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EXPOSURE TO MECHANICAL VENTILATION PROMOTES TOLERANCE TO VENTILATOR-INDUCED LUNG INJURY BY *Ccl3* DOWNREGULATION

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Running head: Preconditioning with mechanical ventilation

Authors' contributions: GMA, JBP, ILA conceived and designed the study. JBP, ILA, LAR, EBS and AGP performed the experiments. GMA, ILA, JBP, LAR and AGP analyzed the data and discussed the results. GMA, ILA and JBP wrote the paper. All authors reviewed and discussed the manuscript.

Abstract

Inflammation plays a key role in the development of ventilator-induced lung injury (VILI). Preconditioning with a previous exposure can damp the subsequent inflammatory response. Our objectives were to demonstrate that tolerance to VILI can be induced by previous low-pressure ventilation, and to identify the molecular mechanisms responsible for this phenomenon. Intact 8-12 week old male CD1 mice were preconditioned with 90 minutes of non-injurious ventilation (peak pressure 17 cmH_2O, PEEP 2 cmH_2O) and extubated. Seven days later, preconditioned mice and intact controls were submitted to injurious ventilation (peak pressure 20 cmH_2O, PEEP 0 cmH_2O) for 2 hours to induce VILI. Preconditioned mice showed lower histological lung injury scores, bronchoalveolar lavage albumin content and lung neutrophilic infiltration after injurious ventilation, with no differences in *Il6* or *Il10* expression. Microarray analyses revealed a downregulation of *Calcb*, *Hspa1b* and *Ccl3,* three genes related to tolerance phenomena, in preconditioned animals. Among the previously identified genes, only *Ccl3*, which encodes the macrophage inflammatory protein 1 alpha (MIP-1α) showed significant differences between intact and preconditioned mice after high-pressure ventilation. In separate, non-conditioned animals, treatment with BX471, a specific blocker of CCR1 (the main receptor for MIP-1α) decreased lung damage and neutrophilic infiltration caused by high-pressure ventilation. We conclude that previous exposure to non-injurious ventilation induces a state of tolerance to VILI. Downregulation of the chemokine gene *Ccl3* could be the mechanism responsible for this effect.

Keywords: Ventilator-induced lung injury, immunotolerance, chemokines, genomics.

Application of positive pressure ventilation may trigger a proinflammatory response within the lungs. This response, which is thought to be detrimental, is an essential component of the so-called ventilator-induced lung injury (VILI)(31). In fact, the development of ventilatory strategies aimed to ameliorate VILI has improved the outcome of patients with the acute respiratory distress syndrome (6). These strategies are also correlated to decreased levels of lung pro-inflammatory mediators (13, 30). Similarly, targeting inflammation is almost uniformly related to a decreased lung damage in experimental models of VILI (33).

One of the most striking characteristics of the inflammatory response is the existence of tolerance phenomena. Preconditioning by previous exposure to a stimulus results in a decreased response after a delayed second-hit. Tolerance to endotoxin is probably the most paradigmatic example of this kind of response (4). However, it has been demonstrated that other stimuli such as ischemia or hyperoxia may have similar effects (7, 32). Recently, using an in-vitro model of VILI, Gao and coworkers have shown the beneficial effects of pre-exposure to moderate stretch, suggesting that mechanical ventilation with low pressures/volumes could induce tolerance to more aggressive ventilatory strategies (9).

The mechanisms behind immunotolerance are not fully elucidated. Low intensity injuries trigger an intracellular response involving a variety of mechanisms such as blunted NF-kB response, changes in intracellular kinases or microRNA production (4). In the case of mechanical ventilation, different clinical and experimental studies have shown that non-injurious ventilation triggers different lung responses including inflammation (11), matrix remodeling (12) or apoptosis (20).

Interestingly, some of these may persist after ventilation, but their impact on tolerance is largely unknown.

The objective of this work is to identify the existence of tolerance to VILI by previous exposure to non-injurious 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 8-12 week 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 than their ventilated counterparts). After anesthesia with intraperitoneal ketamine and xylazyn, mice were intubated with a 20G orotracheal catheter and ventilated in pressure-controlled mode (peak inspiratory pressure 17 cmH_2O, PEEP 2 cmH_2O, respiratory rate 100 breaths/min) for 90 minutes. 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 hours in pressure-controlled mode with higher driving pressures (peak inspiratory pressure 20 cmH_2O, PEEP 0 cmH_2O, respiratory rate 50 breaths/min). Compared to preconditioning, these settings result in a driving

pressure 5 cmH_2O 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). FiO_2 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 microliters 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 a week after low-pressure ventilation and after high-pressure ventilation. Under anesthesia, a laparotomy was performed, the animals were exanguinated by section of the renal artery, the thorax opened and the lungs removed. The left lung was fixated with the intratracheal injection of 250 microliters of 4% phosphate-buffered paraformaldehyde, and immersed in the same fixative for 24 hours, 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, USA).

