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

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***Pseudomonas aeruginosa*, is an opportunistic bacterium with a high prevalence in diverse pulmonary infections. Although several genes are involved in the system of resistance and evasion of the immunological response of the host, little is known about the inflammatory, degradative, and cell-binding response induced by *P. aeruginosa* in human lung alveolar epithelial cells. The purpose of this study was to determine the cytokine expression (IL-1β and TNFα), pro matrix metalloproteinases activation (*pro*MMP-2 and *pro*MMP-9), and the effects on the cell-binding adhesion protein (E-cadherin) in an *in vitro* model of human lung alveolar epithelial cells. A549 cells were stimulated with a different number of colony-forming units of *P. aeruginosa* for 3, 6, and 24 hours. Subsequently, the culture medium was collected, IL-1β and TNFα levels were evaluated by ELISA; *pro*MMP-2 and -9 levels were determined by substrate gel zymography, and the MMP-9 and E-cadherin assessed by immunostaining of A549 cells. Our results demonstrated that *P. aeruginosa* induces mainly the secretion of TNFα, increases *act*MMP-9 level, and significantly reduces the level of E-cadherin in the A549 cells. In summary, the inflammatory/degradative process induced by *P. aeruginosa* modulates the expression of the E-cadherin protein. The probable clinical implications of this study suggest the use of inhibitors that reduce the degradative activity of *pro*MMP-9 which will be further explored in the next phase of this study.**

Keywords: E-cadherin, human lung alveolar epithelial cells, matrix metalloproteinase, *Pseudomonas aeruginosa*, proinflammatory cytokine.

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Abbreviations: MMP, matrix metalloproteinases; E-cadherin, epithelial cadherin; IL-1β, interleukin-1beta; CFU, colony-forming units; TNF-α, tumor necrosis factor-alpha; NFκβ, nuclear factor-kappa B

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic Gram-negative 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; Kownatzki *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 (Pitt & 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 (Kargozaran *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 *pro*MMP-2 and -9 produced by human pneumocytes secretory 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 *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 33258, 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% CO_2. 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% CO_2.

Bacterial strain and preparation

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

Cell stimulation

After reaching 95% confluence, A549 cells were washed twice with sterile saline solution to remove RPMI-FBS and 1 mL of RPMI with 0.2% lactoalbumin hydrolysate (RPMI-LHA; Gibco) was added before incubation at 37°C in 5% CO_2. Next A549 cells were infected with live *P. aeruginosa* in serial dilutions (10^2, 10^4, 10^5, and 10^6 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 *P. aeruginosa* we used the colorimetric assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MMT) as previously described by Zeng *et al.* (2017). Cells were washed twice with sterile saline solution to remove RPMI-LHA and *P. aeruginosa*, and then cultured for 3 hours in presence of 20 μl (5 mg/mL) of MMT in 5% CO_2 at 37°C. Subsequently, 150 μl of Dimethyl sulfoxide (DMSO; Merck 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% CO_2, 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 *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 *pro*MMP-2 and *pro*MMP-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.

Inmunodetection of MMP-9 and E-cadherin in the A549 cells

To localize MMP-9 in A549 cells after infection with *P. aeruginosa* we used immunefluorescence 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 33258 (Sigma-Aldrich). In another set of experiments, E-cadherin was immu-

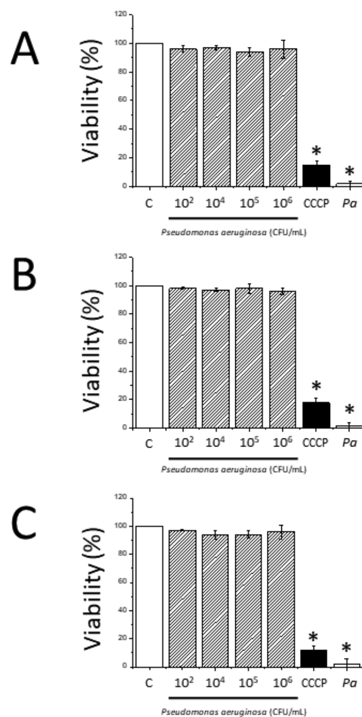

Figure 1. A549 cell viability assay.
Effect of different number of colony-forming units (CFU/mL) of *Pseudomonas aeruginosa* at 3 (**A**), 6 (**B**), and 24-hours (**C**) of stimulated of A549 cell (ashurated bars), the viability was determined with MMT assay. We included two negative controls: carbonyl 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 ± standar deviation. Statistically significant difference *$p<0.05$ *vs*. control group.

