

# Cystic fibrosis airway epithelial cell polarity and bacterial flagellin determine host response to *Pseudomonas aeruginosa*

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## Summary

The role of epithelial polarity and bacterial factors in the control of the innate immune response of airway epithelial cells to *Pseudomonas aeruginosa* PAK was investigated using a human, nasal cystic fibrosis ( $\Delta F508/\Delta F508$ ) epithelial cell line CF15 grown as confluent layers on permeable supports. Addition of PAK to the basal surface of CF15 layers caused significant expression changes in 1525 different genes (out of 12 625 examined), including the cytokines IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$ , as well as genes associated with leucocyte adhesion, antibacterial factors, and NF- $\kappa$ B signalling. Confocal microscopy showed that nuclear migration of NF- $\kappa$ B in all CF15 cells was preceded by PAK binding to the basal and lateral surfaces of some cells. Addition of PAK to the apical surface of CF15 monolayers elicited changes in expression of only 602 genes, including 256 not affected during basolateral PAK exposure. Over time, cytokine expression during apical PAK was similar to that exhibited by basal PAK, but the magnitudes during apical treatment were much smaller with little/no nuclear migration of NF- $\kappa$ B in CF15 cells. Furthermore, these responses depended on the presence of flagellin, but not pili on the bacteria. Thus, *P. aeruginosa* triggered a strong innate immune response that depended on the apical versus basolateral polarity of CF15 cells and the presence of flagellin on the bacteria.

## Introduction

The lung airways in healthy individuals normally remain sterile through the action of the mucociliary escalator, secretion of antibacterial factors and actions of patrolling macrophages (Crystal, 1991). In cystic fibrosis (CF) these defences are compromised, and the upper airways become colonized with a number of pathogens, most frequently by *Pseudomonas aeruginosa* (Gilligan, 1991). The destruction of the airways in chronically colonized CF individuals is due to the massive influx of polymorphonuclear neutrophils (PMNs) in response to continuous stimulation by bacteria or their products. It is generally accepted that CF airway epithelia are triggered to produce and secrete pro-inflammatory cytokines, including interleukins 6 and 8 (IL-6 and IL-8). These in turn recruit PMNs to transmigrate from the blood to the serosal space and then across the epithelial monolayer to the airway surface liquid (ASL) to combat the bacteria (Bonfield *et al.*, 1999). Thus, airway epithelial cells are likely to be central players in mediating the inflammatory response to *P. aeruginosa*. However, key questions remain unanswered about the *P. aeruginosa* factors, epithelial cell receptors and signalling pathways that govern the initiation of this response.

Transcriptional profiling in cultured A549 pneumocyte cells showed that changes in gene transcription in A549 cells were associated with *P. aeruginosa* binding and required that *P. aeruginosa* express a type IV pilus, although it was unclear whether the response of the epithelial cells to *P. aeruginosa* was specifically mediated by adherence *per se* (Ichikawa *et al.*, 2000). Others have also emphasized the importance of pili in *P. aeruginosa* binding to epithelial cells (Immundo *et al.*, 1995) and the resulting activation of NF- $\kappa$ B and upregulation of IL-8 gene expression and secretion (DiMango *et al.*, 1998; Ratner *et al.*, 2001; Weber *et al.*, 2001). It has been further proposed that *P. aeruginosa* bind to and trigger NF- $\kappa$ B activation and IL-8 upregulation more vigorously in CF versus CFTR-corrected airway epithelial cells (DiMango *et al.*, 1995, 1998; Ratner *et al.*, 2001; Weber *et al.*, 2001). In contrast to these studies, structural investigations and other evidence suggest that pili and asialo-GM1 may play only minor roles in the inflammatory response of epithelia to *P. aeruginosa* (Keizer *et al.*, 2001; Schroeder *et al.*,

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2001). Moreover, the hypothesis that CF airway epithelial cells exhibit hyper-inflammatory responses in response to bacteria has also been questioned (Pizurki *et al.*, 2000; Aldallal *et al.*, 2002; Hybiske *et al.*, 2003).

Several reports have emphasized the important role of *P. aeruginosa* flagella during infection, including their role in motility, adherence and activation of host inflammation. Flagella appear to mediate cell adherence and colonization for a large number of pathogens (McSweeney and Walker, 1986; Eaton *et al.*, 1996; Rabaan *et al.*, 2001; Sousa *et al.*, 2001; Tasteyre *et al.*, 2001; Giron *et al.*, 2002). Components of flagella from *P. aeruginosa* bind to both respiratory mucin (Ramphal *et al.*, 1996) and asialoGM1 of epithelia (Feldman *et al.*, 1998), and may be critical in *P. aeruginosa*-induced pneumonia in mice (Feldman *et al.*, 1998). Flagellin activated NF- $\kappa$ B and upregulated IL-8 gene expression and secretion in airway epithelial cells (Ratner *et al.*, 2001; Weber *et al.*, 2001; also see McNamara *et al.* 2001). Flagellin has also been shown to be an important *P. aeruginosa* determinant of the expression of the matrilysin gene in the serous-like cell line Calu-3 (Lopez-Boado *et al.*, 2001). The potential role of bacterial flagella as a trigger for inflammation in airway epithelia is likely to be mediated through toll-like receptor 5 (TLR5), a pathogen-associated molecular pattern (PAMP) receptor specific for bacterial flagellin (Hayashi *et al.*, 2001).

Many of these apparent conflicts over the roles of bacterial ligands in binding to and activating the host response of airway epithelial cells may have arisen from subtle differences related to epithelial polarization. For example, some investigators have used airway epithelial cell preparations that were incompletely polarized, and bacterial binding and epithelial cell responsiveness may depend critically on whether the bacteria gain access to the apical versus basolateral surfaces of the epithelia. Recent work has shown that most or all bacterial binding occurs at the basolateral surfaces of airway epithelia and that there is likely to be little *P. aeruginosa* binding to the apical surfaces *in vivo* (Ulrich *et al.*, 1998; Lee *et al.*, 1999; also see Apodaca *et al.*, 1995; Fleiszig *et al.*, 1997; Jacob *et al.* 2002; however, see also Immundo *et al.*, 1995; Pier *et al.*, 1997). Because flagellin effects on intestinal epithelia appear to be mediated through basolateral TLR5 (Gewirtz *et al.*, 2001a,b), it might be expected that there would be a similar role for a polarized response to flagellin in airway epithelia.

We hypothesized that exposure of bacteria to the different surfaces of polarized epithelial cells would result in the activation of different signalling pathways which would be detectable by gene expression profiling. We also hypothesized that the stimulatory effects of *P. aeruginosa* pili and flagella would be polarized. We addressed these issues by analysing responses (gene

expression, IL-8 secretion, activation of NF- $\kappa$ B) of polarized human airway epithelial cells infected with *P. aeruginosa* (and isogenic mutants defective in production of flagella and pili) selectively on either their apical or basal side. We utilized JME/CF15 cells, a CF ( $\Delta$ F508/ $\Delta$ F508) nasal cell line that grows as a monolayer with properties expected for airway surface cells: they achieve transepithelial resistances characteristic of the presence of tight junctions ( $R_T \geq 600 \Omega \cdot \text{cm}^2$ ) and exhibit apical-to-basolateral  $\text{Na}^+$  transport that is blocked selectively by low doses (10  $\mu\text{M}$ ) of apical amiloride, indicative for the presence of apical ENaC channels (Boucher *et al.*, 1988; Willumsen and Boucher, 1991; Stutts *et al.*, 1997) and basolateral Na/K-ATPase characteristic of epithelial cells that line the surfaces of the lung airways (Willumsen and Boucher, 1991; Jiang *et al.*, 1993). CF15 cells also respond to TNF- $\alpha$  by producing and releasing their own cytokines (Pizurki *et al.*, 1999), indicating that the cells express receptors and functions expected for airway epithelia. Here we show that the innate immune response of polarized airway epithelial cells is critically dependent on whether *P. aeruginosa* gain access to the apical or basal membranes of the epithelial cells and also on the presence of flagella on the bacteria.

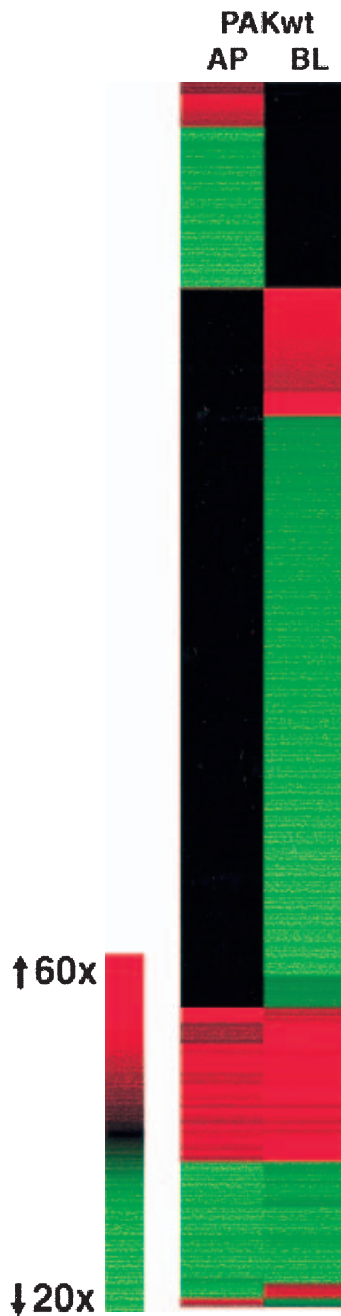
## Results

### *Microarrays to investigate host response to apical versus basal P. aeruginosa*

We examined the global transcriptional response of CF15 cells to exposure to either apically or basally applied *P. aeruginosa*. CF15 cells were grown to confluence on permeable membranes then exposed to strain PAK for 4 h on either the apical or basal surface. As a control, CF15 cells were exposed to assay media without bacteria. Total RNA isolated from 24 transwells was combined for mRNA purification and used as template to generate cRNA targets for hybridization onto Affymetrix U95Av2.0 microarrays. Three separate RNA isolations and microarray hybridizations were accomplished for the final data analysis. Determination of significant expression changes due to either apical or basal *P. aeruginosa* exposure relative to control was made by parametric comparison of the mean signal from each of the infected samples to the control by Student's *t*-test with a *P*-value cut-off of 0.05. We further applied a ratio cut-off of 1.6-fold change in gene expression.

