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Short communication

Escherichia coli lipopolysaccharide induces alveolar epithelial cell stiffening

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ABSTRACT

Introduction: Application of lipopolysaccharide (LPS) is a widely employed model to mimic acute respiratory distress syndrome (ARDS). Available data regarding LPS-induced biomechanical changes on pulmonary epithelial cells are limited only to *P. aeruginosa* LPS. Considering that LPS from different bacteria could promote a specific mechanical response in epithelial cells, we aim to assess the effect of *E. coli* LPS, widely employed as a model of ARDS, in the biomechanics of alveolar epithelial cells.
Methods: Young's modulus (E) of alveolar epithelial cells (A549) was measured by atomic force microscopy every 5 min throughout 60 min of experiment after treatment with LPS from *E. coli* (100 μg/mL). The percentage of cells presenting actin stress fibers (F-actin staining) was also evaluated. Control cells were treated with culture medium and the values obtained were compared with LPS-treated cells for each time-point.
Results: Application of LPS induced significant increase in E after 20 min (77%) till 60 min (104%) in comparison to controls. Increase in lung epithelial cell stiffness induced by LPS was associated with a higher number of cells presenting cytoskeletal remodeling.
Conclusions: The observed effects of *E. coli* LPS on alveolar epithelial cells suggest that this widely-used LPS is able to promote a quick formation of actin stress fibers and stiffening cells, thereby facilitating the disruption of the pulmonary epithelial barrier.

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1. Introduction

Acute respiratory distress syndrome (ARDS) is a critical pathological condition with high clinical burden and mortality rates in intensive care medicine (Pham and Rubenfeld, 2017). ARDS pathophysiology typically includes lung inflammation and non-cardiogenic pulmonary edema primarily caused by increased permeability to proteins across lung endothelial and epithelial barriers (Huppert and Matthay, 2017).

Lipopolysaccharide (LPS) challenge is a model closely mimicking ARDS because epithelial cells can be directly (*eg.* pneumonia) and indirectly (*eg.* sepsis) targeted by this molecular agent (Chen et al., 2010). Despite the broad use of this model, available data focusing on LPS-induced biomechanical changes on pulmonary epithelial cells are very limited, with the exception on a report describing an increase in the elastic modulus of alveolar epithelial cells (A549) in response to *Pseudomonas aeruginosa* (Byfield et al., 2011). Information on cell mechanics is crucial since the integrity of the alveolocapillary barrier could be compromised, depending on the dynamic balance between inward forces – cell mechanical tension generated by actomyosin contraction – and outward forces exerted by cell-cell and/or cell-matrix adhesions (Puig et al., 2013). In this context, it is important to note that the cellular effects elicited by exposure to LPS challenge is bacteria-dependent (Raoust et al., 2009; Matsushita et al., 2007; Chuang et al., 2009), and also depends on cell type (Raoust et al., 2009) and animal strain (Alm et al., 2010). In particular, epithelial injury and disruption could depend on the type of LPS employed. Despite these evidences, most authors only use one type of LPS as a surrogate model of ARDS

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constituting a potential limitation. Accordingly, we aimed at testing the hypothesis that the clinically relevant *E. coli* LPS would increase the epithelial cell stiffness upon cytoskeleton remodeling, hence potentially contributing to alveolo-capillary damage in ARDS.

2. Methods

The study was carried out with alveolar lung epithelial cells (A549 cell line, ATCC® CCL-185) cultured using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin, streptomycin and amphotericin B, 10 mM HEPES and 1 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained in an incubator with humidified environment containing 5% CO_2 at 37 °C.

Cells were plated on 60-mm-diameter culture dishes (TPP, Trasadingen, Switzerland) at 80% confluence. Twenty-four to forty-eight hours after plating, cell stiffness was measured by atomic force microscopy (AFM). To this end, each sample was placed onto a custom-built AFM (Alcaraz et al., 2003) attached to an inverted optical microscope (Eclipse TE2000; Nikon, Melville, NY, USA). Force-indentation curves were obtained with a V shape Au-coated cantilever (thermal tune calibrated, nominal spring constant = 0.03 N/m) with a spherical tip (4.5 μm-diameter) on its apex (Novascan Technologies, Inc, Boone, IA, USA). The cantilever was placed on the cell perinuclear region of each sample for determination of cell stiffness in baseline (mean value of measurements every 5 min during 20 min). Then, the cells where exposed to LPS (100 μg/mL) from *E. coli* (L2880, Sigma Aldrich), or culture medium only. Straight after the challenge (LPS group, n = 12 samples; Control group, n = 11 samples), measurements were taken every 5 min in single cells, keeping the cantilever at the perinuclear region, throughout 60 min of experiment. Each measurement consisted of five force-displacement curves (F-z) (triangular ramp, 1 Hz oscillation, 4 μm peak-to-peak ramp amplitude, and a maximum indentation of 1000 nm). Young's modulus (E) was computed by fitting the force-indentation (F-δ) curve with the Hertz contact model (Alcaraz et al., 2003) using a custom analysis program in MATLAB (Mathworks; Natick, MA, USA):

$$F = \frac{4E}{3(1-\upsilon^2)}\sqrt{R}\delta^{\frac{3}{2}}$$

where υ represents Poisson's ratio (typically 0.5) and R the radius of the indenter (2250 nm).

