. (A) Induction of NF- $\kappa$ B-mediated transcription in vitro. For a luciferase assay, IB3-1, C38, and S9 cells were transiently transfected with an NF- $\kappa$ B-responsive luciferase reporter plasmid and TLR2 or DN-TLR2 and then incubated for 6 h in the presence of 5  $\mu$ g of lipopeptide/ml. The data were normalized to a cotransfected  $\beta$ -galactosidase construct. (B) *P. aeruginosa*-based lipopeptides induce inflammatory IL-8 chemokine production by NHBEs. Confluent monolayers of NHBEs were left unstimulated (in medium) or were incubated for 24 h with *P. aeruginosa* LPS, 1 or 10  $\mu$ g of lipopeptide/ml [sBLP, LPTA(6), or LPTB(6)], or a palmitylated cysteine control. (C) NHBEs were stimulated with LPTA(6) and LPTB(6) in the absence or presence of 20  $\mu$ g of TLR2-blocking antibody (TL2.1)/ml. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

pathogen and the host conspire to bring about excessive inflammation in CF. The differences in TLR2 responsiveness between CF and CFTR-corrected cells cannot be explained, however, by differences in the expression levels of TLR2 in CF and CFTR-corrected cells, as we did not observe any dissimilarity between IB3-1, C38, and S9 cells (Fig. 2B). Thus, it is likely that other parts of the signaling pathway downstream of or parallel to the TLR2 function differ between CF and CFTR-corrected cells. For example, recent data that were published while this study was under review suggest that TLR2 may be distributed slightly differently within plasma membrane domains in CF and normal cells (25). Furthermore, TLR2 activity may be additionally amplified in CF by asialoganglioside gangliotetraosylceramide (aGM1) (38), as aGM1 is increased in CF respiratory epithelial cells (18) due to hyperacidification of the *trans*-Golgi network in CF cells (29, 30). Also, note that in our experiments, transfection with DN-TLR2 did not eliminate the endogenous TLR2 response (Fig. 2C). One explanation for this observation is that the endogenous, preassembled signaling complexes responding to lipopeptides [Fig. 2C, control and LPTA(6)] may be resistant [Fig. 2C, LPTA(6) versus DN-TLR2+LPTA(6)] in respiratory epithelial cells to the superimposed expression of DN-TLR2 (defined as dominant negative in myeloid cells).

While in most challenges the TLR response may serve to limit infections, in other situations it can result in tissue destruction, as in CF and other diseases (2). The pattern recognition receptors of the host play a beneficial role in inducing innate clearance mechanisms. However, when preexisting conditions preclude smooth clearance of the invading pathogens, the same receptors may become targets for the destructive action of bacterial toxins such as LPTs, as in the case of *P. aeruginosa* in CF.

The microarray data and follow-up experiments presented here help to explain critical aspects of the runaway inflammatory processes in the CF lung. Other factors linked to the genetic lesion in CFTR and other modifier genes in CF patients certainly contribute to the colonization and establishment of *P. aeruginosa* in the lung (16). Superimposed on such preconditioned milieu, the previously unrecognized massive induction of genes encoding proinflammatory lipoproteins is likely responsible for the tissue destruction that causes high morbidity and mortality of CF patients. We envision that a simultaneous induction of a large number of lipoprotein genes may result in an additive action, although additional more complex synergisms cannot be excluded.

The knowledge of the molecular basis for the excessive inflammation in CF via bacterial lipoproteins and TLR2 may provide targets for blocking or dampening the signaling cascade, including potential applications of neutralizing or blocking antibodies, thus eliminating the factors that are ultimately responsible for respiratory failure in CF patients.

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