

## RESEARCH PAPER

# Pattern recognition receptors and interleukin-8 mediate effects of Gram-positive and Gram-negative bacteria on lung epithelial cell function

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**Background and purpose:** Lung epithelial cells express pattern recognition receptors, which react to bacteria. We have evaluated the effect of Gram-positive and Gram-negative bacteria on interleukin-8 (CXCL8) release from epithelial cells and the integrity of the epithelial barrier.

**Experimental approach:** Primary cultures of human airway epithelial cells and the epithelial cell line A549 were used, and CXCL8 release was measured after exposure to Gram-negative or Gram-positive bacteria. Epithelial barrier function was assessed in monolayer cultures of A549 cells.

**Results:** Gram-positive bacteria *Staphylococcus aureus* or *Streptococcus pneumoniae*, induced release of CXCL8 from human airway epithelial cells. These bacteria also disrupted barrier function in A549 cells, an effect mimicked by CXCL8 and blocked by specific binding antibodies to CXCL8. Gram-negative bacteria *Escherichia coli* or *Pseudomonas aeruginosa* induced greater release of CXCL8 than Gram-positive bacteria. However, Gram-negative bacteria did not affect epithelial barrier function directly, but prevented disruption induced by Gram-positive bacteria. These effects of Gram-negative bacteria on barrier function were mimicked by FK565, an agonist of the nucleotide-binding oligomerization domain 1 (NOD1) receptor, but not by the Toll-like receptor (TLR) 4 agonist bacterial lipopolysaccharide. Neither the Gram-negative bacteria nor FK565 blocked CXCL8 release.

**Conclusions:** These data show differential functional responses induced by Gram-negative and Gram-positive bacteria in human lung epithelial cells. The NOD1 receptors may have a role in preventing disruption of the epithelial barrier in lung, during inflammatory states.

*British Journal of Pharmacology* (2008) **154**, 864–871; doi:10.1038/bjp.2008.139; published online 21 April 2008

**Keywords:** Gram-positive bacteria; Gram-negative bacteria; Toll-like receptors (TLRs); nucleotide-binding oligomerization domain (NODs) proteins; interleukin-8 (CXCL8)

**Abbreviations:** CXCL8, human interleukin-8; FSL-1, lipopeptide Pam2Cys-GDPKHPKSF; LPS, lipopolysaccharide; NOD1, nucleotide-binding oligomerization domain 1; Pam<sub>3</sub>CSK4, triacylated synthetic lipoprotein; PAMPs, pathogen-associated molecular patterns; PPRs, pathogen response receptors; TLRs, Toll-like receptors

## Introduction

Epithelial cells line the airways forming a physical and metabolic barrier between the environment and the host. Innate immune responses of the host are initiated by activation of pattern recognition receptors (PRRs), which include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Human airway epithelium has been shown to express

functional TLR1–6 and TLR9 (Armstrong *et al.*, 2004; Platz *et al.*, 2004; Greene *et al.*, 2005). Gram-positive bacteria contain within their structure pathogen-associated molecular patterns (PAMPs) specific for TLR2, which heterodimerizes with TLR1 or TLR6 (Mitchell *et al.*, 2007). Gram-positive bacteria also contain peptidoglycans (PGN), comprising the L-lysine (Lys)-type PGN, which is a ligand for NOD2 receptors. Gram-negative bacteria contain PAMPs for a number of TLRs, the most notable being lipopolysaccharide (LPS), which is a ligand for TLR4. Gram-negative bacteria also contain PGN comprising the *meso*-diaminopimelic acid (mDAP)-type PGN, which is a ligand for NOD1 receptors (Chaput and Boneca, 2007). Lung epithelium has been shown to sense whole bacteria *in vitro* resulting in the release

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Received 11 December 2007; revised 15 February 2008; accepted 3 March 2008; published online 21 April 2008

of inflammatory biomarkers. Interestingly, we (Duffin *et al.*, 2005) and others (Mayer *et al.*, 2007) have found that Gram-positive bacteria activate lung epithelium to release human interleukin-8 (CXCL8), substantially less than Gram-negative bacteria. CXCL8 is a chemokine that promotes the recruitment of neutrophils, which are considered the first line of defence against infection.

In this study, we have extended our previous work and that of other groups in this area investigating the ability of whole Gram-positive and Gram-negative bacteria to activate primary human lung epithelial cells, or the well-described human lung epithelial cell line A549 cells. To understand the role of individual PRRs, we have also used ligands specific for TLRs and NLRs. We have measured CXCL8 release and expression, as well as barrier function in the presence and absence of bacteria and selected PAMPs. Our data implied a role for CXCL8 in the loss of barrier function induced by bacteria and a role for NOD1 receptors in preserving the integrity of this barrier.

## Materials and methods

### *Culture and preparation of bacteria*

Clinical blood culture isolates of *Staphylococcus aureus* strain H380 and *Escherichia coli* 0111.B4 were stored frozen in 15% glycerol. To culture, they were first streaked onto agar plates from which single colonies were inoculated into RPMI 1640 medium with 10% fetal bovine serum and glutamine. Cultures were incubated at 37 °C overnight and then centrifuged at 800g to pellet the bacteria. Bacteria were washed twice, and re-suspended, in sterile saline. Aliquots of the bacterial suspension were serially diluted and plated onto agar to quantify the cell density. The bacterial suspensions were then heat treated for 45 min at 80 °C to kill all bacteria (Jimenez *et al.*, 2005). Sterility was confirmed by plating the resultant suspension. Suspensions were adjusted to  $10^{10}$ – $10^{12}$  colony forming units per mL (CFU mL<sup>-1</sup>) and stored frozen at -20 °C in saline containing 15% glycerol.