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 intraalveolar 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 (x200) 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).

Quantitative PCR. RNA was extracted from lung tissue samples after homogenization with TRIzol (Sigma, Poole, UK) and precipitation by adding isopropanol. After centrifugation and washing with ethanol, the pellet containing the RNA was resuspended in water. Complementary DNA was synthesized from 1 µg of total RNA using a standard RT-PCR kit (High capacity cDNA rtKit, Applied Biosystems). Quantitative PCR was carried out in triplicate for each sample using 40 ng of cDNA. EURx qPCR master mix and 10 µM of the specific primers were used for the genes encoding macrophage inflammatory protein-1 alpha (*Ccl3* FW 5'-CCAAGTCTTCTCAGCGCCAT-3' / RV 5'-TCCGGCTGTAGGAGAAGCAG-3'),

chemokine (C-C motif) receptor 1 (*Ccr1*, FW 5'-CTCATGCAGCATAGGAGGCTT-3' / RV 5'-ACATGGCATCACCAAAAATCCA-3'), heat shock protein 70 (*Hspa1b* FW 5'-CAACGGCATCCTGAACGTCAC-3' / RV 5'-TGTTGAAGGCATAGGACTCGAGC-3'), calcitonin-related polypeptide beta (*Calcb* FW 5'-CAGGCCTGAGTCACTAGCAG-3' / RV 5'-TCCTTGAGGCCTTCACATCG-3'), interleukin-10 (*Il10* FW 5'-CTGTTTCCATTGGGGACACTT-3' / RV 5'-CAAGTGTGGCCAGCCTTAGA-3'), interleukin-6 (*Il6* FW 5′-ACCACTTCACAAGTCGGAGG-3′ / RV 5′-TGCAAGTGCATCATCGTTGT-3′) and glyceraldehyde 3-phosphate dehydrogenase as endogenous control (*GAPDH* FW 5'-GTGCAGTGCCAGCCTCGTCC-3′ / Rv 5′-GCCACTGCAAATGGCAGCCC-3') (all from Sigma-Aldrich, USA). The relative expression of the analyzed genes was calculated as $2^{\Delta CT(\text{gene of interest})-\Delta CT(GAPDH)}$.

Western blotting. Equal amounts of proteins were loaded in standard SDS-PAGE gels, electrophoresed, and transferred to PVDF membranes. These membranes were blocked with non-fat milk or bovine serum albumin (BSA) in a TBS-T buffer and incubated overnight with antibodies against phosphorylated extracellular signal-regulated kinases (pERK, Cell Signalling, USA) and macrophage inflammatory protein 1 alpha (MIP-1α, Abcam, UK). Afterwards, the antibody was detected by chemoluminiscence using an appropriate peroxidase-conjugated secondary antibody. Actin (Santa Cruz Biotechnology SC-1616, USA) was used as loading control. Images were acquired by a Chemidoc Imaging system (UVP, USA), and the intensity of each band quantified using the ImageJ software (NIH, USA).

Microarray processing and data analysis. One week after preconditioning or a sham procedure (only anesthesia), RNA was extracted from homogenized tissues as

described above and purified using the RNeasy minikit (Qiagen, USA). RNA quality was confirmed by capillary electrophoresis (Agilent 2100 Bioanalyzer). Affymetrix Mouse Gene 2.0 ST microarrays were processed following manufacturer's instructions and scanned to obtain the raw gene expression values. All the data is available at the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo, accession number GSE65783). After background correction and normalization using the Robust Multichip Average (RMA) algorithm, gene expression was calculated. Differences in gene expression between intact and preconditioned lungs were computed by fitting expression data to a linear model and computing the F statistics using empirical Bayes moderation of the standard errors. P values were adjusted according to the expected false discovery rate using the Benjamini and Hochberg method. In these differentially expressed genes, a gene enrichment analysis was performed to identify the Gene Ontology groups with a significant representation, according to a hypergeometric test. All the analyses were performed using the R statistical software (R foundation for statistical computing, Viena, Austria, available at http://www.R-project.org) and the *oligo*, *limma* and *GOstats* packages from the Bioconductor Project (10).

CCR1 blockade. Additional animals were treated with the CCR1 blocker BX471 (two intraperitoneal doses of 25 mg/kg each 24 hours, purchased from Tocris, UK). The dose was chosen based on previously published data, aimed to ameliorate the acute inflammatory response (2). Control mice received equivalent doses of vehicle. Twelve hours after the last dose, animals were anesthetized and ventilated using high pressures for 120 minutes. Afterwards, lungs were extracted and

histological injury, neutrophilic infiltration and ERK phosphorylation were measured as previously described.

