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Activation of human macrophages by mechanical ventilation in vitro

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Pugin, Jérôme, Irène Dunn, Philippe Jolliet, Didier Tassaux, Jean-Luc Magnenat, Laurent P. Nicod and Jean-Claude Chevrolet. Activation of human macrophages by mechanical ventilation in vitro. *Am. J. Physiol. Cell. Mol. Physiol.* 19: L1040–L1050, 1998.—Positive-pressure mechanical ventilation supports gas exchange in patients with respiratory failure but is also responsible for significant lung injury. In this study, we have developed an in vitro model in which isolated lung cells can be submitted to a prolonged cyclic pressure-stretching strain resembling that of conventional mechanical ventilation. In this model, cells cultured on a Silastic membrane were elongated up to 7% of their initial diameter, corresponding to a 12% increase in cell surface. The lung macrophage was identified as the main cellular source for critical inflammatory mediators such as tumor necrosis factor- α , the chemokines interleukin (IL)-8 and -6, and matrix metalloproteinase-9 in this model system of mechanical ventilation. These mediators were measured in supernatants from ventilated alveolar macrophages, monocyte-derived macrophages, and promonocytic THP-1 cells. Nuclear factor- κ B was found to be activated in ventilated macrophages. Synergistic proinflammatory effects of mechanical stress and molecules such as bacterial endotoxin were observed, suggesting that mechanical ventilation might be particularly deleterious in preinjured or infected lungs. Dexamethasone prevented IL-8 and tumor necrosis factor- α secretion in ventilated macrophages. Mechanical ventilation induced low levels of IL-8 secretion by alveolar type II-like cells. Other lung cell types such as endothelial cells, bronchial cells, and fibroblasts failed to produce IL-8 in response to a prolonged cyclic pressure-stretching load. This model is of particular value for exploring physical stress-induced signaling pathways, as well as for testing the effects of novel ventilatory strategies or adjunctive substances aimed at modulating cell activation induced by mechanical ventilation.

alveolar macrophages; tumor necrosis factor- α ; interleukin-8; interleukin-6; nuclear factor- κ B; metalloproteinases; ventilator-induced lung injury; acute respiratory distress syndrome

THE OUTCOME OF PATIENTS with acute respiratory distress syndrome (ARDS) and other types of respiratory failure has been markedly improved by the utilization of positive-pressure mechanical ventilation (19). However, mechanical ventilation can itself lead to serious complications due to increased intrathoracic pressure and air space distension. Direct injuries can occur, such as blood-gas barrier disruption and pneumothorax (10, 13), but the lung can also be injured indirectly by

the recruitment of inflammatory cells to the lungs (21, 22). Two different types of stress induced by mechanical ventilation are thought to occur: 1) overstretching of small airways and alveoli due to increased volumes in some regions of the lung and 2) cycles of repeated opening and closing of alveolar groups in other regions, causing severe wall stress and surfactant depletion (29, 32).

Cell responses to mechanical stress such as shear stress have been studied during the past decade. It has been demonstrated that sheared endothelial cells produce inflammatory mediators (43, 53, 62). Shear stress-responding elements have been identified in the promoter regions of inflammatory genes (43, 52). The effects of cell stretching have also been studied in various cell systems (20, 24, 28, 48, 58, 60). On the other hand, positive-pressure mechanical ventilation induces a complex pressure-stretching strain in the cells different from this well-studied shear stress. Nonetheless, recent animal studies suggest that mechanical ventilation may induce the production of inflammatory mediators by lung cells. For example, injurious ventilatory strategies applied to isolated rodent lungs lead to the alveolar secretion of various proinflammatory mediators such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and IL-8 (57, 59). In a model of surfactant depletion in rabbits, it was shown that granulocytes participated in ventilator-induced lung injury (21, 22).

The aim of our study was to determine the capacity of lung cells to secrete inflammatory mediators when subjected to a cyclic pressure-stretching strain resembling that induced by mechanical ventilation. Using an in vitro model of mechanical ventilation of cell monolayers, we have identified the macrophage as the likely cellular source for the secretion of proinflammatory mediators in response to mechanical ventilation. Macrophages respond to pressure-stretching strain by secreting the chemokine IL-8 and matrix metalloproteinase (MMP)-9. Mechanical ventilation does not directly induce TNF- α and IL-6 but synergizes with other inflammatory stimuli such as bacterial endotoxin to stimulate the secretion of these mediators.

MATERIALS AND METHODS

Plastic Lung Model

A "plastic lung" was designed to reproduce the cyclic pressure-stretching regimen induced by mechanical ventilation. This plastic lung was made of transparent Plexiglas (Fig. 1). The bottom of the chamber was engineered to reproduce a double six-well plate (12 wells of 3.5-cm diameter each), with the exception that the bottom of the wells was