### *Culture of human primary airway epithelial cells*

Primary bronchial epithelial cells were obtained by protease digestion of bronchial tissue during resection of tumours from patients with lung cancer with their permission. The collection of human samples was approved by the Ethics Committees of the Royal Brompton Hospital and of St Mary's Hospital. All tissue samples used to isolate airway epithelial cells were morphologically normal, that is, non-tumour tissue. The primary epithelial cells isolated in this way were grown in T25 or T75 flasks in Airway Epithelial Cell Growth Medium (Promocell, Berlin, Germany) supplemented with bovine pituitary extract (0.4%), epidermal growth factor (0.5 ng mL<sup>-1</sup>), insulin (5 µg mL<sup>-1</sup>), adrenaline (0.5 µg mL<sup>-1</sup>), triiodothyronine (6.7 ng mL<sup>-1</sup>), transferrin (10 µg mL<sup>-1</sup>), retinoic acid (0.1 ng mL<sup>-1</sup>), penicillin (100 U mL<sup>-1</sup>), streptomycin (100 µg mL<sup>-1</sup>) and amphotericin (1.5 µg mL<sup>-1</sup>). This medium is hereafter referred to as 'complete medium'. For experimental procedures, airway epithelial cells were seeded at  $3.5 \times 10^3$  cells per well of

48-well plates and maintained in complete medium. Primary cultures sometimes include infected cells. To identify and exclude any cells with underlying infections, antibiotics were removed from the culture medium up to 48 h before the cultures were used experimentally. Within this time, any pre-existing infections were obvious and any such cultures with infections were not used in this study. At approximately 80% confluence, airway epithelial cells were treated with medium,  $10^7$  or  $10^8$  CFU mL<sup>-1</sup> heat-killed *E. coli* or *S. aureus*, and supernatants collected after 24, 48 and 72 h for the measurement of CXCL8 release. Cells were used at passage 4 or less.

### *Culture of human lung epithelial cells (A549)*

The human lung alveolar epithelial cell line A549 was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U mL<sup>-1</sup>), streptomycin (100 µg mL<sup>-1</sup>) and Modified Eagle's Medium (MEM) non-essential amino acids (1% v/v) in an atmosphere of 5% CO<sub>2</sub> at 37 °C. Before experimentation, cells were seeded into 96-well plates at a concentration of  $10^5$  cells per well and allowed to rest for 24 h before stimulation with whole bacteria or selective TLR (Alexander *et al.*, 2007) and/or NOD ligands for 24 h. Supernatants were removed and used for the measurement of CXCL8 levels. The effect of treatments on A549 metabolism was assessed by measuring the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 20 µg mL<sup>-1</sup>; Sigma, Poole, UK) to formazan. Medium was removed and formazan dissolved by the addition of dimethyl sulphoxide (100 µL per well). Formazan levels were quantified by measuring absorbance at 550 nm. This was performed after all the treatments, and unless stated, no effects were noted.

### *Measurement of CXCL8 production*

CXCL8 (Alexander *et al.*, 2007) content in cell-free supernatant was determined by ELISA using commercially available matched antibody pairs following a protocol supplied by the manufacturers (R&D Systems, Oxford, UK). CXCL8 concentrations were calculated using a standard curve with authentic CXCL8, provided by the above-described company and results expressed as pg mL<sup>-1</sup> as described previously (Stanford *et al.*, 2000).

### *Assessment of the effects of Gram-positive S. aureus on CXCL8 stability*

Recombinant human CXCL8 (R&D Systems Ltd, Abingdon, UK) was diluted serially in 5% DMEM to concentrations of 2000, 1000, 500 and 250 pg mL<sup>-1</sup>. A 90 µL volume of each standard was added to wells containing A549 cells and also onto a naked plastic culture plate containing no cells. *S. aureus* was diluted in 5% DMEM from their stock concentrations to produce bacterial densities of  $10^8$  CFU mL<sup>-1</sup> mixed with standard CXCL8. The plates were incubated for 24 h at 37 °C and 5% CO<sub>2</sub>. After 24 h, supernatants were transferred into fresh 96-well plates and stored at -20 °C until recover could be measured after analysis of medium for CXCL8 by ELISA.