Statistical analysis. All data are presented as mean±standard error of the mean. Results were compared using Student's T tests or an analysis of the variance (for two and more than two groups respectively). When appropriate, post-hoc tests were done using Bonferroni's correction. Analysis of the microarray data is detailed in the online supplement. A $p \leq 0.05$ was considered significant. All the analyses were done using the R statistical software (R foundation for statistical computing, Viena, Austria, available at http://www.R-project.org).

Results

Ninety-eight mice were used. Eighty were included in the main protocol (14 at baseline conditions, 12 at the end of low-pressure ventilation, 14 one week after low-pressure ventilation, 20 preconditioned and 20 intact mice were submitted to high-pressure ventilation, see Figure 1 for details). Six animals (3 sham and 3 preconditioned mice one week after low-pressure ventilation) 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 to intact, low-pressure ventilation induced a small, non-significant, increase in lung injury score. After 1 week, this mild damage was completely repaired. This finding correlated with

similar respiratory system compliances in baseline and preconditioned animals (25.5±2.5 vs 27.8±2.0 microliters/cmH_2O respectively, p=0.64). As expected, high-pressure ventilation caused a severe damage within the lungs. However, mice preconditioned with prior low-pressure ventilation showed a significantly lower lung injury (Figure 2A). After high-pressure ventilation, respiratory system compliances were 21.8±0.9 and 18.3±0.6 microliters/cmH_2O 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 (Figure 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 (PaO_2 108±19 vs 57±8 mmHg, p=0.04), with lower $PaCO_2$ (32±2 vs 52±6 mmHg, p=0.03) and a trend towards higher pH (7.36±0.01 vs 7.23±0.06, p=0.09).

In order 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 (Figure 3A-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 (Figure 3C), with no differences between preconditioned and non-preconditioned animals. There was a substantial variability in *Il10* expression, so the differences were not significant (p=0.16, Figure 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 (Figures 3E-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 to animals submitted to the sham procedure. The main differentially expressed genes are shown in Figure 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 responsible 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 Figure 5A-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 (Figure 5D). Similarly, protein levels of the *Ccl3* product MIP-1α were lower in preconditioned animals (Figure 5E-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 (Figure 5F-G). Collectively, these results suggest that downregulation of *Ccl3* is one of the mechanisms responsible for tolerance to injurious ventilation.

Blockade of CCR1 ameliorates ventilator-induced lung injury

As MIP-1α exerts its effects by binding to the CCR1 receptor, we blocked this signaling pathway with the CCR1 antagonist BX471. Mice treated with the drug showed a decreased lung injury after high-pressure ventilation, mimicking the effects induced by preconditioning (Figure 6A-B). Similarly, MPO-positive cell count was decreased in treated animals (Figure 6C-D). Finally, phosphorylation of ERK was decreased after BX471 treatment (Figure 6E-F). These results demonstrate that CCR1 blockade mimics the preconditioning effect of previous exposure to 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 genome-wide search revealed significant differences in a group of genes. Although preconditioning induced a down-regulation in the 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 hours (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. In spite of 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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Figure legends.

Figure 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).

Figure 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.

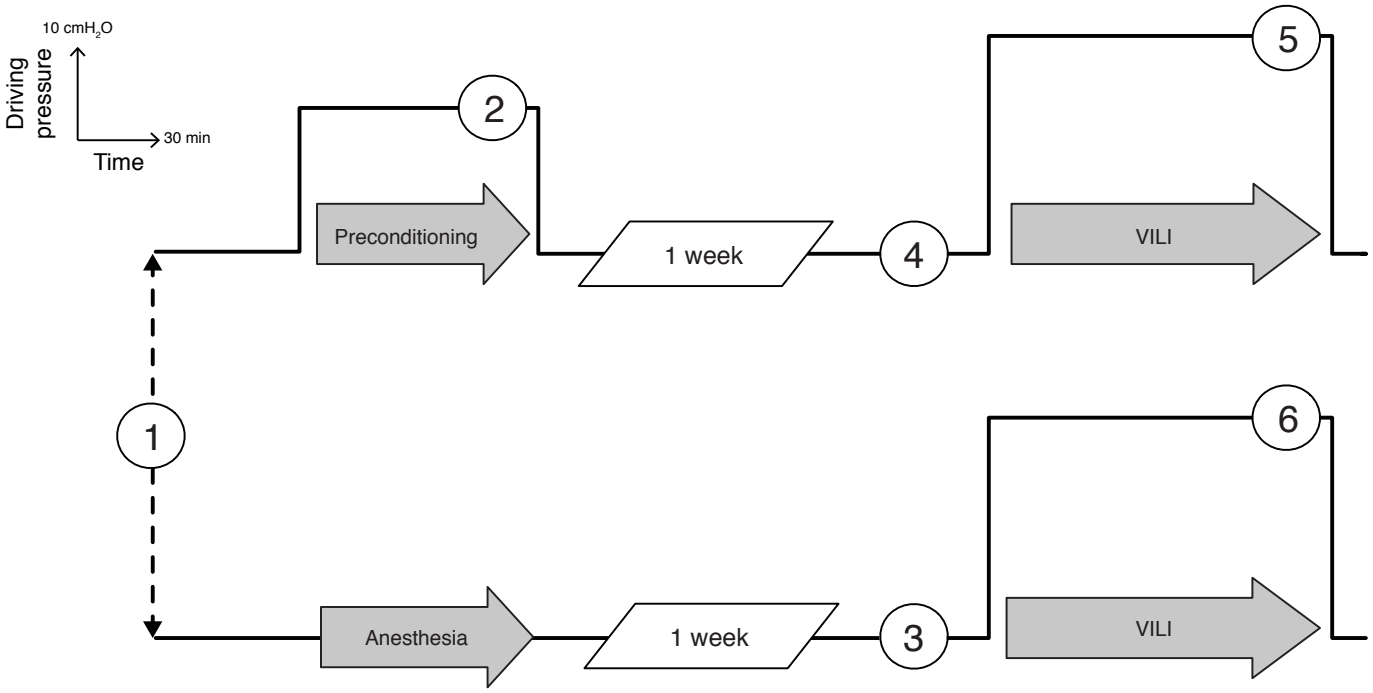
Figure 3: Differences in lung inflammation. Preconditioned mice showed a decreased neutrophilic infiltrate within the lungs, demonstrated by the lower myeloperoxidase-positive cell count (A). Panel B shows representative immunohistological sections. Mechanical ventilation induced an increase in lung *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-F).