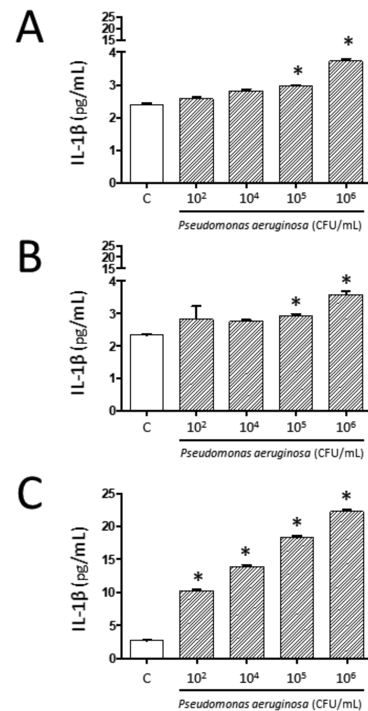

Figure 2. Secretion of IL-1β by A549 cells stimulated with *P. aeruginosa*.
After 3 (**A**), 6 (**B**), and 24 hours (**C**) of stimulation with or without *P. aeruginosa* (differentnumber of colony-forming units; CFU/mL), the culture medium of A549 cells was recovered and analyzed using ELISA. The concentration of IL-1β was expressed as pg/mL. The assay was performed in 8 independent experiments with duplicates. Data represent the mean ± standard deviation. Statistically significant difference *$p\leq0.05$ *vs*. control group.

nodetected using mouse anti-human E-cadherin (clone NCH-38) antibody at a 1:100 dilution. The nucleus was stained using 4',6-diamidino-2-phenylindole (DAPI) for 7 minutes. Negative controls consisted of cells without primary antibody, and, as expected, they did not exhibit any staining (not shown). The immunostaining was analyzed using an epi-fluorescence microscope (Olympus, IX-81, Tokyo, Japan) and photographed with a CCD camera (Hamamatsu, ORCA-Flash 2.8, Tokio, Japan).

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 *pro*MMP-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 10^5, and 10^6 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; $p\leq0.05$, 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; $p\leq0.05$, 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; $p\leq0.05$, Fig. 3A). A similar secre-

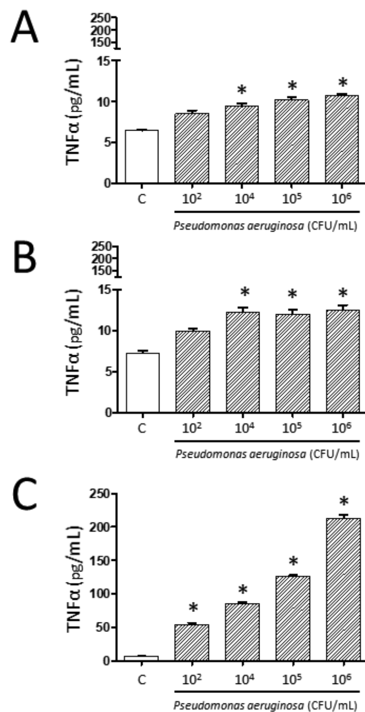

Figure 3. Secretion of TNFα by A549 cells stimulated with *P. aeruginosa*.
After 3 (**A**), 6 (**B**), and 24 hours (**C**) of stimulation with or without *P. aeruginosa* (different number of colony-forming units; CFU/mL), the culture medium of A549 cells was recovered and analyzed using ELISA. The concentration of TNFα was expressed as pg/mL. The assay was performed in 8 independent experiments with duplicates. Data represent the mean ± standard deviation. Statistically significant difference $^*p\leq0.05$ *vs*. control group.

tion profile was observed after 6 hours of stimulation (Fig. 3B). Maximal secretion was observed 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 (6.7±1.1; $p\leq0.05$, Fig. 3C).