*Assessment of epithelial barrier integrity*

A549 cells were grown to confluence on cell culture inserts (PET membrane, 3.0  $\mu\text{m}$  pore size; BD Falcon, BD-Biosciences Discover Labware, Bedford, MA, USA) in studies to assess barrier integrity. Cells were plated 48 h before the treatment. Bacteria or PAMPs were added to cells for 30 min, after which horseradish peroxidase (HRP, 1 mU mL<sup>-1</sup>; Sigma) was added to the upper compartment. Aliquots of 50  $\mu\text{L}$  were collected from the lower compartment 1 h after HRP addition. HRP concentration was measured by its ability to convert pyrogallol (5% w/v, freshly prepared; Sigma), dissolved in 100 mM potassium phosphate buffer (pH 6.0; Sigma), into purpurogallin in the presence of H<sub>2</sub>O<sub>2</sub> (0.5% w/w; Sigma) (Chance and Maehly, 1955). The total volume of the solution was 1 mL and the incubation time was 5 min; the conversion was measured by absorbance at 405 nm read in a standard spectrophotometer. The amount of purpurogallin was stoichiometrically correlated to the amount of HRP detected in the supernatant. A standard curve of HRP (0.8–62.5 mU mL<sup>-1</sup>) was used to calculate concentrations. The results were expressed as HRP U mL<sup>-1</sup>.

*Measurement of CXCL8 gene expression by real-time PCR*

After treatments, medium was removed from cultures, cells frozen until mRNA was extracted and cDNA generated as previously described (Paul-Clark *et al.*, 2006). Transcript levels were determined by real-time PCR (Rotor Gene 6; Corbett Research, Sydney, NSW, Australia) using Taqman Universal PCR master mix and commercially available primers for CXCL8 (Hs00174103\_m1; Applied Biosciences, Foster City, CA, USA). PCR conditions were as follows: step 1, 10 min at 95 °C; step 2, 15 s at 95 °C; step 3, 60 s at 60 °C and repeated for 40 cycles. Data from the reaction were collected and analysed (Corbett Research). Relative quantifications of gene expression were calculated using standard curves and normalized to GAPDH (NM\_002046.3; Applied Biosystem).

*Statistical analysis*

All data shown are means  $\pm$  s.e.mean. Statistical analysis was performed using Student's *t*-test, one-way ANOVA, followed by post-tests or one-sample *T*-test as appropriate and as described in each figure legend using GraphPad software. Experimental *n*-values generally refer to data obtained in separately treated wells of cells. In most cases, experiments were performed on at least three separate experimental days. For experiments using primary cultures of human bronchial airway epithelium, cells from at least two separate donors were used. Experiments shown in Figure 3 were performed in duplicate. Values of  $P \leq 0.05$  were considered statistically significant.

*Reagents*

Bacterial suspensions (10<sup>7</sup> or 10<sup>8</sup> CFU mL<sup>-1</sup>) were prepared as described previously. LPS (*E. coli*-derived, 1  $\mu\text{g}$  mL<sup>-1</sup>) was purchased from Sigma. MDP-Lys18 (1  $\mu\text{M}$ ), FK565 (1  $\mu\text{M}$ ), Pam2CGDPKHPKSF (FSL-1, 100 ng mL<sup>-1</sup>) and Pam<sub>3</sub>CSK4 (300 ng mL<sup>-1</sup>) were purchased from Invivogen (San Diego,

CA, USA). The working concentration for each compound was chosen from a concentration–response curve to evaluate the suboptimal effect on CXCL8 release from human bronchial epithelial cells and A549 cells (data not shown).