Figure 4: Differences in gene expression between intact (n=3) and preconditioned mice (n=3) before injurious ventilation. Gene expression within lung tissue was assessed using microarrays. The genes with a higher differential expression are presented in the heatmap (A) and in the table (B).

Figure 5: Gene expression after ventilator-induced lung injury. Expression of *Hspa1b*, *Calcb* and *Ccl3* was studied in lung tissue from preconditioned (n=10) and intact (n=10) mice submitted to high-pressure ventilation. There were no differences in *Hspa1b* (A) or *Calcb* (B). However, *Ccl3* expression was significantly lower in preconditioned animals (C). The protein levels of MIP-1α, the product of

Ccl3 (D), and phosphorylated ERK (E) were also significantly lower. Panel F shows representative western blots.

Figure 6: Effects of CCR1 blockade. Mice treated with BX417, a CCR1 blocker (n=6), and with vehicle (n=6) were submitted to injurious ventilation. CCR1 blockade ameliorated ventilator-induced lung injury, as demonstrated by lower histological injury scores (A-B) and decreased myeloperoxidase (MPO)-positive cell counts (C-D). Phosphorylation of ERK was decreased in treated mice, suggesting an effective blockade of the receptor (E-F).



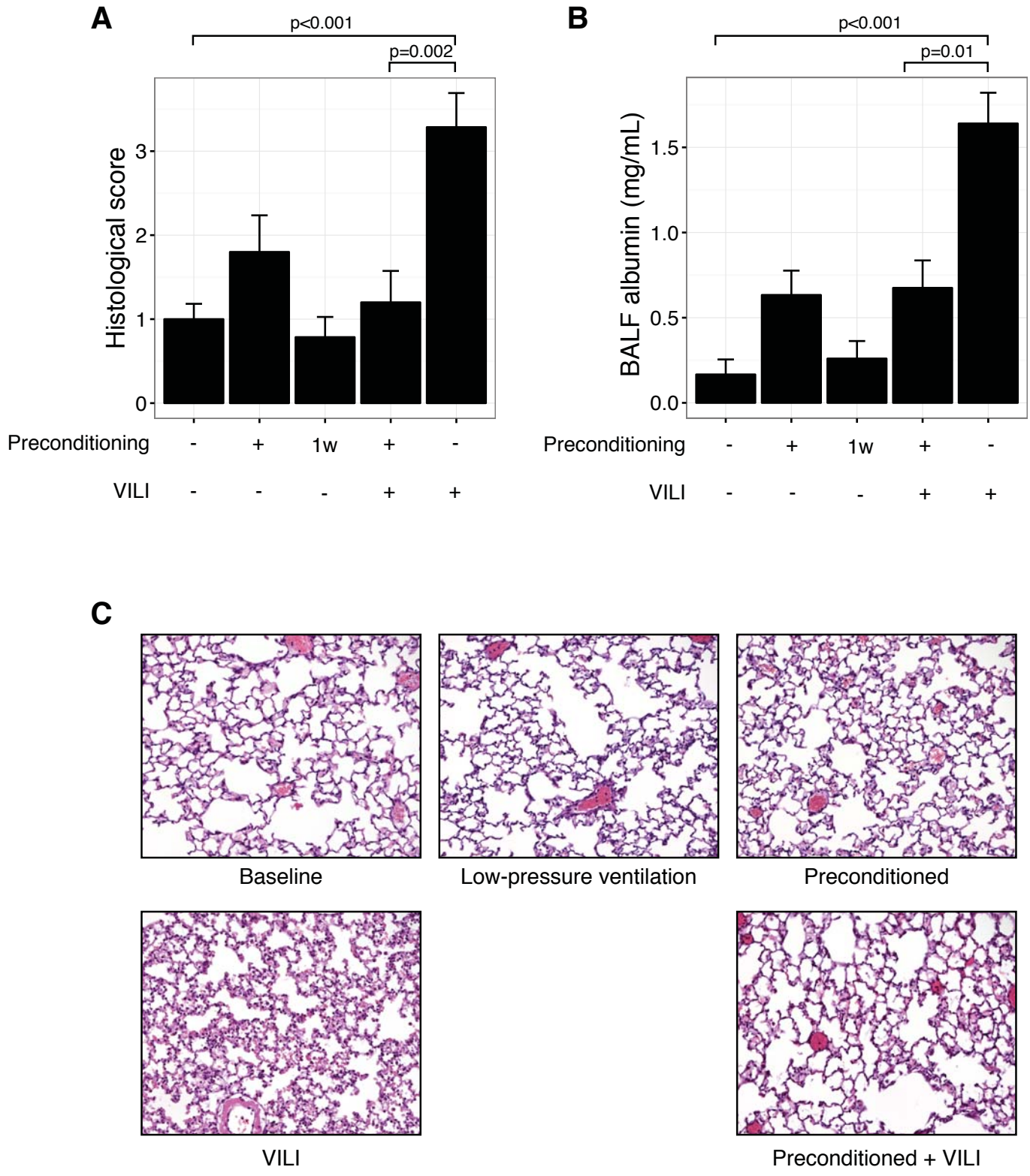
1: Baseline
Tissue injury (n=6) Compliance (n=4) BALF albumin content (n=4) *Il6, Il10* expression (n=6)