Of the 12 625 genes represented on the U95Av2 GeneChip, 1525 genes displayed a differential transcriptional response in either *P. aeruginosa* infection. A cluster analysis of these genes is shown in Fig. 1 (raw data are accessible in *Supplementary material*, Table S1). This clustering diagram emphasizes the genes that were

uniquely up- or downregulated only in response to *P. aeruginosa* on the basal surface (893 genes, or 58% of the 1525 genes that responded to the bacteria), or only to bacteria on the apical surface (256 genes, or 17% of the 1525 responding genes). Data in Fig. 1 also show genes that responded similarly when bacteria were present on either the apical or basal surface (346 genes, or 23% of the 1525 responding genes). Only 30 genes (2% of the 1525 responding genes) exhibited opposite responses during exposure of bacteria to the apical or basal surface of the CF15 cells.



*Cytokines are upregulated more strongly in response to basal versus apical P. aeruginosa*

Although the data showed that there were different epithelial responses in terms of altered gene expression during bacterial exposure to the apical versus basolateral surfaces, there were no clear patterns (e.g. gene categories or cell signalling pathways) that emerged from careful inspection of the apical or basal datasets (see *Supplementary material*, Table S1). However, cytokine genes were all regulated during apical or, even more prominently, basal exposure of CF15 monolayers to *P. aeruginosa*. Although the epithelial responses were similar during basolateral and apical exposure for a few cytokine genes (see Table 1), the vast majority of the cytokine genes were upregulated most strongly in response to basal *P. aeruginosa* exposure compared with apical exposure. Production of many of these cytokines has been reported previously as being upregulated or, in the case of IL-10, downregulated in CF airways (Bonfield *et al.*, 1999). These data indicated that, for physiologically relevant cytokine genes, the host response to *P. aeruginosa* was much stronger when the epithelia were presented with bacteria on their basal surface.

We used RT-PCR to confirm the microarray data and also to determine the timing of the responses of IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  to apical versus basal *P. aeruginosa* over the course of 0.5–3 h. To test the roles of *P. aeruginosa* flagella and pili in these responses, we also compared the epithelial cell gene responses to those obtained during incubation with *P. aeruginosa*, lacking flagella or pili, two previously reported bacterial signalling ligands using the mutants PAK $\Delta$ fliC and PAK $\Delta$ pilA respectively. After exposing monolayers to *P. aeruginosa* on either the apical or basal sides, cells were cleared of bacteria by thorough rinsing with PBS, and total RNA was isolated and subjected to RT-PCR analysis.

Apical PAK triggered a small increase in expression of IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  genes (Fig. 2A, 1st column), with initial detectable upregulation occurring as early as 1 h

**Fig. 1.** Clustering of apical versus basal *Pseudomonas aeruginosa* microarray data. The mean signal intensities from three independent infection and microarray experiments were used for all calculations. The expression ratios were determined by comparing the signal from CF15 cells exposed either on the apical (AP) or basal (BL) surface relative to the uninfected control signal. Separate lists of genes in each sample displaying significant changes relative to control were determined by Student's *t*-test with a *P*-value cut-off of 0.05 and a 1.6- or larger fold change. Genes that had a significant change in only one of the two samples were given a value of 1.0 for the non-significant ratio as some genes were in one, but not both lists. The resulting 1525 genes were organized by hierarchical clustering. Genes that had an increase in expression relative to the control are shown in red and a decrease in green. Expression changes that were less than 1.6-fold were set to black. The colour scale is set such that the brightest red or green signifies a 60-fold larger or 20-fold smaller change respectively. All microarray data were analysed utilizing the Genespring software package.

**Table 1.** Cytokine gene expression changes with apical versus basal *Pseudomonas aeruginosa*.

| Gene ID  | GeneGene  | Ratio versus control |      |
|----------|---|----------------------|------|
|          |   | AP                   | BL   |
| M36821   | GRO-gamma (GRO3)  | 14.0                 | 26.7 |
| M36820   | GRO-beta (GRO2)   | 13.8                 | 24.0 |
| U64197   | Macrophage inflammatory protein 3 alpha, Exodus-1 (SCYA20)  | 13.1                 | 17.9 |
| U81234   | Granulocyte chemotactic protein 2 (SCYB6)                   | 5.9                  | 16.0 |
| M13207   | Granulocyte-macrophage colony-stimulating factor (CSF2)     | 4.8                  | 16.3 |
| X03656   | Granulocyte colony-stimulating factor (CSF3)                | 5.7                  | 13.6 |
| M13207   | Granulocyte-macrophage colony-stimulating factor (CSF2)     | 5.2                  | 13.9 |
| J04130   | Macrophage inflammatory protein 1-beta (SCYA4)              | 3.0                  | 13.6 |
| M28130   | Interleukin 8 (IL-8)  | 10.5                 | 12.7 |
| X04430   | Interleukin 6 (IL-6)  | 4.1                  | 9.0  |
| X54489   | Melanoma growth stimulatory activity, alpha (GRO1)          | 6.3                  | 8.8  |
| M26683   | Monocyte chemotactic protein 1 (SCYA2)                      | 3.1                  | 8.8  |
| M28225   | Monocyte chemotactic protein 1 (SCYA2)                      | 2.6                  | 7.3  |
| U43185   | Stat5A  | 5.2                  | 6.8  |
| M15330   | Interleukin-1, beta (IL-1 $\beta$ )                         | 3.7                  | 7.1  |
| X02530   | Interferon-gamma induced protein (SCYB10)                   | 5.4                  | 6.0  |
| X04500   | Interleukin-1, beta (IL-1 $\beta$ )                         | 4.1                  | 5.8  |
| U43185   | Stat5A  | 2.8                  | 5.1  |
| X02910   | Tumour necrosis factor, alpha (TNF- $\alpha$ )              | 2.7                  | 4.1  |
| AF030514 | Interferon-inducible T-cell alpha chemoattractant (SCYB11)  | 2.6                  | 4.1  |
| AJ001634 | Monocyte chemoattractant protein 4 (MCP4)                   | 2.4                  | 2.6  |
| X78686   | Epithelial-derived neutrophil activating protein 78 (SCYB5) | 3.5                  | 2.4  |
| M21121   | RANTES (SCYA5)  | 1.0                  | 3.6  |
| M21121   | RANTES (SCYA5)  | 1.0                  | 3.3  |
| U84487   | CX3C membrane-anchored chemokine, Neurotactin (SCYD1)       | 1.0                  | 2.7  |
| M29696   | Interleukin 7 (IL-7)  | 1.0                  | 2.3  |
| M20137   | Interleukin 3; colony-stimulating factor, multiple (IL-3)   | 1.0                  | 2.3  |
| X70340   | Transforming growth factor alpha (TGFA)                     | 1.0                  | 2.0  |
| Y13710   | Macrophage inflammatory protein 4 (MIP4)                    | 1.0                  | 0.3  |
| U16031   | Stat6, IL-4 induced (STAT6)                                 | 1.0                  | 0.4  |
| U16720   | Interleukin 10 (IL-10)                                      | 0.6                  | 1.0  |
| X58377   | Interleukin 11 (IL-11)                                      | 0.6                  | 2.5  |

