Increased IL-8 production in human bronchial epithelial cells after exposure to azithromycin-pretreated *Pseudomonas aeruginosa* in vitro

Li Fan¹, Qian Wang¹, César de la Fuente-Núñez², Feng-Jun Sun³, Jian-Guo Xia², Pei-Yuan Xia³ & Robert E.W. Hancock²

¹Department of Pharmacy, Xinqiao Hospital, the Third Military Medical University, Chongqing, China; ²Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, BC, Canada; and ³Department of Pharmacy, Southwest Hospital, the Third Military Medical University, Chongqing, China

Correspondence: Qian Wang, Department of Pharmacy, Xinqiao Hospital, the Third Military Medical University, Chongqing 400037, China. Tel.: 86 23 68774725; fax: 86 23 68755401; e-mail: xqwq411@126.com

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Keywords

*Pseudomonas aeruginosa* PAO1; clinical isolates; cytotoxicity; type III secretion system effector genes.

Abstract

Although *Pseudomonas aeruginosa* is not typically susceptible to azithromycin (AZM) in *in vitro* tests, AZM improves the clinical outcome in patients with chronic respiratory infections, in which both the modulation of the host immune system and of bacterial virulence by AZM are thought to play an important role. However, there is currently little direct evidence showing the impact of bacteria pretreated with AZM on epithelial cells, which represents the first barrier to infecting *P. aeruginosa*. In this study, we pretreated *P. aeruginosa* PAO1 and six different clinical isolates with AZM and subsequently infected human bronchial epithelial cells (HBEs) in the absence of AZM. The results showed that AZM-pretreated *P. aeruginosa* (PAO1 and six different clinical isolates) significantly stimulated HBE cells to release IL-8, a crucial pro-inflammatory cytokine. This effect was not observed in a *P. aeruginosa* PAO1 mutant strain unable to produce the type III secretion system effector gene *pcrV* (strain PW4017). Our results suggest that AZM-pretreated *P. aeruginosa* could indirectly exacerbate pro-inflammation by inducing IL-8 production in HBEs.

Introduction

*Pseudomonas aeruginosa*-related infections and inflammation are responsible for airway damage and functional decline in most patients with cystic fibrosis (CF), many with hospital-acquired pneumonia and a small percentage with severe community-acquired pneumonias (Camposdónico et al., 2008; Fujitani et al., 2011). *Pseudomonas aeruginosa* is able to initiate a vigorous inflammatory response characterized by a massive influx of neutrophils into the airway lumen, and the concentration of neutrophils and neutrophil products correlates with the concentration of IL-8, a crucial chemoattractant in the CF airway (López-Cortés et al., 1995; Tabary et al., 1998; Verhaeghe et al., 2007). IL-8, which is present at high concentrations in the airways of CF patients, can be produced by both stimulated macrophages and neutrophils, but the major source of IL-8 is likely to be the bronchial epithelial cells that have been shown to be induced by *P. aeruginosa* (Chmiel et al., 2002).

Antibiotics are usually given whenever pneumonia is suspected or when a noticeable decline in lung function has been observed. Among the drugs commonly used, the macrolide azithromycin (AZM) is often prescribed. *Pseudomonas aeruginosa* is unsusceptible to AZM during *in vitro* tests; however, this antibiotic has shown clinical effectiveness as a maintenance therapy in CF and other chronic respiratory infections such as chronic bronchitis (Blasi et al., 2005; Yousef & Jaffe, 2010; Saiman et al., 2012). Although its mechanisms still remain unclear, the immunomodulatory activity of AZM on host cells and the inhibition of bacterial virulence are thought to represent two major factors (Feola et al., 2010; Saiman et al., 2010). Reports have shown that AZM can inhibit
type IV pili protein synthesis (Wozniak & Keyser, 2004), alginate release (Hoffmann et al., 2007) and quorum-sensing (QS) systems (Hoffmann et al., 2007; Hall dorsson et al., 2010). Conversely, recent studies have indicated that the exposure of pathogens to subinhibitory AZM may result in increased virulence and resistance (Mulet et al., 2009; Köhler et al., 2010; Wright et al., 2013). Transcriptional analysis has shown that the type III secretion system (T3SS) was induced by AZM in P. aeruginosa PAO1 and have suggested that this may result in increased cytotoxicity towards macrophages (Nalca et al., 2006). To establish infections, P. aeruginosa is known to utilize its type III secretion apparatus to inject effector proteins (toxins) into host cells, thus triggering the host immune response (Hausser, 2009).

