TLR-Induced Inflammation in Cystic Fibrosis and Non-Cystic Fibrosis Airway Epithelial Cells

Catherine M. Greene, Tomás P. Carroll, Stephen G. J. Smith, Clifford C. Taggart, James Devaney, Siobhan Griffin, Shane J. O'Neill and Noel G. McElvaney

J Immunol 2005; 174:1638-1646; doi: 10.4049/jimmunol.174.3.1638
http://www.jimmunol.org/content/174/3/1638

References
This article cites 59 articles, 32 of which you can access for free at:
http://www.jimmunol.org/content/174/3/1638.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
TLR-Induced Inflammation in Cystic Fibrosis and Non-Cystic Fibrosis Airway Epithelial Cells

Catherine M. Greene,2* Tomás P. Carroll,* Stephen G. J. Smith,† Clifford C. Taggart,* James Devaney,* Siobhan Griffin,* Shane J. O’Neill,* and Noel G. McElvaney*

Cystic fibrosis (CF) is a genetic disease characterized by severe neutrophil-dominated airway inflammation. An important cause of inflammation in CF is *Pseudomonas aeruginosa* infection. We have evaluated the importance of a number of *P. aeruginosa* components, namely lipopeptides, LPS, and unmethylated CpG DNA, as proinflammatory stimuli in CF by characterizing the expression and functional activity of their cognate receptors, TLR2/6 or TLR2/1, TLR4, and TLR9, respectively, in a human tracheal epithelial line, CFTE290, which is homozygous for the ΔF508 CF transmembrane conductance regulator mutation. We also characterized TLR expression and function in a non-CF airway epithelial cell line 16HBE14o. Using RT-PCR, we demonstrated TLR mRNA expression. TLR cell surface expression was assessed by fluorescence microscopy. Lipopeptides, LPS, and unmethylated CpG DNA induced IL-8 and IL-6 protein production in a time- and dose-dependent manner. The CF and non-CF cell lines were similarly in their TLR expression and relative TLR responses. ICAM-1 expression was also up-regulated in CFTE290 cells following stimulation with each agonist. CF bronchoalveolar lavage fluid, which contains LPS, bacterial DNA, and neutrophil elastase (a neutrophil-derived protease that can activate TLR4), up-regulated an NF-κB-linked reporter gene and increased IL-8 protein production in CFTE290 cells. This effect was abrogated by expression of dominant-negative versions of MyD88 or Mal, key signal transducers for TLRs, thereby implicating them as potential anti-inflammatory agents for CF.


---

*Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin, Ireland; and †Moyné Institute of Preventive Medicine, Trinity College, Dublin, Ireland

Received for publication October 31, 2003. Accepted for publication November 15, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was funded by research grants from Enterprise Ireland (SC/2001/104), Programme from Research in Third Level Institutes administered by Higher Education Authority, Cystic Fibrosis Association of Ireland, Alpha One Foundation, and Programme from Research in Third Level Institutes administered by Higher Education Authority.

2 Address correspondence and reprint requests to Dr. Catherine M. Greene, Respiratory Research Division-Department of Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland. E-mail address: cmgreene@rcsi.ie

3 Abbreviations used in this paper: CF, cystic fibrosis; BALF, bronchoalveolar lavage fluid; CFTR, CF transmembrane conductance regulator; IRF, IFN-regulatory factor; MALP-2, macrophage-activating lipopeptide-2; NE, neutrophil elastase; Pam3, triacylated lipopeptide; PAO1, *P. aeruginosa* strain O1; PAM, PAO1-conditioned medium; PI, propidium iodide; TBE, Tris-borate-EDTA; TIB, TLR- and IL-1R-related; TRIF, TIR domain-containing adaptor inducing IFN-β; uCpG, unmethylated CpG.

---

Copyright © 2005 by The American Association of Immunologists, Inc.
intracellular signaling molecules represent key inhibitory targets for therapeutic drug design.

