**Pseudomonas aeruginosa** induces spatio-temporal secretion of IL-1β, TNFα, proMMP-9, and reduction of epithelial E-cadherin in human alveolar epithelial type II (A549) cells

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**INTRODUCTION**

*Pseudomonas aeruginosa* is an opportunistic bacterium that has been associated with chronic infections in airways (Beaudoin et al., 2012), cystic fibrosis (Holm et al., 2013) and pulmonary inflammation (Park et al., 2013). The pathogenicity of *P. aeruginosa* is mediated by several factors, including the production of diffusible molecules controlled by a mechanism known as quorum sensing (Chugani et al., 2012; Kowratzki et al., 1987; Perez et al., 2013; Rada and Leto, 2013). It was shown that lipopolysaccharides of *P. aeruginosa* induce in the alveolar and bronchial epithelium the secretion of nitric oxide (Pit & St Croix, 2002), inflammatory cytokines (Wong & Johnson, 2013) and production of matrix metalloproteinases (MMPs) (Frisdal et al., 2001; Okamoto et al., 2002; Yao et al., 1996). MMPs are a family of zinc neutral endopeptidases produced in several pathological conditions (Churg et al., 2007; Holm et al., 2013) by a wide variety of cell types, including neutrophils (Bradley et al., 2012; Louhelainen et al., 2010), alveolar macrophages (Churg et al., 2007), and bronchial epithelial cells (Yao et al., 1996). MMPs induce degradations of various structural components of the extracellular matrix including collagen type I, IV, V, VII, X, fibronectin, elastin, proteoglycan (Woessner, 1991), basement membrane (Karzoxan et al., 2007) as well as cell-binding adhesion proteins (Allport et al., 2002; Nawrocki-Raby et al., 2003). Although the secretion of MMPs is well known in various lung diseases: bronchopulmonary dysplasia (Mizikova & Morty, 2015), adenocarcinomas (Canete-Soler et al., 1994), and chronic obstructive pulmonary disease (Louhelainen et al., 2010), the secretion profile of proMMP-2 and -9 produced by human pneumocytes secretes type II cells during infection with *Pseudomonas aeruginosa* is unknown. We chose the A549 cell line as it is a model of human lung alveolar epithelium which plays an important role in the immune response. We hypothesized that an increase in IL-1β and TNFα concentrations would be accompanied by a parallel increase...
in collagenolytic activity of MMP-2 and -9 in the culture medium, and thereby would induced changes in epithelial cadherin (E-cadherin) in A549 cells during transient \textit{P. aeruginosa} stimulation.

**MATERIALS AND METHODS**

**Antibodies and reagents**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, Mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone, Hoechst 333258, and 4′,6-diamidino-2-phenylindole were obtained from Sigma-Aldrich (St Louis, MO, USA). IL-1β and TNFα were purchased from R&D Systems (Minneapolis, MN, USA). Anti-MMP-9 antibodies were purchased from Calbiochem (Darmstadt, Germany). Anti-human E-cadherin antibody was purchased from BD Bioscience (San Jose, CA, USA).

**Cell lines and culture**

A549 cell line (American Type Culture Collections, Rockville, MD, USA) was obtained and its genetic profile corroborated by the amplification of 21 specific markers. The result showed a complete match with the A549 line (ATTC, CCL-185). A549 cells were cultivated on 12 well plates (Corning, Darmstadt, Germany) in RPMI 1640 medium (Roswell Park Memorial Institute; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), an antibiotic-antimycotic solution (penicillin 100 U/mL, streptomycin 100 µg/mL; Gibco) and incubated at 37°C in 5% CO₂. After reaching 95% of confluence, A549 cells were washed twice with sterile saline solution to remove RPMI-FBS, and 1 mL of RPMI with 0.2% lactoalbumin hydrolyzated (RPMI-LHA; Gibco) was added with subsequent incubation at 37°C in 5% CO₂.