Based on these previous studies, we hypothesized here that there might be a selected immune response exerted by the bronchial epithelium, the first barrier faced by infecting microorganisms, to adapt to AZM-pretreated P. aeruginosa, which may further affect the processes of bacterial colonization and infection. Limited evidence has been provided in relation to how human bronchial epithelial cells (HBEs) respond during acute exposure to AZM-treated P. aeruginosa. Here, we tested this hypothesis by performing in vitro co-culture experiments using P. aeruginosa (PAO1 or clinical isolates) and HBEs and excluded the possible interference of AZM–HBE interaction by removing AZM prior to the infection.

Materials and methods

Bacterial strains

Pseudomonas aeruginosa wild-type PAO1, its transposon mutants (Jacobs et al., 2003) and clinical P. aeruginosa strains from pulmonary specimens collected at Xinqiao Hospital (Chongqing, China) were used in this study. All strains used are shown in Table 1. Four known T3SS effectors genes (exoS, exoT, exoU and exoY) and pcrV were individually amplified using PCR with the pairs of specific primers for each gene. Six clinical isolates were selected from a total of 62 identified isolates. Each of the six isolates carried a different genotype of T3SS effectors. All bacterial cells were grown overnight in brain heart infusion (BHI) medium at 37 °C with shaking. Before infecting HBEs, P. aeruginosa (1.0 × 10^7 CFU mL^{-1}) cells were either pretreated with AZM (Sigma-Aldrich) or left untreated for 6 h, then centrifuged and washed with PBS three times to ensure AZM elimination. Bacterial pellets were then suspended in serum-free RPMI 1640 medium (GIBCO, without antibiotic) and adjusted to a final concentration of 1.0 × 10^8 CFU mL^{-1}.

### Table 1. Strains and primers used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>T3SS effectors genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>wild type, carries exoS, T, Y</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>PW1059</td>
<td>PAO1 exoT::SlacZ/hah</td>
<td>R.E.W. Hancock laboratory</td>
</tr>
<tr>
<td>PW4017</td>
<td>PAO1 pcrV::SlacZ/hah</td>
<td>R.E.W. Hancock laboratory</td>
</tr>
<tr>
<td>PW4736</td>
<td>PAO1 exoY::SlacZ/hah</td>
<td>R.E.W. Hancock laboratory</td>
</tr>
<tr>
<td>PW7478</td>
<td>PAO1 exoS::IphAah/hah</td>
<td>R.E.W. Hancock laboratory</td>
</tr>
<tr>
<td>SW003</td>
<td>exoS, exoT, exoU, exoY</td>
<td>Clinical isolates</td>
</tr>
<tr>
<td>SW005</td>
<td>exoS, exoT, exoU, exoY</td>
<td>Clinical isolates</td>
</tr>
<tr>
<td>SW021</td>
<td>exoS, exoT, exoU, exoY</td>
<td>Clinical isolates</td>
</tr>
<tr>
<td>SW042</td>
<td>exoS, exoT, exoU, exoY</td>
<td>Clinical isolates</td>
</tr>
<tr>
<td>SW055</td>
<td>exoS, exoT, exoU, exoY</td>
<td>Clinical isolates</td>
</tr>
<tr>
<td>SW058</td>
<td>exoS, exoT, exoU, exoY</td>
<td>Clinical isolates</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
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<tr>
<td>P. ae exoTF</td>
<td>5'-CGGTAGAGAGCGAGTAAAGGG - 3'</td>
</tr>
<tr>
<td>P. ae exoTR</td>
<td>5'-TGTTCCGAGTGTTCCGAGTACGTC - 3'</td>
</tr>
<tr>
<td>P. ae exoYT</td>
<td>5'-CGACACGGTACCTGCAACGACGTC - 3'</td>
</tr>
<tr>
<td>P. ae exoYR</td>
<td>5'-CAGGTCGTCTGGTGGATAGGC - 3'</td>
</tr>
<tr>
<td>P. ae exoSF</td>
<td>5'-CAGGTAGTGGAAGTCTTGCTTGTT - 3'</td>
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<tr>
<td>P. ae exoSR</td>
<td>5'-GGGTACCGAGGAGGAGGAA - 3'</td>
</tr>
<tr>
<td>P. ae exoLF</td>
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<tr>
<td>P. ae exoUR</td>
<td>5'-CAGGCGCTGACAGAGGGAGGAGA - 3'</td>
</tr>
<tr>
<td>P. ae pcrVF</td>
<td>5'-CCCCACGCTATATGGTCTATGC - 3'</td>
</tr>
<tr>
<td>P. ae pcrVR</td>
<td>5'-CTTGAAGGCGCTGTCTTCTGAT - 3'</td>
</tr>
<tr>
<td>P. ae_16S mRNA</td>
<td>5'-CCGACGGGGTAAGTACTGCTTCTG - 3'</td>
</tr>
<tr>
<td>P. ae_16S nRNA</td>
<td>5'-CATCTGATAGCTGAGTGGACG - 3'</td>
</tr>
<tr>
<td>Human_b-actinF</td>
<td>5'-GGCATGAGTCTGTGG - 3'</td>
</tr>
<tr>
<td>Human_b-actinR</td>
<td>5'-AGAAGCTATGCTGTGG - 3'</td>
</tr>
<tr>
<td>Human_IL-8F</td>
<td>5'-AAGACATACCTCAACTTCTACC - 3'</td>
</tr>
<tr>
<td>Human_IL-8R</td>
<td>5'-GTCTTTGCTGGTGCTAACGACAT - 3'</td>
</tr>
</tbody>
</table>