Subsequently to cell stiffness measurements, a study on the changes induced in the organization of cytoskeleton fibers was performed. Cells were washed twice with PBS and fixed with 4% formaldehyde-PBS solution for 10 min at room temperature. After two additional washes with PBS, cells were permeabilized with a solution of 0.25% Triton X-100 in PBS for 10 min and washed again with PBS. Incubation was done with a mixture of phalloidin-TRITC (0.15 μM) (P1951, Sigma-Aldrich) and NucBlue™ Live Cell Stain (Life Technologies, Grand Island, NY, USA), following the manufacturer's protocol, in PBS 1% BSA for 20 min at room temperature. After three washes with PBS, dishes were dried and then mounted on a slide containing mounting medium (Mowiol; Calbiochem, La Jolla, CA, USA). Imaging was performed with a confocal laser scanning microscope (Leica TCS-SP5, Leica Microsystems, Wetzlar, Germany) at x63 magnification in samples corresponding to the 0–15 min (n = 3) and 45–60 min (n = 7) after LPS application. The number of cells presenting prominent actin stress fibers characterized by thick bundles at the cell periphery were quantified in percentage of the total number of cells per image. Cell staining, as well as stress fiber quantification were carried out under experimental blind conditions.

In an independent batch of experiments, cell viability was assessed by using a live/dead kit assay (L3224, Thermofisher Scientific, CA, USA) following the manufacturer's instructions. Briefly, at end-point of experiments, cells exposed to either LPS or control for 1 h were washed twice with PBS 1X and incubated for 30 min with 4 μM of EthD-1 and 2 μM calcein AM. Finally, cells were washed again with PBS 1X and images were taken by an inverted microscope. Images were quantified by calculating the percentage of dead cells respect to total cells.

Results are expressed as mean ± standard error. SigmaPlot 11 statistical package (SYSTAT Software, Chicago, IL, USA) was used. The normality of the data (Shapiro-Wilk test) and the homogeneity of variances were tested. If both conditions were satisfied, two-way repeated measures analysis of variance, followed by Student-Newman-Keuls test were used to assess differences among groups ($p < 0.05$). To compare the percentage of cells presenting actin stress fibers, a two-way analysis of variance, followed by Student-Newman-Keuls test was performed.

3. Results

Cell stiffness outcomes are depicted in Fig. 1. As expected, no changes in baseline were found between groups. After *E. coli* LPS or control exposures, the Young's modulus of alveolar epithelial cells was significantly increased only in cells treated with LPS from 20 min (7.35 ± 1.40 kPa) till 60 min (10.07 ± 1.90 kPa) in comparison to controls (4.14 ± 0.86 and 4.93 ± 0.98 kPa, 20 and 60 min, respectively). The LPS dose employed did not induce cell death.

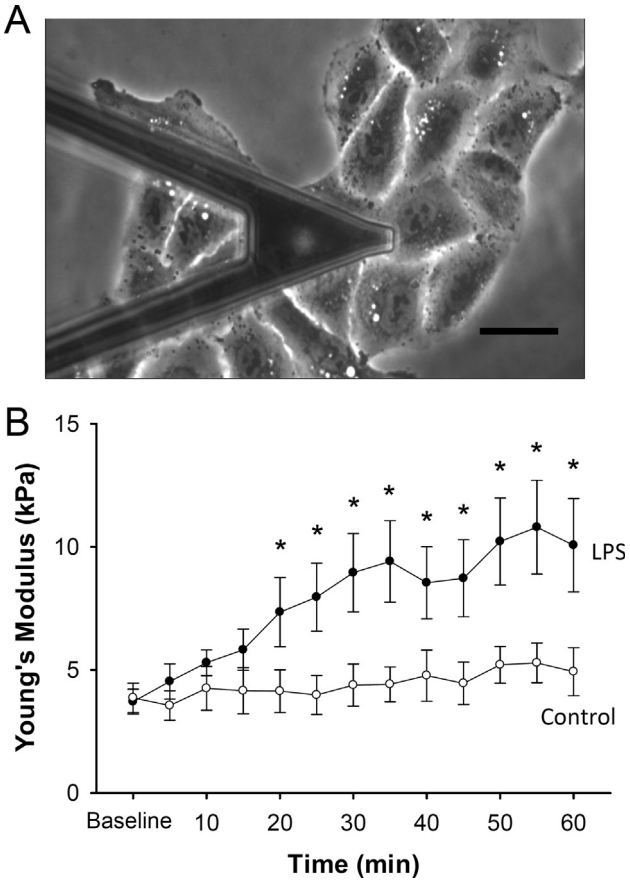

Fig. 1. (A) Phase contrast image of alveolar epithelial cells measured with atomic force microscopy. Bar = 25 μm. (B) Cell Young's modulus time-course after exposure to *E. coli* LPS (black circles, n = 12) or culture medium (white circles, n = 11). Data are mean ± SE. * Statistical significance ($p < 0.05$) compared with control.