Patterns of TLR expression have been studied in many different tissues and cell types; however, until recently, TLR expression and function in airway epithelial cells (26, 27), particularly CF airway epithelial cells, remained largely unexplored (28). Furthermore, the contribution of so-called nonimmune epithelial cells to the inflammatory response in the CF lung deserves more detailed investigation. Therefore, given that there are a number of potential TLR agonists in the CF lung (e.g., NE, Pseudomonas lipopolysaccharide, LPS, and DNA), we decided to evaluate TLR expression in CF airway epithelial cells, to use these cells as a model to determine the contribution made by CF epithelium to inflammation in the CF lung, and evaluate the potential of TLR-specific inhibitors to ameliorate the inflammatory response of CF airway epithelial cells.

Materials and Methods

Cell culture and treatments

CFTE29o and CFB614o cells are AF508 homozygous tracheal and bronchial epithelial cell lines, respectively. The 16HBE14o is a non-CF human bronchial epithelial cell line. These were obtained as a gift from D. Gruenert (University of Vermont, Burlington, VT) (29–31). The cells were cultured on plated plates (fibronectin; 1 mg/ml; Sigma-Aldrich), collagen (Vitrogen 100, 2.9 mg/ml; Cohesion Technologies), and BSA (1 mg/ml; Sigma-Aldrich) in Eagle’s MEM (Invitrogen Life Technologies) supplemented with 10% FCS, 1% l-glutamine, and 1% penicillin/streptomycin (Invitrogen Life Technologies). Twenty-four hours before agonist treatment, cells were washed with serum-free Eagle’s MEM and placed under serum-free conditions or in serum containing 1% FCS for LPS or macrophage-activating lipopeptide-2 (MALP-2) stimulations. Human myelomonocytic U937 cells (European Collection of Cell Culture) were cultured in RPMI 1640 containing 10% FCS, 1% l-glutamine, and 1% penicillin/streptomycin (Invitrogen Life Technologies), and were maintained at 37°C in a humidified atmosphere of 5% CO2. Agonist treatments were performed in serum-free or 1% FCS conditions, as appropriate.

Diacylated lipopeptide from Mycoplasma fermentans (MALP-2 (5,5,5,5-tetraacylshikimate-3,4,5-trihydroxycarboxylic acid)-Ala-Gly-OH) (Pam3; Bachem), triacylated lipopeptide (palmitoyl-Cys((RS)-2,3-di((palmitoyloxy)-propanoyl)-Ala-Gly-OH) (Pam3; Bachem), P. aeruginosa lipopolysaccharide (PAM-3), LPS, uCpG, control DNA, PMA, TNF-α, CF bronchoalveolar lavage fluid (BALF), PC, or vehicle controls, as indicated. P. aeruginosa strain 01 (PAO1) was a gift from R. Hancock (University of British Columbia, Vancouver, British Columbia, Canada). PAO1-conditioned medium (PCM) was prepared by filter sterilizing culture supernatants from 72-h PAO1 trypticase soy broth cultures.

TLR mRNA analysis

Total RNA was isolated from 1 x 10^6 cells using TRI reagent (Sigma-Aldrich). Contaminating DNA was removed using DNase 1 treatment. For RT-PCR, 1 µg of total RNA was reverse transcribed into cDNA with an oligo(dT)15 primer using first strand cDNA synthesis kit (Roche). The integrity of RNA extraction and cDNA synthesis was verified by PCR by multiplex PCR were also performed to detect GAPDH, TLR1, TLR3, TLR5, and TLR6 (Santa Cruz Biotechnology, mouse IgG2A anti-human TLR2.1 (eBioscience); mouse IgG2A anti-human TLR4 (Srecot); mouse IgG1 anti-human TLR6 (Alexis Biochimica); or mouse IgG1 anti-human TLR9 (Imgenex)) for 30 min at 4°C. Following three washes, cells were incubated with 10 µg/ml FITC-labeled secondary Ab (anti-goat IgG or anti-mouse F(ab')2 (DakoCytomation)). Cells were counterstained with propidium iodide (PI) (Probes Plus), and laser-scanning cytometry (Compucyte) was used to quantify cell surface TLR expression, as previously described (17). FITC and PI cellular fluorescence of at least 3 x 10^4 cells were measured. TLR expression was quantified using Compucyte software on the basis of integrated green fluorescence. CD14 expression was quantified using a PE-conjugated mouse anti-human CD14 IgG2A Ab (DakoCytomation), and PE cellular fluorescence was measured in all cells. IAC-1 expression was quantified using an FITC-conjugated anti-human IAC-1 Ab (R&D Systems). Appropriate goat, mouse IgG2A, or mouse IgG1 isotype controls (R&D Systems) were prepared for all samples.