**Results***The effect of E. coli or S. aureus on CXCL8 release by human primary or A549 lung epithelial cells*

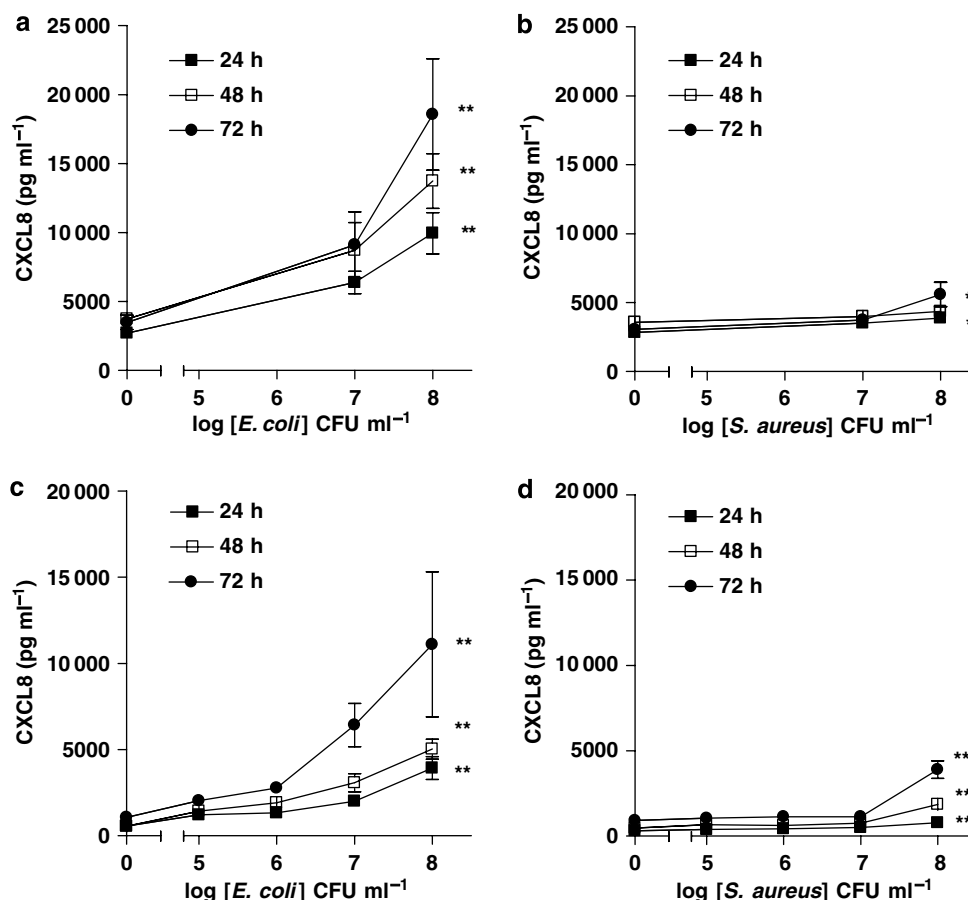
Primary cultures of human bronchial epithelial cells released low, but detectable levels of CXCL8 under control culture conditions. However after stimulation with the Gram-negative bacteria, *E. coli* (10<sup>7</sup> and 10<sup>8</sup> CFU mL<sup>-1</sup>), primary cultures released increased levels of CXCL8 in a concentration- and time-dependent manner (Figure 1a). By contrast, when cells were stimulated with *S. aureus* (10<sup>7</sup> and 10<sup>8</sup> CFU mL<sup>-1</sup>) (Figure 1b), the release of CXCL8, although statistically significant at 24 and 72 h after stimulation, was much lower than that detected when cells were stimulated with *E. coli* at each concentration (Figure 1b). As observed with primary cultures of human bronchial epithelial cells, the human lung epithelial cell line A549 released low or undetectable levels of CXCL8 under control culture conditions. Again, as with primary cells, A549 lung epithelial cells released increased levels of CXCL8 in response to Gram-negative *E. coli* (10<sup>5</sup>–10<sup>8</sup> CFU mL<sup>-1</sup>) or *Pseudomonas aeruginosa* (Figure 2) in a concentration- and time-dependent manner (Figure 1c). However, and as was the case with primary cells, A549 lung epithelial cells released much lower levels of CXCL8 when stimulated with Gram-positive *S. aureus* (Figure 1d) or *S. pneumoniae* (Figure 2). Nevertheless, despite levels being relatively low, and again as seen with primary cells, the release induced by *S. aureus* from A549 cells was statistically significant at each time point (Figure 1d). Specific PAMPs for TLR4 (LPS), TLR2/1 (Pam<sub>3</sub>CSK4) or TLR2/6 (FSL-1) activated A549 epithelial cells to release increased levels of CXCL8 (Table 1).

*Effect of S. aureus on stability of CXCL8 in culture medium*

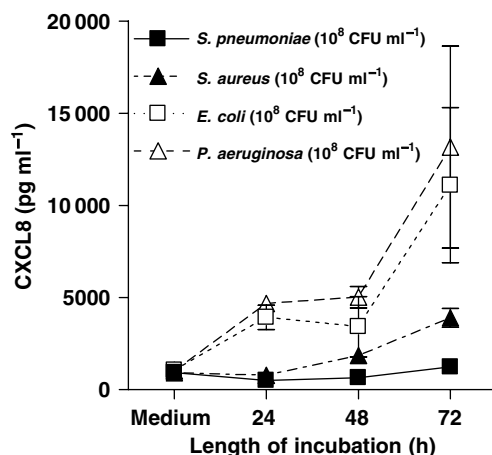
Bacteria may contain molecules that can neutralize CXCL8 protein (Edwards *et al.*, 2005). To be sure that the low yield of CXCL8 from airways cells co-incubated with Gram-positive bacteria was not due to degradation, we performed experiments where known concentrations of recombinant CXCL8 (0.25–1 ng mL<sup>-1</sup>) were incubated with *S. aureus* (10<sup>8</sup> CFU mL<sup>-1</sup>) for 24 h under identical culture conditions as those described above. *S. aureus* had no effect on CXCL8 stability (Figure 3).

*Effect of Gram-negative E. coli and Gram-positive S. aureus alone and in combination on CXCL8 release and gene induction*

As seen above, Gram-positive *S. aureus* induced low but significant levels of CXCL8 from A549 epithelial cells after 24 h, whereas *E. coli* stimulation induced the release of large amounts of chemokine (Figure 4a). When *S. aureus* and *E. coli* were added together, there was a marked increase in CXCL8 release (Figure 4a). In line with observations made with



**Figure 1** Effect of *E. coli* and *S. aureus* on human interleukin-8 (CXCL8) release from human primary airway epithelial cells ( $10^7$  and  $10^8$  CFU mL $^{-1}$ , a–b) and A549 cells ( $10^5$ – $10^8$  CFU mL $^{-1}$ , c–d), respectively. Cells were treated for 24, 48 and 72 h. Lower release of CXCL8 was detected after *S. aureus* than *E. coli* treatment in both type of cells. Data represent mean  $\pm$  s.e.mean,  $n = 6$  (a–b) and  $n = 8$  (c–d), respectively. Statistical differences (compared with control) are denoted by \* $P < 0.05$  and \*\* $P < 0.01$ ; one-way ANOVA, followed by Dunnett's multiple comparison test.