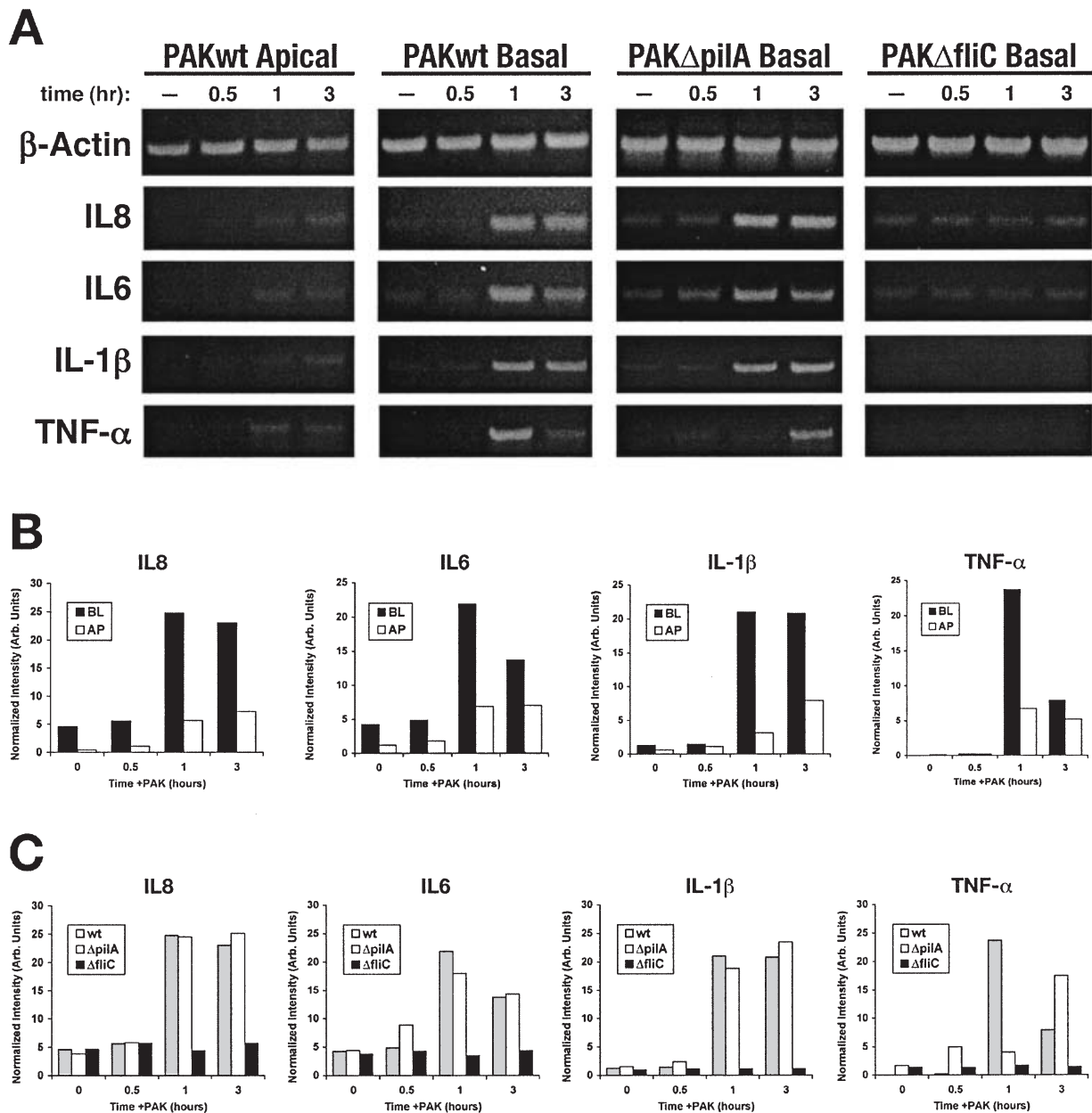
Genes which encode known cytokines or chemokines were manually extracted from the microarray dataset (Fig. 1), and are displayed as expression change ratios versus control (ratio values of 1 correspond to no change). Gene ID contains the corresponding GenBank Accession Numbers.

post-infection. Exposure of CF15 monolayers to wild-type *P. aeruginosa* PAK on the basal side elicited increases in expression of the same genes with a similar time course as with apical *P. aeruginosa* but the magnitude of upregulation was much larger at each time point (Fig. 2A, 2nd column). The differences in upregulation of IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  during *P. aeruginosa* exposure to the apical versus basal sides of the monolayers were also quantified from the gels using densitometric analysis of products generated by RT-PCR (Fig. 2B): band intensities were measured, background-subtracted and normalized to  $\beta$ -actin expression. Results of these experiments showed the following: (i) the similar time course of the upregulation of these genes to the presence of *P. aeruginosa* on the apical and basal sides and (ii) the larger upregulation of these genes to the presence of basal compared with apical *P. aeruginosa*.

Infection of monolayers from the basal side with *P. aeruginosa* that lacked pili (PAK $\Delta$ pilA) gave nearly identical results for both extent and time course of upregulation of the IL-8, IL-6 and IL-1 $\beta$  genes (Fig. 2A, 3rd column and C). The lone difference between the wild type and the PAK $\Delta$ pilA bacteria was for TNF- $\alpha$ , where upregulation at

3 h post-infection appeared to be abrogated, perhaps due to a slower response to the mutant compared with wild-type bacteria. Wild-type *P. aeruginosa* (Fig. 2A, 1st column) and PAK $\Delta$ pilA (not shown) elicited almost identical small effects on cytokine gene expression during exposure of bacteria to the apical surfaces of CF15 cells. In contrast to the results with the pilin mutant, exposure of CF15 cells to basal *P. aeruginosa* which lacked flagella (PAK $\Delta$ fliC) resulted in large reductions of transcriptional upregulation for all four genes (Fig. 2A, 4th column and C), though the small responses of the IL-8 and IL-6 genes to PAK $\Delta$ fliC may indicate responsiveness of CF15 cells to other unidentified *P. aeruginosa* factors. There was no upregulation of cytokine gene expression during exposure of PAK $\Delta$ fliC to the apical surface of CF15 monolayers (not shown). The decreased effectiveness of PAK $\Delta$ fliC did not seem to be attributable to a motility defect, as similar reductions in triggering of cytokine gene expression were obtained with PAK $\Delta$ fliC that were allowed to settle onto the basolateral surfaces of inverted CF15 cultures (not shown).

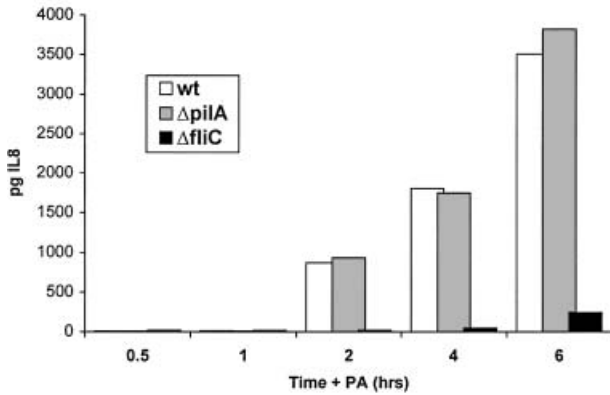
Complementary experiments were performed to test for the ability of airway epithelia to produce and secrete IL-8



**Fig. 2.** Upregulation of cytokine genes in response to *P. aeruginosa* is larger during basal versus apical bacterial exposure and also requires bacterial flagella but not pili. Polarized CF15 monolayers grown on permeable filters were incubated with PAK either apically or basally for 0.5–3 h. Responses were compared among wild-type *P. aeruginosa*, PAK $\Delta$ pilA and PAK $\Delta$ fliC. Control CF15 cell monolayers were treated identically only without bacteria. CF15 cells were then lysed, and extracted RNA was subjected to RT-PCR analysis for IL-8, IL-6, IL-1 $\beta$ , TNF- $\alpha$  and  $\beta$ -actin. Amplified products were visualized using gel electrophoresis and representative data from three separate experiments are shown for the effects of exposure to (A) apical wild-type *P. aeruginosa*, basal wild-type *P. aeruginosa*, basal PAK $\Delta$ pilA and basal PAK $\Delta$ fliC. Band intensities were quantified, background subtracted normalized to  $\beta$ -actin expression for comparisons of the responses to apical versus basal wild-type *P. aeruginosa* (B), or basal PAK versus PAK $\Delta$ pilA versus PAK $\Delta$ fliC (C).

into the basal compartment during exposure of CF15 cells to *P. aeruginosa* on the apical versus basal surfaces. Cell supernatants were collected from the basal compartment after exposure of CF15 monolayers to wild-type *P. aeruginosa* PAK, PAK $\Delta$ pilA or PAK $\Delta$ fliC on the apical side for 0.5–6 h, and the samples were analysed for IL-8. During

exposure to apical *P. aeruginosa*, CF15 cells secreted IL-8 into the basal compartment beginning after 2 h, and this secretion continued for up to 6 h, attaining levels 370-fold higher than uninfected cells (Fig. 3). CF15 cells secreted equivalent amounts of IL-8 in response to PAK $\Delta$ pilA, while the flagellin mutant (PAK $\Delta$ fliC) induced only a small (6.5%)

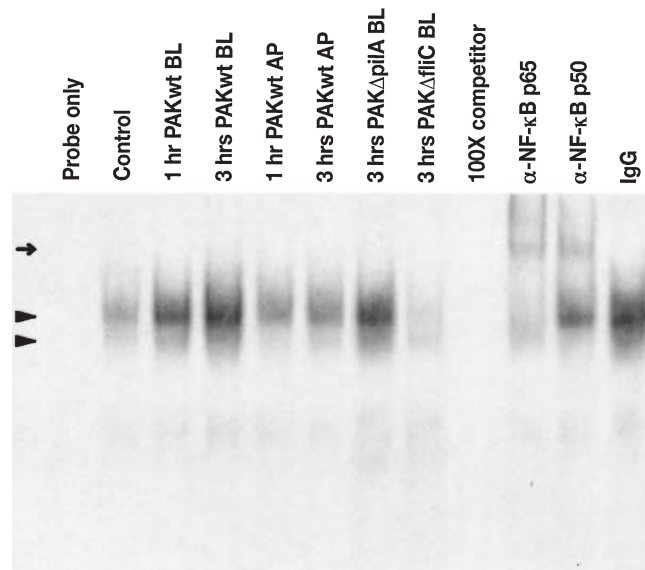


**Fig. 3.** *P. aeruginosa* flagellin-specific induction of IL-8 secretion. Confluent CF15 cells growing on permeable supports were infected for 0.5–6 h with wild-type *P. aeruginosa*, PAK $\Delta$ pilA or PAK $\Delta$ fliC on their apical surface. Cell supernatants were collected from the basal side at the start of the experiment ('0') and after 6 h of bacteria exposure ('6'), cleared of bacteria and cellular debris, and analysed for IL-8 (ELISA). Values from uninfected samples (0.5–6 h) were subtracted from all measurements. Data are an average of ELISAs performed in duplicate and are representative of four similar experiments.

increase in IL-8 secretion over the amount secreted in response to wild type or PAK $\Delta$ pilA (Fig. 3). Secretions of IL-8 from CF15 monolayers infected with PAK, PAK $\Delta$ pilA and PAK $\Delta$ fliC on the basal surface (data not shown) were similar to those induced by the bacteria exposed to the apical surfaces.

