Exposure to mechanical ventilation promotes tolerance to ventilator-induced lung injury by Ccl3 downregulation

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The objective of this work is to identify the existence of tolerance to VILI by previous exposure to noninjurious mechanical ventilation. We developed an animal model of preconditioning by mechanical ventilation and used microarrays to characterize the response of preconditioned mice. Finally, validation of the identified targets was done in additional experiments.

METHODS

Animals. All experiments were performed in 8- to 12-wk-old male CD1 mice. All mice were kept under specific pathogen-free conditions with free access to food and water. The experiments were approved by the Ethics Committee of the Universidad de Oviedo, Oviedo, Spain.
Protocol overview. Mice were randomly assigned to receive a short ventilatory course or a sham procedure (receiving the same dose of anesthesia as their ventilated counterparts). After anesthesia with intraperitoneal ketamine and xylazine, mice were intubated with a 20G orotracheal catheter and ventilated in pressure-controlled mode [peak inspiratory pressure 17 cmH2O, positive end-expiratory pressure (PEEP) 2 cmH2O, respiratory rate 100 breaths/min] for 90 min. After this time, animals were extubated and returned to their cages for recovering. One week later, preconditioned and control mice were anesthetized, tracheostomized, and ventilated for 2 h in pressure-controlled mode with higher driving pressures (peak inspiratory pressure 20 cmH2O, PEEP 0 cmH2O, respiratory rate 50 breaths/min). Compared with preconditioning, these settings result in a driving pressure 5 cmH2O higher and for a longer time than during preconditioning. Without the protective effects of PEEP (34), these settings induce a moderate VILI (1, 11, 15), as the magnitude of tissue injury is proportional to the area under the pressure-time curve (34). FiO2 was 0.21 during all the protocol. During all the ventilatory periods, temperature was maintained using a heating pad. Figure 1 shows the timeline of the study.

Linear compliance was measured by insufflation of a fixed amount of 500 µl of air and recording of the increase in airway pressure using a calibrated pressure transducer. Blood gases were measured using a NPT7 gasometer (Radiometer) using samples drawn from the aorta at the end of the ventilatory period. Tissue samples were obtained from each experimental group. In additional animals, a bronchoalveolar lavage was performed after the study.

Tissue sampling. Mice were studied in baseline conditions, immediately and 1 wk after low-pressure ventilation and after high-pressure ventilation. Under anesthesia, a laparotomy was performed, the animals were exsanguinated by section of the renal artery, the thorax opened, and the lungs removed. The left lung was fixated with the intratracheal injection of 250 µl of 4% phosphate-buffered paraformaldehyde, immersed in the same fixative for 24 h, and then stored in 50% ethanol. The right lung was immediately frozen at −80°C. For biochemical analysis, tissues were mechanically homogenized in standard RIPA buffer (21). The protein content of the homogenates was measured (BCA kit, Pierce).

Histological studies. Lungs were embedded in paraffin, and three histological sections stained with hematoxylin and eosin. Tissue damage was evaluated by two observers, blinded to the experimental conditions, using a predefined score (0: Normal lungs; 1: Capillary congestion; 2: Alveolar wall thickening; 3: Inflammatory infiltrates or intra-alveolar flooding; 4: Massive disruption of the lung structure).

Immunohistochemistry. Myeloperoxidase-positive cells were recognized in paraffin-embedded sections by using a specific antibody (Dako). The number of positive cells was counted for three random fields (×200) and averaged.

Bronchoalveolar lavage. In separate experiments, a bronchoalveolar lavage (BAL) was performed at the end of the ventilatory period, before animals were killed. Three aliquots (0.7 ml) of saline were injected through the tracheostomy tube and recovered to obtain BAL fluid (BALF). Albumin content in BALF was measured using a COBAS 8000 automated analyzer (Roche Diagnostics).

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**Fig. 1.** Schematic representation of the experimental design and study groups, including sample size and measurements in each one. BALF, bronchoalveolar lavage fluid; VILI, ventilator-induced lung injury; MPO, myeloperoxidase.
Mice were randomized to the following 3 groups: group A sham, group B lowpressure ventilation, and group C high-pressure ventilation, see Fig. 1 for details). Six animals (3 sham and 3 preconditioned mice each) were used for microarray studies. The remaining 12 were used to assess the effect of BX471 treatment (6 treated with the drug and 6 treated with vehicle).