Cytokine protein production

Cells (1 x 10^6) were left untreated or stimulated with lipopeptide (MALP-2 or Pam3), LPS, uCpG, control DNA, PMA, TNF-α, CF bronchoalveolar lavage fluid (BALF), PC, or vehicle controls, as indicated. In some experiments, cells were pretreated with actinomycin D (10 µg/ml) or TLR2.1 (eBioscience) (5 µg/ml) 1 h before agonist treatment. IL-8 and IL-6 concentrations in the supernatants were determined by ELISA (R&D Systems). All assays were performed in duplicate or triplicate a minimum of three times.

CF BALF

BALF was collected from individuals with CF (n = 7) following informed consent using a protocol approved by Beaumont Hospital Ethics Committee (32). Samples were filtered through gauze and centrifuged at 1000 x g for 10 min, and cell-free supernatants were aliquoted and stored at -80°C. Antigenic NE levels and NE activity were calculated in pooled BALF, as previously described (33, 34). LPS levels were quantified using the QCL 1000 Luminex amebocyte lysate assay (Cambrex). Pseudomonas DNA was detected in individual BALF samples by PCR using PAO1-specific gene primers for flc (forward, 5'-AACACTGACGCTATCCGGAA-3' and reverse, 5'-CACTGATGTTCTGATGCTTTGAAAT-3'), product 111 bp) and lasB (forward, 5'-CAAATCCCAGCTTAAGTTC-3' and reverse, 5'-CAAGTGGTACCGACG-3'), product 103 bp). Thermocycling conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A final extension step of 72°C for 10 min was performed. Products were resolved on 1.5% TBE agarose gels containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich), and images were captured using the GeneGenius Gel Documentation and Analysis System (Syngene).

Table 1. TLR gene-specific primers

<table>
<thead>
<tr>
<th>Gene (Accession no.)</th>
<th>Primers (5'-3')</th>
<th>Bases</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2 (U88878)</td>
<td>Forward: CCTACATTAGCAAGCACAGTGCACATC</td>
<td>323–346</td>
<td>477</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATCTCCGACGTTCCAAACTCTCA</td>
<td>822–800</td>
<td>418</td>
</tr>
<tr>
<td>TLR4 (U88880)</td>
<td>Forward: CCAGACCAAGAACGATGGAC</td>
<td>954–927</td>
<td>383</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTTCTCCGACGAAAGTG</td>
<td>148–164</td>
<td>293</td>
</tr>
<tr>
<td>TLR6 (AB020807)</td>
<td>Forward: GACCTGAATCCAGAAG</td>
<td>551–517</td>
<td>438–423</td>
</tr>
<tr>
<td>TLR9 (AB045180)</td>
<td>Forward: ATGGTGTTTCTCGCCG</td>
<td>145–159</td>
<td>483–471</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAAGAGATGCCCGAGG</td>
<td>483–471</td>
<td>402–390</td>
</tr>
</tbody>
</table>
Transfection and reporter gene studies