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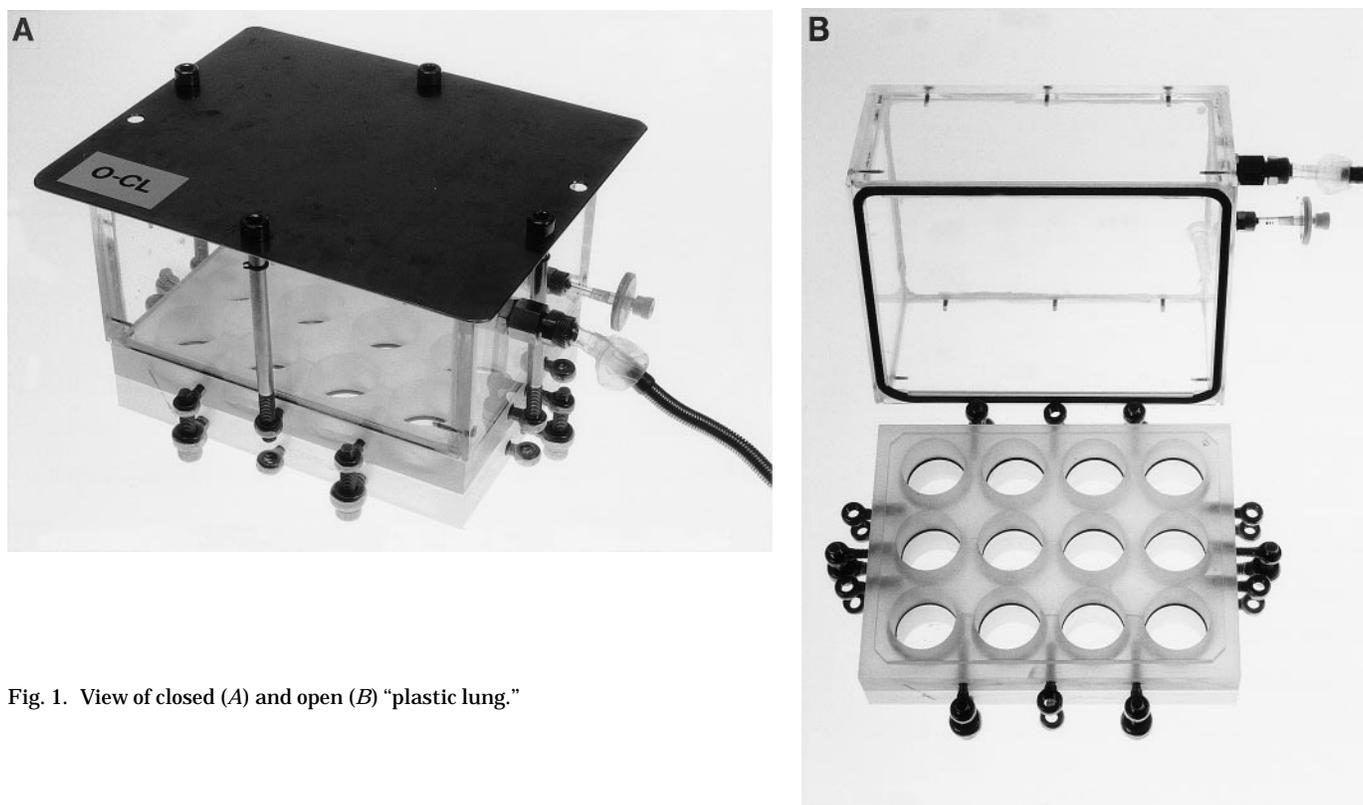
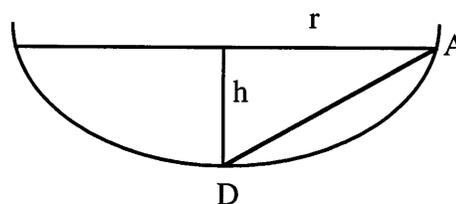


Fig. 1. View of closed (A) and open (B) "plastic lung."

composed of a Bioflex Silastic, distensible, optically clear membrane (Flexcell International, McKeesport, PA). The same membrane is used in the Flexercell strain system, a stretching device with BioFlex plates bottomed with these membranes, and used for cyclic cell stretching (Flexcell International) (20, 60). Two holes were made in the lateral wall of the chamber. The first was connected to a noncollapsible size 6.0 endotracheal tube (Laserflex tracheal tube, Mallinckrodt Medical, St. Louis, MO), and the second was connected to a pressure transducer and a graphic recorder (Gould Instrument Systems, Valley View, OH), allowing on-line monitoring of the pressure regimen inside the chamber. For culturing cells before mechanical ventilation in a 5% CO₂, 100% humidity atmosphere, the first hole was obturated and the second hole was connected to a 0.2- μ m filter (Millex GV, Millipore, Molsheim, France). During mechanical ventilation experiments, the chamber was bolted down to the bottom part in an airtight manner and connected to a regular intensive care adult ventilator (Evita 2, Drägerwerk, Lübeck, Germany). CO₂ was added to the inspiratory circuit of the ventilator, adjusted to an inspired fraction of 5%, and monitored in the expired air with a Normocap apparatus (Datex Instrumentarium, Helsinki, Finland) throughout the protocol. The inspired fraction of O₂ of the ventilator was adjusted to ensure a 21% O₂ concentration in the plastic lung to compensate for the CO₂ admixture. The inspired air was kept moist at 37°C with a heatable respiratory humidifier (MR700, Fisher & Paykel Healthcare, Auckland, New Zealand) and a Pall heat and moisture exchange filter (BB25 filter, Pall, Muttens, Switzerland) connected between the inspiratory limb of the ventilator and the Y piece. The plastic lung was kept in a 37°C incubator throughout cell ventilation. The temperature inside the chamber was checked several times and was always found to be 37°C. The Pall filter also prevented potential bacterial contamination of the plastic

lung chamber during ventilation. The ventilatory circuit, the Y piece, and the chamber of the respiratory humidifier were treated with 70% ethanol overnight before each ventilation experiment. Only endotoxin-free sterile distilled water was used for humidification of the inspired air. The day before cell ventilation, the plastic lung was sterilized with 70% ethanol and ultraviolet exposure (>1 h), and rinsed three times with sterile distilled water and once with PBS, pH 7.4. The cells were then seeded in the wells of the plastic lung or in BioFlex plates for control static conditions (see *Cells* for cell types and specific culture conditions) and cultured in a 37°C, 5% CO₂, 100% humidity atmosphere. The next day, the plastic lung was connected to the ventilator. The cells were ventilated in a controlled mode at a frequency of 20 cycles/min with an inspiratory-to-expiratory time ratio of 1:1, an inspiratory flow of 90 l/min, and a maximum pressure limit of 80 cmH₂O. This corresponded to a peak and a plateau pressure of \sim 70 cmH₂O, a tidal volume of 280–300 ml, and a minute ventilation of 5.6–6 l/min in the plastic lung. Experiments were also performed with pressures of 40 and 20 cmH₂O in the plastic lung. Mean stretching of the Silastic membrane was calculated according to the following formula: mean stretching = [surface of the sphere zone ($=\pi AD^2$)/surface of the disk ($=\pi r^2$)] - 1; because $AD^2 = r^2 + h^2$, then stretching = h^2/r^2 , where r is the radius and h is the height.



Using a small plastic stick of negligible mass with a millimetric scale, we measured a central vertical excursion of 6 mm during the "inspiratory pressure plateau" of 70 cmH₂O, which corresponded to a mean stretching of 12%; i.e., the surface of the inflated membrane was 12% greater than that of the noninflated membrane. Because of the physical properties of the Silastic membrane and the limitation of pressures generated by the ventilator, maximum mean stretch was limited to 12–15% in this model. In other systems of cell stretching, this amount of stretch is associated with maximal biological cell responses (20, 24, 28, 58). In the Silastic membrane used in our studies, the radial strain profile is not uniform. Cells are more elongated in the periphery compared with those in the center (15). A pressure-volume curve of the system was established, and the compliance of the plastic lung was measured at 10 ml/cmH₂O, which corresponds to a "stiff lung." According to the pressure-volume curve, ventilation of the plastic lung was performed in the linear portion of the curve.