Interestingly, it was shown that IL-1β and TNFα induce the secretion of MMPs (Roomi *et al*., 2013). Therefore, our next step was to determine the effect of the inflammatory responses induced by *P. aeruginosa* on the secretion of extracellular matrix metalloproteases into the culture medium from A549 cells. Lysis bands for *pro*MMP-2 and -9 were identified by taking the mobility of U937 standard as a reference point, as previously reported and validated by our research group (Flores-Herrera *et al*., 2012).

Production of metalloproteinases by A549 cells

Figure 4 shows the lysis bands of *pro*MMP-2 and -9 secreted by A549 cells after stimulation with *P. aeruginosa* for 3 (4A), 6 (4E), and 24 hours (4I). The relative densitometric analysis indicated that after 3 (Fig. 4B) and 6 hours (Fig. 4F) of stimulation, significantly higher levels of *pro*MMP-2 were detected when compared to the control. Maximal secretion of *pro*MMP-2 was observed after 24 hours of stimulation with *P. aeruginosa* at 10^5, and 10^6 CFU/mL, with a 1.3-fold increase compared to the control (48.7±2.8; $p\leq0.05$ Fig. 4J). After the same period of stimulation, we observed a band of 66-KDa that corresponded to the MMP-2 active form (Fig. 4J).

The relative densitometric analysis indicated that after 3 (Fig. 4B) and 6 hours (Fig. 4F) of stimulation with *P. aeruginosa*, A549 cells secreted *pro*MMP-9 in a dose-dependent manner. Maximal secretion was detected after incubation with 10^4, 10^5 and 10^6 CFU/mL with 1.8-, 1.7-, and 2.0-fold increase, respectively, in comparison to the control (21.7±2.4; $p\leq0.05$, Fig. 4F). Interestingly, after 24 hours of stimulation, we did not detect the lysis band corresponding to *pro*MMP-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 *pro*MMP-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, *pro*MMP-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, V, 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 *pro*MMP-9 immunoreactivity, we complemented our approach by analyzing E-cadherin using immunodetection.

proMMP-9 reduces E-cadherin immunofluorescence in A549 cells

A549 cells incubated for 24 hours with *P. aeruginosa* showed very low immunostaining intensity for E-cadherin compared to the respective controls (Fig. 6). Immunoreactivity was located around the cells and the nuclei.

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 *pro*MMPs (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