Indeed, after 1 h exposure to either LPS or control conditions, dead cells were negligible in both groups (0.14 ± 0.03 and 0.19 ± 0.05%, respectively).

F-actin was organized in thick bundles at the cell periphery and this rearrangement was more markedly observed 45–60 min after LPS challenge (Fig. 2). Specifically, at the beginning of the LPS-exposure the percentage of cells presenting actin stress fibers was 6.1 ± 6.1% and after 45–60 min this percentage increased to 74.8 ± 9.4% ($p < 0.001$) which also was significantly different respect to the control group (28.5 ± 9.1%) ($p < 0.001$). Accordingly, no changes in F-actin polymerization were observed along time in the control group.

4. Discussion

The present study describes for the first time the considerable time-dependent cell stiffening induced by *E. coli* LPS in alveolar epithelial cells. These results concur with cytoskeletal remodeling and formation of prominent stress fibers observed along time exposure. Our results showing that *E.coli* LPS induces a response similar to *P. aeruginosa* LPS (Byfield et al., 2011) add important information to better characterize the *in vitro* model of ARDS since these two bacteria cover most community-acquired and nosocomial pneumonias.

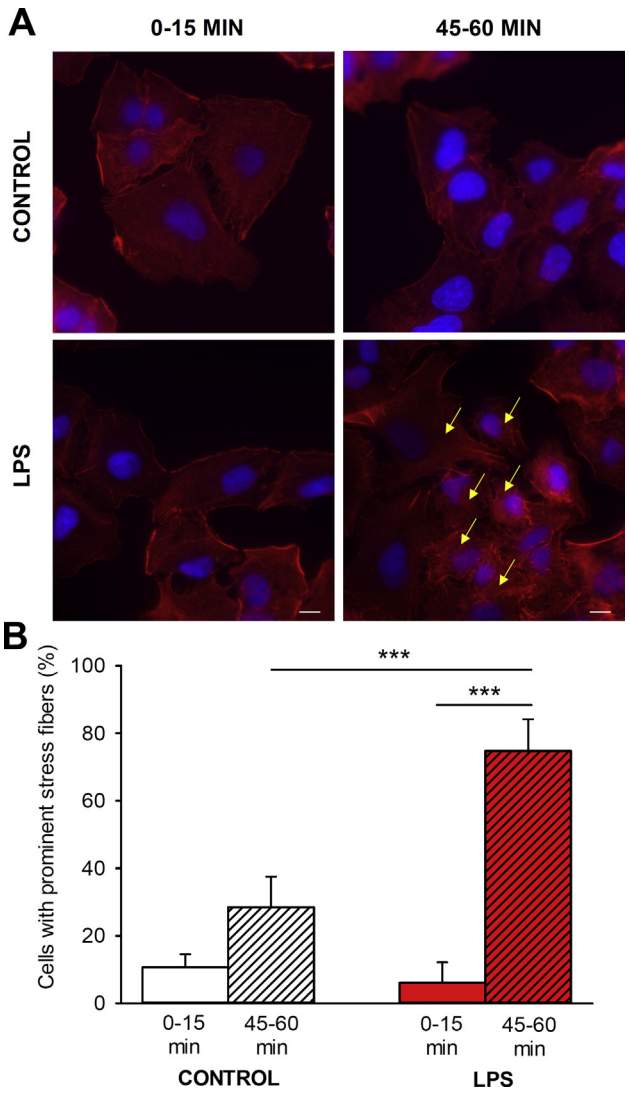

Fig. 2. (A) Representative images of cytoskeleton staining of alveolar epithelial cells 0–15 min and at 45–60 min after LPS exposure or control. F-actin was stained with phalloidin (red) and nuclei with Hoechst dye (blue). Bar = 10 μm. Peripheral F-actin stress fibers were more markedly observed 45–60 min after LPS challenge (yellow arrows). (B) Percentage of cells presenting prominent actin stress fibers. Data are mean ± SE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