Validation of Cell Stretching

To validate our plastic lung model, primary alveolar macrophages (AMs) were cultured on Silastic membranes in BioFlex six-well plates (Flexcell International) and stretched under direct microscopic vision. The lid from the six-well plate was sealed to the bottom part with silicon glue in an airtight fashion, and a hole was drilled into the lid. A rigid plastic catheter was connected to it and also sealed with silicon glue. This catheter was connected to a syringe and a pressure-monitoring system. Sealed BioFlex plates were then put under an Axiovert 100TV phase-contrast microscope (Zeiss) in a 37°C heated chamber and connected to a digital-imaging system (Hamamatsu digital camera 4742-95). The Silastic membranes were progressively "inflated" with the syringe with increasing air volumes to obtain the following pressure increments inside the "chamber": 0, 12, 25, 45, 60, and 75 cmH₂O. One AM was imaged at a time. The focus was constantly readjusted during membrane inflation, and the vertical excursion was recorded for each pressure level. At maximal pressure, vertical excursions were on the order of 5–7 mm, corresponding to those measured in the plastic lung with similar pressures. Images of stretched AMs were recorded at all pressures. Cell diameter and surface were measured in pixels with the Openlab software (Improvision) and converted to micrometers and square micrometers, respectively, according to the magnification factor used to view the cell (at $\times 32$ magnification, 478 pixels = 100 μm). Percent cell stretch (elongation) at a given pressure was defined as $[(d \text{ at a given pressure} - d \text{ at zero pressure}) / d \text{ at zero pressure}] \times 100$, where d is the cell diameter. For cells imaged on the periphery of the Silastic membrane, the correction of the cell surface due to the angle of the Silastic membrane with that of the horizontal plane due to membrane inflation was neglected.

Cells

Human AMs were obtained by lavage from lungs removed from patients with lung cancer as described elsewhere (34). All donors were current smokers or had quit smoking recently. Briefly, healthy lobes of these lungs were lavaged with 0.15 M NaCl shortly after the operation. The cells were then pelleted, washed with RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U/ml of penicillin, and 50 $\mu\text{g/ml}$ of streptomycin (culture medium; GIBCO, Basel, Switzerland). The cells were then seeded onto plastic surfaces

(6-well regular plastic Falcon plates or BioFlex plates with a Silastic bottom membrane; Flexcell International) at a density of 2×10^5 cells/cm². This procedure yielded >95% pure AMs as determined by a specific esterase-staining method. Human monocyte-derived macrophages (MDMs) were obtained from buffy coats of healthy blood donors. Peripheral blood monocyte cells were purified with a classic Ficoll technique, washed in culture medium, and distributed onto plastic or Silastic surfaces to obtain a macrophage cell density comparable to that of AMs (see above). After a 45-min incubation at 37°C, lymphocytes were washed away, leaving adherent MDMs. MDMs rested 24 h before change of medium and cell stimulation. In some experiments, the glucocorticoid (GC) dexamethasone (1 $\mu\text{g/ml}$; Organon) was used to pretreat (2 h) MDMs and AMs.

Nonadherent promonocytic human THP-1 cells [2×10^5 cells/cm²; American Type Culture Collection (ATCC), Manassas, VA] (2) were seeded onto Silastic membranes in the presence of 10 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO). This treatment rendered cells firmly adherent. After 18 h, the medium was changed, and the cells rested for 30 h, the time necessary for chemokine and cytokine levels to return to their basal levels. Mechanical ventilation and stimulation with agonists was then performed in fresh medium. *Escherichia coli* 0111:B4 lipopolysaccharide (LPS; 200 ng/ml; List, Campbell, CA) was used as a positive control for MDM, AM, and THP-1 cell stimulation.

In other experiments, the following adherent cell types were tested: human type II-like epithelial A459 cells (ATCC), EA.hy926 endothelial cells (12) cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics, human bronchial BET-1A cells (ATCC) cultured in LHC-9 medium (Biofluids, Rockville, MD) (49), and human primary lung fibroblasts cultured in their corresponding medium (Clonetics, Walkersville, MD). PMA and/or recombinant IL-1 β (kindly provided by J.-M. Dayer, University of Geneva, Switzerland) were used as positive controls for endothelial cells, epithelial cells, and fibroblasts.

Mechanical Ventilation of Cells

On the day of assay, the cells were washed with RPMI 1640 medium and ventilated for 32 h in 3 ml of RPMI 1640 medium containing 2% fetal bovine serum. This volume of medium covered the cells during the whole cycle of mechanical ventilation. Experiments were performed in triplicate for each condition and time point. Each experiment was performed most often three times but at least two times. Data are expressed as means \pm SD. Conditioned medium was sampled at 8, 24, and sometimes 32 h and centrifuged at 4°C. Supernatants were divided into aliquots and kept frozen at -70°C until the day of assay. In all experiments, cells were seeded at the same density in parallel onto six-well BioFlex plates (same Silastic membrane as in the plastic lung) and kept in a 37°C incubator (static controls). Positive controls (addition of 200 ng/ml of LPS for macrophages and 2 ng/ml of IL-1 or 100 ng/ml of PMA for the other cell types at *time 0*) were performed in parallel with the mechanical ventilation both in the plastic lung and in static control conditions. Cells submitted to ventilation or in the static plates were found to be >95% viable after 32 h as assessed by the trypan blue exclusion method. In addition, lactate dehydrogenase activity (measured by the classic pyruvate-to-lactate method) in supernatants from macrophages ventilated for up to 32 h did not increase significantly, indicating the absence of a cytotoxic effect of mechanical ventilation in our model system.

Measurement of Inflammatory Markers

ELISAs. Human TNF- α levels in conditioned supernatants were measured by a sandwich ELISA technique with a pair of monoclonal antibodies according to the manufacturer's protocol (PharMingen, San Diego, CA). The limit of detection of the TNF- α assay was 40 pg/ml. Human IL-8 levels in conditioned supernatants were measured by a sandwich ELISA technique with a polyclonal rabbit anti-human IL-8 antibody (a kind gift of S. L. Kunkel, University of Michigan, Ann Arbor, MI) as described elsewhere (38). Human IL-6 and IL-10 were measured in conditioned supernatants with ELISA techniques based on monoclonal antibody pairs and protocols obtained from Endogen (Woburn, MA). The limit of detection was 10 pg/ml for the three assays.

MMPs. Levels of MMP-9 were determined by the quantification of the gelatinolytic band obtained in gelatin zymograms of conditioned supernatants (8, 41). Quantification was performed with a Molecular Dynamic densitometer and Image-Quant software, and relative MMP-9 concentrations are expressed as arbitrary density units.