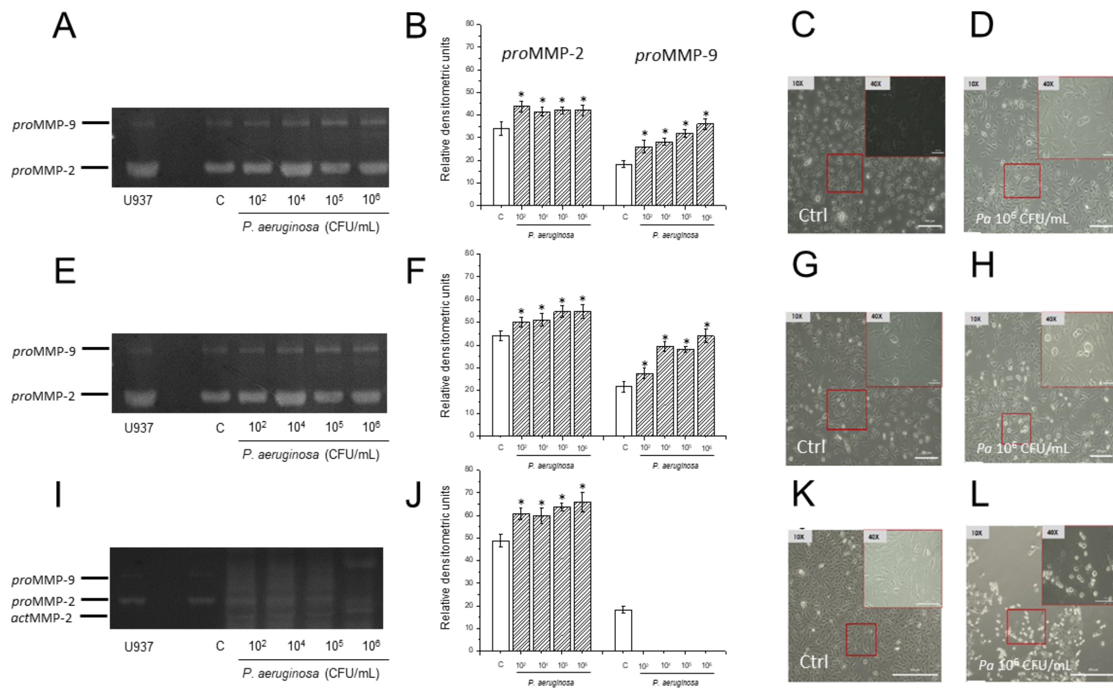

Figure 4. Secretion of *pro*MMP-2 and *pro*MMP-9 by A549 cells after *P. aeruginosa* stimulation.
Representative gelatin-gel zymography (**A, E, and I**) showing enzymatic activity of *pro*MMP-2 and *pro*MMP-9 secreted into the culture medium by A549 cells after stimulation with or without *P. aeruginosa* (different number of colony-forming units; CFU/mL). After 24 hours of stimulation with *P. aeruginosa,* we detected *act*MMP-2. The *pro*MMP-9 form was not clearly visualized (**I**). Each lysis band was quantified by densitometric analysis after bacterial stimulation (**B, F, and J**). The baseline activity of media was evaluated using a promyelocyte cell line (U937, ATCC Manassas, VA, USA). The assay was performed of 8 independent experiments. Data represent the mean ± standard deviation. Statistically significant difference $^*p \leq 0.05$ *vs*. control group. Phase-contrast images showing the change in the morphology of A549 cells after stimulation with 10^6 CFU/mL of *P. aeruginosa* (**D, H, L**) *vs*. control group (**Ctrl, C, G, and K**). The magnification of the main image is 10x and of the box is 40x. Scale bar=100 µm.

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 (NFκβ) 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 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 (NFκβ) could also explain the link between IL-1β and TNFα (Fig. 7). NFκβ plays an important role in the immunological pathway (Tak & Firestein, 2001), and mutations of cellular NFκβ induced changes in this immunological response (Picard *et al*., 2011; Sung *et al*., 2014). NFκβ 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 *pro*MMP-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

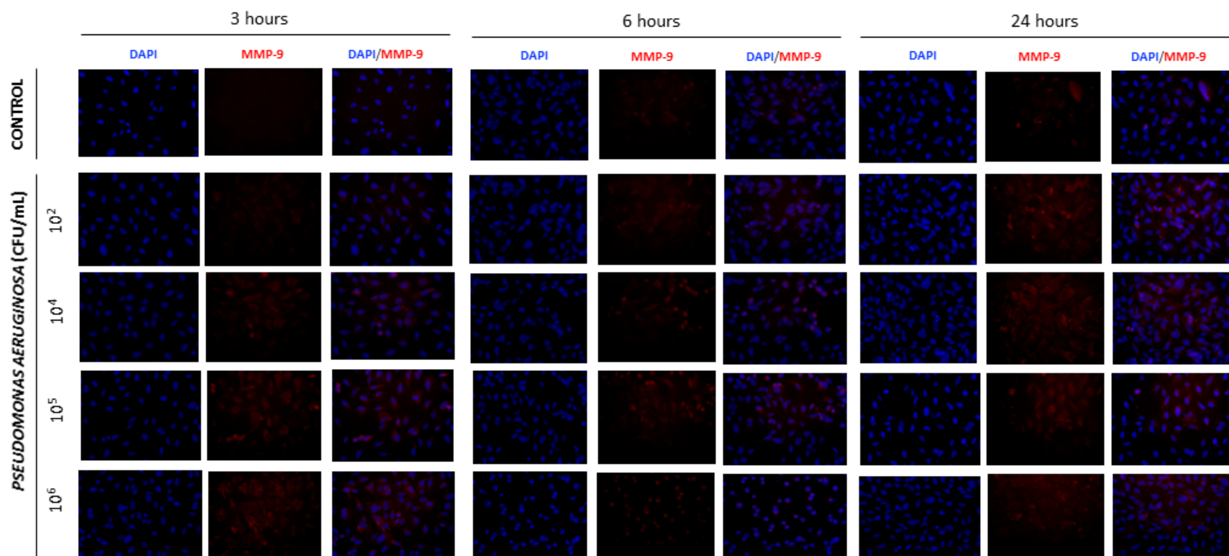

Figure 5. Immunoreactivity of *pro*MMP-9 in A549 cells.
Increased immunoreactivity of *act*MMP-9 was observed after 3, 6, and 24 hours of stimulation with *P. aeruginosa* when compared to the control group. In these assays, the nucleus was stained using Hoechst (blue color) and colocalization with *act*MMP-9 immunostaining (red color) was shown. The assay was performed in five independent experiments. The magnification is 20x.