Preconditioning ameliorates ventilator-induced lung injury. Lung injury was scored in histological sections. Compared with intact, low-pressure ventilation induced a small, nonsignificant increase in lung injury score. After 1 wk, this mild damage was completely repaired. This finding correlated with similar respiratory system compliances in baseline and preconditioned animals (25.5 $\pm$ 2.5 vs. 27.8 $\pm$ 2.0 $\mu$l/cmH2O, respectively, $P = 0.64$). As expected, high-pressure ventilation caused severe damage within the lungs. However, mice preconditioned with prior low-pressure ventilation showed a significantly lower lung injury (Fig. 2A). After high-pressure ventilation, respiratory system compliances were 21.8 $\pm$ 0.9 and 18.3 $\pm$ 0.6 $\mu$l/cmH2O in preconditioned and intact mice, respectively ($P = 0.04$). The albumin content in the bronchoalveolar lavage fluid was significantly lower in preconditioned animals, suggesting a decreased alveolar permeability (Fig. 2B). Figure 2C shows representative histological sections of each experimental group. In line with these findings, oxygenation after injurious ventilation was better in preconditioned animals [arterial Po2 (PaO2) 108 $\pm$ 19 vs. 57 $\pm$ 8 mmHg, $P = 0.04$], with lower arterial Pco2 (PaCO2) (32 $\pm$ 2 vs. 52 $\pm$ 6 mmHg, $P = 0.03$) and a trend toward higher pH (7.36 $\pm$ 0.01 vs. 7.23 $\pm$ 0.06, $P = 0.09$).

To demonstrate the existence of an inflammatory response within the lungs (22), MPO-positive cell counts were performed in histological sections from mice submitted to high-pressure ventilation, either after preconditioning or not. The myeloperoxidase-positive cell count was lower in lungs from preconditioned animals (Fig. 3, A and B). Additionally, gene expression and protein levels of Il6 and Il10 were quantified, as canonical examples of pro- and anti-inflammatory cytokines, respectively. Il6 expression significantly increased in all the experimental groups submitted to mechanical ventilation (Fig. 3C), with no differences between preconditioned and nonpreconditioned animals. There was a substantial variability in Il10 expression, so the differences were not significant ($P = 0.16$, Fig. 3D). Protein levels followed the changes in gene expression, with significant increases in IL-6 after VILI, and
no significant differences among groups in IL-10 (Figs. 3, E and F).

Preconditioned mice show a differential lung gene expression. To explore the genomic mechanisms behind tolerance to VILI, we used microarrays to characterize the gene expression in lung tissue from preconditioned mice before high-pressure ventilation, compared with animals submitted to the sham procedure. The main differentially expressed genes are shown in Fig. 4. In a Gene Ontology enrichment analysis, an overrepresentation of the category “Response to abiotic stimulus” was detected (OR 12.67, \( P = 0.00016 \)), reinforcing the idea that the differences in gene expression were caused by the previous exposure to mechanical ventilation.

Ccl3 is downregulated in preconditioned mice after VILI. Among the differentially expressed genes, we focused on the genes Hspa1b, Calcb, and Ccl3 as possible mechanisms re-

Fig. 2. Assessment of lung injury. A: histological lung injury, showing an ameliorated lung injury in preconditioned animals. B: quantification of bronchoalveolar lavage fluid (BALF) albumin content, a marker of alveolar permeability. C: representative histological sections of each experimental group.
sponsible for the ameliorated lung injury in preconditioned mice. These genes were significantly downregulated in lungs from preconditioned mice, according to the microarray data. Then their expression was assessed in lungs from mice submitted to high-pressure ventilation, with or without preconditioning. As shown in Fig. 5, A–C, only Ccl3 was significantly lower in tissues from preconditioned mice after injurious ventilation. Neither preconditioning nor injurious ventilation induced a significant change in Ccr1 expression (Fig. 5 D). Similarly, protein levels of the Ccl3 product MIP-1α/H9251 were lower in preconditioned animals (Fig. 5, E and G). In line with this finding, phosphorylation of ERK, a transcription factor linked to CCR1 activation by MIP-1α (14), was significantly decreased in these mice (Fig. 5, F and G). Collectively, these results suggest that downregulation of Ccl3 is one of the mechanisms responsible for tolerance to injurious ventilation.

**DISCUSSION**

The results reported herein show that previous exposure to low-pressure mechanical ventilation results in tolerance to high-pressure-induced lung injury. Exposure to a low-intensity stimulus can lead to tolerance to further insults. This phenomenon has been widely studied in endotoxemia and our findings extend it to ventilator-induced lung injury. A genomewide search revealed significant differences in a group of genes. Although preconditioning induced a downregulation in the antagonist BX471. Mice treated with the drug showed a decreased lung injury after high-pressure ventilation, mimicking the effects induced by preconditioning (Fig. 6, A and B). Similarly, MPO-positive cell count was decreased in treated animals (Fig. 6, C and D). Finally, phosphorylation of ERK was decreased after BX471 treatment (Fig. 6, E and F). These results demonstrate that CCR1 blockade mimics the preconditioning effect of previous exposure to ventilation.

