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## PHARMACOLOGICAL MODULATION OF C-X-C MOTIF CHEMOKINE RECEPTOR 4 INFLUENCES DEVELOPMENT OF ACUTE RESPIRATORY DISTRESS SYNDROME AFTER LUNG ISCHEMIA-REPERFUSION INJURY

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### Abstract

Activation of CXC motif chemokine receptor 4 (CXCR4) has been reported to result in lung protective effects in various experimental models. The effects of pharmacological CXCR4 modulation on the development of acute respiratory distress syndrome (ARDS) after lung injury, however, are unknown. Thus, we studied whether blockade and activation of CXCR4 influences development of ARDS in a unilateral lung ischemia-reperfusion injury rat model. Anesthetized, mechanically ventilated animals underwent right lung ischemia (series 1: 30 min; series 2: 60 min) followed by reperfusion for 300 min. In series 1, animals were treated with vehicle or 0.7  $\mu\text{mol/kg}$  of AMD3100 (CXCR4 antagonist) and in series 2 with vehicle, 0.7 or 3.5  $\mu\text{mol/kg}$  ubiquitin (non-cognate CXCR4 agonist) within 5 min of reperfusion. AMD3100 significantly reduced  $\text{PaO}_2/\text{FiO}_2$  ratios, converted mild ARDS with vehicle treatment into moderate ARDS ( $\text{PaO}_2/\text{FiO}_2$  ratio < 200) and increased histological lung injury. Ubiquitin dose-dependently increased  $\text{PaO}_2/\text{FiO}_2$  ratios, converted moderate-to-severe into mild-to-moderate ARDS and reduced protein content of bronchoalveolar lavage fluid (BALF). Measurements of cytokine levels (TNF $\alpha$ , IL-6, IL-10) in lung homogenates and BALF showed that AMD3100 reduced IL-10 levels in homogenates from post-ischemic lungs, whereas ubiquitin dose-dependently increased IL-10 levels in BALF from post-ischemic lungs. Our findings establish a cause-effect relationship for the effects of pharmacological CXCR4 modulation on the development of ARDS after lung ischemia-reperfusion injury. These data further suggest CXCR4 as a new drug target to reduce the incidence and attenuate the severity of ARDS after lung injury.

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The authors have no conflicts of interest.

## Keywords

AMD3100; ARDS; drug target; lung injury; ubiquitin

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## Introduction

Despite recent advances in critical care medicine, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) remain major contributors to morbidity and mortality in critically ill patients (1–7). The American European Consensus Conference definition of ALI/ARDS from 1994 has recently been revised in the 2012 Berlin definition of ARDS, which now defines ALI as mild ARDS (8). Although the etiology of ARDS is multifactorial, it is generally accepted that mild ARDS and its progression into moderate and severe ARDS is caused by local and systemic inflammation in response to the insult, which leads to increased pulmonary capillary permeability, third spacing of fluids into the lung and formation of lung edema, the hallmark of ARDS (9, 10).

While several independent risk factors for development of ARDS have been identified, such as pneumonia, non-pulmonary-sepsis or severe injuries, the molecular events leading to ARDS remain poorly understood (4, 9, 11, 12). Thus, therapeutic options are currently limited to lung-protective ventilation strategies (5, 6, 13). Pharmacological interventions that may attenuate progression of mild ARDS to moderate or severe ARDS are currently not available, but urgently needed.

The G protein-coupled receptor C-X-C motif chemokine receptor 4 (CXCR4) is abundantly expressed in tissues, including lung epithelial and vascular endothelial cells (14, 15). CXCR4 is essential during embryonic development, has pleiotropic roles in the immune system, during tissue repair (16–19) and gained particular attention as a drug target through its role in cancer metastases and HIV infection (20–22). More recently, it has been reported that administration of the cognate CXCR4 agonist CXCL12 (stromal cell-derived factor-1 $\alpha$ ) and of the non-cognate CXCR4 agonist ubiquitin result in lung protective effects in various experimental models (23–28). While these data suggest CXCR4 as a potential therapeutic target to attenuate lung injury, the effects of pharmacological activation and blockade of CXCR4 on the development of ARDS after lung injury have not been systematically studied. Such data, however, are essential prerequisites to establish CXCR4 as a possible new drug target. Thus, in the present study we tested how blockade of endogenous CXCR4 with the selective CXCR4 antagonist AMD3100 and activation of CXCR4 with the non-cognate CXCR4 agonist ubiquitin modulate development and severity of ARDS in a unilateral lung warm ischemia-reperfusion injury rat model.

