Impaired lung repair during neutropenia can be reverted by MMP-9

Jorge Blázquez-Prieto, MSc, Inés López-Alonso, MSc, Laura Amado-Rodríguez, MD, PhD, Covadonga Huidobro, PhD, Adrián González-López, PhD, Wolfgang M Kuebler, MD, PhD, Guillermo M Albaiceta, MD, PhD.

Online Data Supplement

DETAILED METHODS

Study animals. Male, 8-12 week old CD1 mice, kept under pathogen-free conditions with free access to food and water, were used in all experiments. The Animal Research Ethics Committee of the Universidad de Oviedo evaluated and approved the study.

Experimental model. Mice were studied at three different time points: baseline, after induction of VILI, and after 48 hours of spontaneous breathing (recovery). During the recovery phase, animals were randomized to receive an anti-neutrophil antibody to induce neutropenia or the corresponding isotype control antibody (*vide infra*). In total, the experimental setup thus included four groups for analysis (baseline, VILI, repair, repair+neutropenia), with 17 animals per group (Figure 1A in the main article). This experimental design allowed us to identify the existence of injury and repair in our model, using both baseline and VILI groups as references. To avoid time-dependent effects, all the mechanistic studies were done by comparing neutropenic and non-neutropenic mice, both at 48 hours after injury).

Mice were anesthetized with intraperitoneal ketamine (80 mg/Kg) and xylazine (25 mg/Kg) and orotracheally intubated using a 20G catheter. Afterwards, animals were connected to a mechanical ventilator (Evita 2 Dura Neoflow, Drager, Germany), in pressure-controlled mode. To induce lung injury, the following settings were used: Peak inspiratory pressure 17 cmH_2O, PEEP 0 cmH_2O, respiratory rate 50 breaths/min, I:E ratio 1:1. Inspired oxygen fraction was 0.21. Based on preliminary experiments, these settings result in a tidal volume of 15 ml/Kg at the onset of ventilation. Respiratory rate was chosen to avoid

hypocapnia. After 90 minutes of ventilation, mice were recovered from anesthesia, extubated and allowed to breath spontaneously for two days.

Neutrophil depletion. After extubation, animals were randomized to receive a dose of 200 μg every 24 hours of anti-Gr1 antibody (that binds to neutrophils, promoting its clearance) or a non-specific immunoglobulin (both administered intraperitoneally). Forty-eight hours after ventilation, cell counts in peripheral blood from the retroorbital plexus were obtained using an Abacus Junior cytometer.

Tissue sampling. Ten mice per group were used for tissue sampling. The lungs were removed from anesthetized mice as previously described (1). The right lung was immediately frozen and stored at -80°C for biochemical analyses, which were carried out in tissues mechanically homogenized and after measuring its protein content (BCA kit, Pierce, USA). For histological studies, the left lung was inflated with 250 microliters of 4% phosphate-buffered paraformaldehyde and immersed in the same fixative. After 24 hours, these samples were stored in 50% ethanol.

Histological studies. Lungs were embedded in paraffin and three slices, with a minimal separation of 1 mm between them, were stained with hematoxylin and eosin. Tissue damage was evaluated by two observers blinded to the experimental conditions, using a predefined score (2) (0: Normal lungs; 1: Capillary congestion; 2: Alveolar wall thickening and inflammatory infiltrates; 3: Intraalveolar flooding; 4: Massive disruption of the lung structure).

MicroCT scans. Lungs from three animals of each experimental group were used for CT scanning. After insuflation with 700 microlitres, the left main bronchus was tied and the left lung removed and placed in a SkyScan-1174 microCT scanner (Bruker. Kontich, Belgium) for image acquisition (50 kV, 800 mA, exposure time 1250 ms). Images (924 images per lung) were reconstructed in DICOM format using the NRecon software, and analyzed using ImageJ software (NIH, USA). The lung contour was drawn and density histograms corresponding to its volume were acquired. The percentage of non-aerated lung (defined as voxels with density between -100 and +200 HU) was computed for each sample. In addition, volumetric rendering images were performed using Horos software.

Immunohistochemistry. Additional lung sections were processed for the detection of myeloperoxidase-positive cells and macrophages. Specific antibodies against myeloperoxidase (MPO, Dako), F4/80 (Santa Cruz Biotechnology SC-71086, USA) and Ki67 (DCS-Innovative Diagnostik-Systeme, Germany) were used, respectively. The number of positive cells was counted in three random fields (x200) and averaged. Again, cell counts were done by two observers blinded to the experimental conditions.