Human cells

Human bronchial epithelial (HBE) cell line 16HBE 140 cells were preserved in the Respiratory Research Institute of Third Military Medical University (Chongqing, China). Cells were cultured at 37 °C with 5% CO₂ in RPMI 1640 medium supplemented with 10% foetal calf serum (GIBCO). Cells were grown to confluence on the surface of 1 cm² coverslips, which were set in 6-well plates for quantitative PCR (qPCR) assays and scanning electron microscopy (SEM) experiments. 24-well and 96-well plates were used for other experiments. Prior to infection, HBE cell monolayers were washed with PBS three times and incubated in serum-free RPMI 1640 for 2 h.

Quantitation and observation of adherence

Quantitation and observation of the adhesion of P. aeruginosa to HBE cells were evaluated after bacterial infection for 2 h, following the method described previously (Larrosa et al., 2012) with some modifications. Briefly, confluent cells grown in 24-well plate (6 × 10^5 per well) were inoculated with bacterial suspensions and grown at 37 °C with...
10% CO₂ (six wells per treatment). Following incubation, the monolayers were gently washed three times with PBS. Cells were then removed using a scraper and suspended in PBS, which contained 2 μL Triton X-100 per well. Cells were subsequently incubated at 37 °C for 2 min and vortexed for 30 s. Detached cells were then serially diluted in peptone water solution and quantified by performing cell counts on LB agar plates after incubation for 48 h. For observation using scanning electron microscopy (SEM) (Hitachi S3400, Japan), cells were gently washed three times with PBS and fixed with 2.5% glutaraldehyde. A series of dehydration and coating steps were performed as described for SEM observation (Wang et al., 2010).

**Cytotoxicity assay**

Release of lactate dehydrogenase (LDH) by HBEs as a result of membrane damaging effects exerted by different treatments was used as a measure of cytotoxicity. After incubation with AZM-pretreated or AZM-untreated bacteria, HBE cells were incubated for 4 h in serum-free RPMI 1640 medium. Wells were rinsed once with sterile PBS, followed by addition of 180 μL per well RPMI 1640 buffered with 25 mM HEPES. Subsequently, 20 μL of each P. aeruginosa isolate, in triplicate, was added at a final concentration of 10⁸ CFU mL⁻¹; negative controls consisted of 20 μL of PBS without bacteria. After infection for 4 h, supernatants were centrifuged and assayed for LDH activity according to the cytotoxicity assay kit protocol (Cytotoxicity Assay kit; Promega).