#### *NF- $\kappa$ B* activation during exposure of CF15 cell monolayers to *P. aeruginosa* from the apical or basal surface

Results of microarray, RT-PCR and IL-8 secretion analyses indicated that *P. aeruginosa* triggered larger increases in cytokine gene expression when the bacteria had access to the basolateral versus apical membranes of CF15 monolayers. Further, NF- $\kappa$ B is activated in airway epithelia during *P. aeruginosa* infection (DiMango *et al.*, 1998), and many cytokine and other inflammatory genes are known to have NF- $\kappa$ B binding sites in their promoters (Pahl, 1999). We have extracted those genes known to be regulated by NF- $\kappa$ B (Pahl, 1999) from the microarray dataset, and these have been summarized in Table 2. Most of these genes exhibited greater responses (either up- or down-regulation) with *P. aeruginosa* exposed to the basal surface of epithelial cells, compared with responses generated by apical exposure. Particularly pronounced



**Fig. 4.** Infection of basal but not apical surface of polarized CF15 cells induces nuclear translocation of NF- $\kappa$ B. Polarized CF15 monolayers grown on permeable filters were incubated with wild-type *P. aeruginosa* either apically or basally for 1–3 h. After infection, cells were rinsed with PBS and nuclear extracts were isolated. Six independently infected samples were combined for analysis. Electrophoretic mobility shift assays were performed on these extracts with  $^{32}$ P-labelled oligonucleotides containing the NF- $\kappa$ B consensus binding sequence. 'Probe Only' (lane 1) contained free probe without any nuclear material. 'Control' (lane 2) is from cells treated identically to the infected cells but without bacteria for 3 h. Lanes 3–6 contained samples from cells exposed to apical or basal wild-type *P. aeruginosa*. Lane 7 contained samples from cells exposed to basal PAK $\Delta$ pilA for 3 h. Lane 8 contained samples from cells infected with basal PAK $\Delta$ fliC for 3 h. '100 $\times$  competitor' (lane 9) contained the reaction mixture from lane 3 plus 100-fold molar excess of unlabelled NF- $\kappa$ B probe. ' $\alpha$ -NF- $\kappa$ B p65' (lane 10) and ' $\alpha$ -NF- $\kappa$ B p50' (lane 11) show data from supershift reactions containing the lane 3 mixture pre-incubated with either an anti-NF- $\kappa$ B p65 or p50 antibody. 'IgG' (lane 12) is a supershift negative control containing the lane 3 mixture plus 2.2 mg ml $^{-1}$   $\alpha$ -rabbit IgG. Upper arrowhead represents NF- $\kappa$ B complexes containing p65 heterodimers; lower arrowhead denotes faint band containing p50 heterodimers. Arrow marks NF- $\kappa$ B supershifted bands. A representative gel from four similar experiments is shown.

**Table 2.** Effect of apical versus basal *P. aeruginosa* on NF- $\kappa$ B-regulated genes in CF15 cells.

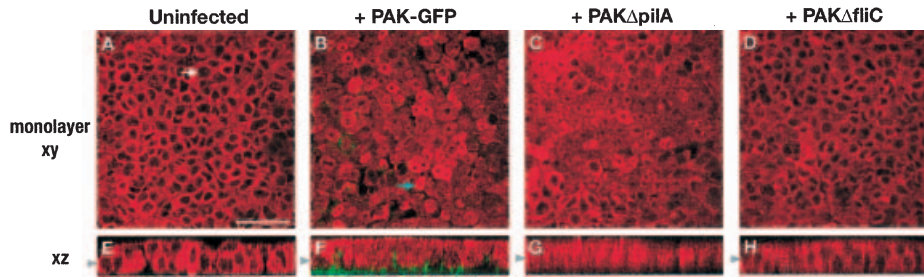
| Gene                | Name/Function                                | Ratio versus Control |      |
|---------------------|--|----------------------|------|
|                     |  | AP                   | BL   |
| IL-8                | Interleukin-8                                | 10.5                 | 12.7 |
| IL-6                | Interleukin-6                                | 3.3                  | 7.8  |
| IL-1 $\beta$        | Interleukin-1 $\beta$                        | 3.7                  | 7.1  |
| IL11                | Interleukin-11                               | 0.7                  | 3.5  |
| TNF- $\alpha$       | Tumour necrosis factor $\alpha$              | 1.3                  | 3.6  |
| Gro- $\alpha$       | Melanoma growth stimulating activity         | 6.3                  | 8.8  |
| Gro- $\beta$        | Macrophage inflammatory protein-2-alpha      | 13.8                 | 24.0 |
| Gro- $\gamma$       | Macrophage inflammatory protein-2-beta       | 14.0                 | 26.7 |
| G-CSF               | Granulocyte colony-stimulating factor        | 5.5                  | 15.6 |
| IFN- $\gamma$       | Interferon-gamma                             | 4.3                  | 11.1 |
| RANTES              | Rantes                                       | 1.3                  | 3.6  |
| MIP-1 $\beta$       | Macrophage inflammatory protein 1-beta       | 4.3                  | 11.1 |
| A20                 | TNF-inducible zinc finger protein            | 6.5                  | 8.2  |
| NFKB1               | NF- $\kappa$ B p105 precursor                | 2.5                  | 3.3  |
| IRF-1               | Interferon regulatory factor 1               | 2.5                  | 2.2  |
| Complement factor B | Complement factor B                          | 3.0                  | 3.9  |
| jun                 | Proto-oncogene                               | 2.7                  | 5.2  |
| c-myc               | Proto-oncogene                               | 0.6                  | 1.5  |
| uPA                 | Urokinase-type plasminogen activator         | 2.0                  | 3.0  |
| COX-2               | Cyclooxygenase                               | 2.4                  | 4.0  |
| CCR5                | Chemokine receptor                           | 2.8                  | 2.7  |
| CD69                | Early T-cell activation antigen              | 4.8                  | 8.5  |
| Serum amyloid A     | Serum component                              | 1.0                  | 3.3  |
| Lipoxygenase        | Leukotriene biosynthesis                     | 1.7                  | 1.8  |
| Mn SOD              | Manganese Superoxide dismutase               | 6.9                  | 2.8  |
| NDUFS2              | NADPH-ubiquinone oxidoreductase              | 0.8                  | 0.6  |
| PAF Receptor        | Platelet activator receptor                  | 3.0                  | 2.8  |
| Bfl-1               | Pro-survival, Bcl-2 related                  | 11.5                 | 15.3 |
| Bcl-XL              | Inhibitor of apoptosis                       | 1.0                  | 0.4  |
| IAP1                | Inhibitor of apoptosis                       | 8.8                  | 15.6 |
| p53                 | Tumor suppressor                             | 1.0                  | 0.4  |
| Thrombospondin      | Adhesive ECM glycoprotein                    | 1.0                  | 0.4  |
| VEGF                | Vascular Endothelial Growth Factor           | 3.5                  | 3.3  |
| APOC3               | Apolipoprotein CIII                          | 2.0                  | 2.3  |
| IGFBP-2             | Insulin-like growth factor binding protein-2 | 1.0                  | 0.2  |
| BDKRB1              | Bradykinin B1 receptor                       | 0.4                  | 1.0  |
| IEX-1               | Immediate early gene                         | 2.1                  | 2.2  |
| EBV                 | Epstein-Barr virus                           | 2.8                  | 5.2  |
| UGCG1               | Ceramide glucosyltransferase                 | 1.2                  | 1.5  |
| Transglutaminase    | Catalyses cross-linking of proteins          | 1.4                  | 1.9  |
| Xanthine oxidase    | Adenine nucleotide catalytic pathway         | 2.0                  | 3.3  |

Genes known to be regulated by NF- $\kappa$ B (Pahl, 1999) and that were among the 1525 genes regulated in response to *P. aeruginosa* were manually extracted. AP and BL values represent expression ratios versus control, with 1 being no change.

was the bacteria-induced response of cytokine and inflammatory genes: IL-8, IL-6, IL-1 $\beta$ , IL-11, TNF- $\alpha$ , Gro- $\alpha$ , Gro- $\beta$ , Gro- $\gamma$ , G-CSF, IFN- $\gamma$ , RANTES, MIP-1, A20, NFKB1 and Complement factor B. We therefore hypothesized that NF- $\kappa$ B activation would occur more prominently during exposure of CF15 monolayers to *P. aeruginosa* on the basal compared with the apical side. To test this hypothesis, we infected CF15 monolayers apically or basally with PAK for up to 3 h and performed electromobility shift assays on the nuclear extracts from these cells. We also tested the roles of pilin and flagellin by performing similar experiments using the corresponding mutant strains PAK $\Delta$ pilA and PAK $\Delta$ fliC respectively.