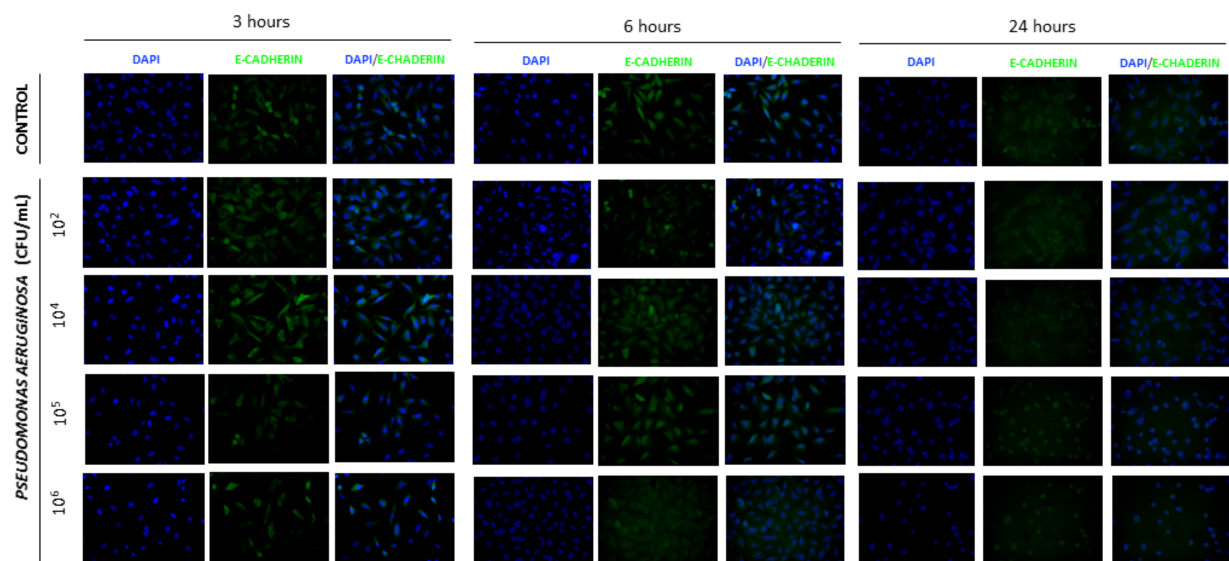

Figure 6. MMP-9 reduced the E-cadherin signal in A549 cells.
A bright signal from E-cadherin immunostaining was detected after 3, 6, and 24 hours in the control group. In contrast, weaker staining was observed after 24 hours of incubation with *P. aeruginosa*. In these assays, the nucleus was stained using 4´,6-diamidino-2-phenylindole (DAPI, blue color) and colocalization with E-cadherin immunostaining (green color) was shown. The assay was performed in five independent experiments. The magnification 20x.

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 expresion. 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 *pro*MMP-2 and a 62-KDa band corresponding to its active form (Fig. 4E). Unfortunately, the activity of MMP-2 could not be determined. Furthermore, *pro*MMP-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-