**qPCR quantification of expression of the IL-8 and T3SS genes**

The expression of IL-8 genes in HBE cells was investigated by qPCR analysis using RNA isolated according to our previously described rapid method (Wang et al., 2010), from cells either treated with 4 or 8 μg mL⁻¹ of AZM or left untreated for 2 h. The amplicon of the constitutively expressed β-actin gene was used as an internal control for data normalization. For P. aeruginosa, the expression of genes exoS, exoY, exoT, exoU and perV was evaluated after treatment with AZM 8 μg mL⁻¹ for 6 h. Briefly, the expression of first-strand cDNA was synthesized followed by PCR amplification according to the manufacturer’s recommended protocol (RT-PCR kit, Promega). The SYBR Green assay was performed using the Bio-Rad Real-time PCR System (CFX96, Bio-Rad). The relative quantitation method was used to evaluate quantitative changes in gene expression. The amplicon of the constitutively expressed 16S rRNA gene was used as an internal control as well as to normalize the data. Primers used for qPCR are shown in Table 1.

**Cytokine IL-8 production assay**

Cytokine IL-8 released from culture supernatants was quantified using enzyme-linked immunosorbent assay (ELISA). First, P. aeruginosa PAO1 was either pretreated or not with AZM for 6 h before infection of HBE cells. The culture supernatants were then harvested at 2-, 4- and 8-h time points. The results indicated that AZM treatment at concentrations of 4–16 μg mL⁻¹ for 8 h significantly enhanced IL-8. AZM (8 μg mL⁻¹) pretreatment was used to investigate IL-8 production in the different clinical isolates and mutant strains. The IL-8 measurement was performed according to the manufacturer’s instructions described in the IL-8 ELISA kit (Becton Dickinson).

**Statistical analysis**

Three independent experiments were performed with similar results. Data from representative experiments are shown. Differences between groups were calculated using Student’s t-test or one-way ANOVA, and statistical significance was defined as *, P < 0.05; **, P < 0.01.

**Results**

**Quality and observation of initial adherence**

After removing AZM prior to infection, no statistically significant difference in bacterial adherence after 2 h was found for AZM-treated or AZM-untreated postinfection based on viable bacterial cell counts on LB agar plates. We quantified the amount of AZM-treated and AZM-untreated bacteria adhered to HBE cells as 8.3 ± 2.7 × 10⁶ per well and 8.7 ± 2.4 × 10⁶ per well, respectively. However, these results differed somewhat from those obtained by scanning electron microscopy (SEM), in which the AZM-treated samples showed overall a larger number of cells than the untreated samples (Fig. 1), likely reflecting differences in internalization of bacteria. Thus, the AZM-treated cells appeared to adhere to the surface of the HBEs (Fig. 1b), while untreated bacteria tended to internalize into the HBEs as opposed to attach to their surface (Fig. 1a). These results implied that AZM-untreated bacteria entered the HBEs, which is consistent with previous reports (Plotkowski et al., 1999).

**AZM-pretreated P. aeruginosa strains (both PAO1 and clinical isolates) exhibited increased cytotoxicity towards HBEs**

HBE cells released significantly more LDH when exposed to AZM-pretreated (4, 8, 16 μg mL⁻¹) P. aeruginosa
PAO1 compared with AZM-untreated PAO1, in a dose-dependent manner (Supporting information, Fig. S1). We further investigated LDH levels released by HBEs after incubation with clinical isolates and PAO1 mutant strains, which had been pretreated with 8 µg mL⁻¹ of AZM. The results showed that all clinical strains significantly increased LDH release except for SW003 (exoS⁺, exoT⁺, exoU⁺, exoY⁻) (Fig. 2), a strain that carries only the T3SS effector gene exoT. Moreover, compared with its wild-type PAO1, the pcrV mutant (PW4017) showed significantly lower cytotoxicity towards HBEs (Fig. 2). In addition, no statistically significant difference in cytotoxicity was observed between the AZM-pretreated and AZM-untreated pcrV mutant groups (Fig. 2). These results demonstrated that overall AZM-pretreated strains tended to cause more membrane damage to HBE cells, due to type III secreted toxins, but suggested strain-dependent effects as shown with the clinical strains (Fig. 2).

