RESEARCH LETTER

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

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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* 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 (Campodónico *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; Halldorsson *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 (Hauser, 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.

Table 1. Strains and primers used in this study

Strains	T3SS effectors genotype	Source
PAO1	Wild type, carries *exoS, T, Y*	Laboratory collection
PW1059	PAO1 *exoT::ISlacZ/hah*	R.E.W. Hancock laboratory
PW4017	PAO1 *pcrV::ISlacZ/hah*	R.E.W. Hancock laboratory
PW4736	PAO1 *exoY::ISlacZ/hah*	R.E.W. Hancock laboratory
PW7478	PAO1 *exoS:: ISphoA/hah*	R.E.W. Hancock laboratory
SW003	$exoS^{-}$, $exoT^{+}$, $exoU^{-}$, $exoY^{-}$	Clinical isolates
SW005	$exoS^{+}$, $exoT^{+}$, $exoU^{-}$, $exoY^{+}$	Clinical isolates
SW021	$exoS^{+}$, $exoT^{+}$, $exoU^{+}$, $exoY^{+}$	Clinical isolates
SW042	$exoS^{-}$, $exoT^{+}$, $exoU^{+}$, $exoY^{+}$	Clinical isolates
SW055	$exoS^{+}$, $exoT^{+}$, $exoU^{-}$, $exoY^{-}$	Clinical isolates
SW058	$exoS^{-}$, $exoT^{+}$, $exoU^{+}$, $exoY^{-}$	Clinical isolates

Primers	Sequence
*P. ae_exoT*F	5′- CGGTAGAGAGCGAGGTAAAGGG - 3′
*P. ae_exoT*R	5′- TGTTCGCCGAGGTACTGCTC - 3′
*P. ae_exoY*F	5′- CGACCAGCATCTCAGCAAGC - 3′
*P. ae_exoY*R	5′- CAGGTCGGTCTGGGTATAGGC - 3′
*P. ae_exoS*F	5′-CAGGTAGTGAAGACTTTCCGTGG - 3′
*P. ae_exoS*R	5′-GGGTTCAGGGAGGTGGAGAGAT - 3′
*P. ae_exoU*F	5′-TAGCATGGTTGGCAGCTGAGTTGA - 3′
*P. ae_exoU*R	5′-CAGGGCGATACAGAGAGGGGAAGA - 3′
*P. ae_pcrV*F	5′- CCCCACGCTATATGGCTATGC -3′
*P. ae_pcrV*R	5′- CTTGAGTTCCCCGCTCTGCT- 3′
*P. ae_16S rRNA*F	5′-CGGACGGGTGAGTAATGCCTA - 3′
*P. ae_16S rRNA*R	5′-CATCTGATAGCGTGAGGTCCGA - 3′
Human_β-*actin*F	5′-GGCATGGAGTCCTGTGG - 3′
Human_β-*actin*R	5′-AGAAGCATTTGCGGTGG - 3′
Human_*IL-8*F	5′-AAGACATACTCCAAACCTTTCCACC - 3′
Human_*IL-8*R	5′-GTTTTCCTTGGGGTCCAGACAG - 3′

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 strains 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}.

Human cells

Human bronchial epithelial (HBE) cell line 16HBE 14o- cells were preserved in the Respiratory Research Institute of Third Military Medical University (Chongqing, China). Cells were cultured at 37 °C with 5% CO_2 in RPMI 1640 medium supplemented with 10% foetal calf serum (GIBCO). Cells were grown to confluence on the surface of 1 cm^2 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% CO_2 (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 2 mL 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 10^8 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 *pcrV* 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 $8.3 \pm 2.7 \times 10^6$ per well and $8.7 \pm 2.4 \times 10^6$ 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*

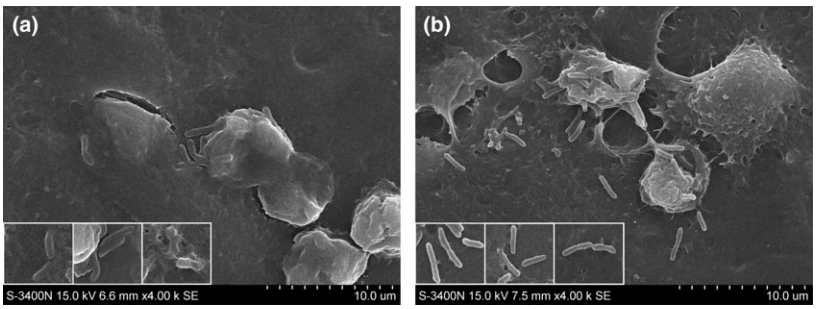

Fig. 1. Adherence to HBEs of AZM-untreated (a) and AZM-pretreated *Pseudomonas aeruginosa* PAO1 (b) as observed by SEM after 2 h of infection. Inset images show additional samples from the same group.