**Bacterial strain and preparation**

Prior to the stimulation experiments, we confirmed \textit{P. aeruginosa} (ATCC 27853, Rockville, MD, USA) identity through the following screening methods: morphology, production of pigments (pyocyanin and fluorescein), and disc method to assess susceptibility-resistance for penicillins (pipracillin, carbenicillin), β-lactam-β-lactamase inhibitors combinations (pipracillin-tazoabactam), cephalos (ceftriaxone, cefoperazone, cefepime, and cefazidime), carbapenems (meropenem), monobactams (aztreonam), aminoglycosides (gentamicin, and amikacin), fluoroquinolones (ciprofloxacin, norfloxacin). These analyses confirmed that \textit{P. aeruginosa} strain maintains all its characteristics. For the stimulation assays \textit{P. aeruginosa} was grown in 5% Blood Agar Base (Becton Dickinson, USA) and harvested in calf medium.

**Cell stimulation**

After reaching 95% confluence, A549 cells were washed twice with sterile saline solution to remove RPMI-LHA and 1 mL of RPMI with 0.2% lactoalbumin hydrolysat (RPMI-LHA; Gibco) was added before incubation at 37°C in 5% CO₂. Next A549 cells were infected with live \textit{P. aeruginosa} in serial dilutions (10⁶, 10⁵, 10⁴, and 10³ colony-forming units (CFU/mL)). The CFU numbers were based on a turbidity equivalent to 0.5 McFarland standard. After the infection, A549 cells were cultured for 3, 6 or 24 hours. At the end of the incubation time, the medium was collected and samples were centrifugated at 1400 rpm at 4°C for 5 min, the supernatants were collected and stored at -70°C until further processing.

**Cell viability assay**

To evaluate A549 cells viability after incubation with \textit{P. aeruginosa} we used the colorimetric assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as previously described by Zeng et al. (2017). Cells were washed twice with sterile saline solution to remove RPMI-LHA and \textit{P. aeruginosa}, and then cultured for 3 hours in presence of 20 µl (5 mg/mL) of MTT in 5% CO₂ at 37°C. Subsequently, 150 µl of Dimethyl sulfoxide (DMSO; Merek KGaA, Darmstadt, Germany) was added into each well (Zeng et al., 2017). For negative control, a mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) was dissolved in dimethylsulfoxide at a concentration of 80 µM (Chaudhari et al., 2008) and added to the cells before the incubation at 37°C with 5% CO₂, 95 % air. Blue formazan product in the culture medium from A549 cells was analyzed by spectrophotometric absorbance reading at 570 nm in Benchmark microplate (model 550; BioRad. Hercules, CA, USA). Five independent experiments were performed, each in duplicate.

**Measurement of proinflammatory cytokines**

To quantify IL-1β and TNFα secreted to the culture medium of A549 cells after each period of incubation with \textit{P. aeruginosa} we used a specific DouSet enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions. This procedure was previously reported by our research group (Flores-Herrera et al., 2012; Osorio-Caballero et al., 2015). For IL-1β (DY201; R&D Systems) and TNF (DY210; R&D Systems), a standard curve was created from 4 to 260 pg/mL and 15 to 960 pg/mL, with a sensitivity of 2.0 and 5.0 pg/mL, respectively. The concentration of IL-1β and TNFα were expressed as pg/mL. The ELISA assay was performed in eight independent experiments.

**Zymography gel activity**

To evaluate the secretion of proMMP-2 and proMMP-9 into the culture media of A549 cells, SDS-polyacrylamide gels with porcine gelatin (1 mg/mL) were used as described previously (Flores-Herrera et al., 2012). Each well was loaded with 0.75 µg of protein and the activity band was determined by optical density using NIH Imagej. We used a culture medium from, promyelocyte cells as a control of electrophoretic mobility (U937, ATCC, CRL-1593.2; Manassas, VA, USA). The gel activity assay was performed in eight independent experiments.

**Immunodetection of MMP-9 and E-cadherin in the A549 cells**

To localize MMP-9 in A549 cells after infection with \textit{P. aeruginosa} we used immunofluorescence as described previously (Flores-Herrera et al., 2012). After fixing the cells with 4% paraformaldehyde for 10 minutes, a primary mouse anti-MMP9 antibody (clone 56-2A4; Calbiochem Darmstadt, Germany) was added at 1:50 dilution. An appropriate fluorescent-labeled secondary antibody (Molecular Probes, USA) was used. The nucleus was stained with 1 ng/mL of Hoechst 333258 (Sigma-Aldrich).