## Results

### Effects of AMD3100 after lung ischemia-reperfusion injury

In the first series of experiments, we tested the effects of the selective CXCR4 antagonist AMD3100 on the development of ARDS after unilateral lung ischemia-reperfusion injury. There were no significant differences for any physiological parameter between animals

treated with vehicle and AMD3100 at baseline. Mean arterial blood pressures (MAP, Fig. 1A), blood lactate concentrations (Fig. 1B), creatinine concentrations (Fig. 1B) and hematocrit values (Fig. 1C) were comparable between vehicle- and AMD3100-treated animals throughout the observation period. As compared with vehicle-treated animals, leukocyte counts in AMD3100-treated animals were significantly increased at the end of the experiment (Fig. 1D). While peak inspiratory pressures required to maintain minute volumes were indistinguishable between the groups (Fig. 1E/F), there were significant differences in  $\text{PaO}_2/\text{FiO}_2$  (P/F)-ratios between animals with vehicle and AMD3100 treatment (Fig. 1G). In vehicle-treated animals P/F-ratios decreased from  $546 \pm 18$  mmHg at baseline to  $299 \pm 12$  mmHg at  $t = 300$  min. With AMD3100-treatment, P/F-ratios were significantly lower at  $t = 60$  min and remained significantly reduced until the end of the experiment, as compared with vehicle-treated animals. With AMD3100 treatment, animals fulfilled criteria for mild ARDS (P/F ratio  $< 300$  mmHg) at  $t = 60$  min and criteria for moderate ARDS (P/F ratio  $< 200$  mmHg) at  $t = 240$  min.

Fig. 2A shows representative images from H&E stained lung slices of the post-ischemic and contralateral lungs from animals with vehicle and AMD3100 treatment and Fig. 2B the corresponding LIS for all animals. While there were no statistically significant differences between LIS of the contralateral lungs between the groups, LIS of the post-ischemic lungs were significantly higher with AMD3100 treatment (LIS, median (min/max): 7 (4/10)), as compared with vehicle treatment (LIS, median (min/max): 3 (2/6)).

There were no significant differences in W/D ratios between the post-ischemic and contralateral lung and between animals treated with vehicle or AMD3100 (Fig. 3A). Furthermore, myeloperoxidase activities, malondialdehyde/4-HAE concentrations and TNF $\alpha$  concentrations in lung homogenates were indistinguishable in the post-ischemic and contralateral lungs from animals treated with vehicle or AMD3100 (Fig. 3B–D). While IL-6 concentrations were significantly increased in homogenates from the post-ischemic lungs, as compared with homogenates from the contralateral lungs (IL-6, pg/mg (median (interquartile range)): vehicle: - contra-lateral 6 (3/19), -post-ischemic 57 (36/80),  $p=0.008$ ; AMD3100: - contra-lateral 12 (10/17), - post-ischemic 41 (24/78),  $p=0.015$ ), there were no differences between animals after vehicle or AMD3100 treatment (Fig. 3E). IL-10 concentrations, however, were significantly lower in homogenates from post-ischemic lungs after AMD3100 treatment, as compared to homogenates from post-ischemic lungs after vehicle treatment (Fig. 3F; IL-10 pg/mg (median (interquartile range)): vehicle - 19 (11/27), AMD3100 - 7 (6/10),  $p = 0.031$ ).