**Figure 2** Effect of Gram-negative bacteria (*E. coli*, *P. aeruginosa*), and Gram-positive bacteria (*S. aureus* and *S. pneumoniae*) on human interleukin-8 (CXCL8) release by A549 cells over 72 h. The bacteria concentration was  $10^8$  CFU mL $^{-1}$ . Data represent mean  $\pm$  s.e.mean,  $n = 8$ .

mature protein release, CXCL8 gene expression was induced by both bacteria, with levels being higher in cells treated with *E. coli* than in *S. aureus* (Figure 4b). When cells were

**Table 1** Release of CXCL8 from A549 cells, stimulated by a range of PAMPs

Treatment	CXCL8 release (pg mL $^{-1}$ )
Medium	930 $\pm$ 146
LPS 1 $\mu$ g mL $^{-1}$	2967 $\pm$ 184**
Pam <sub>3</sub> CSK4 300 ng mL $^{-1}$	2360 $\pm$ 201**
FSL-1 100 ng mL $^{-1}$	1898 $\pm$ 392*
FK565 1 $\mu$ M	1500 $\pm$ 196
MDPLys18 1 $\mu$ M	1774 $\pm$ 265
FK565 1 $\mu$ M + LPS 1 $\mu$ g mL $^{-1}$	4250 $\pm$ 457**

Abbreviations: CXCL8, human interleukin-8; FSL-1, lipopeptide Pam2Cys-GDPKHPKSF; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular pattern; Pam<sub>3</sub>CSK4, triacylated synthetic lipoprotein.

Effect of different PAMPs on CXCL8 release from A549 cells. Cells were treated for 24 h. LPS (1  $\mu$ g mL $^{-1}$ ), FSL-1 (100 ng mL $^{-1}$ ) and Pam<sub>3</sub>CSK4 (300 ng mL $^{-1}$ ) induced a release of CXCL8 compared with control treated cells. No increase in CXCL8 release was seen when cells were treated with FK565 or MDPLys18. Data represent mean  $\pm$  s.e.mean,  $n = 5$ . Statistical differences (compared with medium control wells) are denoted by \* $P < 0.05$  and \*\* $P < 0.01$ ; one-way ANOVA, followed by Dunnett's multiple comparison test.

treated with a combination of *E. coli* and *S. aureus*, the CXCL8 gene expression was synergistically increased, compared with when cells were treated with each bacterium separately (Figure 4b).

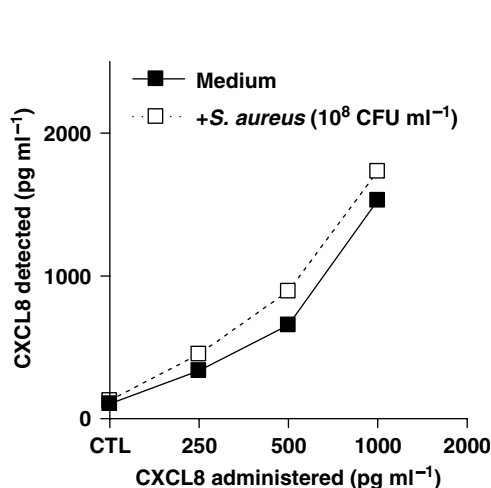
### Effect of CXCL8 on barrier function in monolayers of A549 epithelial cells

Under control culture conditions, a confluent monolayer of epithelial cells form a competent barrier, that is, impermeable to large proteins. CXCL8 ( $3 \text{ ng mL}^{-1}$ ) added to cells for 1 h caused a disruption of the barrier function resulting in a significant increase in the passage of HRP through the monolayer (Figure 5). The effects of CXCL8 on barrier function in this study were specific, as they were blocked by a specific binding antibody to CXCL8 (Figure 6c).

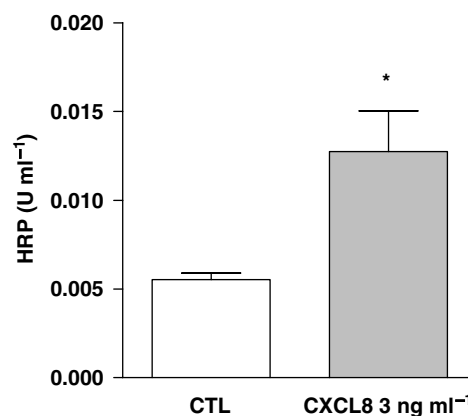
### Effect of Gram-negative *E. coli*, Gram-positive *S. aureus* or specific PAMPs on barrier function in A549 epithelial cells: role of CXCL8

Gram-negative *E. coli* ( $10^8 \text{ CFU mL}^{-1}$ ) had no significant effect on barrier function in epithelial cells (Figure 6a). By contrast, *S. aureus* ( $10^8 \text{ CFU mL}^{-1}$ ) caused a significant