Nuclear factor- κ B activation. Electrophoretic mobility shift assays were performed as previously described (39). Briefly, MDMs in static conditions and MDMs ventilated in the presence and absence of LPS were collected by cell scraping at different times (30, 60, and 120 min) and chilled in ice-cold PBS, pH 7.3. Nuclear proteins were obtained with a first extraction with an anti-protease- and Triton X-100-containing lysis buffer and a second extraction with a high salt-containing buffer. Three micrograms of nuclear proteins were mixed with trace amounts of 32 P-labeled nuclear factor- κ B (NF- κ B) oligonucleotide probe (Promega) in the presence of salmon sperm DNA (Sigma) and poly(dI-dC) (Pharmacia) and were analyzed on 5% acrylamide gels made in Tris-glycine-EDTA. Electrophoresed gels were then transferred onto Whatman paper, dried, and subjected to autoradiography.

RESULTS

Visualization of Cell Stretching

Using direct microscopic observation and recording of macrophages cultured on Silastic membrane submitted to stretch, we could demonstrate that the cell diameter and surface progressively increased with volume-pressure load increments to a level similar to that of the membrane itself (Fig. 2). At 75 cmH₂O pressure, macrophages were elongated by a factor of $7.1 \pm 0.4\%$ (cell diameter), corresponding to an increase in cell surface of $11.8 \pm 2\%$. This value of cell stretching is in accordance with the calculated Silastic membrane stretching when similar pressures were applied to the plastic lung (see MATERIALS AND METHODS).

Cytokine Response to Mechanical Ventilation

AMs. Of the different cell types submitted to mechanical ventilation, AMs were the cells responding the most. IL-8 secretion was chosen as a marker for ventilator-induced lung inflammation because this chemokine is the most potent chemoattractant for neutrophils and because neutrophil recruitment has been observed in lungs submitted to positive-pressure mechanical ventilation. AMs produced elevated IL-8 levels in response to pressure-stretching cyclic strain (Fig. 3), with a maximal response as early as 8 h after initiation of the

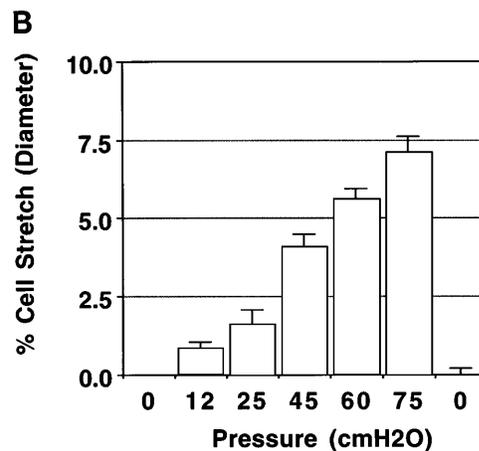
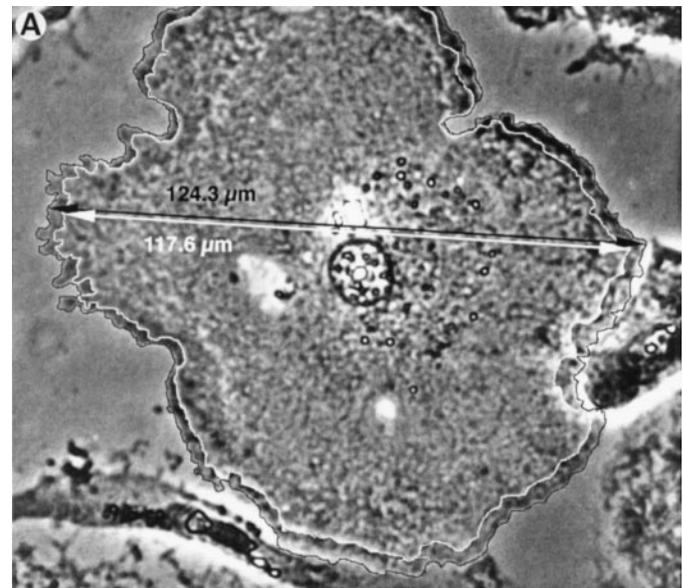
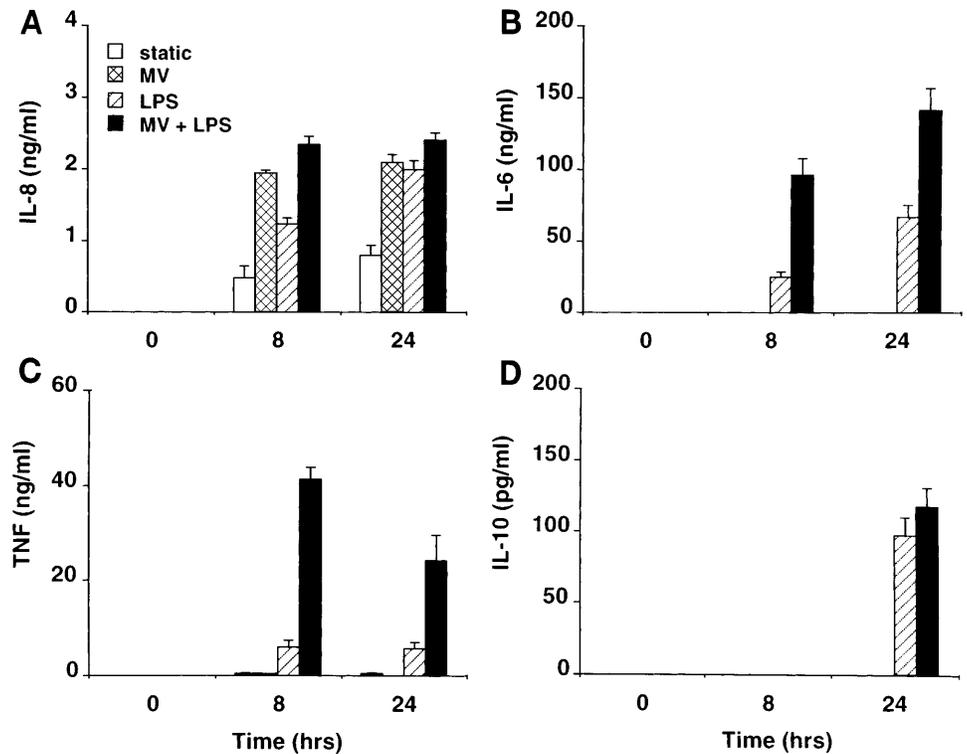


Fig. 2. *A*: superimposed phase-contrast microscopic images ($\times 32$ magnification) of an alveolar macrophage adherent to a Silastic membrane in static conditions (white line) and submitted to a pressure of 60 cmH₂O (black line). Arrows, cell diameters. In this representative case, cell elongation was 5.7%. *B*: effect of different pressures [starting and ending with static (0) condition] applied to macrophages ($n = 4$) cultured on Silastic membranes on cell elongation. Values are means \pm SD.