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**P. aeruginosa affects the E-cadherin in A549 cells**

Volunteer A549 cell viability assay.

Effect of different number of colony-forming units (CFU/mL) of *Pseudomonas aeruginosa* at 3 (A), 6 (B), and 24-hours (C) of stimulated A549 cell (ashered bars), the viability was determined with MMT assay. We included two negative controls: carbonyl cyanide m-chlorophenylhydrazone as mitochondrial inhibitor incubated with A549 cells (CCCP, 80 µM; black bar) and *Pseudomonas aeruginosa* (Pa). The assay was performed in five independent experiments with duplicates. Data represent the mean ± standard deviation. Statistically significant difference *p*<0.05 vs. control.

Statistical analysis

Data were analyzed by one-way ANOVA with multiple comparisons followed by Tukey’s test using SigmaPlot version 11.0 (San Jose, CA, USA). Results are expressed as mean ± S.E.M. *p*<0.05 was considered significant. Immunostainings of proMMP-9 and E-cadherine were performed five times.

**RESULTS**

**Effects of P. aeruginosa on A549 cells viability**

Figure 1 shows the viability of A549 cells with and without *P. aeruginosa* stimulation after 3 (1A), 6 (1B) and 24 hours (1C). The viability was not affected by the different doses of *P. aeruginosa* when compared to the control group (*p*=0.65). In the same experiments, we included the mitochondrial inhibitor (CCCP), which significantly reduced the viability of A549 cells in comparison to the control group (*p*<0.05). Finally, MMT was not metabolized by *P. aeruginosa* (Fig. 1). These experiments demonstrated that infection with *P. aeruginosa* did not affect the viability of A549 cells. We then assessed the effect of *P. aeruginosa* on the secretion of IL-1β and TNFα.

**Secretion of proinflammatory cytokines by A549 cells**

**IL-1β**

Figure 2 shows that the stimulation of A549 cells with *P. aeruginosa* significantly increased the secretion of IL-1β in a dose-dependently manner. After 3 hours of stimulation with *P. aeruginosa* at 10^6^, and 10^7^ CFU/mL, A549 cells significantly increased the secretion of IL-1β by 1.2- and 1.6-fold, respectively, in comparison to the control (2.3±0.7; *p*≤0.05, Fig. 2A). A similar secretion profile was observed after 6 hours of stimulation (Fig. 2B). Maximal secretion of IL-1β was detected after 24 hours of stimulation with *P. aeruginosa* at 10^2^, 10^4^, 10^5^, and 10^6^ CFU/mL, with 3.8-, 5.1-, 6.7-, and 8.1-fold increase, respectively when compared to the control (2.7±0.216; *p*≤0.05, Fig. 2C).

**TNFα**

Figure 3 shows that stimulation of A549 cells with *P. aeruginosa* increased the secretion profile of TNFα in a dose-dependent manner. After 3 hours of stimulation with *P. aeruginosa* at 10^4^, 10^5^, and 10^6^ CFU/mL, A549 cells significantly increased the secretion of TNFα by 1.4-, 1.5-, and 1.6-fold, respectively when compared with the control (6.4±0.4; *p*≤0.05, Fig. 3A). A similar secre-
Production of metalloproteinases by A549 cells

Figure 4 shows the lysis bands of proMMP-2 and -9 secreted by A549 cells after stimulation with *P. aeruginosa* for 3 (4A), 6 (4F), and 24 hours (4I). The relative densitometric analysis indicated that after 3 hours (Fig. 4B) and 6 hours (Fig. 4F) of stimulation with *P. aeruginosa*, A549 cells secreted proMMP-9 in a dose-dependent manner. Maximal secretion was detected after incubation with $10^2$, $10^3$ and $10^6$ CFU/mL with 1.8-, 1.7-, and 2.0-fold increase, respectively, in comparison to the control ($21.7\pm2.4$; $p\leq0.05$, Fig. 4F). Interestingly, after 24 hours of stimulation, we did not detect the lysis band corresponding to proMMP-9 (Fig. 4J).