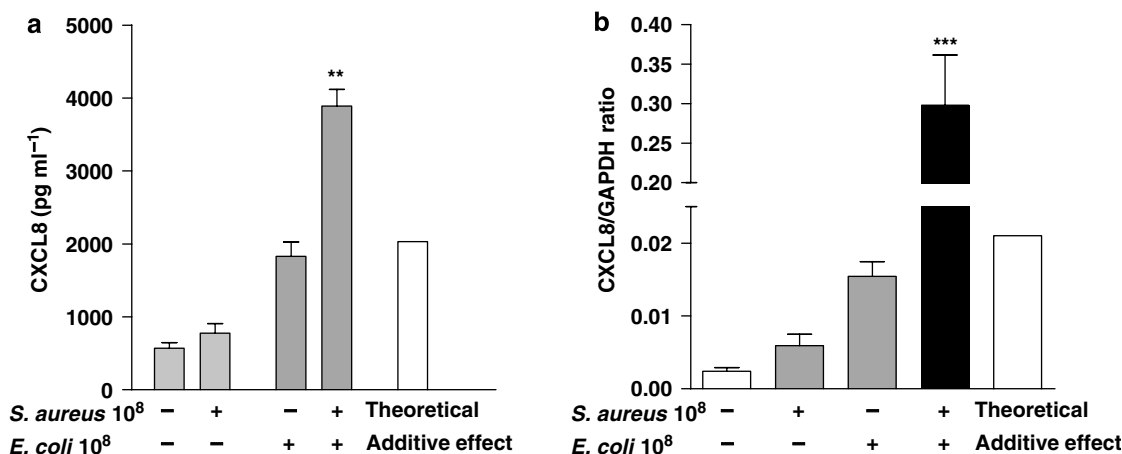
disruption of barrier function (Figure 6a). The effects of *S. aureus* were mimicked by FSL-1, which activates the TLR2/6 heterodimer. Pam<sub>3</sub>CSK4 had a weak tendency on barrier function, which did not reach statistical significance (Figure 6b). Interestingly, the effects of *S. aureus* on barrier function were prevented by co-treatment with *E. coli* (Figure 6a). The activity of *S. aureus* on barrier function was mediated by CXCL8, as neutralizing antibodies to CXCL8 prevented its effects (Figure 6c). In line with observations made with *S. aureus*, the effects of authentic CXCL8 on barrier function were blocked by co-treatment with *E. coli* (Figure 7a). LPS induced significant CXCL8 release from A549 cells, but FK565, which activates NOD1 receptors, did not. (Table 1). In the present set of experiments, barrier function of A549 cells was disrupted by LPS but not by FK565 (Figure 7b). Furthermore, FK565 prevented the disruptive effects of LPS on barrier function, when they were given



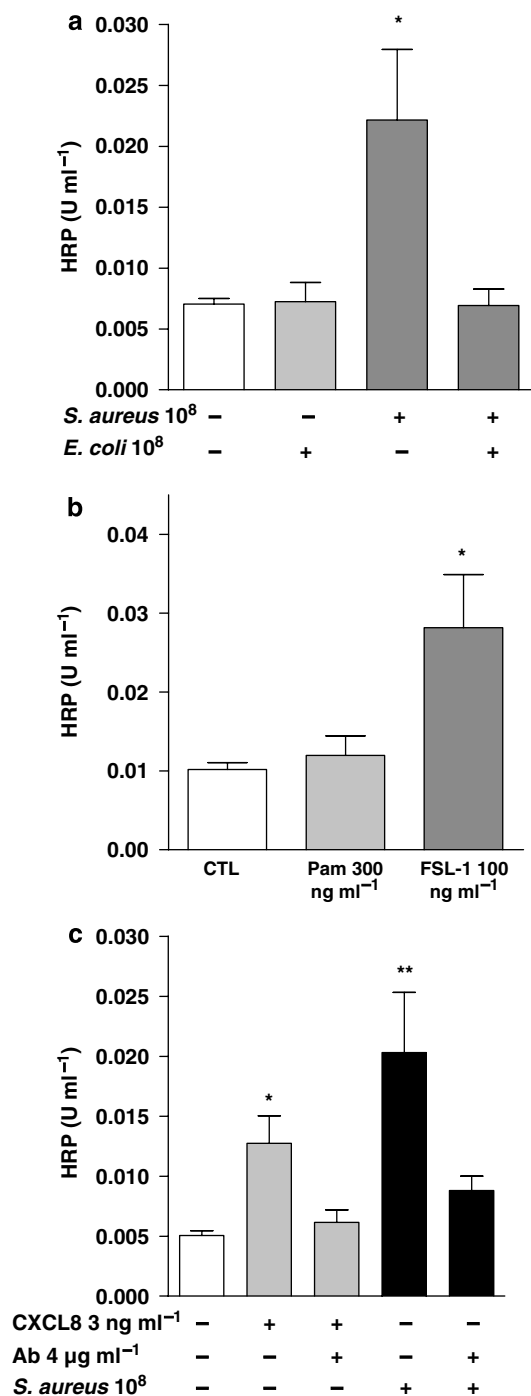
**Figure 3** Effect of *S. aureus* ( $10^8 \text{ CFU mL}^{-1}$ ) on human interleukin-8 (CXCL8) stability in culture medium over 24 h. *S. aureus* did not induce CXCL8 degradation. Data represent mean  $\pm$  s.e.mean,  $n = 2$ .



**Figure 5** Effect of human recombinant human interleukin-8 (CXCL8) ( $3 \text{ ng mL}^{-1}$ ) on barrier integrity. The detection of horse-radish peroxidase (HRP) in the lower insert compartment was increased after CXCL8 treatment compared with medium alone. Data represents mean  $\pm$  s.e.mean,  $n = 16$ . Statistical difference is denoted by  $*P < 0.01$ ; Student's *t*-test.