mechanical stress. TNF- α and IL-6 were produced by AMs in response to LPS but not after mechanical ventilation, suggesting a differential response to mechanical ventilation between IL-8 and both TNF- α and IL-6. A marked synergistic effect on the secretion of both TNF- α and IL-6 was observed when LPS was added to ventilated cells (Fig. 3). Importantly, the absence of TNF- α measured in ventilated macrophages over a period of 32 h made very unlikely the possibility of LPS contamination of the plastic lung. In addition, endotoxin levels were consistently found to be ≤ 0.05 endotoxin unit/ml (*Limulus* assay lysate; BioWhittaker) in supernatants from ventilated macrophages. The anti-inflammatory cytokine IL-10 was produced only after 24 h by AMs in response to LPS (Fig. 3). There was no significant additive effect of mechanical ventilation on the LPS-induced secretion of this anti-

Fig. 3. Interleukin (IL)-8 (A), IL-6 (B), tumor necrosis factor- α (TNF; C), and IL-10 (D) secretion by human primary alveolar macrophages submitted to a pressure-stretching strain [mechanical ventilation (MV)] for 8 and 24 h in absence and presence of 200 ng/ml of lipopolysaccharide (LPS). Static control cells are macrophages plated on a similar Silastic membrane but not submitted to a mechanical stress. Values are means \pm SE of pooled experiments with alveolar macrophages from 3 different donors.



inflammatory cytokine. Treatment of macrophages with recombinant TNF- α (10 ng/ml) or IL-1 β (2 ng/ml) induced IL-8 responses 2–10 times smaller than those with LPS (200 ng/ml) or mechanical ventilation (data not shown). No difference in cell response was observed when cells were plated onto an uncoated Silastic surface or collagen 1-, fibronectin-, or gelatin-treated Silastic membranes.

MDMs. Because of the inconsistent availability of AMs for our experiments, we tested the hypothesis that MDMs produced similar responses and could serve as surrogate cells for AMs. The IL-8 response of MDMs shown in Fig. 4 indicated a very similar IL-8 secretion

pattern when compared with that of AMs. Ventilated MDMs produced IL-8 levels similar to those induced by LPS in the same cells under static conditions. A small additive effect of the two stimuli was observed. Similar to observations made in AMs, mechanical ventilation per se did not induce TNF- α and IL-6 in MDMs, but the secretion of these cytokines induced by LPS was markedly enhanced by mechanical ventilation (data not shown). MDMs ventilated with different driving pressures indicated that 20 cmH₂O induced only slightly elevated IL-8 levels at 24 h, whereas 40 and 80 cmH₂O induced similar elevated levels of IL-8 at 8 and 24 h (Fig. 5). MMP-9 was also secreted by MDMs in response

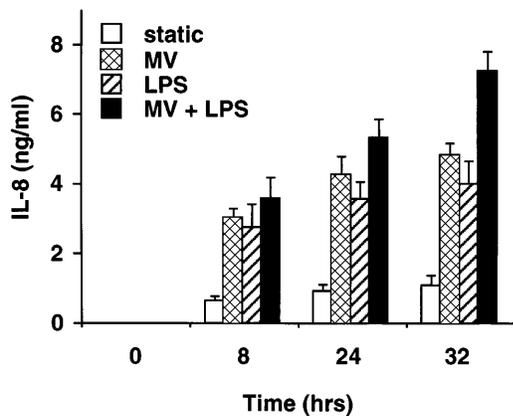


Fig. 4. IL-8 secretion by human monocyte-derived macrophages submitted to a pressure-stretching strain (MV) for 8, 24, and 32 h in absence and presence of 200 ng/ml of LPS. Static control cells are macrophages plated on a similar Silastic membrane but not submitted to a mechanical stress. LPS represents IL-8 response of macrophages stimulated by LPS under static conditions. Values are means \pm SE of pooled experiments with monocyte-derived macrophages from 4 different donors.

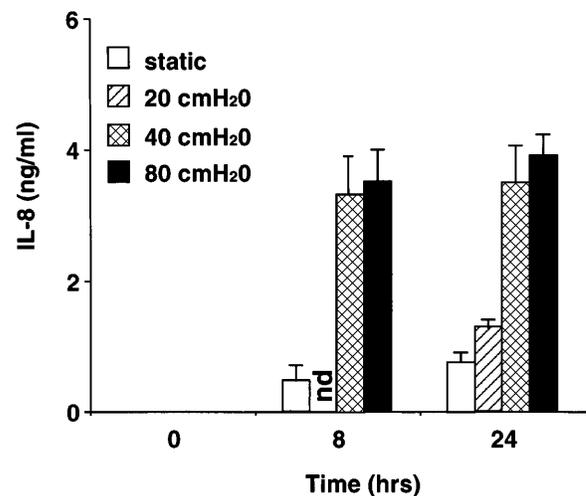


Fig. 5. IL-8 secretion by monocyte-derived macrophages plated on Silastic membranes in plastic lung submitted to various pressure levels for 8 and 24 h. nd, Not done. Values are means \pm SE from 4 independent experiments performed in triplicate.

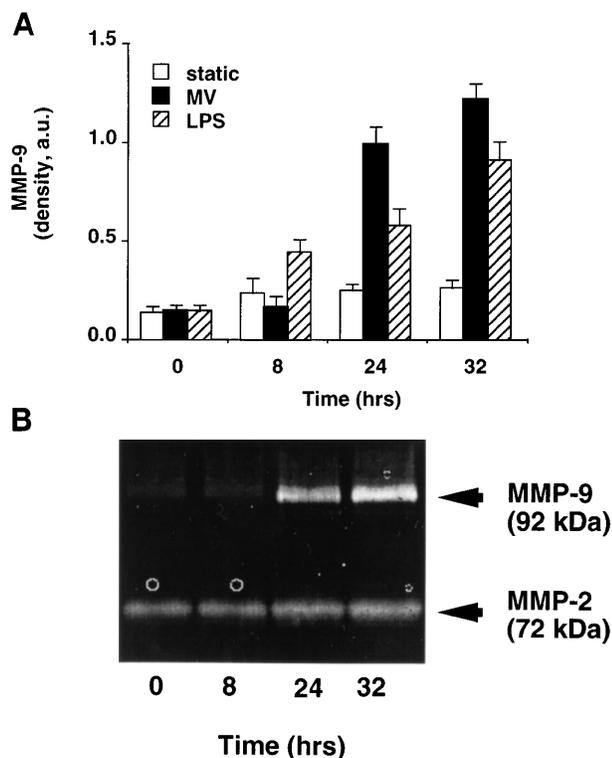


Fig. 6. *A*: matrix metalloproteinase (MMP)-9 production by monocyte-derived macrophages submitted to pressure-stretching strain (MV) for 8, 24, and 32 h. LPS represents IL-8 response of macrophages stimulated by LPS under static conditions. MMP-9 levels were determined by quantification of 92-kDa gelatinolytic band on gelatin zymograms of conditioned supernatants (see MATERIALS AND METHODS). a.u., Arbitrary units. Values are means \pm SE of 3 different experiments. *B*: representative gelatin zymography of conditioned supernatant from monocyte-derived macrophages submitted to MV for various times. Note upregulation of MMP-9 (92 kDa) at 24- and 32-h time points.

to mechanical ventilation and reached a maximum after 24 h at levels comparable to those induced by LPS (Fig. 6).