Consistent with these findings, we observed morphological changes in A549 cells characterized by an increase in the number of spherical cells (Fig. 4L), when compared to the control group (Fig. 4K). This finding, together with the absence of the proMMP-9 band in activity gels, suggested that this enzyme can be located in the extracellular matrix of A549 cells, as previously reported under other pathological conditions (Flores-Herrera et al., 2012; Nawrocki-Raby et al., 2003). To explore this hypothesis, we performed immunolocalization with specific antibodies.

**proMMP-9 detection in A549 cells by immunofluorescence**

As shown in Fig. 5, proMMP-9 was immunodetected in the extracellular matrix of A549 cells after stimulation with $10^6$ CFU/mL of *P. aeruginosa*. We observed a significant increase in immunoreactivity after 3, 6 and 24 hours compared to the respective controls (Fig. 5). As it was previously demonstrated in another cellular system, the active isoform of MMP-9 is able to degrade different support components, including collagen type I, IV, VI, XI, elastin, and proteoglycan of the extracellular matrix (Morrison et al., 2009; Woessner, 1991), as well as cell-binding proteins such as vascular endothelial-cadherin (Allport et al., 2002) and E-cadherin (Nawrocki-Raby et al., 2003). After observing a change in the morphology of A549 cells, a reduction in the number of adhered cells (data not shown), and a decrease of proMMP-9 immunoreactivity, we complemented our approach by analyzing E-cadherin using immunodetection.

**DISCUSSION**

Several *in vivo* and *in vitro* models of infection are able to release a diverse set of molecules that are associated with cellular stress (Osorio-Caballero et al., 2015), and the reduction of chemotactic (Henriquez et al., 2015) and proinflammatory cytokines (Keyel, 2014; van de Veerdonk et al., 2011), which are involved in the next phase of the inflammatory response through the secretion of degradatives enzymes, such as proMMPs (Flores-Herrera et al., 2012). In *in vitro* models, the induction of the degradative response affects cell integrity by decreasing the expression of cell-cell adhesion proteins, like E-cadherin and vascular endothelial-cadherin (Allport et al., 2002; Nawrocki-Raby et al., 2003). However, little evidence is available on the effect of *Pseudomonas aeruginosa* on the inflammatory-degradative response in human lung alveolar epithelial type II (A549 line) cells.

Our results showed that *P. aeruginosa* was able to increase the secretion of 1) the proinflammatory cytokines IL-1β and TNFα; and 2) the prodegradative enzyme...
MMP-9 in a time- and concentration-dependent manner. This proinflammatory/prodegradative environment compromised cell viability through changes in cell morphology and decrease of E-cadherin expression in the A549 cells.

IL-1β is a pivotal cytokine in several second messenger signaling pathways. It is involved in the activation of the inflammatory response (Chen et al., 2017; Ledesma et al., 2004), acts as a modulator of the specialized cells of the immune system (Gabay et al., 2010; Rubartelli et al., 1990), and induces the expression of MMPs (Eberhardt et al., 2000; Nam & Kwon, 2014). The production of IL-1β by alveolar macrophages and epithelial cells is induced by different bacterial components that interact with Toll-like receptors 4 (TLR4). Interestingly, this receptor has high homology with the IL-1R receptor which amplifies the inflammatory response and promotes the activation of transcription factors, such as nuclear factor kappa-beta (NFĸβ) and activator protein (AP-1), inducing the expression of genes related to the inflammatory response (Armstrong et al., 2004; Parker et al., 2016). Wong and others (Wong et al., 2012) showed that alveolar type I cells obtained from rats that were stimulated with LPS from E. coli for 18 hours, show high expression of TNFα and IL-1β, but a low expression of IL-6 (Wong & Johnson, 2013). Similarly, in our experiments A549 cells stimulated during with P. aeruginosa for 24 hours showed a 10-fold increase in secretion of TNFα (Fig. 3C) in comparison to IL-1β (Fig. 2C).

Saperstein and others (Saperstein et al., 2009) and Thorley and others (Thorley et al., 2007), demonstrated that the IL-1β signaling pathways modulate TNFα secretion. They used mouse lung epithelial cell type II and primary human alveolar type II cells to show that increase of TNFα can be reversed by using small interfering RNA and by neutralizing IL-1β with a specific antibody, respectively.