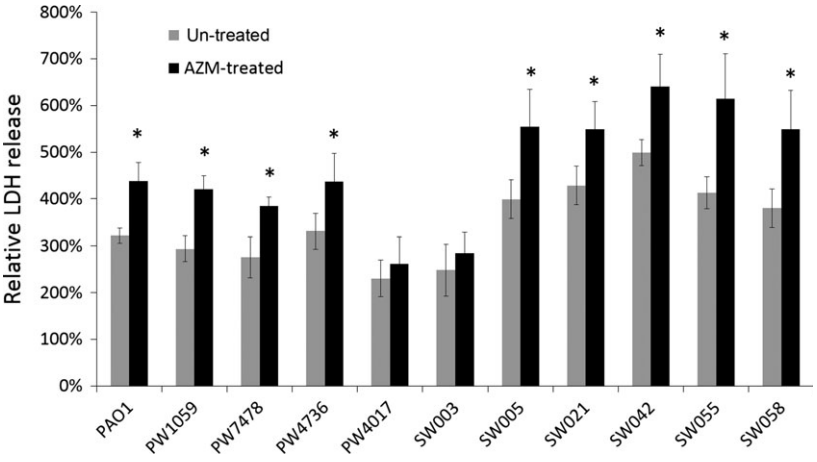

Fig. 2. LDH release by HBEs upon exposure to *Pseudomonas aeruginosa* pretreated with 8 μg mL^{-1} of AZM in relation to LDH release of uninfected HBE cells. Statistical significance of LDH release between HBEs infected with AZM-pretreated or AZM-untreated *P. aeruginosa* strains was determined using Student's *t*-test (*, $P < 0.05$).

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 *P. aeruginosa* 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, except for the *pcrV* mutant of PAO1 (strain PW4017) that in fact showed a significantly weaker stimulation of IL-8 production 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 weaker than in all other strains tested ($P < 0.05$), except for strains SW005 (*exoS*$^+$, *exoT*$^+$, *exoU*$^-$, *exoY*$^+$) and SW058 (*exoS*$^-$, *exoT*$^+$, *exoU*$^+$, *exoY*$^-$) ($P > 0.05$). Intriguingly, the *pcrV* mutant, along with the SW003 clinical isolate, exhibited significantly reduced cytotoxicity (Fig. 2). Furthermore, we observed

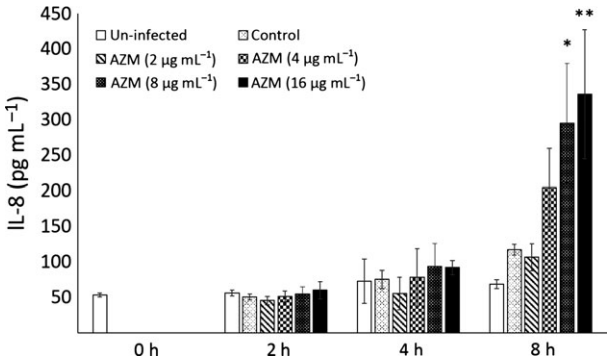

Fig. 3. *Pseudomonas aeruginosa* PAO1 pretreated with increasing concentrations of AZM (2, 4, 8 and 16 μg mL^{-1}) induced IL-8 production in HBEs over time as determined by ELISA. Statistical significance between treated and untreated PAO1-infected controls was determined using one-way ANOVA, *, $P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

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 mL^{-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*.

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

	Fold change				
Strains	*exoS*	*exoT*	*exoU*	*exoY*	*pcrV*
PAO1	26.85	24.75	N/A	20.33	17.58
SW003	N/A	12.76	N/A	N/A	31.98
SW042	N/A	7.36	10.92	4.32	12.71