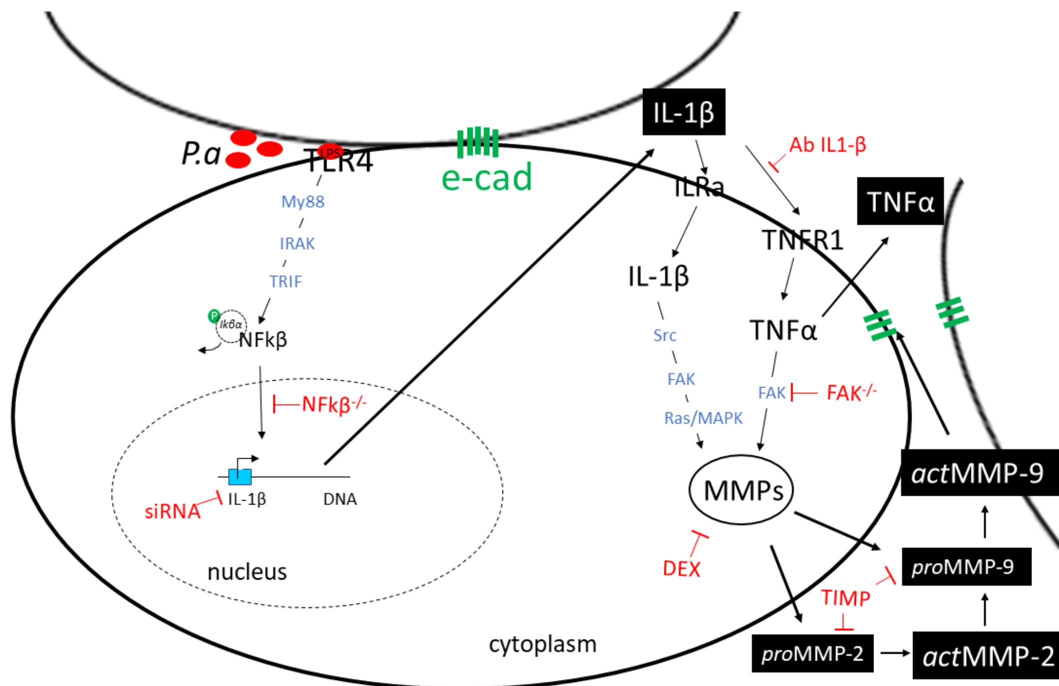

Figure 7. Network of inflammatory cytokine signaling and activation of *pro*MMP-9 in A549 cells.
The interaction of the structural components of *P. aeruginosa* lipopolisaccharide (LPS) with Toll-like receptor 4 activates a series of intracellular signals (myeloid differentiation primary response protein; *MyD88* and TIR domain-containing adaptor inducing interferon-beta; *TRIF*) leading to the phosphorylation of the inhibitory protein (Iκβα) that induces the activation of the nuclear transcription activator NFκβ and as a consequence increases IL-1β expression (Liu *et al.* 2017). IL-β interacts with the receptor for IL-1β and with the receptor type-1 of TNF (TNFR1) (Jayaraman *et al*., 2013; Jackson *et al*., 2010), modulating the secretion of extracellular matrix metalloproteases (MMPs). At the extracellular level, *pro*MMP-2 is modified by the membrane type-1 matrix metalloproteinase (MT1-MMP) increasing its degradative capacity (Fig. 4I). *act*MMP-2 is able to cut specific regions of *pro*MMP-9 transforming it into its active form with the capability to degrade different substrates; among them, the cell-binding protein of the E-cadherin type (Fig. 6).

pression of MMP-2 and -9 during pulmonary pathological. During physiological development, MMPs are secreted into the extracellular space in the form of *pro*MMPs 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 *pro*MMP-2 (72 KDa) and *pro*MMP-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, *act*MMP-9 degrades the E-cadherin involved in cell-cell adhesion (Allport *et al*., 2002; Nawrocki-Raby *et al*., 2003). Using immunohistochemistry, Shaco-Levy *et al.* (2008) showed that an increase in the secretion of *act*MMP-9 reduces the level of E-cadherin and intracellular β-catenin protein. Our results showed a reduction of the E-cadherin with relation to the concentration of *P. aeruginosa* used for stimulation and the time of stimulation (Fig. 7). Carayol *et al.* (2002) and Kim *et al.* (2018) used human nasal epithelial cell to demonstrated the association between an increase of MMP-9 expression and a decrease in E-cadherin levels. Interestingly, the activity of MMP-9 was inhibited by preincubation with dexamethasone which was accompanied by increased levels of E-cadherin (Carayol *et al*., 2002; Kim *et al*., 2018).

Although in this study we did not examine the expression of NFκβ, we are planning to do it as part of our research project in order to explore the potential links between inflammasome (IL-1β/TNFα) and NFκβ.

The studies reported here demonstrated that *P. aeruginosa* induces mainly the secretion of TNFα, increasing the *act*MMP-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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