Recently, Jayaraman and others (Jayaraman et al., 2013) proposed a hypothetical mechanism by which IL-1β increases the secretion of TNFα via interacting with the type-1 form of the TNF receptor (TNFR1) and increasing the secretion of the soluble form of TNFα (Jayaraman et al., 2013; MacEwan, 2002). However, a alternative mechanisms mediated by nuclear factor kappa-beta (NFĸβ) could also explain the link between IL-1β and TNFα (Fig. 7). NFĸβ plays an important role in the immunological pathway (Tak & Firestein, 2001), and mutations of cellular NFĸβ induced changes in this immunological response (Picard et al., 2011; Sung et al., 2014). NFĸβ and mitogen-activated protein kinases (MAPKs) knockout mice displayed an altered inflammatory response of chemokines and cytokines after LPS stimulation (Picard et al., 2011; Sung et al., 2014).

The next phase of the inflammatory response promoted by IL-1β/TNFα is the expression and secretion of MMPs (Fang et al., 2006; Flores-Herrera et al., 2012). Our results suggest that an infectious and inflammatory process modulates the secretion of proMMP-2 and -9 in a dose-dependent manner and in relation to the stimulation time (Fig. 4).

There is evidence of the mechanism through which IL-1β (Eberhardt et al., 2000; Mon et al., 2017; Ruhul Amin et al., 2003) and TNFα (Fang et al., 2006; Jayaraman et al., 2013; Mon et al., 2006; Tsai et al., 2014) increase the activity of MMP-9 (Fig. 7). Recently, Mon et al. (2017) demonstrated that IL-1β activates MMP-9.
through a series of intracellular signals initiated by the activation of the proto-oncogene tyrosine-protein kinase Src (Src) which phosphorylates two tyrosines (Y397 and Y925), activating the system mediated by the growth factor receptor-bound protein 2 (Grb2) and Ras-dependent MAPK protein. This complex activates the MMP-9 (Mon et al., 2017). In addition, it was also shown by Mon et al. (2006) that TNFα interacts with the focal adhesion kinase (FAK) directly involved in the MMP-9 expression. FAK activation is mediated by the TNFR2 receptor in two tyrosine (Y398, and Y925). These findings were confirmed using an antibody against TNFR2, which inhibited FAK phosphorylation and by using FAK−/− cells, which prevented the degradative activity of MMP-9 (Mon et al., 2006).

Finally, after 24 hours of stimulation with *P. aeruginosa* we observed a 72 KDa band corresponding to proMMP-2 and a 62-KDa band corresponding to its active form (Fig. 4E). Unfortunately, the activity of MMP-2 could not be determined. Furthermore, proMMP-9 (92 KDa) could not be clearly identified in the activity gels (Fig. 4E and F), but it was clearly detected in the extracellular matrix of A549 cells using a specific antibody (Fig. 5). Alterations in the morphology of A549 cells were also evident (Fig. 6). Frisdal et al. (Frisdal et al., 2001) and Jackson and others (Jackson et al., 2010) have shown higher ex-
pression of MMP-2 and -9 during pulmonary pathological. During physiological development, MMPs are secreted into the extracellular space in the form of proMMPs and are bound to specific tissue inhibitors (TIMPs), as well as to the membrane-type metalloproteases (MT-MMP) (Somerville et al., 2003). Their activation is triggered by the removal of the peptides associated with the active site of the proMMP-2 (72 KDa) and proMMP-9 (92 KDa), inducing conformational change (Defawe et al., 2005; Koo et al., 2012; Somerville et al., 2003) (Fig. 7). Moreover, evidence from different sources suggests that in pathological processes, actMMP-9 degrades the E-cadherin involved in cell-cell adhesion (Allport et al., 2002; Nawrocki-Raby et al., 2018). The studies reported here demonstrated that P. aeruginosa induces mainly the secretion of TNFα, increasing the actMMP-9, and significantly reduces the level of E-cadherin in the A549 cells.  

**Conflicts of Interest**

The authors declare no financial or commercial conflict of interest.

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