### Effects of ubiquitin after lung ischemia-reperfusion injury

In the second series of experiments, we tested the effects of the non-cognate CXCR4 agonist ubiquitin. To be able to assess whether activation of CXCR4 with ubiquitin could attenuate ARDS development after lung ischemia-reperfusion injury, we increased the ischemic time period from 30 min to 60 min. There were no significant differences in any physiological parameter between the vehicle-treated and the ubiquitin-treated groups at baseline. Similar to series 1, MAP (Fig. 4A), lactate concentrations, creatinine concentrations (Fig. 4B) and hematocrit values (Fig. 4C) were indistinguishable between the groups throughout the

experiment. Furthermore, there were no significant differences in leukocyte counts between groups (Fig. 4D). Peak inspiratory pressures to maintain minute ventilation during the experiment were comparable with vehicle- and ubiquitin-treatment (Fig. 4E/F).

The P:F ratios of the animals are shown in Fig. 4G. With vehicle treatment, P:F ratios decreased continuously to  $111 \pm 10$  mmHg at  $t = 300$  min. Vehicle-treated animals fulfilled criteria for mild ARDS (P:F ratio  $< 300$  mmHg) at  $t=90$  min and criteria for moderate ARDS (P:F ratio  $< 200$  mmHg) at  $t = 180$  min. With  $0.7 \mu\text{mol/kg}$  ubiquitin, P:F ratios were significantly higher until  $t = 120$  min ( $t = 90$  min:  $378 \pm 20$  mmHg,  $t = 120$  min:  $240 \pm 27$ ), as compared with vehicle-treated animals. With this dose of ubiquitin, animals fulfilled criteria for mild ARDS at  $t = 180$  min and criteria for moderate ARDS only at the end of the observation period ( $t = 300$  min). With  $3.5 \mu\text{mol/kg}$  ubiquitin, P:F ratios were significantly higher throughout the entire reperfusion period, as compared with vehicle-treated animals. These animals fulfilled criteria for mild ARDS at  $t = 180$  min and did not progress to moderate ARDS during the observation period.

Histomorphological analyses did not show obvious differences between the experimental groups (Fig. 5A). LIS of the contralateral and post-ischemic lungs were comparable between the groups (Fig. 5B). While W/D ratios of the lungs were also comparable between groups (Fig. 6A), measurements of protein content in BAL fluid revealed significant differences between vehicle- and ubiquitin-treated animals (Fig. 6B/C). With both doses of ubiquitin, protein concentrations in BAL fluids from the contralateral and post-ischemic lungs were significantly lower than in corresponding BAL fluids from vehicle-treated animals. Measurements of IL-10, TNF $\alpha$  and IL-6 concentrations per mg of protein in BAL fluid from contralateral lungs and of TNF $\alpha$  and IL-6 concentrations per mg of protein in BAL fluid from post-ischemic lungs did not show significant differences among the groups (Fig. 6D–F). IL-10 concentrations per mg of protein in BAL fluids from the post-ischemic lungs increased dose-dependently with ubiquitin treatment (Fig. 6D). There were no significant differences in IL-10, TNF $\alpha$  and IL-6 concentrations in lung homogenates between the groups (Fig. 6G–I).

## Discussion

In the present study, we tested how pharmacological modulation of CXCR4 influences the development of ARDS after unilateral lung ischemia-reperfusion injury. Our findings suggest that blockade of endogenous CXCR4 after lung ischemia-reperfusion injury promotes development of ARDS, whereas activation of CXCR4 delays development and attenuates severity of ARDS.

While there is no single animal model that is able to resemble all aspects of the human pathophysiology, lung-ischemia reperfusion injury is a well-accepted model that reflects key features of human ARDS (29, 30). The current Berlin definition of ARDS grades the severity of ARDS based on the P/F ratio under PEEP or continuous positive airway pressure of equal or greater than  $5 \text{ cmH}_2\text{O}$  (mild –  $200 < \text{P/F} < 300$  mmHg, moderate –  $100 < \text{P/F} < 200$  mmHg, severe –  $\text{P/F} < 100$  mmHg) (8). Thus, we ventilated animals with a PEEP of  $5 \text{ cmH}_2\text{O}$  during the reperfusion period to be able to monitor the temporal pattern of ARDS