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

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

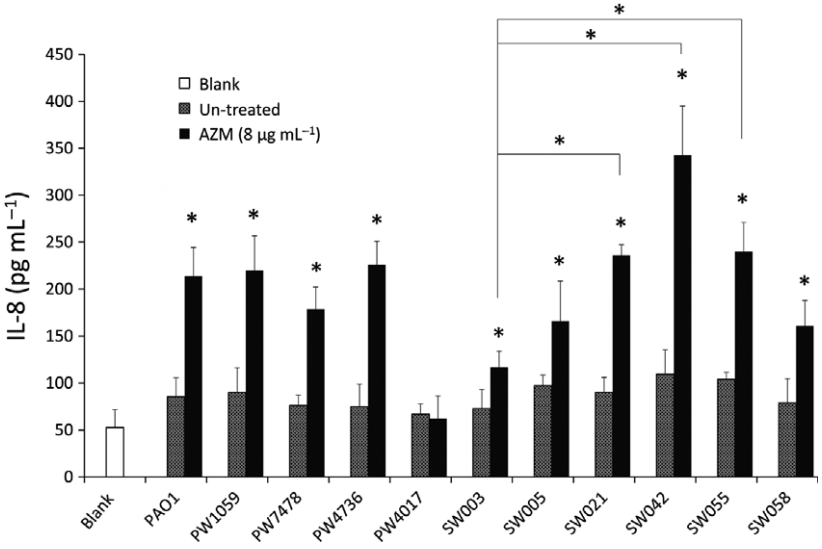

Fig. 4. HBEs were infected for 8 h with *Pseudomonas aeruginosa* PAO1 wild-type, PAO1 mutants and different *P. aeruginosa* clinical isolates that were either pretreated with 8 μg mL^{-1} of AZM or left untreated. All AZM-pretreated strains significantly induced IL-8 production in HBEs as determined 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.

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References

Baumann U, King M, App EM, Tai S, König A, Fischer JJ, Zimmermann T, Sextro W & von der Hardt H (2004) Long term azithromycin therapy in cystic fibrosis patients: a study on drug levels and sputum properties. *Can Respir J* **11**: 151–155.

Blasi F, Cazzola M, Tarsia P, Cosentini R, Aliberti S, Santus P & Allegra L (2005) Azithromycin and lower respiratory tract infections. *Expert Opin Pharmacother* **6**: 2335–2351.

Campodónico VL, Gadjeva M, Paradis-Bleau C, Uluer A & Pier GB (2008) Airway epithelial control of *Pseudomonas aeruginosa* infection in cystic fibrosis. *Trends Mol Med* **14**: 120–133.

Carfartan G, Gerardin P, Turck D & Husson MO (2004) Effect of subinhibitory concentrations of azithromycin on adherence of *Pseudomonas aeruginosa* to bronchial mucins collected from cystic fibrosis patients. *J Antimicrob Chemother* **53**: 686–688.

Chmiel JF, Berger M & Konstan MW (2002) The role of inflammation in the pathophysiology of CF lung disease. *Clin Rev Allergy Immunol* **23**: 5–27.

Cohen TS & Prince A (2012) Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat Med* **18**: 509–519.

Cruz AC, Neves BC, Higa LY, Folescu T, Marques EA & Milagres LG (2012) Type III apparatus of *Pseudomonas aeruginosa* as a tool to diagnose pulmonary infection in cystic fibrosis patients. *APMIS* **120**: 622–627.

Dacheux D, Toussaint B, Richard M, Brochier G, Croize J & Attree I (2000) *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. *Infect Immun* **68**: 2916–2924.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW & Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**: 295–298.

de la Fuente-Núñez C, Reffuveille F, Fernández L & Hancock RE (2013) Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr Opin Microbiol* **16**: 580–589.

Favre-Bonté S, Köhler T & Van Delden C (2003) Biofilm formation by *Pseudomonas aeruginosa*: role of the C4-HSL cell-to-cell signal and inhibition by azithromycin. *J Antimicrob Chemother* **52**: 598–604.

Feola DJ, Garvy BA, Cory TJ, Birket SE, Hoy H, Hayes DJ & Murphy BS (2010) Azithromycin alters macrophage phenotype and pulmonary compartmentalization during lung infection with *Pseudomonas*. *Antimicrob Agents Chemother* **54**: 2437–2447.

Fujitani S, Sun HY, Yu VL & Weingarten JA (2011) Pneumonia due to *Pseudomonas aeruginosa*: part I: epidemiology, clinical diagnosis, and source. *Chest* **139**: 909–919.

Galle M, Jin S, Bogaert P, Haegman M, Vandenabeele P & Beyaert R (2012) The *Pseudomonas aeruginosa* type III secretion system has an exotoxin S/T/Y independent pathogenic role during acute lung infection. *PLoS ONE* **7**: e41547.

Halldorsson S, Gudjonsson T, Gottfredsson M, Singh PK, Gudmundsson GH & Baldursson O (2010) Azithromycin maintains airway epithelial integrity during *Pseudomonas aeruginosa* infection. *Am J Respir Cell Mol Biol* **42**: 62–68.

Hauser AR (2009) The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat Rev Microbiol* **7**: 654–665.

Hoffmann N, Lee B, Hentzer M, Rasmussen TB, Song Z, Johansen HK, Givskov M & Høiby N (2007) Azithromycin blocks quorum sensing and alginate polymer formation and increases the sensitivity to serum and stationary-growth-phase killing of *Pseudomonas aeruginosa* and attenuates chronic *P. aeruginosa* lung infection in Cftr(-/-) mice. *Antimicrob Agents Chemother* **51**: 3677–3687.