Promonocytic THP-1 cells. THP-1 cells have been used in a variety of studies as a model for monocyte/macrophage activation (2). We investigated whether

this cell line could be used as a model for mechanical stress-induced macrophage activation. This would have the advantage of setting a reproducible model independent of interindividual differences in cellular responses and that could generate genetically modified cells with transfection, for example (39). We established conditions in which THP-1 could be rendered adherent to the Silastic membrane with the phorbol ester PMA, without persistent cell activation. Adherent THP-1 cells submitted to mechanical ventilation showed activation patterns very similar to those observed in AMs and MDMs (Fig. 7). Ventilated THP-1 cells secreted IL-8 but not TNF- α or IL-6. The addition of LPS and mechanical ventilation resulted in a marked enhancement of TNF- α , IL-8, and IL-6 production compared with LPS alone (Fig. 7). Seventy-two-hour macrophage differentiation of THP-1 cells by 1,25-dihydroxyvitamin D₃ (2) did not modify the pattern of cell response to mechanical ventilation but increased cytokine levels by a factor of \sim 3 (data not shown).

NF- κ B Activation by Mechanical Ventilation

The secretion of many proinflammatory cytokines depends on the activation of nuclear factors such as NF- κ B. Once activated, the latter translocates into the nucleus and binds to specific NF- κ B DNA sequences present in the promoter region of cytokine genes (27, 31). NF- κ B has been implicated in the activation of cells in response to mechanical stress such as endothelial shear stress (23). We tested the hypothesis that NF- κ B could be activated by a cyclic pressure-stretching force applied to MDMs and thereby be implicated in cellular responses to cyclic pressure-stretching strain. In Fig. 8, we show that NF- κ B was activated in MDMs as early as 30 min after the initiation of mechanical ventilation and persisted for as long as 2 h.

Blocking Mechanical Ventilation-Induced Cell Activation With GCs

GCs exert their anti-inflammatory effects, at least in part, via the inhibition of NF- κ B activation in response to various proinflammatory stimuli (1). We observed

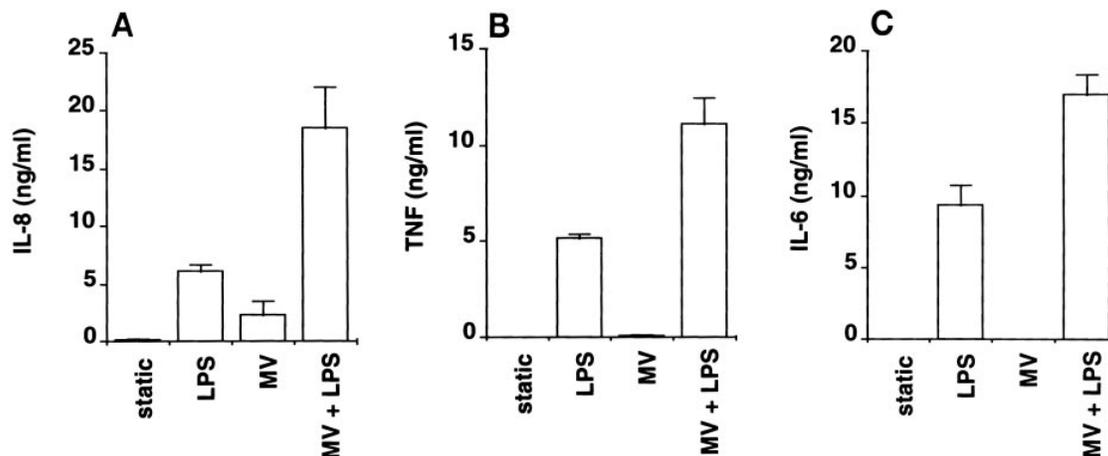
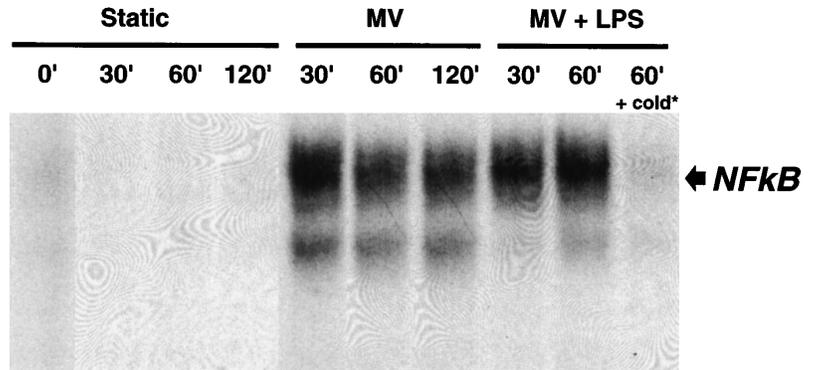


Fig. 7. IL-8 (*A*), TNF (*B*), and IL-6 (*C*) secretion by adherent promonocytic THP-1 cells submitted to MV in plastic lung for 24 h at 80 cmH₂O driving pressure in absence and presence of 200 ng/ml of LPS. Values are means \pm SD of a representative experiment done in triplicate.

Fig. 8. Representative electrophoretic mobility shift assay of nuclear factor- κ B (NF- κ B) activation in monocyte-derived macrophages under static conditions or submitted to MV in absence and presence of 200 ng/ml of *Escherichia coli* LPS for 0, 30, 60, and 120 min (0', 30', 60', and 120', respectively). Arrow, complex of ³²P-labeled NF- κ B consensus DNA oligoprobe and activated NF- κ B proteins. +cold*, 60 \times excess unlabeled NF- κ B oligoprobe.



that the mechanical ventilation-induced NF- κ B signal decreased after GC pretreatment (data not shown) and, more importantly, that the mechanical ventilation-induced secretion of IL-8 and TNF was abolished by GC treatment (Fig. 9). Dexamethasone also inhibited IL-8

and TNF secretion induced by LPS plus mechanical ventilation (Fig. 9).