development in our model. In series 1, control animals barely reached the criteria for mild ARDS at the end of the observation period. In contrast, AMD3100 treatment shortly after reperfusion accelerated development of ARDS and increased the severity of the reperfusion injury to moderate ARDS. In combination with the observation of increased histological lung injury with AMD3100 treatment, these findings imply that endogenous CXCR4 mediates lung protective effects in the pathophysiology of ARDS. Furthermore, our finding that AMD3100 treatment promotes ARDS development is in agreement with previous reports that CXCR4 blockade increases mortality and tissue injury in models of endotoxemia and polymicrobial sepsis, and that administration of AMD3100 impairs hemodynamic stability in models of traumatic-hemorrhagic shock (31–36). Collectively, these data indicate that endogenous CXCR4 plays important roles in infectious and non-infectious inflammation, and provides tissue/organ protection, including lung protection. The observation that leukocyte counts at the end of the observation period were significantly higher with AMD3100 treatment, as compared with vehicle-treatment, is in line with its known pharmacological properties (32, 37).

In contrast to AMD3100 treatment, ubiquitin treatment dose-dependently increased P/F ratios and converted moderate-to-severe ARDS into mild-to-moderate ARDS. Although we were unable to differentiate the degree of histological lung injury between vehicle- and ubiquitin-treated animals, measurements of BAL fluid protein content showed that protein leakage into the alveolar space was significantly reduced with ubiquitin treatment. As this effect was detectable in BAL fluid from the post-ischemic lung and from the contralateral lung, these findings suggest that CXCR4 activation stabilizes permeability of the alveolar-capillary membrane, through which it reduces direct and remote lung injury, respectively.

Despite significant changes in P/F ratios between vehicle- and AMD3100- or ubiquitin-treated animals, lung W/D ratios were indistinguishable at the end of the observation period. We have shown previously that ubiquitin-treatment significantly reduced W/D ratios of the uninjured lung in a polytrauma (bilateral femur fracture plus unilateral lung contusion) and fluid resuscitation model 7 hours post injury and W/D ratios of the post-ischemic lung after 90 min of ischemia (23, 27). Thus, it appears likely that the combination of a shorter ischemic insult and a shorter observation period in the present study did not provide sufficient time for the development of lung edema that can be differentiated by W/D measurements. This assumption is further supported by the observation that W/D ratios of post-ischemic and contralateral lungs were not significantly different. In addition, the finding that ubiquitin treatment significantly improved P/F ratios while lung histology was not obviously altered indicates that functional and histo-morphological consequences of lung ischemia-reperfusion are not directly correlated with each other.

Protective and therapeutically relevant effects of natural and synthetic CXCR4 agonists have been reported in multiple previous pre-clinical studies, including animal models of endotoxemia, sepsis, ischemia-reperfusion injury, trauma and hemorrhagic shock (23–27, 32–34, 38–44). The findings of the present study confirm that CXCR4 agonists have therapeutic potential, demonstrate that administration of the non-cognate CXCR4 agonist ubiquitin delays and attenuates development of ARDS after lung ischemia-reperfusion injury and provide initial information on its dose-effect profile.

While our observations on the effects of pharmacological blockade and activation of CXCR4 are consistent with the assumption that CXCR4 fulfills protective roles during inflammation and development of tissue injury, the molecular mechanisms underlying the observed effects in the present and in previous studies are not well understood. Although single time point measurements of surrogate markers of inflammation and tissue injury in BAL fluid or lung extracts are inherently difficult to interpret, such measurements may provide initial insights into possible mechanisms. Similar to our observations in previous animal models, we did not detect differences in lipid peroxidation products and MPO activities in lung extracts between groups (27). The finding that there were no differences in TNF $\alpha$  or IL-6 levels in lung homogenates and BAL fluids was not surprising due to their large inter-individual variability, their dynamic nature and the small sample size in each group. Thus, we cannot exclude that larger sample sizes or detailed time course measurements would demonstrate differences between vehicle-treated and AMD3100- or ubiquitin-treated animals. Nevertheless, our observations that AMD3100-treatment reduced IL-10 concentrations per mg of protein in homogenates from post-ischemic lung, whereas ubiquitin-treatment dose-dependently increased IL-10 concentrations per mg of protein in BAL fluid from post-ischemic lungs, could point towards anti-inflammatory and immune modulatory effects of CXCR4 as a contributing mechanism. This assumption is supported by previous observations suggesting enhanced pro-inflammatory responses after pharmacological CXCR4 blockade and enhanced anti-inflammatory responses following CXCR4 activation (23, 31, 32, 41). As CXCR4 has been reported to regulate vascular endothelial barrier function, direct effects of pharmacological CXCR4 modulators on pulmonary vascular permeability, independent of the cytokine network, may also account for the observed effects (45). To delineate the exact underlying mechanisms and target cell populations, detailed mechanistic studies will be required. Such studies, however, are beyond the scope of the present study.