Transfections were performed into cells (1 × 10⁶) with TransFast Reagent (Promega) in a 1:1 ratio, according to the manufacturer’s instructions, using 100 ng of NF-κB-linked luciferase reporter plasmid and 200 ng of either pCDNA3.1 (Invitrogen Life Technologies) or a ΔMyD88 expression plasmid (35, 36) or pDC304 or a Mal P/H expression vector (a gift from A. Bowie, Dublin, Ireland) (37). ΔMyD88 contains only a functional TIR domain and lacks the death domain required for downstream signaling, while Mal P/H is a dominant-negative version of Mal with a Pro125His point mutation in box 2 of the TIR domain. Uniform transfection efficiencies were achieved by initially optimizing transfection conditions using a constitutive luciferase expression vector, pGL3-control (Promega). Cells were then supplemented with additional growth medium for 24 h at 37°C before being left untreated or stimulated, as indicated. Cells were lysed with reporter lysis buffer (Promega), and reporter gene activity was quantified by luminometry (PerkinElmer Wallac Victor², 1420 multilabel counter) using the Promega luciferase assay system, according to the manufacturer’s instructions.

Statistical analysis

Data were analyzed with GraphPad Prism 3.0 software package (GraphPad). Results are expressed as mean ± SE, and were compared by Mann-Whitney U test. Differences were considered significant when the p value was ≤ 0.05.

Results

TLR expression by CF and non-CF airway epithelial cells

CFTE29o⁻ and 16HBE14o⁻ cells were assessed for GAPDH, CD14, TLR1–6, and TLR9 RNA expression by RT-PCR (Fig. 1). Similar TLR and CD14 RNA expression profiles were also seen in CFBE41o⁻ cell lines (data not shown).

TLR1–6, TLR9, and CD14 cell surface protein expression was assessed in CFTE29o⁻ and 16HBE14o⁻ cells using laser-scanning microscopy. Cell surface TLRs1–5 and TLR9, but not TLR6, were detected by microscopy on both the CFTE29o⁻ and 16HBE14o⁻ cell lines (Fig. 1). Both airway epithelial cell lines also expressed only low levels of CD14 on the surface. Western blotting of membrane fractions also detected TLR2, TLR4, TLR5, and TLR9 expression in both cell lines (data not shown).

Diacylated and triacylated lipopeptide, LPS, and uCpG dinucleotides induce IL-8 expression from CFTE29o⁻ cells

The effect of TLR2/6, TLR2/1, TLR4, and TLR9 agonists on IL-8 protein production in CFTE29o⁻ cells was assessed. Diacylated lipopeptide (MALP-2) dose dependently induced IL-8 expression from CFTE29o⁻ cells after 24 h at 4, 40, and 400 ng/ml (p ≤ 0.05). Triacylated lipopeptide (Pam3) and LPS also induced IL-8 expression from CFTE29o⁻ cells after 24 h at doses of 1, 10, and 50 μg/ml (p ≤ 0.05) (Fig. 2A). uCpG DNA induced IL-8 expression from CFTE29o⁻ cells only after 48 h at 10 and 100 μg/ml (p ≤ 0.05). However, uCpG did significantly increase IL-8 expression from myelomonocytic U937 cells at both 24 and 48 h (83 ± 2 vs 120 ± 19 and 106 ± 4 pg/ml IL-8 for control vs uCpG (30 μg/ml) at 24 and 48 h, respectively, p ≤ 0.05). Both phosphodiester- and phosphorothioate-modified uCpG DNA induced similar effects; however, phosphodiester-containing oligonucleotides were used because high concentrations of phosphorothioate-containing oligonucleotides can induce CpG-independent effects (38). The lipopeptide effect was inhibited by pretreatment with a TLR2-neutralizing Ab (Fig. 2B); the LPS effect was inhibited by polymixin B (Fig. 2C); a control oligonucleotide that did not contain uCpG dinucleotides failed to increase IL-8 expression from CFTE29o⁻ (Fig. 2D); and all effects were blocked by pretreatment with actinomycin D (Fig. 2E) (p ≤ 0.05). PCM could also dosedependently increase IL-8 expression from CFTE29o⁻ cells at 24 h at doses of 1, 5, or 10% (v/v) (132 ± 13 vs 201 ± 20, 342 ± 75, and 452 ± 44 pg/ml IL-8 for control vs 1, 5, or 10%, respectively, p ≤ 0.05).