**Increased IL-8 release during acute exposure to AZM-pretreated strains**

We first determined IL-8 gene expression in HBE cells after challenge for 2 h with PAO1 that had been either pretreated with 4 or 8 µg mL⁻¹ AZM for 6 h or left untreated. The results showed 1.42-fold and 3.14-fold increases in IL-8 expression by AZM-pretreated PAO1, compared with that in the non-AZM-treated bacterial controls (Fig. S2). Further assays quantifying IL-8 production were performed using ELISAs and showed that after 8 h of infection, both AZM-pretreated and AZM-untreated *P. aeruginosa* PAO1 had the ability to stimulate HBEs to secrete IL-8, but AZM-pretreated PAO1 showed a significantly stronger ability to induce IL-8 production at concentrations of 8 and 16 µg mL⁻¹ (Fig. 3). Further experiments revealed that all the AZM-pretreated strains tested were capable of significantly enhancing IL-8 production at concentrations of 8 and 16 µg mL⁻¹ (Fig. 3). However, we observed no statistically significant difference in cytotoxicity compared with its PAO1 parent strain (Fig. 4). The SW003 (exoS⁺, exoT⁺, exoU⁺, exoY⁻) clinical isolate pretreated with AZM did enhance IL-8 production (Fig. 4), but the increase was significantly lower than in all other strains tested (*P < 0.05*). Intriguingly, the pcrV mutant, along with the SW003 clinical isolate, exhibited significantly reduced cytotoxicity (Fig. 2). Furthermore, we observed

Fig. 1. Adherence to HBEs of AZM-untreated (a) and AZM-pretreated *Pseudomonas aeruginosa* PAO1 (b) as observed by SEM after 2 h of infection. Inset images show additional samples from the same group.

Fig. 2. LDH release by HBEs upon exposure to *Pseudomonas aeruginosa* pretreated with 8 µg mL⁻¹ of AZM in relation to LDH release of uninfected HBE cells. Statistical significance of LDH release between HBEs infected with AZM-pretreated or AZM-untreated *P. aeruginosa* strains was determined using Student’s t-test (*, *P < 0.05*).
that *P. aeruginosa* PAO1 pretreated with subinhibitory concentrations of the macrolide antibiotic erythromycin, but not other antibiotic classes, induced IL-8 production in HBEs (Fig. S3).

Previous global transcriptional analyses have described that T3SS genes of *P. aeruginosa* PAO1 are up-regulated by AZM treatment (Nalca et al., 2006). Based on these results, using qPCR, we further analysed the expression of T3SS genes in representative clinical isolates SW003 and SW042 (the ones that, after AZM treatment, induced IL-8 production in HBEs the least and the most), between AZM (8 μg L⁻¹)-pretreated and AZM-untreated conditions. In bacteria pretreated with AZM, all T3SS effector genes (exoS, exoT, exoY, exoU and pcrV) had increased expression (from 4.32-fold to 26.85-fold change) in comparison with those grown without the AZM pretreatment after 6 h post-AZM treatment (Table 2), showing an overall induction of expression of T3SS genes by AZM in clinical isolates of *P. aeruginosa*.

**Discussion**

The bronchial epithelium was selected as an infection model for this study as it is known to play a predominant role in early stages of airway infections. Following introduction into the airways, the first line of defence faced by *P. aeruginosa* is the bronchial epithelia, which produce IL-8, a crucial pro-inflammatory factor during pulmonary infection when exposed to pathogens such as *P. aeruginosa* and their characteristic toxins (e.g. LPS). Expression of IL-8 by airway epithelial cells has been implicated in leucocyte migration associated with airway inflammatory disease such as CF (Terheggen-Lagro et al., 2005). Both the pro-inflammatory state and the inflammatory cascade are substantially induced upon exposure to infection. This scenario leads to the production of cytokines and chemokines such as IL-8 and neutrophils in the early stages of infections, which in turn boosts the innate immune defences. Up-regulation of IL-8 may cause inflammatory