Bronchoalveolar lavage. In four animals per group, a bronchoalveolar lavage (BAL) was performed. Three aliquots (0.7 ml) of saline were injected through the tracheal tube and recovered to obtain BAL fluid (BALF). The collected BALF was centrifuged at 1500 rpm for 15 minutes to remove cells and stored at -80°C for subsequent analyses. Albumin content in BALF was measured as absorbance at 629 nm using the bromocresol green technique.

Quantitative PCR. RNA was obtained 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 RNAse free water. Complementary DNA was synthesized from 1,000 ng 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 myelocytomatosis oncogene (*Myc* FW 5'-TTCATCTGCGATCCTGACGAC-3' / RV 5'-CACTGAGGGGTCAATGCACTC-3'), cyclin-D1 (*Ccnd1* FW 5'-GCGTACCCTGACACCAATCTC-3' / RV 5'-CTCCTCTTCGCACTTCTGCTC-3') and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH* FW 5'-GTGCAGTGCCAGCCTCGTCC-3' / RV 5'-GCCACTGCAAATGGCAGCCC-3') (all from Sigma-Aldrich, USA). The relative expression of the analyzed genes was calculated as $2^{\Delta \mathrm{CT(experimental)} - \Delta \mathrm{CT(reference)}}$.

Western blot. 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 macrophage inflammatory protein-2 (MIP-2) (AbD Serotec, USA), tissue inhibitor of metalloproteinases-1 (TIMP-1) (Abcam, UK), matrix metalloproteinase-8 (MMP-8), interleukin-6 (IL-6) (Santa Cruz Biotechnology SC-1265, USA), interferon gamma (INF γ) (Abcam, UK), interleukin-10 (IL-10) (Abcam, UK), or tumor necrosis factor alpha (TNF α)

(PromoKine, Germany). 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).

MMP-9 activity. MMP-9 activity was measured in BALF of both neutropenic and healthy patients by gelatin zymography (3). Twelve µl of BALF were loaded in an 8% SDS-polyacrylamide gel containing 0.2% gelatin and electrophoresed. Afterwards, gels were washed twice in 2.5% Triton X-100 for 15 min, then washed again with deionized water until the complete removal of Triton X-100 and incubated overnight at 37ºC in a buffer containing 20 mM Tris-HCl, 5 mM $CaCl_2$, pH=7.4. Then the gels were stained using Commassie blue, destained with a mixture of acetic acid and methanol, and scanned. Intensity of the gelatinolytic bands was quantified (in arbitrary density units) using ImageJ software (NIH, USA).

Cytokines in patient lavage samples. Mini-bronchoalveolar lavages were performed in 4 non-neutropenic and 4 neutropenic patients under mechanical ventilation during the early phase of ARDS (less than 3 days from meeting ARDS criteria), as previously described (4). The collected bronchoalveolar fluid was filtered, centrifuged at 1500 rpm for 15 minutes to remove cells and the supernatants stored at -80°C for subsequent analyses. Different pro- (TNF α, IL-8, IL-6, INF γ) and anti-inflammatory (IL-10) cytokines were measured using a multiplexing technique (Flowcytomix kit, Bender Medysystems, Austria) in a flow cytometer

(Cellular Cytomics FC500, Beckman Coulter, USA). Clinical and laboratory data, including diagnoses and severity, was collected and is presented in Supplementary Table 1. This part of the protocol was reviewed and approved by the regional ethics committee (Comité Ético de Investigación Clínica del Principado de Asturias, Spain). Signed informed consent was obtained from each patient's next of kin.

Wound healing assay. The human bronchial epithelial cell line BEAS-2b (ATCC, USA) was used in all *in vitro* experiments. Cells were grown in DMEM High Glucose medium with L-glutamine (Biowest, France) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 20% FBS. After confluence, the insets were removed to create a wound, washed with PBS and then cultured using the same medium as before but supplemented with 10% BALF from neutropenic patients or patients with normal peripheral neutrophil counts undergoing mechanical ventilation (both filtered using an Acrodisc 0.2-μm filter, Pall Life Sciences). Each sample was studied in duplicate and the results averaged. The assays were performed in plates with and without a type-I collagen coating (Oris Cell Migration Assays, AMS Biotechnology, UK). In separate experiments, 15 ng of exogenous MMP-9 were added to the BALF in collagen-coated plates. Cultures were photographed using an Olympus BH-2 microscope and an Olympus C-5060 camera. Images were obtained immediately after wounding and every 6 h for a total of 72 h. Wound area was measured using ImageJ software and the percentage of the initial wound area covered by cells over time was computed.