Effect of Mechanical Ventilation on Other Cell Types

Several other human cell types, which might theoretically be of importance in biological responses to distended airways, were tested in the plastic lung. These included endothelial cells, epithelial cells, and fibroblasts. Only the type II-like A549 epithelial cells responded to mechanical ventilation by secreting low levels of IL-8 after 24 h, whereas the same cells produced high IL-8 levels in response to PMA (Fig. 10). With identical cell culture and ventilation protocols as those used for macrophages, neither IL-8 nor IL-6 were produced in response to mechanical ventilation by endothelial EA.hy926 cells, bronchial BET-1A cells, and primary lung fibroblasts, whereas all these cells were capable of secreting IL-8 in response to other stimuli (recombinant IL-1 β or PMA; data not shown). Fibroblasts and endothelial and epithelial cells do not produce TNF- α .

DISCUSSION

Herein, we describe an in vitro model of mechanical ventilation of lung cells and provide insight into a

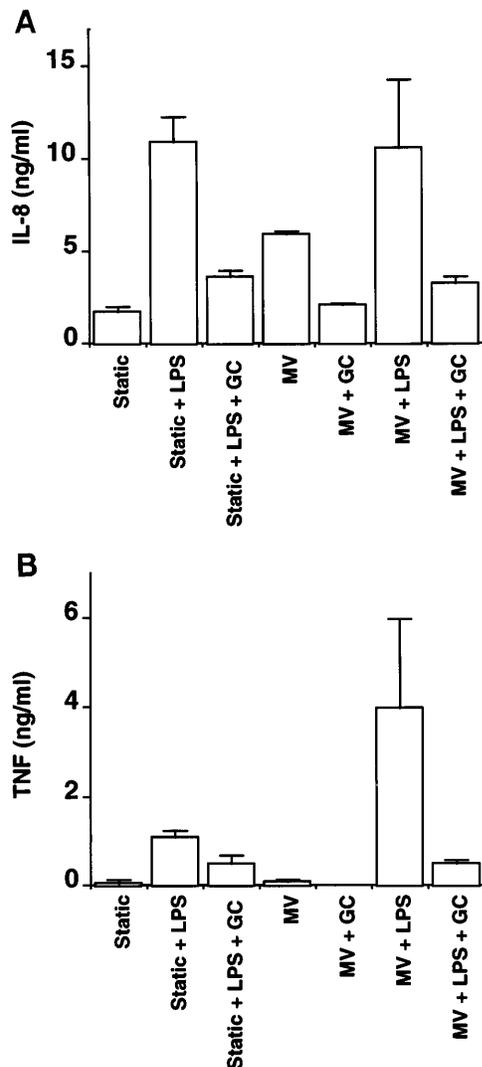


Fig. 9. Effect of glucocorticoid (GC) dexamethasone on IL-8 (A) and TNF (B) secretion by monocyte-derived macrophages under static conditions or submitted to MV in plastic lung for 24 h in absence and presence of 200 ng/ml of *E. coli* LPS. Values are means \pm SD of a representative experiment done in triplicate.

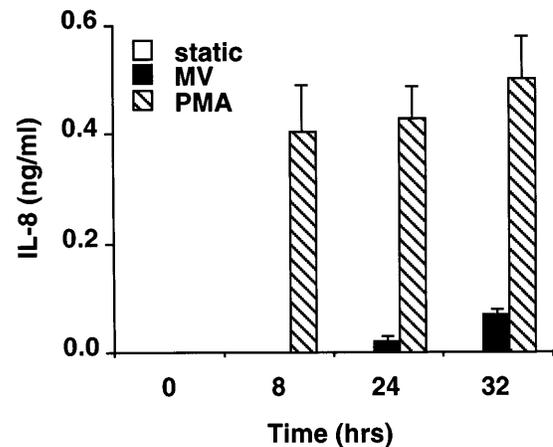


Fig. 10. IL-8 secretion by human alveolar type II-like A549 cells submitted to a cyclic pressure-stretching strain (MV) in plastic lung for 8, 24, and 32 h. Phorbol 12-myristate 13-acetate (PMA) indicates IL-8 response of A549 cells stimulated by PMA in static conditions. Values are means \pm SD of a representative experiment done in triplicate.

cellular basis for ventilator-induced lung inflammation. The lung macrophage was identified as an important mechanosensor, responding to pressure-stretching cyclic load by secreting critical inflammatory mediators such as IL-8, TNF- α , IL-6, and MMP-9. A key transcription factor, NF- κ B, was also activated in these cells.

In ARDS, large portions of the lung consist of alveoli that are collapsed at end expiration and open during inspiration, whereas other areas are condensed and never open and others still are close to normal. Thus, during mechanical ventilation, the former lung zones undergo cyclic opening and closing and probably sustain damage due to high wall stress forces, whereas the latter receive most of the tidal volume and are at risk of overdistension during inspiration (14, 46). New ventilatory strategies were recently developed with the aim of minimizing overdistension (pressure-targeted ventilation, low-volume ventilation) (29, 30) and opening-closing cycles of alveolar groups (positive end-expiratory pressure) (14, 25, 61).

Experimental data exist to support lung injury induced by both phenomena. In various animal models and in vitro cell experiments, including some with lung-derived cells, cell stretching induces proinflammatory mediators, cellular proliferation, or cellular injury (18, 20, 24, 28, 48, 58, 60). Conversely, in animal models where rapid alveolar opening was induced, the lungs were either significantly injured (61) or responded by the secretion of proinflammatory mediators (59). In the latter situation, it has been calculated that wall stress during alveolar opening corresponded to a transmural pressure of ~ 140 cmH₂O applied to the cells (32). In an ex vivo isolated rat lung model, Tremblay et al. (57) recently measured increased TNF- α , IL-1 β , IL-6, and IL-8 in lung lavages after injurious ventilatory regimens. Maximum cytokine production was observed in lung lavage from animals ventilated with large volumes, with an end-expiratory lung volume below the lower inflection point of the pressure-volume curve, where cycles of opening and closing of alveolar groups occur (57).