In conclusion, the present study confirms previous reports suggesting therapeutic potential of CXCR4 agonists to attenuate lung injury in various animal models, establishes cause-effect relationships for the effects of pharmacological CXCR4 modulation on the development of ARDS after lung ischemia-reperfusion injury and provides initial pre-clinical evidence that CXCR4 agonists could be used to attenuate progression of mild ARDS to moderate and severe ARDS. Our findings point towards CXCR4 as a drug target to reduce the incidence and attenuate the severity of ARDS. Future studies delineating the molecular mechanisms underlying the protective effects of CXCR4 activation as well as pre-clinical efficacy studies in other ARDS models will be required to characterize and better understand the translational potential of this new therapeutic approach.

## Methods

### Lung ischemia-reperfusion injury model

All procedures were performed according to National Institutes of Health Guidelines for Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago and the Animal Care and Use Review Office of the U.S. Army Medical Research and Materiel Command. Male Sprague-Dawley rats (300 –

350 g) were purchased from Harlan (Indianapolis, IN, USA). Anesthetized (isoflurane inhalation) animals were oro-tracheally intubated with a 16-gauge EXEL disposable safelet angiocatheter (EXELINT International, Los Angeles, CA, USA) and mechanically ventilated with a SomnoSuite small animal anesthesia system (Kent Scientific Corporation, Torrington, CT, USA). Animals were ventilated with an initial positive end expiratory pressure (PEEP) of 2 mmHg, a fraction of inspired oxygen ( $\text{FiO}_2$ ) of 1.0 and anesthetized with isoflurane at 2.5%. Tidal volumes were titrated to maintain normal  $\text{PaCO}_2$  (35–45 mmHg). The femoral artery was then cannulated with a 24-gauge BD angiocath shielded IV catheter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to allow for monitoring of arterial blood pressure, blood withdrawal, and drug administration. Animals underwent a right lateral thoracotomy and a suture was placed around the hilum of the right lung. Five units of heparin in 500  $\mu\text{L}$  normal saline (NS) solution was administered and allowed to circulate for 5 min. The suture was then tied around the hilum of the right lung, occluding the pulmonary artery, vein, and right main stem bronchus. After 30 min (AMD3100 treatment) or 60 min (ubiquitin treatment) of ischemia, the suture was removed and animals were ventilated with  $\text{FiO}_2$  1.0, PEEP 5 mmHg. A sigh breath was administered every 15 breaths for the first 10 min following reperfusion and then every 90 breaths until completion of the experiment. Hemodynamics were continuously monitored with the surgivet invasive blood pressure monitor (Med-Electronics, Beltsville, MD, USA) and blood pressures values were recorded every 5 min throughout the experiment. Arterial blood gases and routine laboratory parameters were determined in regular intervals throughout the experiment. At  $t=300$  min, animals were euthanized (5% isoflurane, bilateral pneumothorax) and both lungs harvested for histology and measurements of inflammatory markers in whole lung homogenates. All experiments were performed randomized and blinded. Animals received vehicle (0.5 mL NS,  $n=5$ ) or AMD3100 (700 nmol/kg,  $n=5$ ) in 0.5 mL NS intra-arterially within 5 min of reperfusion after a 30 min period of ischemia. Animals received vehicle (0.5 mL NS,  $n=6$ ), 0.7  $\mu\text{mol/kg}$  ubiquitin ( $n=6$ ) or 3.5  $\mu\text{mol/kg}$  ubiquitin ( $n=5$ ) intra-arterially within 5 min of reperfusion following a 60 min period of ischemia. Dosing of AMD3100 and ubiquitin was selected based on our previous studies in other animal models (27, 33, 34, 46).