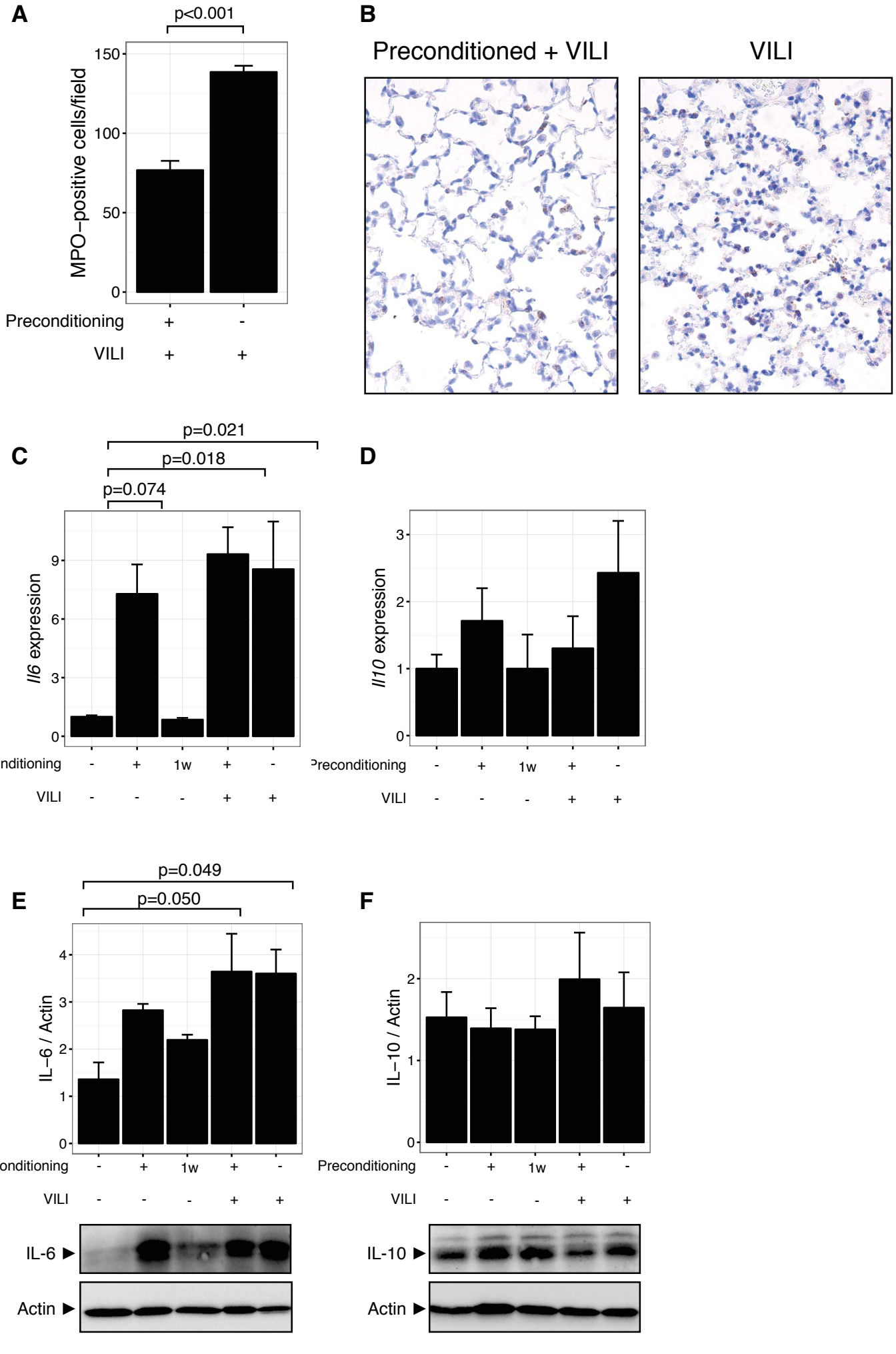
2: Non-injurious ventilation
Tissue injury (n=6) BALF albumin content (n=4) *Il6, Il10* expression (n=6)