Nuclear NF- $\kappa$ B levels in uninfected cells were virtually undetectable (Fig. 4). Basal infection of the CF15 monolayers with wild-type *P. aeruginosa* caused a significant

increase in the amount of NF- $\kappa$ B in the nuclei within 1 h post-infection, and this migration increased even further at 3 h post-infection (Fig. 4, upper band). In contrast, apical *P. aeruginosa* stimulated only a small amount of nuclear accumulation of NF- $\kappa$ B. Co-incubation with unlabelled NF- $\kappa$ B probe (Fig. 4, lane 9) reduced the labelling of the  $^{32}$ P-containing band, thus demonstrating competition and specificity of the response. Furthermore, addition of an anti-NF- $\kappa$ B p65 antibody to the EMSA reaction led to a supershift of the upper band, showing that the active NF- $\kappa$ B complex contained p65 heterodimers, while incubation with an anti-NF- $\kappa$ B p50 antibody resulted in a supershift of the faint lower band, indicating that p50 heterodimers were also present, albeit in a much smaller amount. Infection with PAK $\Delta$ pilA for 3 h on the basal side led to an equivalent NF- $\kappa$ B response compared with wild-



**Fig. 5.** Nuclear translocation of NF- $\kappa$ B in individual CF15 cells after basal infection with *P. aeruginosa* and flagellin/pilin deficient mutants. Basal *P. aeruginosa* induce cytosol-to-nucleus migration of NF- $\kappa$ B in all cells throughout the monolayer of polarized CF15 cells; activation occurs normally with PAK $\Delta$ pilA but is prevented with PAK $\Delta$ fliC. Polarized CF15 cells were left untreated or infected basally with PAK-GFP, PAK $\Delta$ pilA or PAK $\Delta$ fliC. After infection, cells were rinsed with PBS, then fixed and stained with an NF- $\kappa$ B p65 antibody. Images (NF- $\kappa$ B, red and PAK-GFP, green) were obtained using a confocal microscope. Lateral XY sections were taken through the monolayer of: uninfected cells (A), cells infected basally for 3 h with PAK-GFP (B), PAK $\Delta$ pilA (C) and PAK $\Delta$ fliC (D). Panels E–H show reconstructed XZ sections from the same fields. The white arrow denotes a typical inactive cell; the light blue arrow shows a typical cell with nuclear NF- $\kappa$ B. Arrowheads in E–H mark sections where lateral slices (A–D) were taken. Results from three representative experiments each. Scale bar = 50  $\mu$ m.

type *P. aeruginosa*, while infection of the basal surface of CF15 monolayers with PAK $\Delta$ fliC resulted in a complete loss of NF- $\kappa$ B activation.

#### Microscopic analysis of NF- $\kappa$ B activation during exposure of CF15 monolayers to apical versus basal *P. aeruginosa*

We used confocal microscopy to test whether the responses (cytokine gene expression and nuclear migration of NF- $\kappa$ B) of CF15 monolayers to basal exposure to *P. aeruginosa* were due to consistent activation in all cells of the monolayer, while the weak response to apical *P. aeruginosa* was due to a small response throughout all the cells of the monolayer or to a large response in only a few cells. *P. aeruginosa*-expressing GFP (PAK-GFP) were used to correlate *P. aeruginosa* binding to the cytosol-to-nucleus movements of NF- $\kappa$ B of the epithelial cells. CF15 monolayers were infected with PAK-GFP for 3 h, cells were fixed and stained with an anti-NF- $\kappa$ B antibody, and images were acquired as 40–50  $\mu$ m stacks (0.5  $\mu$ m slice interval) using a confocal microscope.

In the absence of bacteria CF15 epithelia grew as confluent layers covering the entire filters. Careful examination showed that CF15 cells grew into multilayers in some regions with 50–100 cells growing as small, flattened islands sitting atop the underlying monolayer (see Fig. 5E). Twenty to 50% of the apical surface of the monolayer was covered with these multilayer cells. As shown in Fig. 5A, NF- $\kappa$ B was localized exclusively to the cytoplasm in untreated, control cells, i.e. there was no NF- $\kappa$ B activation under resting conditions.

Exposure to PAK-GFP on the basal surface of the monolayers for 3 h resulted in many bacteria having migrated through the filter and accumulated along basolateral membranes of the monolayer cells (Fig. 5B and F). Although PAK-GFP were occasionally observed to have invaded into the lateral spaces between adjacent epithelial cells

(Fig. 5F), they were never observed inside CF15 cells. Infection with PAK-GFP on the basal side of CF15 cultures led to nuclear migration of NF- $\kappa$ B in 88% of the monolayer cells and 40% of the multilayer cells (Table 3). Activation of multilayer cells in the absence of bacteria that have penetrated the monolayer suggests that a bacteria- or CF15-derived soluble factor may be responsible. Activation of NF- $\kappa$ B in the monolayer typically occurred in groups of 10–30 cells; there were few isolated, active cells.

We also tested the responses of individual CF15 cells to basal infection with PAK $\Delta$ pilA and PAK $\Delta$ fliC. Seventy one per cent of monolayer cells exhibited nuclear (active) NF- $\kappa$ B in response after 3 h exposure to basal *P. aeruginosa* that lacked pili (PAK $\Delta$ pilA in Fig. 5C and G), similar to the 88% for wild-type *P. aeruginosa* (PAK-GFP) infected cells, and 31% of multilayer cells were activated (Table 3). In contrast, infection of CF15 monolayers basally with the flagellin mutant PAK $\Delta$ fliC elicited no activation of NF- $\kappa$ B in either monolayer (Fig. 5D and H) or multilayer cells (Table 3).

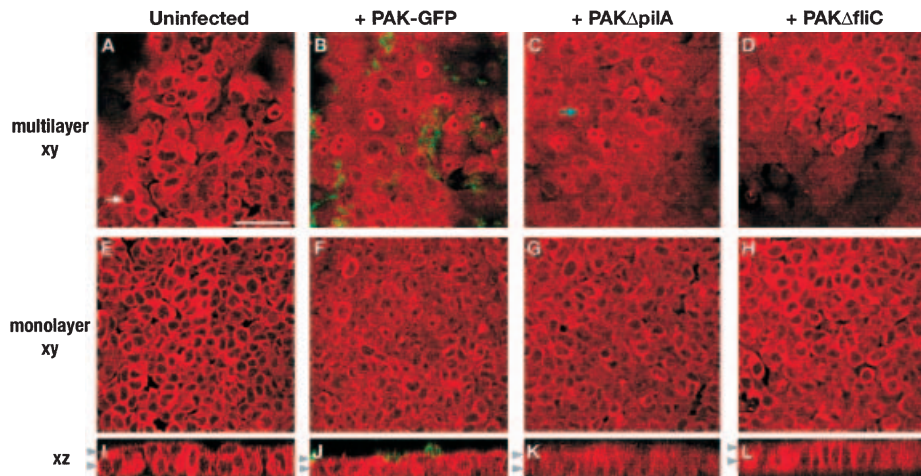
**Table 3.** CF15 cells with nuclear NF- $\kappa$ B after *P. aeruginosa* infection.

|                             | Monolayers                    |    | Multilayers                   |    |
|-----------------------------|-------------------------------|----|-------------------------------|----|
|                             | No. active cells <sup>a</sup> | %  | No. active cells <sup>a</sup> | %  |
| No bacteria                 | 0/1036                        | 0  | 0/348                         | 0  |
| Basal <i>P. aeruginosa</i>  |                               |    |                               |    |
| +3 h PAK-GFP                | 361/408                       | 88 | 86/212                        | 40 |
| +3 h PAK $\Delta$ pilA      | 343/485                       | 71 | 55/177                        | 31 |
| +3 h PAK $\Delta$ fliC      | 0/499                         | 0  | 0/161                         | 0  |
| Apical <i>P. aeruginosa</i> |                               |    |                               |    |
| +3 h PAK-GFP                | 17/456                        | 4  | 93/186                        | 50 |
| +3 h PAK $\Delta$ pilA      | 13/478                        | 3  | 61/223                        | 27 |
| +3 h PAK $\Delta$ fliC      | 0/509                         | 0  | 4/200                         | 2  |

a. Expressed as (number of cells with nuclear NF- $\kappa$ B)/(total number of cells).

Four random XY fields (similar to and including those in Fig. 5 and Fig. 6) were chosen from uninfected cells and from cells infected with PAK-GFP, PAK $\Delta$ pilA or PAK $\Delta$ fliC for 3 h. Total numbers of nuclei and those with nuclear NF- $\kappa$ B were counted.