**Table 2.** Expression of T3SS genes in *Pseudomonas aeruginosa* PAO1 and representative clinical isolate strains after treatment with 8 μg mL⁻¹ of AZM for 6 h

<table>
<thead>
<tr>
<th>Strains</th>
<th>exoS</th>
<th>exoT</th>
<th>exoU</th>
<th>exoY</th>
<th>pcrV</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>26.85</td>
<td>24.75</td>
<td>N/A</td>
<td>20.33</td>
<td>17.58</td>
</tr>
<tr>
<td>SW003</td>
<td>N/A</td>
<td>12.76</td>
<td>N/A</td>
<td>N/A</td>
<td>31.98</td>
</tr>
<tr>
<td>SW042</td>
<td>N/A</td>
<td>7.36</td>
<td>10.92</td>
<td>4.32</td>
<td>12.71</td>
</tr>
</tbody>
</table>

N/A, Not applicable as the strain lacks this gene.

Fig. 3. *Pseudomonas aeruginosa* PAO1 pretreated with increasing concentrations of AZM (2, 4, 8 and 16 μg mL⁻¹) induced IL-8 production in HBEs over time as determined by ELISA. Statistical significance between treated and untreated PAO1-infected controls was determined using one-way ANOVA, *, P < 0.05; **, P < 0.01.

Fig. 4. HBEs were infected for 8 h with *Pseudomonas aeruginosa* PAO1 wild-type, PAO1 mutants and different *P. aeruginosa* clinical isolates that were either pretreated with 8 μg mL⁻¹ of AZM or left untreated. All AZM-pretreated strains significantly induced IL-8 production in HBEs as determined by ELISA methodology, except for strain PW4017 (pcrV mutant). The production of IL-8 in AZM-treated clinical isolate SW003 was compared statistically with that of clinical isolate strains SW005, SW021, SW042, SW055 and SW058 also treated with AZM. Statistical significance was observed in all cases, except for SW005 and SW058 (P > 0.05). *, Statistical analysis was performed using Student’s t-test, P < 0.05.
exacerbation in HBE cells, and prolonged inflammation could eventually lead to lung damage (Verhaeghe et al., 2007). Our results indicate that there is an IL-8 enhancement in human bronchial epithelial cells during acute exposure to P. aeruginosa pretreated with 8 μg mL⁻¹ of AZM [a concentration that can be easily attained in sputum (Baumann et al., 2004)], which may result in an inflammatory response of host cells in the airway.

We did not observe a significant decline or increase in adhesion of AZM-pretreated P. aeruginosa to HBEs, which contrasts previous reports, in which AZM showed a significant inhibition of adhesion to polystyrene or bronchial mucins (Vranes, 2000; Carfartan et al., 2004). These differences likely relate the specific adherence substrate utilized but may also be due to variations in the methodologies used in the different studies. For example, in our experiments, bacteria that adhered to HBEs were not in the presence of AZM. Also, our observations suggested the internalization of bacterial cells by human bronchial epithelial cells, a process that is known to occur during the initial hours of P. aeruginosa colonization (Plotkowski et al., 1999). Furthermore, a recent study demonstrated that AZM maintains the host airway epithelial integrity during P. aeruginosa infection (Halldorsson et al., 2010). Further research is needed to clarify the direct effect of AZM on P. aeruginosa internalization by HBEs, but mutant studies indicated that it was dependent on type II secretion (not present in the pcrV mutant) but not any one of the individual secreted toxins (but rather likely a mixture).