FIGURE 1. TLR expression by CFTE29o⁻ and 16HBE14o⁻ cells. TLR RNA and protein expression were assessed in CFTE29o⁻ (A) and 16HBE14o⁻ (B) cells by RT-PCR and laser-scanning microscopy, respectively. Total RNA was extracted from 1 × 10⁶ cells, reverse transcribed into cDNA, and used as a template in PCR using gene-specific primers. Products were electrophoresed in 1.5% TBE agarose gels containing 0.5 μg/ml ethidium bromide and visualized under UV. For fluorescence microscopy, cells (2 × 10⁶) were grown in chamber slides, Fc blocked, and labeled with anti-TLR (1–6, 9) and anti-CD14 (solid) or isotype control Abs (clear) and fluorophore-conjugated detection Abs. Assays were performed in duplicate.
Similar to their effects in CFTE29o− cells, triacylated lipopeptide, LPS, and uCpG also increased IL-8 protein production from 16HBE14o− cells (p ≤ 0.05) (Fig. 2F). Diacylated lipopeptide failed to increase IL-8 (or IL-6) expression from 16HBE14o− cells after 24 h (data not shown). The relative amounts of IL-8 induced by 16HBE14o− and CFTE29o− cells were similar, although the CFTE29o− cells expressed higher levels of IL-8 basally and following agonist treatment than the 16HBE14o− cells.
IL-6 and ICAM-1 expression are increased by lipopeptides, LPS, and uCpG DNA in CFTE290° cells

Diacetylated and triacetylated lipopeptides, LPS, and uCpG DNA, but not a control oligonucleotide, induced IL-6 expression from CFTE290° cells similar to TNF-α after 48 h (data not shown, p ≤ 0.05) (Fig. 3A). As before, these effects were blocked by polymixin B or actinomycin D, as appropriate (p ≤ 0.05, Fig. 3B). PCM (1% v/v) also increased IL-6 production from CFTE290° cells compared with untreated cells after 24 h (259 ± 34 vs 386 ± 12 ng/ml IL-6 for control vs PCM, p ≤ 0.05).

Expression of the adhesion molecule ICAM-1 on CFTE290° cells was quantified in response to 48 h of stimulation with lipopeptide, LPS, uCpG, or TNF-α (Fig. 3C). All agonists significantly up-regulated ICAM-1 compared with unstimulated control cells (p ≤ 0.05).

ΔMyD88 inhibits lipopeptide-, LPS-, and CF BALF-induced NF-κB reporter gene expression in CFTE290° cells

BALF isolated from individuals with CF (CF BALF) contains proinflammatory factors present within the CF lung, including LPS (39), bacterial DNA, and NE, among others. The seven pooled CF BALF samples contained 677 endotoxin U/ml, equivalent to 1.4 μg/ml LPS, and 743 μg/ml NE, which had 14.3% activity and is equivalent to 3.7 μM NE. Given that the process of CF BALF collection involves a 25- to 50-fold dilution of epithelial lining fluid, these values correspond to ~35–70 μg/ml LPS and 92–185 μM NE on the respiratory surface in vivo. PAO1-specific gene primers were used in PCR to detect the Pseudomonas flagellin subunit gene, fliC, and the Pseudomonas elastase gene, lasB, in each CF BALF sample (Fig. 4A). fliC and lasB were detected in four BALF samples (lanes 1, 2, 4, and 7). We assessed the ability of CF BALF (10 μl) to induce IL-8 protein production from CFTE290° cells and compared its effects with those of NE and LPS alone and in combination (Fig. 4B). Stimulation with both NE (10 nM) and LPS (10 μg/ml) together induced higher levels of IL-8 production compared with each agonist used alone, and their combined effects were less than those induced by 10 μl of CF BALF, which contains 37 nM NE and 14 ng of LPS, and many other proinflammatory factors.

