1 Complete Material and Methods

1.1 Animals

Female C57BL/6 N mice (aged 8-12 weeks, weighing 20-25 grams) were obtained from Charles River (Charles River; Sulzfeld, Germany) and were kept under standard conditions. All experimental procedures were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Recklinghausen, Germany. Permission number: AZ 84-02.04.2013.A131).

1.2 Experimental setup

Surgery was performed as previously described.[14] In short, mice were initially anaesthetized by intraperitoneal bolus injection of pentobarbital sodium (75 mg/kg) + fentanyl (40 µg/kg). Anaesthesia was maintained with pentobarbital sodium (20 mg/kg) via an intraperitoneal catheter every 30-60 minutes and fentanyl (20 µg/kg) every 3 hours. Mice were placed in a supine position and body temperature was kept between 36.5°C and 37.5°C by a biofeedback-controlled homeothermic blanket system (Harvard Apparatus; Holliston, MA, United States). A catheter was inserted into the carotid artery to permanently monitor blood pressure (AP_{mean}) and to supply saline (300 µL/h) to prevent hypovolaemia and thrombus formation. Heart rate (HR) was calculated online from a 3-lead ECG and pulse oxymetry was performed with a thigh clip (MouseOx®, STARR Life Sciences; Oakmont, PA, United States).

Mice were ventilated for seven hours via a tracheal catheter with a mechanical ventilator (MidiVent Type 849, Hugo Sachs Elektronik - Harvard Apparatus GmbH; March-Hugstetten,

Germany) equipped with a flow-pressure sensor (Type 382 Mouse, Hugo Sachs Elektronik). Dynamic pulmonary compliance (C_{dyn}) and resistance were calculated online from flow-pressure values by the Pulmodyn Software v 1.1 (Hugo Sachs Elektronik). Δ C_{dyn} (C_{dyn} end of ventilation - C_{dyn} start of ventilation) was calculated at the end of the experiments. Control animals were ventilated with a plateau airway pressure (p_{plat}) of 10 cmH_2O at 180 breaths / min with recruitment manoeuvres every 20 min to prevent atelectasis [14]. Repeated recruitment manoeuvres (1 s duration and 30 cmH_2O peak pressure) were performed every 20 minutes to maintain lung mechanics in their physiological range by preventing atelectasis.[14] Groups with p_{plat} > 10 cmH_2O were ventilated at a frequency of 90 breaths / min. End-tidal carbon-dioxide ($p_{et}CO_2$) waveform was monitored with a micro-capnograph (Type 340, Hugo Sachs Elektronik). A positive end-expiratory pressure (PEEP) of 2 cmH_2O was applied, the fraction of inspired oxygen (F_iO_2) was set to 0.3 and the I:E ratio (inspiration to expiration ratio) was 1:1 with a sinus-shaped waveform in all experiments.

At the end of experiment, mice were sacrificed by exsanguinations via the carotid artery. Blood samples were analysed for s_aO_2, p_aO_2, p_aCO_2 and pH by an ABL700 or ABL800 blood gas analyser (Radiometer; Copenhagen, Denmark); the Horovitz-ratio was calculated as p_aO_2 / F_iO_2.

1.2.1 One-hit model of ventilator-induced lung injury

We performed and analysed two different series of experiments separately. In the first series (series_1), the following plateau airway pressures were used in the ventilation protocol: 10 cmH_2O (p10), 24 cmH_2O (p24), 27 cmH_2O (p27) and 30 cmH_2O (p30).

In the second series of experiments (series_2), a respiratory pressure monitor and limiter (I) (IPML Type 870/01 for mouse, Hugo Sachs Elektronik) with pressure release above

34 cmH_2O (valve open time: 150 ms) was used to limit the maximum pressure in all animals. This value was chosen because clinical data suggest that pressures above 35 cmH_2O favour pneumothorax formation.[6] The following plateau airway pressures were used during ventilation with the pressure release valve: 24 cmH_2O (p24 I), 27 cmH_2O (p27 I) and 30 cmH_2O (p30 I).

1.2.2 Treatments

The steroid dexamethasone was used to assess the inflammatory part in our model. Depending on group assignment, animals in the second series randomly received dexamethasone (D, 1mg/kg i.v.) directly after start of mechanical ventilation. The following additional groups were examined: 24 cmH_2O (p24 I D), 27 cmH_2O (p27 I D) and 30 cmH_2O (p30 I D).

1.3 Sample preparation

The right superior lobe was fixed in 4 % formalin for histopathology, the right middle and inferior lobes were snap frozen in liquid nitrogen and stored at -80°C for real-time qPCR measurements. The wet/dry-ratio was obtained from the right post-caval lobe after weight constancy at 42°C. Bronchoalveolar lavage was performed of the left lung by instilling twice 200 µL ice cold saline via a tracheal catheter. From each lung, about 350 µL bronchoalveolar lavage fluid (BALF) was recovered, centrifuged and the supernatant was stored at -80°C until quantification of cytokines. All samples were analysed in a blinded fashion.