The mechanisms behind how subinhibitory concentrations of AZM act on P. aeruginosa remain unclear. Interestingly, it has been noted that AZM inhibits biofilm formation by inhibiting quorum sensing (QS) in P. aeruginosa (Favre-Bonté et al., 2003). QS is an interbacterial communication system by which signal molecules act as autoinducers and trigger a variety of biological functions when microbial populations reach a certain cell density. QS controls not only virulence factor production but also biofilm formation in P. aeruginosa (Davies et al., 1998) and thus contributes significantly to pathogenesis and persistence of P. aeruginosa infections (de la Fuente-Núñez et al., 2013). In vitro studies have shown that AZM inhibited biofilms formed by P. aeruginosa strains isolated from CF patients (Wagner et al., 2005). In addition, P. aeruginosa utilizes a complex type III secretion apparatus to inject effector proteins (toxins) from the bacterial cytoplasm into the cytoplasm of its host’s cells. These T3SS effectors disrupt endothelial barriers, which allows bacteria and pro-inflammatory cytokines to escape to the bloodstream, leading to bacteraemia and septic shock (Galle et al., 2012). T3SS of P. aeruginosa play a dominant role in acute infections, and emerging evidence suggests that they are also involved in chronic infections such as those present in CF patients (Dacheux et al., 2000; Cruz et al., 2012). Secretion of T3SS effector proteins by strains isolated from lower respiratory and systemic P. aeruginosa infections has been shown to be associated with increased death of patients (Roy-Burman et al., 2001). Many patients with chronic P. aeruginosa infections produce antibodies against T3SS effector proteins ExoU, ExoS, ExoT and ExoY (Hauser, 2009), the only four that have been identified thus far. The pcrV gene encodes a protein that is necessary to translocate these effector proteins into host cells (Dacheux et al., 2000).

Our results showed that a pcrV mutant (strain PW4017) exhibited significantly lower cytotoxicity towards HBEs compared with its parent strain P. aeruginosa PAO1 (Fig. 2). Furthermore, this mutant was the only strain tested that did not induce IL-8 production in HBEs when pretreated with AZM (Fig. 4). Another strain that, when pretreated with AZM, led to only slightly increased IL-8 levels was a community-acquired pneumonia clinical isolate SW003, which carries the T3SS effector gene exoT but not exoS, exoU or exoY (Fig. 4). The levels of IL-8 induced by strain SW003 (pretreated with AZM) were significantly lower than every other strain except for SW005 (exoS⁺, exoT⁺, exoU⁻, exoY⁻) and SW058 (exoS⁻, exoT⁻, exoU⁺, exoY⁻) (Fig. 4). These results suggest that cytotoxicity (Fig. 2) and production of pro-inflammatory cytokine IL-8 (Figs 3 and 4) induced by AZM-pretreated P. aeruginosa on HBEs may depend on the expression of pcrV and combinations of different T3SS effector genes (exoS, exoU and exoY), depending on the specific strain. Consistent with this, we confirmed previous microarray results (Nalca et al., 2006) (Table 2), by showing an overall 4.3- to 26-fold induction of expression of T3SS genes by AZM in clinical isolates of P. aeruginosa. These results suggest that AZM stimulates a more virulent phenotype both in PAO1 and clinical strains through the exacerbation of T3SS effector genes. To reveal in detail the complex relationship between different T3SS genotypes and AZM-altered pathogenesis, comparisons among different genotypes in multiple clinical isolate strains are needed.

It is clear that not only the adaptation of the infecting organism to the host but also the failure of the host’s immune system to eradicate bacterial pathogens contributes to the establishment of chronic infections (Cohen & Prince, 2012). AZM activity towards the immune system of the host coupled with its antimicrobial and antibiofilm properties makes it an interesting anti-infective agent. The purpose of the present study was to provide direct evidence that AZM-pretreated P. aeruginosa exhibited increased cytotoxicity and that co-culture of these bacte-
ria with HBEs in the absence of AZM triggered the production of the pro-inflammatory cytokine IL-8. These results may improve our understanding about the influence of AZM on colonization and establishment of infections by bacterial pathogens.

Acknowledgements

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** LDH release in HBEs by different concentrations of AZM (0, 2, 4, 8, 16 μg mL⁻¹)-treated PAO1, compared with LDH levels from uninfected HBE cells. *, using Student’s *t*-test, *P* < 0.05.

**Fig. S2.** IL-8 expression in HBEs infected for 2 h with PAO1. *, compared with untreated PAO1, using Student’s *t*-test, *P* < 0.05.

**Fig. S3.** IL-8 production in HBEs infected for 8 h with PAO1, which was pretreated with antimicrobials at concentrations of 1/4 MIC. *, using Student’s *t*-test, *P* < 0.05.