During the last two decades different studies have pointed out the crucial role played by cell mechanical forces on the integrity of the alveolar capillary barrier (Trepat and Farre, 2008). In fact, changes on cell stiffness, actin reorganization and other mechanical parameters in epithelial and endothelial cells have been studied in response to a large array of inflammatory mediators and reactive oxygen species (Puig et al., 2013; Ivanov et al., 2010; Okamoto et al., 2017). Interestingly, most of these studies have reported changes in cell stiffness compromising the integrity of the alveolocapillary barrier (Puig et al., 2013; Ivanov et al., 2010; Okamoto et al., 2017). Specifically, these biomechanical changes have been suggested to be produced in part by (i) focal adhesion formation, (ii) tight junction recruitment and, (iii) intrinsic mechanisms driving cytoskeletal rearrangement such as activation of the Rho-actomyosin which enhances the formation of stress fibers (Okamoto et al., 2017) as we observed in this work.

LPS is a major component in gram-negative bacteria and has been widely employed in experimental models of ARDS *in vivo* and *in vitro* (Chen et al., 2010). These studies have contributed to better understanding the mechanisms involved in ARDS and other inflammatory systemic diseases. Indeed, LPS is known to promote a large spectrum of biological responses in different cell types (Isowa et al., 1999; Isowa and Liu, 2001; Raoust et al., 2009; Martin and Matute-Bello, 2011; Chen et al., 2010). However, the increasing knowledge available on LPS effects on cell biomechanics has revealed some controversial data, including the fact that changes induced on cell stiffness is cell type-dependent. On the one hand, LPS has been reported to reduce stiffness in human macrophages and cardiomyocytes (Leporatti et al., 2006; Wang et al., 2013), and on the other hand, stiffness is increased in blood monocytes, murine macrophages (RAW264.7) and in live lung slices (Doherty et al., 1994; Pi et al., 2014; Meng et al., 2015) subjected to LPS challenge. Recent studies employing LPS synthetized by different bacteria have described that the cell response to LPS exposure is bacteria-dependent (Raoust et al., 2009; Matsushita et al., 2007; Chuang et al., 2009). Thus, the use of different types of LPS in each study could be the reason of those controversial findings in the literature.

Regarding the alveolar epithelium, only one study showed that exposure to *P. aeruginosa* LPS promotes an increase in cell stiffness in A549 cells (Byfield et al., 2011). In our study, we found that a similar response is induced after exposure to *E. coli* LPS. These data are therefore relevant when considering that *E. coli* is one of the most employed models for ARDS (Huppert and Matthay, 2017; Ballard-Croft et al., 2012). Increased cell stiffness has been associated with augmented centripetal forces of the cell, which along with cyclic stretch exerted to cells during spontaneous breathing, and specially mechanical ventilation, could lead to disruption of the cell-cell tight junctions and cell-matrix adhesions thereby compromising the alveolocapillary barrier integrity (Ballard-Croft et al., 2012). Interestingly, the barrier disruption that occurs in the alveolocapillary barrier in ARDS is similar to that described in the gut mucosal barrier, which has been speculated to facilitate the initial bacterial translocation to the organism (Wells et al., 1993). The LPS dose employed in this work, although similar than values reported in other studies (Du et al., 2012), was relatively high in order to activate the inflammatory response of alveolar epithelial cells (Boots et al., 2012; Palmberg et al., 1998). However, further works on dose-response are still needed.

In agreement with cell stiffness measurements, we found that *E. coli* LPS exposure induced a fast F-actin network reorganization in alveolar epithelial cells. Actin is the most abundant cytoplasmic protein and regulates important cellular functions including integrin-mediated adhesion, motility and proliferation (Du et al., 2012). This cytoskeletal reorganization, which has been proposed as a key step at the initiation of lung inflammation in the systemic inflammatory response syndrome, suggests that F-actin could be a potential target for treatment and prevention of lung inflammation characterizing this clinical condition (Du et al., 2012). Moreover, cytoskeleton microfilaments have been proposed to be involved in LPS-induced TNF-α and MIP-2 production in rat type I pneumocytes (Isowa et al., 1999; Isowa and Liu, 2001).

In conclusion, this study provides new evidence that *E. coli* LPS promotes an increase in alveolar epithelial cell stiffness, likely orchestrated by F-actin remodeling. This mechanical change is fast (within the first hour after LPS exposure), thus explaining the early development of ARDS few hours after exposure to LPS in animals (Ballard-Croft et al., 2012; Bastarache and Blackwell, 2009). However, further studies focused on the effects of LPS-induced changes in cytoskeletal reorganization and cell stiffness in other cell types, such as endothelial and immune cells are still needed to understand in depth their contribution to the development of ARDS manifestations and to delineate potential future treatments to counteract bacterial LPS damage elicited in cell mechanics.

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Conflict of interest statement

The authors confirm that they have no financial affiliation or involvement with any commercial organization that has direct financial interest in any matter included in this manuscript.

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