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**Fig. 3** Differences in lung inflammation. Preconditioned mice showed a decreased neutrophilic infiltrate within the lungs, demonstrated by the lower myeloperoxidase-positive cell count (A). B shows representative immunohistological sections. Mechanical ventilation induced an increase in lung Il6 expression (C). Il10 expression showed a substantial variability among groups, with no statistically significant differences (D). Protein levels of IL-6 and IL-10 followed these changes in gene expression (E and F).
majority of the genes, some were also upregulated, in line with previous findings (5). Among those, further studies after VILI revealed Ccl3 as one gene involved in the induction of tolerance. Moreover, blockade of CCR1, the main receptor of the Ccl3 product, permitted us to mimic the observed effect. Overall, these findings point to a new pathway that may be useful for prevention of VILI and show that a previous short ventilatory course may modify its later occurrence.

The induction of tolerance to inflammatory responses is a well-known phenomenon. Classically, low-dose LPS or ischemia are known to induce a preconditioned state that results in a dampened inflammatory response to the same or different stimuli (homo- and heterotolerance, respectively). The time frame of this state has not been fully described: In other models of tolerance, a normal inflammatory release is recovered after 8 days (24). However, differences in gene expression have been described even months after the initial challenge (3). Our experimental design cannot help to identify the optimal timing after preconditioning.

The mechanisms responsible for immunotolerance remain elusive. Different pathways could be activated (4) depending on the experimental model. Using a genome-wide search, we identified some differences between intact and preconditioned mice. Although we cannot discard a significant effect caused by any of the genes identified by our microarray analysis, we focused on Hspa1b, Calcb, and Ccl3, as these have been implicated in ischemic or endotoxic preconditioning (18, 29, 35). The contribution of other genes to the pathogenesis of VILI or immunotolerance should be demonstrated in further studies.

Among the three selected genes, only Ccl3 showed a significant difference after VILI. The product encoded by Ccl3 is macrophage inflammatory protein-1 alpha. MIP-1α is a cytokine that belongs to the CC chemokine subfamily. This chemokine is produced by a great variety of cell types, including macrophages, neutrophils, epithelial cells, and fibroblasts. MIP-1α can bind to both type 1 and type 5 chemokine receptors (CCR1 and CCR5, respectively). Whereas binding to CCR1 leads to the recruitment of inflammatory cells, followed by extracellular matrix deposition, binding to CCR5 results in anti-inflammatory effects (23). Different studies have shown that Ccl3 play a significant role in tolerance to LPS (18). Regarding lung injury, Ccl3 is one of the genes needed for lung recruitment of circulating neutrophils in LPS- or bleomycin-induced lung injury (28). Similar results have been found in experimental models of ventilator-induced lung injury: lung stretch results in an increase in Ccl3 levels, whereas anti-inflammatory drugs such as steroids ameliorate this increase and the subsequent damage (16). Our data highlight that Ccl3 downregulation may be a relevant step in tolerance to mechanical ventilation. Therefore, targeting this chemokine could be an effective approach to avoid VILI in this context. Other authors have used genomic studies to identify therapeutic targets, resulting in attenuation of VILI (26). However, it must
be noted that our experimental approach precludes a firm causative relationship between Ccl3 and tolerance to VILI, and the involvement of other pathways cannot be discarded.

Using a cellular model, it has been recently demonstrated that moderate cyclical stretch can induce tolerance to overstretching by a Rac/Rho dependent mechanism involving apoptosis inhibition and cytoskeleton rearrangement (9). However, we did not identify a differential expression of these genes in our in vivo model.

Our results show that lung response to mechanical ventilation could be conditioned by the previous ventilatory history. A recent study has shown that the impact of high tidal volumes on
mortality decreases over ventilation time (25). Additionally, our results suggest that a previous exposure to ventilation leads to a different response when a second exposure is performed. Up to 10–15% of the critically ill patients that are extubated need reintubation in the next 48 h (27). Moreover, reintubation is related to a worse outcome (8). In these preconditioned patients, the fine-tuning of the ventilatory parameters could be different than the previous settings. Finally, the data reinforce the concept of dampened inflammatory response in critically ill patients after an episode of acute inflammation (17). It must be noted that our experiments were performed in juvenile mice, which have been reported to be more resistant to VILI (19). The impact of these findings in adult or elderly mice is to be demonstrated. Despite this limitation, all these phenomena illustrate the impact of mechanical ventilation on the course of critical illness.

In conclusion, our results show the existence of tolerance to VILI by exposure to previous ventilation. Moreover, the study of the mechanisms involved in this phenomenon allowed us to identify a potential therapeutic target to prevent VILI by targeting the Ccl3-MIP-1α-CCR1 pathway. This mechanism illustrates the complex nature of the lung inflammatory response to mechanical ventilation and could help to design novel therapeutic approaches.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


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