3: Sham
Microarray (n=3)

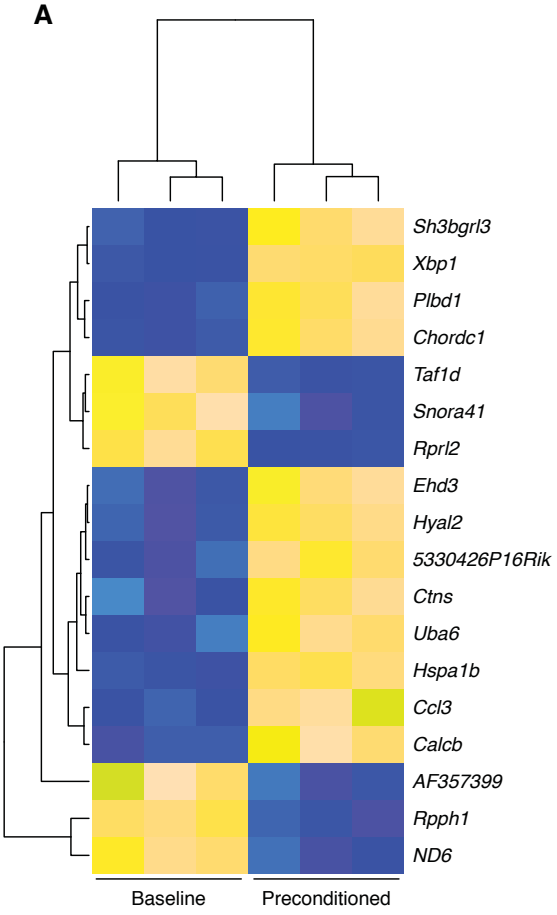
4: Preconditioned
Tissue injury (n=6) Compliance (n=4) BALF albumin content (n=6) *Il6, Il10* expression (n=6) Microarray (n=3)

5: Preconditioned+VILI 6: VILI
Tissue injury (n=10) Compliance (n=4) Blood gases (n=4) BALF albumin content (n=6) MPO staining (n=10) *Il6, Il10* expression (n=10) *Hspa1b, Calcb, Ccl3, Ccr1* expression (n=10) MIP-1a signaling (n=10)


A
p<0.001
p=0.002
Histological score
0
1
2
3
Preconditioning - + 1w + -
VILI - - - + +
B
p<0.001
p=0.01
BALF albumin (mg/mL)
0.0
0.5
1.0
1.5
Preconditioning - + 1w + -
VILI - - - + +
C
Baseline
Low-pressure ventilation
Preconditioned
VILI
Preconditioned + VILI