NF-κB reporter gene expression in CFTE290° cells was measured in response to stimulation with pooled CF BALF or lipopeptide, LPS, or PMA for 18 h. Each of these agonists significantly increased NF-κB reporter gene expression (p < 0.05) compared with untreated cells (Fig. 5). uCpG DNA did not increase reporter gene expression after 18 h (data not shown). Cotransfection with a plasmid expressing a signaling-deficient mutant of MyD88, termed ΔMyD88, inhibited the stimulatory effects of lipopeptide, LPS, and CF BALF by 40 ± 15, 43 ± 4, and 33 ± 1%, respectively (p < 0.05). PMA-induced NF-κB reporter gene activation was not inhibited by ΔMyD88.

ΔMyD88 and ΔMal inhibit lipopeptide-, LPS-, and CF BALF-induced IL-8 protein expression in CFTE290° cells

IL-8 protein production by CFTE290° cells was also measured in response to stimulation with lipopeptide, LPS, CF BALF, or PMA for 18 h. Each of these agonists significantly increased IL-8 protein production (p < 0.05) compared with untreated cells (Fig. 6). Overexpression of ΔMyD88 or an inactive mutant of the MyD88 adaptor protein Mal, Mal P/H, inhibited the lipopeptide, LPS, and CF BALF (p < 0.05), but not the PMA-induced effects.

Discussion

Inflammation in the CF lung is a neutrophil-dominated event; however, the epithelium plays an important role by regulating neutrophil recruitment, and therefore contributes significantly to the overall inflammatory response within the lung. In light of the current intensive research in the area of TLRs and general widespread interest in innate immunity, we thought it was timely to investigate in more detail innate immune responses of CF airway epithelial cells, in particular those activated by specific TLRs.

TLRs represent a major arm of the innate immune system. Their principal role is to recognize and discriminate microbial components and mount a rapid protective inflammatory response (8–25). TLR function has been most widely studied in immune cells to date; however, given their broad tissue distribution, it is likely that TLRs fulfill their known roles in other cell types as well. In this study, we have evaluated the roles of specific TLRs in CF airway epithelial cells, in particular those relevant to key proinflammatory agents present in the CF lung. Our studies found that TLRs 1–5 and TLR9 are expressed on the surface of CF and non-CF tracheal and bronchial epithelial cell lines. Others have reported similar findings in 16HBE14o− cells and primary CF nasal polyp tissue in culture (28); however, it has been reported that TLR4 is not surface expressed on BEAS-2B and A549 airway epithelial cells (27), suggesting heterogeneity of TLR expression exists between different airway epithelial cell types. Although mRNA for TLR6 was present in all of the epithelial cell types examined, we failed to detect TRL6 protein expression; nonetheless, the CFTE290° cells were responsive to the TLR2/6 agonist MALP-2. Interestingly, MALP-2 failed to induce a response in the non-CF cell line, suggesting that MALP-2 responsiveness may be enhanced in cells with impaired CFTR and raises the question of whether TLR2 may be a modifier gene for CF. Many studies have proposed the linkage between mutations in CFTR and other genetic loci (40), and it is possible that mutations in CFTR and TLR genes could be linked given the important role of TLR agonists in CF lung disease. The enhanced MALP-2 responsiveness of the CF cell line compared with non-CF cells could also be due to the less tight junctions of CF airway epithelial cells, thus enabling MALP-2 to access additional TLR2 proteins on the basolateral surface of the CFTE290° cells (30). Alternatively, the differential responses observed may be due to tissue specificity given that CF cell line tested was derived from trachea, whereas the non-CF cell line was bronchial derived. Differential responses in these two cell types have been documented in the past (41). Our finding that TLR9 is expressed on the surface of epithelial cells is in contrast to studies performed using macrophages in which TLR9 was found to be localized to endosomes and shown to become activated following internalization of uCpG DNA (42). Similar to a previous report, we found that expression of CD14 by CF and non-CF airway epithelial cells was low (27).