**Fig. 6.** Nuclear translocation of NF- $\kappa$ B in individual CF15 cells after apical infection with *P. aeruginosa* and flagellin/pilin deficient mutants. Apical *P. aeruginosa* induce cytosol-to-nucleus migration of NF- $\kappa$ B only in poorly polarized multilayers; this activation requires flagellin but not pilin. Polarized CF15 cells were left untreated or infected apically with PAK-GFP, PAK $\Delta$ pilA or PAK $\Delta$ fliC for 3 h. After infection, cells were rinsed with PBS, then fixed and stained with an anti-NF- $\kappa$ B p65 antibody. Images (NF- $\kappa$ B, red and PAK-GFP, green) were obtained using a confocal microscope. Lateral XY sections were taken through the multilayer regions of: uninfected cells (A), cells infected apically for 3 h with PAK-GFP (B), PAK $\Delta$ pilA (C) and PAK $\Delta$ fliC (D), and monolayer regions of: uninfected cells (E), cells infected apically with PAK-GFP (F), PAK $\Delta$ pilA (G) and PAK $\Delta$ fliC (H). Panels I–L show reconstructed XZ sections from the same fields. The white arrow denotes a typical inactive cell; the light blue arrow shows a typical cell with nuclear NF- $\kappa$ B. Arrowheads in I–L mark sections where lateral slices (A–H) were taken. Results from three representative experiments each. Scale bar = 50  $\mu$ m.

Interaction of *P. aeruginosa* with CF15 monolayers from the apical side yielded more complicated responses. Numerous PAK-GFP remained attached after thorough rinsing, and these bacteria were found almost exclusively bound to the multilayer cells (Fig. 6B). Nuclear migration of NF- $\kappa$ B occurred primarily in these multilayer cells after infection with apical PAK-GFP for 3 h: 50% of the cells in the multilayer exhibited nuclear NF- $\kappa$ B, compared with just 4% of the cells in the monolayer (Table 3). The underlying monolayer region of apically infected CF15 cells showed few or no cells with nuclear NF- $\kappa$ B (Fig. 6F), even in regions devoid of the multilayer cells (Fig. 6J, far left). In this regard, confluent monolayers infected with PAK-GFP were indistinguishable from uninfected cells (Fig. 6E) in that they exhibited little or no nuclear migration of NF- $\kappa$ B (Table 3). Infection of CF15 monolayers on the apical surface for 3 h with PAK $\Delta$ pilA resulted in nuclear migration of NF- $\kappa$ B in 27% of multilayer cells (Fig. 6C, Table 3) and just 3% of monolayer cells (Fig. 6G, Table 3). Infections with apical PAK $\Delta$ fliC resulted in only 2% NF- $\kappa$ B activation of CF15 multilayer cells (Fig. 6D, Table 3) and no activation of monolayer cells (Fig. 6H, Table 3).

## Discussion

### *P. aeruginosa* elicit different responses of CF15 cells depending on apical versus basal exposure

One of the most important conclusions to emerge from

these studies is that *P. aeruginosa* elicited different patterns of gene expression in CF15 cell monolayers depending on the accessibility of the apical or basal surface to the bacteria. Many genes exhibited altered expression during the physiological circumstance of having *P. aeruginosa* present on the apical surface, and the expression of an even larger number of genes was influenced when *P. aeruginosa* were exposed to the basal surface. Still other genes exhibited altered regulation when *P. aeruginosa* were present on either side of the monolayers. These results suggested differential expression of receptors for bacterial ligands based on cellular polarity, and involvement of different signalling pathways in sensing apical versus basal *P. aeruginosa*. However, this issue of different signalling and receptor pathways being activated by apical versus basal bacteria remains to be clarified in future studies because, although the basal pathway appears to be the most important in terms of total number of regulated genes (particularly for inflammation-related genes), there was no clear distinction in terms of classes of genes that were up- or downregulated by apical versus basal *P. aeruginosa*.

There were, though, clear similarities of the responses of CF15 cells to *P. aeruginosa* and the responses of both macrophages and monocytes to a variety of pathogens (*Bordetella pertussis*, *Staphylococcus aureus* and *Escherichia coli*) and LPS (Ichikawa *et al.* 2000; Detweiler *et al.* 2001; Boldrick *et al.* 2002), particularly those genes associated with antibacterial properties (Muc1, Muc2,

Muc3, Muc5AC, Muc6, lactotransferrin,  $\beta$ -defensin 2,  $\beta$ -defensin 6, Toll/IL1R-like protein 4), inflammation and cytokines (IL-8, IL-6, IL-1 $\beta$ , Gro1, Gro2, Gro3, CSF2, CSF3, MCP, RANTES), leucocyte adhesion (ICAM1, ICAM3, lipocalin, cadherins and integrins) and NF- $\kappa$ B and MAPK signalling (IKB- $\epsilon$ , TRAF6, MAD3, NF- $\kappa$ B p105, NF- $\kappa$ B p50, JNK3, MKK6, MAPKKK5, MEK5b, c-Jun, JunD, p38-beta, MTK1, MAPKAPK2). In addition, there were similar *P. aeruginosa*-induced increases in IL-8, TNF- $\alpha$ , Gro1, Gro2, Gro3, GM-CSF, G-CSF, RANTES, IRF1 and Cox-2 transcript levels in both the pneumocyte cell line A549 (Ichikawa *et al.*, 2000) and in CF15 cells relative to uninfected controls (Table 1). Thus, it seems likely that a major portion of the inflammatory response observed during exposure of *P. aeruginosa* to CF15 and A549 is characteristic of the 'stereotypical' responses reported for monocytes/macrophages to a variety of pathogens (Boldrick *et al.*, 2002). Future studies will be required to determine whether the similar patterns of pathogen-induced inflammatory gene expression in epithelial cells and macrophages/monocytes are due to the activation of the same cellular receptors and signalling pathways in these different cell types. Further microarray studies should also help to determine whether any of the responses noted for *P. aeruginosa*-triggered CF15 cells are unique to this specific pathogen and to only airway epithelial cells and only during the initial stages of infection – such findings may yield insights into the pathophysiological consequences and disease progression in cystic fibrosis.

*Effects of apical versus basal exposure to P. aeruginosa on NF- $\kappa$ B activation and cytokine gene expression and secretion*

Although the responses (gene microarrays, RT-PCR and nuclear migration of NF- $\kappa$ B) of CF15 cells to basal *P. aeruginosa* were consistently large, the responses to apical *P. aeruginosa* were inconsistent, with somewhat larger responses to apical bacteria in some experiments compared with others. It is unlikely that these differences were due to a few apically added bacteria migrating to the basal side to elicit the observed changes because the CF15 monolayers remained intact with unchanged transepithelial resistance for 5–6 h of apical exposure. Another possibility is that CF15 cells have a smaller and variable number of apical receptors than basolateral receptors with which to respond to *P. aeruginosa* ligands. However, an even more likely explanation emerges from careful examination of the confocal images taken from polarized CF15 cells exposed to apical or basal bacteria: monolayer cells that were directly exposed to basal *P. aeruginosa* exhibited consistent nuclear migration of NF- $\kappa$ B throughout the monolayer, while apical *P. aeruginosa* triggered NF- $\kappa$ B

migration mostly in multilayer cells, where most apical *P. aeruginosa* bound. Thus, the small and somewhat variable cytokine responses to apical *P. aeruginosa* may have been due to the (variable) presence of poorly polarized multilayer cells while few or none of the monolayer cells contributed to the response to apical *P. aeruginosa*.

Yet, if the innate immune response of the CF15 monolayers to apical *P. aeruginosa* was solely due to poorly polarized multilayer cells that have exposed basolateral membranes, then what was the explanation for the microarray data (Fig. 1) showing that there were some unique genes upregulated in response to apical *P. aeruginosa* – shouldn't the bacteria have triggered the same sets of genes during exposure to the apical or basolateral surfaces? One possible explanation for this apparent contradiction is that apical *P. aeruginosa* triggered the basolateral receptors in the exposed multilayer cells, which then produced and secreted cytokines and other factors that activated the underlying monolayer cells, and the net response of the entire epithelium was different than that seen in response to basal *P. aeruginosa*. Consistent with this hypothesis is the observation that cells with active NF- $\kappa$ B were almost always observed as patches of 10–30 cells, never as single cells, suggesting that *P. aeruginosa* might directly activate only a small number of cells, which then in turn induce inflammation in neighbouring cells by a paracrine mechanism.

The cause of the multilayer growth pattern of CF15 cells noted in the confocal images (Fig. 6) is unknown, but one possibility is the tendency of airway epithelial cells to grow into pseudo-stratified epithelia, similar to the *in vivo* state. However, in contrast to *in vivo* airway epithelia where the basal cells appear to be less differentiated than the mucus-secreting and ciliated cells at the surface, our multilayer cells appeared to be incompletely polarized cells growing on top of the intact monolayer. We have observed similar cellular growth patterns in primary cultures of human tracheal and bronchial epithelial cells, so this growth pattern is not restricted to cell lines, and may be a more universal artefact of growing cells in culture.