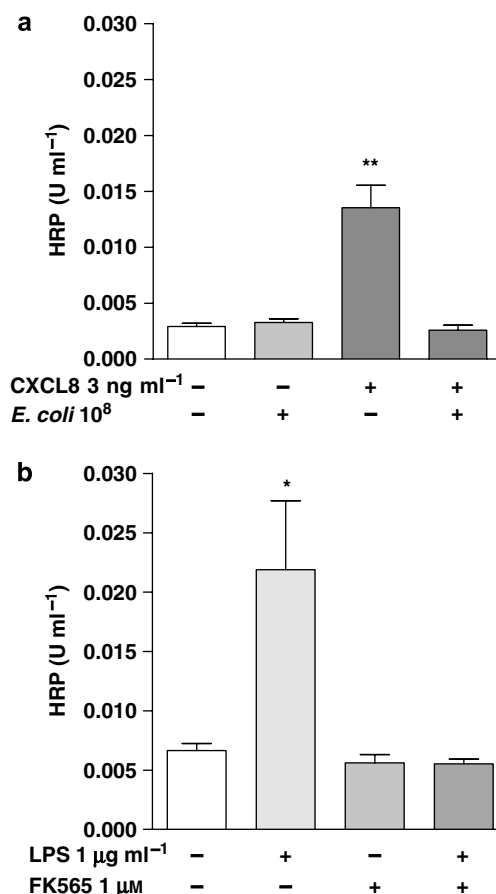


**Figure 4** Effect of *E. coli* ( $10^8 \text{ CFU mL}^{-1}$ ) and *S. aureus* ( $10^8 \text{ CFU mL}^{-1}$ ) on human interleukin-8 (CXCL8) production (a) and gene expression (b) over 24 and 3 h, respectively. The theoretical additive levels calculated for CXCL8 release is denoted by the white bar and was calculated by adding the mean amount of CXCL8 detected after treatment of the A549 cells with *E. coli* and *S. aureus* alone, either for the protein release (a) or for the mRNA levels (b). Statistical differences are denoted by  $**P < 0.0005$  (a) and  $***P < 0.0001$  (b); one sample *T*-test, comparing the actual CXCL8 release to the theoretical additive effect. Data represent mean  $\pm$  s.e.mean,  $n = 6$  (a) and 3 (b), respectively.



**Figure 6** Effect of *E. coli* (10<sup>8</sup> CFU mL<sup>-1</sup>) and *S. aureus* (10<sup>8</sup> CFU mL<sup>-1</sup>) (a) or Toll-like receptor (TLR)2 ligands, lipopeptide Pam2Cys-GDPKHPKSF (FSL-1; 100 ng mL<sup>-1</sup>) and triacylated synthetic lipoprotein (Pam3CSK4; 300 ng mL<sup>-1</sup>) (b) on barrier integrity. (c) The effect of the neutralizing antibody for human interleukin-8 (CXCL8) on barrier function after CXCL8 (3 ng mL<sup>-1</sup>) and *S. aureus* treatment. Data represent mean  $\pm$  s.e.mean,  $n = 16$  (a),  $n = 12$  (b),  $n = 8$  (c), respectively. Statistical difference is denoted by \* $P < 0.05$  and \*\* $P < 0.01$ ; Student's  $t$ -test and one way ANOVA, followed by Dunnett's multiple comparison test.

together (Figure 7b). Co-treatment of A549 cells with this combination of LPS and FK565 resulted in an additive increase in CXCL8 release (Table 1).



**Figure 7** (a) Effect of *E. coli* (10<sup>8</sup> CFU mL<sup>-1</sup>) on barrier disruption induced by human interleukin-8 (CXCL8). (b) Effect of FK565 (1 µM) on the barrier disruption induced by LPS (1 µg mL<sup>-1</sup>). FK565 mimicked the effect of *E. coli* in preventing barrier disruption induced by human interleukin-8 (CXCL8) and Lipopolysaccharide (LPS) respectively. Data represent mean  $\pm$  s.e.mean,  $n = 4$  (a),  $n = 8$  (b), respectively. Statistical difference is denoted by \* $P < 0.05$  and \*\* $P < 0.01$ ; one-way ANOVA, followed by Dunnett's multiple comparison test.

## Discussion

In this study, we have shown that primary cultures of human airway epithelium detect and react selectively to Gram-positive and Gram-negative bacteria and to a range of selected PAMPs. Both Gram-positive and Gram-negative bacteria induced CXCL8 release by human bronchial epithelial cells, although the effect of Gram-negative bacteria was more pronounced. Gram-positive bacteria, but not Gram-negative bacteria, disrupted barrier function, which was mediated by CXCL8. Interestingly, Gram-negative bacteria prevented the effects of Gram-positive bacteria on barrier function, and this prevention was mediated independent of the effects on CXCL8 release. The effects of Gram-negative bacteria on barrier function were mimicked by specific PAMPs for NOD1, but not TLR4.