Administration of exogenous MMP-9. Additional animals were treated with exogenous MMP-9. Mice were intubated and connected to a mechanical ventilator

(ventilator parameters were the same as before: PIP 17 cmH_2O, PEEP 0 cmH_2O, respiratory rate 50 breaths/min, I:E ratio 1:1). After ventilation and extubation, all mice received a dose of 200 μg every 24 hours of anti-Gr1 antibody and were randomly assigned to receive MMP-9 (six intranasal doses of 25 ng/8 hours, n=6) or the equivalent volume of saline (10 microliters, n=6). The dose was chosen based on preliminary experiments. Forty-eight hours after ventilation, mice were anesthetized, tracheostomized and sacrificed for tissue sampling (n=6 per group) and CT scanning (n=3 per group).

Statistical analysis. Data are presented as mean±SEM. Histological score, BALF albumin concentration and tissue cell counts were compared using an ANOVA. When significant, pairwise comparisons were done between all the possible pairs using Holm's correction for multiple tests. Bivariate comparisons were done using a T-test. Differences in wound closure rates were assessed using a repeated-measurements ANOVA (including time and neutropenia as within-group and between-group factors respectively), and half-lives calculated after non-linear fitting to a exponential decay model. When appropriate, post-hoc comparisons were done using Holm's correction. A p value lower than 0.05 was considered statistically significant. All the analyses were done using the R 3.2.1 statistical package.

REFERENCES

1. Blazquez-Prieto J, Lopez-Alonso I, Amado-Rodriguez L, et al. Exposure to mechanical ventilation promotes tolerance to ventilator-induced lung injury by Ccl3 downregulation. *Am J Physiol Lung Cell Mol Physiol* 2015;309(8):L847-856.
2. Lopez-Alonso I, Aguirre A, Gonzalez-Lopez A, et al. Impairment of autophagy decreases ventilator-induced lung injury by blockade of the NF-kappaB pathway. *Am J Physiol Lung Cell Mol Physiol* 2013;304(12):L844-852.
3. Amado-Rodriguez L, Gonzalez-Lopez A, Lopez-Alonso I, et al. Anti-inflammatory effects of clarithromycin in ventilator-induced lung injury. *Respir Res* 2013;14(1):52.
4. Gonzalez-Lopez A, Garcia-Prieto E, Batalla-Solis E, et al. Lung strain and biological response in mechanically ventilated patients. *Intensive Care Med* 2012;38(2):240-247.

Supplementary Table 1. Clinical data of the patients included in the study. All the neutropenic patients received induction chemotherapy before ICU admission, and the acute respiratory distress syndrome was diagnosed in the context of febrile neutropenia. APACHE-II scores were calculated at ICU admission, and the respiratory SOFA score was calculated at the time of bronchoalveolar lavage. All patients were receiving broad-spectrum antibiotics at the time of study, and all the bronchoalveolar lavage cultures were negative. None of the neutropenic patients were receiving G-CSF at the time of the study. Neutropenia refers to the number of days with a neutrophil count in peripheral blood below 0.5 x $10^3/mm^3$. PMN: Polymorphonuclear neutrophils. Lymph: Lymphocytes. Mono: Monocytes

	Age	Sex	Neutropenia	PMN $x10^3/mm^3$	Lymph $x10^3/mm^3$	Mono $x10^3/mm^3$	Comorbidities	Main diagnoses	APACHE-II score	Respiratory SOFA	Outcome
1	50	Female	Yes (5 days)	0.04	0.17	0.03	None	NHL	26	2	Survivor
2	68	Female	Yes (6 days)	0.18	0.22	0.01	None	AML	37	3	Dead
3	54	Male	Yes (6 days)	0.01	0	0.02	Liver cirrhosis	AML	20	4	Dead
4	55	Male	Yes (4 days)	0.21	0.01	0.03	Smoking	AML	28	4	Survivor
5	61	Female	No	10.93	1.05	0.35	Hypothyroidism	Pneumonia	21	4	Dead
6	83	Female	No	28.42	0.18	0.64	Arterial hypertension	Abdominal sepsis	28	2	Survivor
7	85	Female	No	16.69	1.01	0.55	Atrial fibrillation, arterial hypertension	Urinary sepsis	27	2	Survivor
8	76	Male	No	13.91	0.78	0.09	Chronic hepatitis	Polytrauma	22	3	Dead

Supplementary Figure 1. Expression of cyclin-D1 (*Ccnd1*) and *Myc* was assessed to characterize the activation of the *Wnt* pathway triggered by neutrophil migration. There were no differences between neutropenic and non-neutropenic mice in the expression of these genes.