*Critical role for P. aeruginosa flagellin, but not pilin, in triggering inflammatory response of CF15 cells*

As shown from experiments on CF15 monolayers using isogenic *P. aeruginosa* mutants, flagellin played a key role in the innate immune response of CF15 monolayers to *P. aeruginosa*: upregulation of cytokines and secretion of IL-8 was completely abolished when infections were performed with PAK $\Delta$ fliC. Furthermore, as shown by electromobility shift assay and single cell analysis, the activation of NF- $\kappa$ B also required flagellin. We also note that one preliminary experiment using a microarray showed that

exposure of epithelial cells to PAK $\Delta$ fliC elicited altered expression of only 169 genes (K.H., J.K.I., S.L. and T.E.M., unpubl. obs.), while wild-type *P. aeruginosa* gave changes in expression for 1525 genes (Fig. 1) – i.e. the absence of flagellin caused the altered regulation of only 11% of the 1525 genes that exhibited altered gene expression in response to wild-type PAK. The critical role of flagellin compared with the apparently limited role of pilin in triggering altered epithelial gene regulation could be due to differences in bacterial binding or to differences in the epithelial receptors present on the epithelial cells. Future experiments utilizing GFP-expressing *P. aeruginosa* mutants should be performed to test this possibility.

While the nature of the requirement for flagellin in the epithelial inflammatory response to *P. aeruginosa* is unknown, we hypothesize that the mechanism may involve *P. aeruginosa* flagellin activating toll-like receptor 5 (TLR5), a highly conserved transmembrane receptor that specifically recognizes flagella of Gram-negative bacteria (Hayashi *et al.*, 2001), on basolateral membranes of CF15 cells. Similar observations have been reported for the pathogenesis of *Salmonella typhimurium* on the colonic epithelial cell line T84 (Gewirtz *et al.*, 2001a, b), where flagella secreted by *S. typhimurium* were apparently transcytosed across the monolayer to the serosal space, where it then acted on basolateral TLR5 to trigger inflammatory reactions and the resulting secretion of IL-8. This notion of epithelia being unresponsive to mucosal/apical pathogens with regards to inflammation until either bacteria or bacterial flagellin have gained access to the basal space may prove to be a paradigm for how epithelia detect mucosal pathogens, for the host may not want to mount a full-fledged, but harmful, inflammatory response until pathogens reach the basal surface, e.g. during conditions where the epithelial barrier has been breached.

In contrast to the critical role for flagellin, our data showed that pilin played only a minor role in triggering the host inflammatory response in CF15 monolayers. The differences between the present data and previous work (Immundo *et al.*, 1995; DiMango *et al.*, 1995, 1998; Ratner *et al.*, 2001) may have arisen partly from the use of isolated pili and antibodies to asialo-GM1 in the hopes that these reagents would be informative about the activities of intact bacteria on epithelial receptors. The difference between the present and previous work may also be partially due to the use of polarized airway epithelial monolayers grown on permeable supports in the present study compared with the use of poorly polarized cells grown on impermeable supports. We further note that addition of 0.01–100  $\mu\text{g ml}^{-1}$  LPS from *P. aeruginosa*, to the apical surface of CF15 monolayers elicited no IL-8 secretion (K.H. and T.E.M., unpubl. obs.), despite the presence of serum (and likely LPS binding protein, LBP) in the

incubation medium. Thus, the contribution of *P. aeruginosa* LPS is minimal to the overall cytokine response of CF15 epithelial cells.

#### *A model for P. aeruginosa activation of CF airway epithelial cells in vivo*

Our data indicate that activation of CF airway epithelia in the intact lung by *P. aeruginosa* is not due to bacteria that have accumulated in the thick, sticky mucus in the ASL and occasionally interact with epithelia cell on the apical surface of the respiratory tract. In contrast, exposure of *P. aeruginosa* to the basal surface (e.g. conditions that might exist in damaged or poorly polarized region of the epithelium) leads to activation of the cells via a flagellin-dependent mechanism. This hypothesis assumes that the CF15 cell line is a good model for events that occur in the airway surface epithelial cells *in vivo*. Although we cannot be sure that all the receptors and other critical components normally present in the apical membranes of airway epithelial cells *in vivo* are also present in the CF15 cell line, we note that, in addition to the many selective responses demonstrated here, CF15 cells also exhibit other characteristics of surface cells in the airways of CF individuals: polarized ion and fluid transport (Jiang *et al.*, 1996) and pH regulation (Wunderlich and Machen, 2003) and secretion of IL-8 in response to TNF- $\alpha$  (Pizurki *et al.*, 2000) and IL-1 $\beta$  (K.H. and T.E.M., unpubl.). We are performing experiments to determine whether similar selective, polarized responses to *P. aeruginosa* occur in airway epithelial cells *in vivo*. Work to be presented elsewhere has shown that the CF15 and CFTR-corrected CF15 cells behave identically with regard to binding of *P. aeruginosa*, activation of NF- $\kappa$ B, regulation of cytokine genes and secretion of IL8 (Hybiske *et al.*, 2003). Thus, results presented here for *P. aeruginosa* activation of the innate immune response of CF15 cells probably pertain equally to CFTR-expressing airway epithelia.

Our data also suggest that *P. aeruginosa* induce an inflammatory response in airway epithelia via flagellin acting on basolateral membranes, consistent with a possible polarized localization of the major flagellin receptor TLR5. The implication of these results presents a paradox. Namely, if airway epithelia do not directly elicit an innate immune response to luminal *P. aeruginosa*, how does the inflammation observed in infected CF airways occur? We propose three possible models to explain how this might happen: (i) airway epithelia *in vivo* may contain occasional damaged cells, and these infrequent regions could allow luminal *P. aeruginosa* to migrate to the basal space; (ii) there may be regions in the epithelia with imperfectly formed tight junctions, and these 'hot-spots' would also provide conduits for *P. aeruginosa* to gain access to baso-

lateral membranes; and (iii) other cell types, such as macrophages, are the initial sensors of luminal *P. aeruginosa*, and factor(s) they secrete in response to bacteria in turn trigger inflammation in the underlying epithelia.

## Experimental procedures

### Reagents

pKT-kan-GFP plasmid was generously donated by B. Quiñones and S. Lindow (University of California, Berkeley). Unless otherwise specified, all reagents and chemicals were obtained from Sigma.

### Tissue culture

Cystic fibrosis airway cells CF15, a continuous SV40 large T antigen-transformed nasal epithelial cell line homozygous for  $\Delta$ F508 CFTR, was grown in culture on 4.2 cm<sup>2</sup> transwell membranes (1  $\mu$ m pore size; BD Falcon) in DMEM:F12 media containing 10% FBS, 2 mM L-glutamine, 1% Pen/Strep, 10 ng ml<sup>-1</sup> EGF, 1  $\mu$ M hydrocortisone, 5  $\mu$ g ml<sup>-1</sup> insulin, 5  $\mu$ g ml<sup>-1</sup> transferrin, 30 nM triiodothyronine, 180  $\mu$ M adenine and 5.5  $\mu$ M epinephrine. All CF15 cells were grown in the absence of the antibiotic resistance marker G418. CF15 cells were grown to confluence on permeable filter inserts until they achieved resistances ( $R_T$ ) > 600  $\Omega$ ·cm<sup>2</sup> as measured with an epithelial voltohm-meter (World Precision Instruments);  $R_T$  usually reached a maximum after 5 days in culture.

### Bacterial strains

*P. aeruginosa* strain PAK was used for all studies and was grown overnight in Luria–Bertani media at 37°C with vigorous aeration. Prior to experiments, bacteria were washed three times with PBS and were resuspended in Assay Media (CF15 media minus antibiotics and supplements) at a concentration of  $5 \times 10^8$  cfu ml<sup>-1</sup> ( $OD_{600} = 0.25$ ). Bacteria were incubated with epithelial cells that had been pre-equilibrated with Assay Media overnight at 37°C + 5% CO<sub>2</sub> for 15 min–6 h with an apical multiplicity of infection (MOI) of 50 and basal MOI of 100. For apical infections, 1 ml of bacteria was applied to the apical chamber of CF15 cultures; for basal infections, CF15 cultures were bathed in 2 ml of bacteria on the basal chamber, and the 1  $\mu$ m pore diameter of the transwell membrane facilitated bacteria access. Isogenic mutants of *P. aeruginosa* PAK lacking flagellin or pili (PAK $\Delta$ fliC and PAK $\Delta$ pilA respectively) were engineered by in frame deletion of the *fliC* and *pilA* genes from the chromosome.

GFP-expressing *P. aeruginosa* (PAK-GFP) were generated by introducing into wild-type *P. aeruginosa* PAK the pKT-kan-GFP plasmid (Brandl *et al.*, 2001; Joyner and Lindow, 2000) by electroporation. Briefly, the bacteria were grown overnight in LB, and 100  $\mu$ l were transferred to 5 ml LB. Inoculates were grown for 5 h at 37°C until  $OD_{600} = 1$ . After the culture was cooled on ice, it was centrifuged (10000 r.p.m., 2 min, 4°C) and the bacterial pellet was resuspended in 1.5 ml of cold 10% glycerol in water. Washes were repeated three times. A final pellet was resuspended in 500  $\mu$ l cold 10% glycerol. Next, 100  $\mu$ l of this suspension was mixed with 8  $\mu$ l (8  $\mu$ g) plasmid DNA and elec-

troporated (2 V, 25  $\mu$ F, 250  $\Omega$ ). Transformed cells were rescued with 900  $\mu$ l SOC medium, grown for 3 h at 37°C and plated onto LB-agar plates supplemented with 30  $\mu$ g ml<sup>-1</sup> kanamycin and colonies were recovered after overnight incubation at 37°C. Glycerol stocks were made of viable colonies and kept frozen at –80°C.