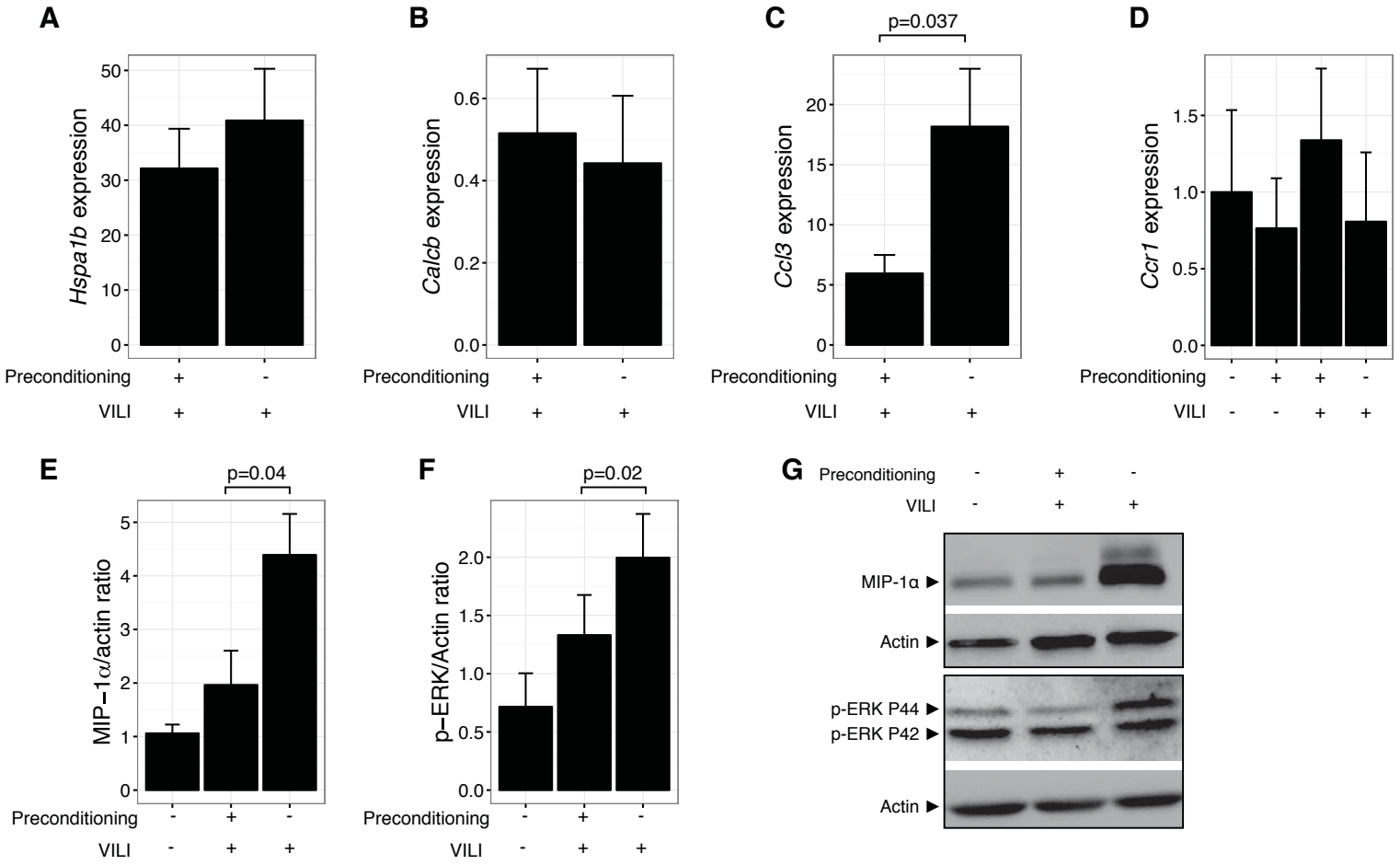

A
p<0.001
MPO−positive cells/field
150
100
50
0
Preconditioning + -
VILI + +
B
Preconditioned + VILI
VILI
C
p=0.021
p=0.018
p=0.074
Il6 expression
9
6
3
0
Preconditioning - + 1w + -
VILI - - - + +
D
Il10 expression
3
2
1
0
Preconditioning - + 1w + -
VILI - - - + +
E
p=0.049
p=0.050
IL−6 / Actin
4
3
2
1
0
Preconditioning - + 1w + -
VILI - - - + +
IL-6 ▶
Actin ▶
F
IL−10 / Actin
2
1
0
Preconditioning - + 1w + -
VILI - - - + +
IL-10 ▶
Actin ▶