1.4 Cytokine detection

BALF cytokine levels of chemokine (C-X-C motif) ligand 1 (CXCL1, KC), chemokine (C-X-C motif) ligand 2 (CXCL2, MIP-2), chemokine (C-X-C motif) ligand 10 (CXCL10, IP-10), interleukin 6 (IL6) and tumor necrosis factor (TNF) were quantified with enzyme-linked immunosorbent assays (ELISA) (R&D Systems GmbH; Wiesbaden-Nordenstadt, Germany) according to the manufacturer's protocols.

1.5 Real-time qPCR analysis

Frozen lung tissues were grinded in liquid nitrogen. Total RNA was isolated from 20 mg lung powder with RNeasy® Mini Kit (QIAGEN GmbH, Hilden, Germany) automated on a QIAcube robot (QIAGEN GmbH) with additional DNase digestion. RNA was quantified in buffered 10 mM TRIS-HCl (pH 7.5) using a spectrophotometer (NanoDrop™ 1000, Thermo Fisher Scientific Inc.; Waltham, MA, USA).

For reverse transcription 600 ng of total RNA were added to 0.75 µL of oligo(dT)18 primer (0.5 µg/µL), 0.25 µL of random hexamer primer (0.2 µg/µL), 1 µL dNTP mix (10mM) and 0.5 µL water. Samples were incubated for 5 min at 65°C to linearize the RNA. 4 µL 5x buffer (incl. 50 mM DTT), 0.5 µL RiboLock RNase inhibitor (40 U/µL) and 1 µL Maxima® reverse transcriptase (200 U/µL) were added on ice. After incubation at 25°C for 10 min, RNA was reverse transcribed at 50°C for 15 min. The heat-inactivation step was performed at 85°C for 5 min. All incubation steps were performed on a Biometra UNO II Thermocycler (Biometra GmbH; Göttingen, Germany). Reagents were supplied by Fisher Scientific - Germany GmbH (Schwerte, Germany). For real-time qPCR 4 µL of cDNA were incubated as template with 0.5 µL sense and antisense primer (6.25 µM) (Eurofins MWG GmbH; Ebersberg, Germany)

and 5 µL SYBR Green I Master (Roche-Diagnostics GmbH; Mannheim, Germany) according to manufacturer's instruction in a LightCycler® 480 (Roche-Diagnostics GmbH). Primer pairs used for the detection of chemokine (C-X-C motif) ligand 1 (*Cxcl1*), (C-X-C motif) ligand 10 (*Cxcl10*), dipeptidylpeptidase 4 (*Dpp4*), interleukin 6 (*Il6*), myeloperoxidase (*Mpo*), neuropeptide Y (*Npy*), TATA box binding protein (*Tbp*) and tumor necrosis factor (*Tnf*) mRNA levels are listed in Tab. S2, Supplemental Digital Content 2.

Quantification of real-time qPCR was performed with Cp values, acquired via the second derivative maximum method. Advanced relative quantification was performed with the LightCycler 480 software v 1.5 (Roche-Diagnostics GmbH) and efficiency-corrected by in-run standard curves from all samples.[17] Inter-run calibrators from unventilated control animals were used to avoid inter-run variations. Data were normalised to *Tbp*, which was validated as a very stably expressed reference gene out of ten candidate genes, as determined by geNorm (average M ≤ 0.2).[18] Gene expression is depicted as fold induction relative to unventilated controls. Real-time qPCR quality control was performed by in-run controls, melting curve profiles using the LightCycler 480 software and product separation in agarose gels.

1.6 Lung histopathology

The right superior lobe was fixed with 4 % formalin and embedded in paraffin for sectioning. Hematoxilin and eosin (HE) staining was performed with 3 mm thick sections. Histopathology was evaluated in a blinded manner as reported earlier.[14] In short: a scoring system based on four criteria was used: neutrophils in the alveolar or interstitial space, alveolar septal thickening, alveolar congestion and formation of hyaline membranes. Each criterion scored one point, if present, resulting in a range of 0 to 4 points.

1.7 Statistical analysis

Based on the data from Protti et al. [10] and on own preliminary data the experiments were planned with a statistical power of 80% and an α-error of 0.05 in order to detect differences in the paO_2/FiO_2 ratio of greater than 80 (JMP 10, SAS Institute Inc., Cary, NC, USA). The data were analysed using SAS® software v 9.4 (SAS Institute Inc., NC, USA) using two-sided tests. Survival analysis was performed with log-rank tests (Proc Lifetest) and p-values corrected by the Bonferroni-Holm procedure. Univariate tests were carried out using general linear mixed model analysis (Proc Glimmix) assuming a log-normal distribution for all cytokine data and a normal distribution for all other parameters and residual plots were used as diagnostics. In case of heteroscedasticity (according to the covtest statement), the degrees of freedom were adjusted by the Kenward-Rogers method. P-values were always adjusted by the simulated-Shaffer procedure. $P<0.05$ was considered significant. Data were plotted with GraphPad Prism® software v 6 (GraphPad Software Inc., La Jolla, CA, USA).