Airway epithelium is continuously exposed to a wide variety of pathogens and these cells are known to express a range of TLRs (Armstrong *et al.*, 2004; Greene *et al.*, 2005), but their role in immune function is unclear. In this study we show that, for two separate types of Gram-positive and

Gram-negative bacteria, airway epithelium reacts by releasing CXCL8. In our study, we have used heat-killed bacteria that retain many of the inflammatory qualities of live bacteria. However, as with all studies using killed pathogens, results can be different when live infections are studied. We found that Gram-negative bacteria induced greater levels of CXCL8 release from lung epithelial cells than Gram-positive bacteria (Duffin *et al.*, 2005). Similar observations have recently been made by others (Mayer *et al.*, 2007) who suggest that a lack of expression of CD36 accounts, at least in part, for the reduced response of these cells to Gram-positive bacteria. CD36 is a scavenger receptor for lipids, which facilitates the sensing of selected PAMPs by the TLR2/6 heterodimer (Hoebe *et al.*, 2005). Gram-positive bacteria contain PAMPs for TLR2/1 and TLR2/6 heterodimer complexes, whereas Gram-negative bacteria contain PAMPs for TLR4 (Mitchell *et al.*, 2007). In our study and that of Mayer *et al.* (2007), lung epithelium released significant amounts of CXCL8 in response to PAMPs for the TLR2/1 (Pam<sub>3</sub>CSK4), TLR2/6 (FSL-1) or TLR4 (LPS). Bacteria also contain PGN that can be broken down to PAMPs for NLRs. Products of PGN from Gram-negative bacteria activate NOD1 (and NOD2), whereas those from PGN from Gram-positive bacteria activate NOD2. In our study, we found that a selective PAMPs for NOD1 (FK565; (Ahmed *et al.*, 1989; Cartwright *et al.*, 2007) had minimal effect on the ability of lung epithelium to release CXCL8. Similarly, we found that muramyl dipeptide (MDP) which activates NOD2 (Fritz *et al.*, 2006) slightly stimulated lung epithelium to release CXCL8.

In other cell types, activation of TLR2 and TLR4 pathways can result in synergistic release of inflammatory mediators (Paul-Clark *et al.*, 2006; Schroder *et al.*, 2006). In this study, we show that Gram-positive and Gram-negative bacteria enhance upregulation of CXCL8 release and gene expression in lung epithelium, which is consistent with observations, made for other genes and cell types.

Others have shown in endothelial cells that CXCL8 disrupts barrier function (Talavera *et al.*, 2004), suggesting that its effects are mediated by the modulation of the tight junctions either by affecting zona occludens-1 protein and/or adhesion molecule expression. Our present data suggest the same phenomenon may exist in human lung epithelial cells. Moreover, we show that Gram-positive *S. aureus* similarly disrupts barrier function. The effects of *S. aureus* in our model were blocked by binding antibodies to CXCL8 and were mimicked by FSL-1, and to a much lesser degree by Pam<sub>3</sub>CSK4. By contrast, *E. coli* did not affect barrier function in these cells. As mentioned above, *E. coli* induced substantially more CXCL8 release than *S. aureus*.

Why then does *E. coli* not induce a disruption in barrier function in lung epithelial cells? One possible explanation for our observations was that *E. coli* was having dual effects on barrier function and the disruption that would be induced by CXCL8 was being prevented. This possibility was confirmed in experiments where the co-treatment with *E. coli* completely prevented barrier function disruption induced by either CXCL8 or *S. aureus*. As mentioned above, *E. coli* contains PAMPs for TLR4 and for NOD1. We found that the activation of TLR4 with LPS mimicked the effects of

*E. coli* on CXCL8 release, but unlike the whole bacteria, LPS induced a clear disruption of barrier function in these cells. By contrast, activation of NOD1 with FK565 had no direct effect on barrier function, and like whole *E. coli*, prevented disruption of the barrier. It is beyond the scope of this current study to establish the physiological relevance of our findings. Clearly, bacterial infection results in inflammation in the lung leading to a disruption in barrier function. This is the case for Gram-negative and Gram-positive bacteria. Our observations using isolated human epithelial cells *in vitro* are therefore simply modelling the response of these stromal cells without the participation of associated adjoining cells and/or recruited leukocytes. The relevance of our data in this context perhaps is an understanding of how individual PAMPs may interact to cause effects in these cells. Our findings that FK565 did not disrupt barrier function by itself and prevented disruption induced by other agonists, is interesting, although its meaning for events *in vivo* is unclear. However, although both LPS and FK565 induced shock and signs of sepsis, only LPS, and not FK565, induced detectable signs of lung inflammation (Cartwright *et al.*, 2007). It is tempting to speculate that these *in vivo* findings may be explained by the observations made here using human cells *in vitro*.

It is also beyond the scope of this study to elucidate the pathways by which bacteria, TLRs and NLRs interact via CXCL8-dependent and CXCL8-independent pathways to modulate barrier function of lung epithelium. However, our data suggest that such interactions are functionally relevant and identify NOD1 as a potential therapeutic target in the prevention of lung inflammation.

## Acknowledgements

We are grateful to Professor Andrew Nicholson from Royal Brompton Hospital and Dr Rex D Stanbridge from St Mary's Hospital, London, United Kingdom. This work was supported by the British Lung Foundation. Dr Stanbridge RD, for the collection of human samples at St Mary's Hospital.

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