Our in vitro model was designed to combine both pressure and stretch forces, simulating the in vivo situation of positive-pressure mechanical ventilation. Using this model, we first tested whether IL-8, a potent neutrophil chemoattractant produced by various lung cell types (7, 40, 54), could be upregulated in response to mechanical forces resembling those of mechanical ventilation. IL-8 was chosen as a relevant mediator because a hallmark of ventilator-induced lung injury is the lung recruitment of neutrophils and neutrophil-mediated tissue injury (21, 22). In addition, elevated IL-8 levels were measured in the alveolar space of patients ventilated for ARDS (9, 33). Of the different cell types tested, the macrophage was the strongest IL-8 producer in response to mechanical stress. This is not a complete surprise because this cell already integrates various other stresses (bacterial molecules, proinflammatory cytokines, osmotic shock, pH variations) (16). Interestingly, TNF- α and IL-6 could not be induced directly by mechanical ventilation of macro-

phages but required an inflammatory costimulus for a synergistic response. Although IL-8 and TNF- α are usually secreted in parallel after myeloid cell activation, a differential regulation of these two cytokines has been described in circulating leukocytes (6). These findings suggest that mechanical ventilation may induce limited inflammation in normal lungs, mainly IL-8-dependent recruitment of neutrophils, but may well augment lung inflammation to clinically important levels in preinjured or infected lungs. Supporting this hypothesis, two groups have demonstrated in rats that a previous lung alteration (oleic acid or the toxic α -naphthylthiourea) was associated with a marked ventilation-induced lung injury, with massive lung edema, compared with that of the toxin alone or high-volume ventilation alone (11, 17).

On the basis of the observations made in our studies, it can be concluded that macrophages are sensitive to cyclic pressure stretching, a mechanical strain resembling that of positive-pressure mechanical ventilation. However, we cannot ascertain that the AM is the cell playing the major role in the initiation of ventilator-induced lung injury. First, these cells are loosely adherent because they are easily collected by bronchoalveolar lavage. Second, it was shown by Tremblay et al. (57) that TNF- α mRNA induced by an injurious ventilatory regimen in isolated rat lungs was not decreased by a depletion of AMs collected by bronchoalveolar lavage. The macrophage seems, however, to be a strong candidate because injurious ventilatory regimens consistently induced TNF- α secretion in animal airways (55, 57, 59) and because TNF- α is essentially produced by myeloid cells. To reconcile these data, we hypothesize that the interstitial macrophage may play an important role as a mechanosensor in the lung.

ARDS is also characterized by profound tissue remodeling in both its early and late phases (37, 45). We tested the hypothesis that mechanical cell stresses induced the secretion of enzymes important for lung remodeling. MMPs are enzymes that degrade extracellular matrix components and basement membranes and are found at elevated levels in the alveolar spaces of patients with ARDS (44, 56). MMP-9 is a type IV collagenase that is produced mainly by neutrophils and macrophages (35) and epithelial cells (63). The epithelial-derived MMP-9 was shown to actively contribute to lung repair processes (5). Whereas neutrophils secrete MMP-9 by degranulation, the MMP-9 secreted by macrophages is tightly regulated and requires *de novo* enzyme synthesis (42, 47). In the present study, we showed that, in addition to known proinflammatory mediators, a pressure-stretching stimulus induced MMP-9 secretion after 24 h in human macrophages. Pardo et al. (36) demonstrated that additional collagenase transcripts (MMP-1 and MMP-2) were upregulated in rat lungs ventilated with high volumes.

It is still unclear what triggers intracellular signaling events, and a membrane or intracellular mechanoreceptor(s) remains to be discovered (3). Second messengers and signal transduction pathways have been studied in some detail in mechanical stresses such as

endothelial fluid shear stress (50). In this latter example, mitogen-activated protein kinase cascades are turned on, leading to the activation of various transcription factors (26). Different DNA "shear stress-responding elements" have been identified in the promoter region of sensitive genes (43, 51), including 12-*O*-tetradecanoylphorbol 13-acetate-responding element (activator protein-1) (52) and NF- κ B DNA binding domain (23). Less is known about activation pathways during cell stretching. In experiments performed in MDMs submitted to cyclic pressure stretching, we observed the activation and nuclear translocation of NF- κ B. This transcription factor has been implicated as a critical factor for the activation of various inflammatory genes including IL-8 and MMP-9 (31), the same genes that were shown to respond to mechanical ventilation. However, it does not seem sufficient because IL-6 and TNF- α , which both have NF- κ B consensus DNA sequences in their promoter region, were not directly activated by cell stretching. We did not observe activated activator protein-1 in nuclear extracts from ventilated macrophages (Dunn and Pugin, unpublished data).

GCs exert their anti-inflammatory properties, at least partially, via blockade of NF- κ B activation and nuclear translocation (4). In the present work, we observed that dexamethasone blocked both NF- κ B activation and cytokine production by cells submitted to mechanical ventilation or to a combined cell stress, i.e., mechanical ventilation and bacterial endotoxin. This indicated that common distal activation pathways were utilized by proinflammatory cell stresses such as LPS and mechanical stresses and that both agonists could be blocked with a GC. This may also have some implications in the pharmacological treatment of ventilator-induced lung inflammation.

Of the various other cell types present in the lung parenchyma, only the alveolar type II-like A549 cells responded, albeit poorly, to pressure stretching. A similar observation was made by another group (18) with stretched A549 cells. Their weak IL-8 response might be related to the loss by these immortalized cells of important functions or molecules critical for mechanical strain responses. Human primary type II or type I cells would be of great interest to test in our model; however, these cells cannot be isolated with the degree of purity needed for such studies. Human bronchial epithelial BET-1A cells were shown to produce IL-8 after cell deformation and detachment (49) but failed to secrete that chemokine when ventilated. The absence of an IL-8 response in endothelial cells is also puzzling, although again the cells that we tested were derived from human umbilical vein endothelial cells and fused with A549 cells (12) and thus might not reflect the response of capillary pulmonary endothelial cells. Supporting this fact, endothelial cells from different vascular beds have different responses to stretching (20).

Using microscopic-imaging technology, we could clearly demonstrate that in our model macrophages cultured on Silastic membranes were actually stretched when the membrane was inflated and that cell stretch-

ing was certainly a main determinant for cell activation. However, this model has limitations: it clearly oversimplifies the lung structure and the complex forces exerted on lung cells during the process of positive-pressure mechanical ventilation. In addition, the true percent stretch of alveolar and interstitial cells during mechanical ventilation is not known and probably varies considerably from one territory to the other. On the other hand, our model has the advantage of simplicity and the potential for addressing focused questions with purified primary cells or cell lines in a mechanically active environment resembling that of positive-pressure mechanical ventilation. It may also allow one to dissect the signal transduction pathway of cells activated by a cyclic pressure-stretching strain.

In conclusion, the model described in this study led to the identification of the AM as a likely important cellular source for proinflammatory mediators produced in response to mechanical ventilation. This provides a potential molecular and cellular basis for the ventilator-induced lung inflammation and remodeling theory. Our model is particularly suitable for further studies on signaling pathways and gene regulation in response to a pressure-stretching strain and to test the effect of ventilator settings and therapeutics on the mechanical stress-induced cell activation.

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