Jacobs MA, Alwood A, Thaipisuttikul I *et al.* (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *P Natl Acad Sci USA* **100**: 14339–14344.

Köhler T, Perron GG, Buckling A & van Delden C (2010) Quorum sensing inhibition selects for virulence and cooperation in *Pseudomonas aeruginosa*. *PLoS Pathog* **6**: e1000883.

Larrosa M, Truchado P, Espín JC, Tomás-Barberán FA, Allende A & García-Conesa MT (2012) Evaluation of *Pseudomonas aeruginosa* (PAO1) adhesion to human alveolar epithelial cells A549 using SYTO 9 dye. *Mol Cell Probes* **26**: 121–126.

López-Cortés LF, Cruz-Ruiz M, Gómez-Mateos J, Viciana-Fernandez P, Martinez-Marcos FJ & Pachón J (1995) Interleukin-8 in cerebrospinal fluid from patients with meningitis of different etiologies: its possible role as neutrophil chemotactic factor. *J Infect Dis* **172**: 581–584.

Mulet X, Maciá MD, Mena A, Juan C, Pérez JL & Oliver A (2009) Azithromycin in *Pseudomonas aeruginosa* biofilms: bactericidal activity and selection of nfxB mutants. *Antimicrob Agents Chemother* **53**: 1552–1560.

Nalca Y, Jänsch L, Bredenbruch F, Geffers R, Buer J & Häussler S (2006) Quorum-sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: a global approach. *Antimicrob Agents Chemother* **50**: 1680–1688.

Plotkowski MC, de Bentzmann S, Pereira SH, Zahm JM, Bajolet-Laudinat O, Roger P & Puchelle E (1999) *Pseudomonas aeruginosa* internalization by human epithelial respiratory cells depends on cell differentiation, polarity, and junctional complex integrity. *Am J Respir Cell Mol Biol* **20**: 880–890.

Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, Sawa T, Frank DW & Wiener-Kronish JP (2001) Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J Infect Dis* **183**: 1767–1774.

Saiman L, Anstead M, Mayer-Hamblett N *et al.* (2010) "Effect of azithromycin on pulmonary function in patients with cystic fibrosis uninfected with *Pseudomonas aeruginosa*: a randomized controlled trial. *JAMA* **303**: 1707–1715.

Saiman L, Mayer-Hamblett N, Anstead M *et al.* (2012) Open-label, follow-on study of azithromycin in pediatric patients with CF uninfected with *Pseudomonas aeruginosa*. *Pediatr Pulmonol* **47**: 641–648.

Tabary O, Zahm JM, Hinnrasky J, Couetil JP, Cornillet P, Guenounou M, Gaillard D, Puchelle E & Jacquot J (1998) Selective up-regulation of chemokine IL-8 expression in cystic fibrosis bronchial gland cells *in vivo* and *in vitro*. *Am J Pathol* **153**: 921–930.

Terheggen-Lagro SW, Rijkers GT & van der Ent CK (2005) The role of airway epithelium and blood neutrophils in the inflammatory response in cystic fibrosis. *J Cyst Fibros* **4** (suppl 2): 15–23.

Verhaeghe C, Delbecque K, de Leval L, Oury C & Bours V (2007) Early inflammation in the airways of a cystic fibrosis foetus. *J Cyst Fibros* **6**: 304–308.

Vranes J (2000) Effect of subminimal inhibitory concentrations of azithromycin on adherence of *Pseudomonas aeruginosa* to polystyrene. *J Chemother* **12**: 280–285.

Wagner T, Soong G, Sokol S, Saiman L & Prince A (2005) Effects of azithromycin on clinical isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients. *Chest* **128**: 912–919.

Wang Q, Sun FJ, Liu Y, Xiong LR, Xie LL & Xia PY (2010) Enhancement of biofilm formation by subinhibitory concentrations of macrolides in icaADBC-positive and -negative clinical isolates of *Staphylococcus epidermidis*. *Antimicrob Agents Chemother* **54**: 2707–2711.

Wozniak DJ & Keyser R (2004) Effects of subinhibitory concentrations of macrolide antibiotics on *Pseudomonas aeruginosa*. *Chest* **125**: 62S–69S; quiz 69S.

Wright EA, Fothergill JL, Paterson S, Brockhurst MA & Winstanley C (2013) Sub-inhibitory concentrations of some antibiotics can drive diversification of *Pseudomonas aeruginosa* populations in artificial sputum medium. *BMC Microbiol* **13**: 170.

Yousef AA & Jaffe A (2010) The role of azithromycin in patients with cystic fibrosis. *Paediatr Respir Rev* **11**: 108–114.

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, $P < 0.05$.