Activation of TLRs 2/6, 2/1, 4, and 9 by their cognate agonists induced proinflammatory responses in the CF airway epithelial cells. Expression of IL-6, IL-8, and ICAM-1, all NF-κB-regulated genes, was increased both time and dose dependently following stimulation with lipopeptides, LPS, or uCpG DNA. MALP-2 is active at very low concentrations and induced functional responses at doses of 4–400 ng/ml. In contrast, the levels of triacylated lipopeptide and LPS required to induce similar responses were significantly higher, 1–50 μg/ml; however, these amounts are physiologically relevant in CF in vivo. In the context of CF, IL-8 is particularly important, as it is a potent neutrophil chemotactic factor (43). It attracts neutrophils to the inflammatory site, where their transepithelial passage is facilitated by ICAM-1 (44). uCpG was a less potent stimulator than lipopeptides or LPS, inducing its effects at 48–72 h rather than 24 h poststimulation. This is a cell-specific phenomenon, as we found that uCpG could strongly induce IL-8 expression in myelomonocytic U937 cells at 24 h.
FIGURE 3. IL-6 and ICAM-1 expression are increased by lipopeptides, LPS, and uCpG DNA in CFTE290− cells. A, CFTE290− cells (1 × 10⁵/ml) were left untreated (−) or stimulated with increasing doses (+, ++, ++++) of diacylated (MALP-2 at 4, 40, and 400 ng/ml) or triacylated (Pam3) lipopeptide or LPS (1, 10, or 50 μg/ml) for 24 h, or uCpG DNA (at 1, 10, or 100 μg/ml) for 48 h. Levels of IL-6 in cell supernatants were measured by ELISA, and values are expressed as picograms per milliliter (+, p ≤ 0.05). B, The effects of polymyxin B (1 μg/ml, as appropriate) and actinomycin D (10 μg/ml, 1 h) on Pam3 (10 μg/ml), LPS (10 μg/ml), or uCpG (100 μg/ml) were also evaluated. C, ICAM-1 expression was quantified on CFTE290− cells stimulated with lipopeptide (Pam3), LPS, uCpG DNA (all at 10 μg/ml), or TNF-α (10 ng/ml) for 48 h. Cells (2 × 10⁵) were grown in chamber slides, Fe blocked, and labeled with FITC-conjugated anti-ICAM-1 or an isotype control Ab. Cells were counterstained with PI, and ICAM-1 surface expression was quantified by laser-scanning microscopy. Assays were performed in duplicate and are representative of at least three separate experiments.
A primary aim of this work was to examine whether so-called nonimmune airway epithelial cells contribute to the inflammatory response in the CF lung. Given that airway epithelial cells do induce functional TLR responses, it appears that they have an important role in pulmonary inflammation in CF given their immense surface area. In addition to lipopeptides, LPS, and uCpG DNA, we also evaluated the proinflammatory properties of PCM and CF BALF on IL-6 and IL-8 protein production by CFTE29o cells. PCM contains factors secreted by stationary phase culture of PAO1, and is highly proinflammatory (45). As well as lipopeptides, LPS, and uCpG DNA, other immunostimulatory factors present in PCM are likely to include flagellin, pyocyanins, Pseudomonas elastases, and exotoxins. Flagellin, the major structural component of flagella, is encoded by the \( fliC \) gene. Purified r\( fliC \) from \( S. \) \textit{enteritidis} is a potent inducer of cytokine synthesis in macrophages (46), and \( S. \) \textit{typhimurium} flagellin has been shown to induce IL-8 production by intestinal epithelial cells (47). Like other members of the TLR family, TLR5 signals via the same intracellular signaling molecules. In this study, we did not directly evaluate the proinflammatory effects of PAO1 flagellin on TLR5-mediated responses because in the CF lung \( Pseudomonas \) exists as a biofilm. Flagellin subunit genes are negatively regulated during biofilm formation (48, 49) and by azithromycin (50), an antibiotic commonly prescribed to individuals with CF, and, as such, flagellin may not be a major proinflammatory stimulus in the CF lung with an established \( Pseudomonas \) biofilm.