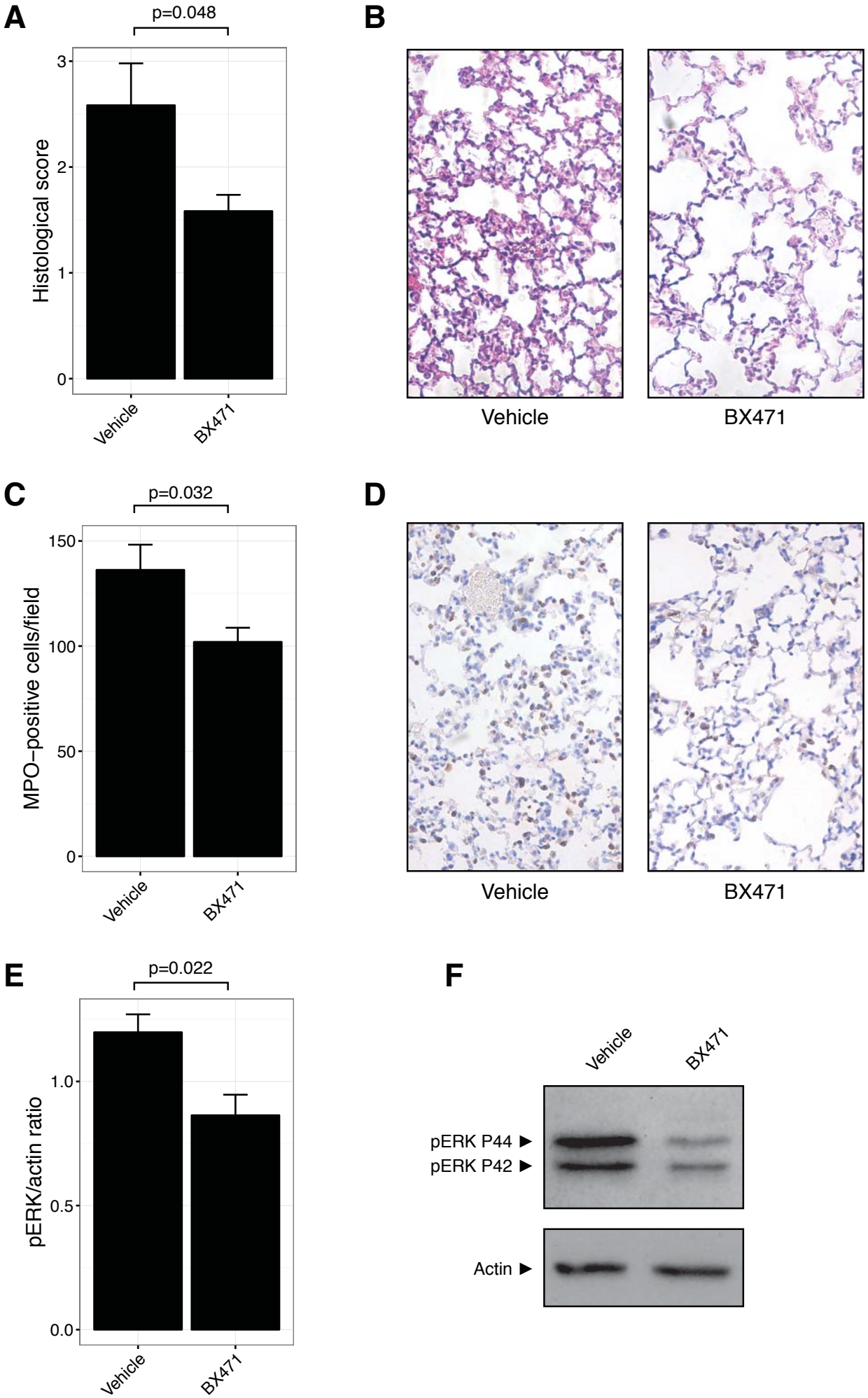
A



B

Gene symbol	Log fold-change	P.Value	adj.P.Val
Hspa1b	-2,555	2,63E-08	0,00066701
Taf1d	1,671	5,40E-06	0,05467777
5330426P16Rik	-1,175	6,47E-06	0,05467777
Chordc1	-0,875	1,35E-05	0,05747564
Calcb	-1,849	1,53E-05	0,05747564
ND6	0,920	1,90E-05	0,05747564
Snora41	1,508	1,92E-05	0,05747564
Sh3bgrl3	-0,890	1,98E-05	0,05747564
Plbd1	-0,824	2,31E-05	0,05747564
Uba6	-0,922	2,44E-05	0,05747564
Hyal2	-0,822	2,71E-05	0,05747564
Ccl3	-1,165	2,72E-05	0,05747564
Rpph1	0,759	2,98E-05	0,05815134
Ehd3	-1,038	3,42E-05	0,05828296
Ctns	-0,897	3,62E-05	0,05828296
Rprl2	0,699	3,69E-05	0,05828296
AF357399	1,836	3,91E-05	0,05828296
Xbp1	-0,650	4,22E-05	0,05947238


A
Hspa1b expression
50
40
30
20
10
0
Preconditioning + -
VILI + +
B
Calcb expression
0.6
0.4
0.2
0.0
Preconditioning + -
VILI + +
C
p=0.037
Ccl3 expression
20
15
10
5
0
Preconditioning + -
VILI + +
D
Ccr1 expression
1.5
1.0
0.5
0.0
Preconditioning - + + -
VILI - - + +
E
p=0.04
MIP−1α/actin ratio
5
4
3
2
1
0
Preconditioning - + -
VILI - + +
F
p=0.02
p−ERK/Actin ratio
2.0
1.5
1.0
0.5
0.0
Preconditioning - + -
VILI - + +
G
Preconditioning - + -
VILI - + +
MIP-1α ▶
Actin ▶
p-ERK P44 ▶
p-ERK P42 ▶
Actin ▶


A
p=0.048
Histological score
3
2
1
0
Vehicle
BX471
B
Vehicle
BX471
C
p=0.032
MPO−positive cells/field
150
100
50
0
Vehicle
BX471
D
Vehicle
BX471
E
p=0.022
pERK/actin ratio
1.0
0.5
0.0
Vehicle
BX471
F
Vehicle
BX471
pERK P44 ▶
pERK P42 ▶
Actin ▶