### RNA isolation

Total RNA was isolated from  $\sim 5 \times 10^6$  CF15 cells per condition using a phenol–chloroform extraction method (Totally RNA, Ambion); RNA was then eluted and stored in RNase-free H<sub>2</sub>O + 10 mM EDTA. For microarray analysis, RNA was subjected to polyA purification (FastTrack 2.0, Invitrogen).

### cRNA target synthesis, GeneChip hybridization and data analysis

For each GeneChip hybridization, 2  $\mu$ g polyA selected RNA was used as a template to synthesize double-stranded cDNA according to the Affymetrix supplied protocol. The first strand synthesis reaction was accomplished using SuperScript II Reverse Transcriptase (Invitrogen) and T7-(dT)<sub>24</sub> primer (Genset Corp.), followed by second strand synthesis using *E. coli* DNA ligase, *E. coli* DNA polymerase I and *E. coli* RNase H (Invitrogen). The phenol-extracted double-stranded cDNA was used as template in an *in vitro* T7 transcription reaction using the MEGAscript T7 high yield transcription kit (Ambion) and biotinylated nucleotides Biotin-11-CTP, Biotin-16-UTP (NEN, Perkin-Elmer). Total cRNA yield was measured spectrophotometrically and a 20  $\mu$ g aliquot was fragmented in 1 $\times$  fragmentation buffer (40 mM Tris-acetate pH 8.1, 100 mM KOAc, 30 mM MgOAc) at 94°C for 35 min. To check the quality of each sample, the cDNA, full length and fragmented cRNA were analysed by electrophoresis on a 1% Agarose-TAE gel. Next, 15  $\mu$ g of the fragmented cRNA was hybridized to a human GeneChip probe array U95Av2 (Affymetrix) for 16 h at 45°C. The arrays were washed, stained in an Affymetrix fluidics station and scanned according to the manufacturer's protocols. Raw image analysis was performed using the Affymetrix Microarray Suite v5.0 software. Downstream data analysis was performed using GeneSpring v5.0 software (Silicon Genetics).

### RT-PCR

Total RNA was extracted as described above, and was immediately cleansed of genomic DNA contamination by 30 min treatment with 2  $\mu$  DNase (DNA-free, Ambion). cDNA was generated from each sample by incubating 2  $\mu$ g RNA with 0.6  $\mu$ l RNase inhibitor (Roche-BM) + 1.2  $\mu$ l 25 mM dNTP (NEB) + 0.5  $\mu$ l Oligo dT<sub>15</sub> (Promega) + 1  $\mu$ l MMuLV RT (NEB) + 3  $\mu$ l 10 $\times$  RT buffer in a 30  $\mu$ l reaction volume for 1 h at 37°C. The PCR reaction consisted of 5  $\mu$ l cDNA + 2  $\mu$ l 50 mM forward primer + 2  $\mu$ l 50 mM reverse primer + 1  $\mu$ l 10 mM dNTP (NEB) + 0.5  $\mu$ l *Taq* (Promega) + 5  $\mu$ l 10 $\times$  *Taq* Buffer in a 50  $\mu$ l reaction volume. Following a 2 min 94°C hot start, reactions proceeded at: 30 s at 94°C, 45 s at 60°C, 60 s at 72°C for either 25 (ActB, IL-8, IL-1 $\beta$ ) or 35 (IL-6, TNF- $\alpha$ ) cycles. This was followed by a 10 min 72°C elongation phase. Amplifications were performed in a thermal cycler (P-E Cetus DNA Thermal Cycler 480, Perkin-Elmer). Next,

20  $\mu$ l amplified product was run on a 1.5% agarose gel containing 500 ng ml<sup>-1</sup> ethidium bromide and images were obtained with a mounted camera (Polaroid). Prior to experiments, PCR reaction conditions were optimized for each primer pair to ensure that they were performed in the linear range. Quantification was performed by measuring the band and gel-background intensities of scanned gels using NIH Image. Intensities of the bands were subtracted from the background, and normalized by expressing them as a ratio over  $\beta$ -actin subtracted intensities for matching samples.

#### Primers used

$\beta$ -Actin TGGCATTGTTACCAACTGGGACGA GCTTCTCTTTGAT GTCACGCACG; IL-8 ATGACTCCAAGCTGGCCGTGGCT TCTCAGCCCTCTCAAAAACCTTCTC; IL-1 $\beta$  AGTACCTGAGC TCGCCAGTG CTGCTTGAGAGGTGCTGATG; IL-6 GTCTCCT CATTGAATCCAGATTGG AGCTCAGCTATGAACTCCTTCTC; TNF- $\alpha$  ATGAGCACTGAAAGCATGATCCGG GCAATGATCCCA AAGTAGACCTGCC.

#### IL-8 enzyme-linked immunosorbent assay (ELISA)

Supernatants from the apical or basal surface of infected cells were collected, cleared of *P. aeruginosa* and cellular debris by centrifugation (5 min, 1000 g), and stored at -20°C until use. Samples were thawed and diluted 1:100 in 100  $\mu$ l Assay Diluent (BD Pharmingen). Diluted samples were run in triplicate according to manufacturer's protocol (OptEIA Human IL8 Set, BD Pharmingen) and read at 450 nm with an EL<sub>x</sub>808 Ultra Microplate Reader (Bio-Tek Instruments). Resulting data were analysed using Excel (Microsoft).

#### NF- $\kappa$ B electromobility shift assays

CF15 cells were infected with *P. aeruginosa* as described above and rinsed three times with PBS. Cells were scraped from transwell membranes and pelleted. Pellets were resuspended in 10 ml of PME (5 mM PIPES pH 7.2, 5 mM MgCl<sub>2</sub>, 5 mM NaCl, 0.5 mM EDTA) and incubated for 10 min with gentle shaking. Intact nuclei were obtained by passing lysed cells twice through a 1.5 in. 23 gauge needle, and centrifugation for 5 min at 500 g. Nuclear pellets were lysed by resuspending in lysis buffer (50 mM Hepes/KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton-100, 0.1% Na-deoxycholate, plus protease inhibitor cocktail - 1  $\mu$ M PMSF, 2 ng ml<sup>-1</sup> aprotinin, 2 ng ml<sup>-1</sup> pepstatin A and 2 ng ml<sup>-1</sup> leupeptin), and gentle rocking for 2 h at 4°C. Nuclear extracts were dialysed overnight at 4°C against 20 mM HEPES pH 7.9 + 1.5 mM MgCl<sub>2</sub> + 20% glycerol + 0.2 mM EDTA + 100 mM KCl, and purified with a YM-3 Microcon (Millipore). Double-stranded probes were generated by end-labelling NF- $\kappa$ B consensus oligos (sense: 5'-AGTTGAGGGACTTCCCAGGC-3') with [ $\gamma$ -<sup>32</sup>P]-ATP using T4 PNK (NEB). Next, 10  $\mu$ g nuclear extracts was incubated with -40 000 cpm labelled probe for 30 min at 4°C in a reaction containing: 20 mM HEPES pH 7.9, 2.5 mM MgCl<sub>2</sub>, 12% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 2  $\mu$ g dIdC. Supershifts were performed by preincubating nuclear extracts with 4  $\mu$ l anti-NF- $\kappa$ B p65 antibody (Santa Cruz Biotech) for 30 min at 30°C. Reactions were run on

a 4% acrylamide-TBE gel, and bands were visualized with autoradiography.

#### NF- $\kappa$ B Immunofluorescence

CF15 cells were infected with PAK-GFP, PAK $\Delta$ fliC or PAK $\Delta$ pilA as described above, and then rinsed three times with PBS. Cells were then fixed for 5 min with 3.7% formaldehyde in PBS, rinsed with PBS and permeabilized with 0.5% Triton X-100 (in PBS) for 15 min. After blocking with 1% BSA-PBS for 20 min, cells were incubated for 1 h at room temperature with an anti-NF- $\kappa$ B p65 antibody (Santa Cruz Biotech), followed with thorough rinses with 1% BSA-PBS. Finally, cells were incubated for 1 h with an Alexa546-anti-rabbit secondary antibody (Molecular Probes) and 5  $\mu$ M DAPI (Molecular Probes). Images were obtained using a confocal microscope (Axioplan LSM 510, Zeiss, Oberkochen, Germany) with a 40 $\times$  water objective (NA = 0.8). Enhancements were performed with both Volocity LE (Improvision) and Photoshop 7 (Adobe) on a Macintosh G4 (Apple). For quantification of NF- $\kappa$ B migration for a particular experimental condition, three random fields were selected and viewed as 2-colour images: NF- $\kappa$ B - red, DAPI (nuclei) - blue. These fields were counted for total cell number (DAPI-stained nuclei) and for cells with active (nuclear) NF- $\kappa$ B. In these two-colour images, active cells exhibited pink nuclei (versus blue for inactive cells) nuclei, which facilitated counting.

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#### Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/cmi/cmi342/cmi342sm.htm>

**Table S1.** CF15 gene expression changes in response to *Pseudomonas aeruginosa*. Raw data from microarray cluster analysis (Values = ratios versus control).

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