CF BALF contains a representative sampling of host and pathogen factors present on the epithelial surface of the lung. In addition to LPS (39), we also detected PAO1 DNA in our CF BALF samples. It has previously been shown that uCpG DNA isolated from CF sputum induces lower respiratory tract inflammation in an animal model (51); however, the epithelium may not play a major role in these events given that TLR9-induced responses were relatively weak in the CFTE29o and 16HBE14o cells. Viruses and ssRNA and dsRNA may also be present in CF BALF. These potentially signal via TLRs 3, 7, 8, and 9 (13, 22, 23, 52) (and TLR4, in the case of respiratory syncytial virus (53)) and are likely to be
important in modulating the immune response in the CF lung during viral infection. Another abundant factor present in the CF BALF was NE, the neutrophil-derived protease that can induce IL-8 gene expression in bronchial epithelial cells via TLR4 (7, 17). Given the heterogeneous composition of CF BALF, it is unlikely that any one single factor is entirely responsible for the proinflammatory properties of CF BALF. Our studies measuring the co-stimulatory effects of different TLR agonists suggest that different components within CF BALF may be additive and combine to induce its potent proinflammatory effects.

We have previously demonstrated that NE-induced IL-8 expression can be inhibited by ΔMyD88 (54). Building on these studies, we show in this study that ΔMyD88 also inhibits NF-κB-linked reporter gene expression in CF airway epithelial cells in response to lipopeptide, LPS, and CF BALF. Furthermore, both ΔMyD88 and a dominant-negative mutant of its adaptor Mal can abrogate IL-8 protein production by each of these stimuli, providing direct evidence of a potential role for these inhibitors as CF therapeutic agents.

Pulmonary inflammation in CF is characterized by production of thick inspissated mucus, bacterial colonization, and severe inflammation, and because of this, many of the efforts directed toward curing CF have targeted the lungs. The results to date with gene therapy have been disappointing, and now many CF researchers are considering a multifaceted approach to curing CF, recognizing that therapies that ameliorate abnormal mucus production, bacterial colonization, and inflammation will almost certainly lead to improved survival.

In addition to MyD88 and Mal, other TIR domain adaptor proteins exist that function in a MyD88-independent fashion. The TRIF protein is responsible for eliciting TLR3 response to viruses and some TLR4 responses to LPS (55, 56). Both TLRs using TRIF to induce IFN-regulatory factor (IRF)-3 or -7 regulated expression of genes encoding IFN-α and -β, IFN-γ-inducible protein-10, and RANTES. Two additional TIR domain-containing adaptors have been identified in humans, termed TRIF-related adaptor molecule and sterile α and HEAT-Armadillo motifs (57). TRIF-related adaptor molecule participates in the TLR4 MyD88-independent pathway, leading to activation of NF-κB and IRF-3 and -7, whereas sterile α and HEAT-Armadillo motifs have yet to be characterized fully. However, it remains to be shown whether targeting the MyD88-independent signaling pathway represents a therapeutic target for preventing inflammation in CF, as there is no information regarding activation of IRF-3/7, IFN-α or -β, or IFN-γ-inducible protein-10 in CF; however, aberrantly low levels of RANTES are secreted by airway epithelial cells, suggesting that this pathway may be impaired in CF cells (58, 59). Nonetheless, it is clear from this and other studies in our laboratory that inhibitors based on the TIR domain of MyD88 or its orthologues could potentially ameliorate pulmonary inflammation induced by TLR and IL-1R agonists. An important challenge for the future will be to develop suitable delivery methods for these inhibitors and determine their compatibility with current conventional CF therapies.

Acknowledgments

We are grateful to A. Bowie (Trinity College) for providing pDC304 and Mal P/H vectors; D. Gruenert (University of Vermont) for the CFTE29o, CFB8E41o, and 16HBE14o cell lines; and R. Hancock (University of British Columbia) for *P. aeruginosa* strain 01.

**References**


Downloaded from http://www.jimmunol.org/ by guest on April 20, 2017