### **Bronchoalveolar lavage**

At the completion of the 60 min ischemia groups ( $t = 300$  min) bronchoalveolar lavages (BAL) were performed from both lungs. After a midline sternotomy, the left main stem bronchus was occluded with a vascular clamp, 1.5 mL of normal saline were injected via the endotracheal tube and the BAL fluid was aspirated. The vascular clamp was then placed on the right main stem bronchus and the BAL procedure repeated. Recovery of BAL fluid was 70–83%. The BAL fluid was then centrifuged ( $0.5 \times g$  for 5 min), the supernatant aspirated, snap frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until further analysis.

### **Arterial blood gases and routine laboratory parameters**

Arterial blood gases, electrolytes, creatinine, lactate, hematocrit and hemoglobin were analyzed using the Element point of care veterinary blood gas, electrolyte and critical care analyzer (Cuattro Veterinary USA, Loveland, CO, USA). Complete blood counts were

analyzed using the Hematrue hematology analyzer (Cuattro Veterinary USA, Loveland, CO, USA).

### **Histopathological analysis of lung tissue**

For histomorphological examination, lung specimens were placed in formalin fixative solution and sent to AML Labs (Saint Augustine FL), where they were embedded in paraffin wax, sliced into 5  $\mu$ m sections and stained with hematoxylin and eosin (H&E). From each lung specimen, 6 slides were prepared. The slides were examined under a light microscope by 3 investigators who were blinded as to the identity of the specimens. Histopathology was assessed using a previously described lung injury score (LIS), with slight modifications (47). In brief, each investigator rendered a score of 0 (no damage) to 4 (maximal damage) based on the following criteria: 1.) alveolar congestion, 2.) presence of hemorrhage, 3.) interstitial edema, and 4.) alveolar wall thickness.

### **Lung Wet Weight to Dry Weight Ratios**

The ratio of the tissue wet weight to dry weight (W/D) was determined gravimetrically, as previously described (23, 48).

### **Lung Extract Preparation**

Snap frozen tissues were homogenized in 1/10 phosphate buffered saline, pH 7.4 (1:5 weight/volume), centrifuged (20,000  $\times$  g, 4°C, 30 min) and supernatants (= extracts) aliquoted, as described (48, 49).

### **Enzyme Linked Immunosorbent Assays (ELISA)**

Interleukin 6 (IL-6), IL-10 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) were measured in lung extracts and BAL samples with commercially available enzyme-linked immunosorbent assay kits (DuoSet ELISA, all from R&D Systems, Minneapolis, MN) according to the manufacturer's protocols.

### **Myeloperoxidase (MPO)**

MPO was measured in lung extracts using a commercially available assay kit (Invitrogen, Carlsbad CA) according to the manufacturer's protocol.

### **Lipid peroxidation assay**

Malondialdehyde (MDA) in combination with 4-hydroxyalkenals (4-HAE) were measured as an indicator of lipid peroxidation in the lung extracts using a commercially available colorimetric microplate assay (No. FR 22, Oxford Biomedical Research, Oxford, MI), as described (23).

### **Data Analyses and Statistics**

Data are presented as mean  $\pm$  standard error or median with interquartile range (25<sup>th</sup>/75<sup>th</sup> percentile). Parametric data were analyzed with unpaired Student's t-test, 1-way ANOVA or 2-way ANOVA with Sidak's multiple comparisons test, as appropriate. Non-parametric data were analyzed with the Mann-Whitney U test. Data analyses were calculated with the



GraphPad Prism program (GraphPad Software). A two-tailed  $p < 0.05$  was considered significant.

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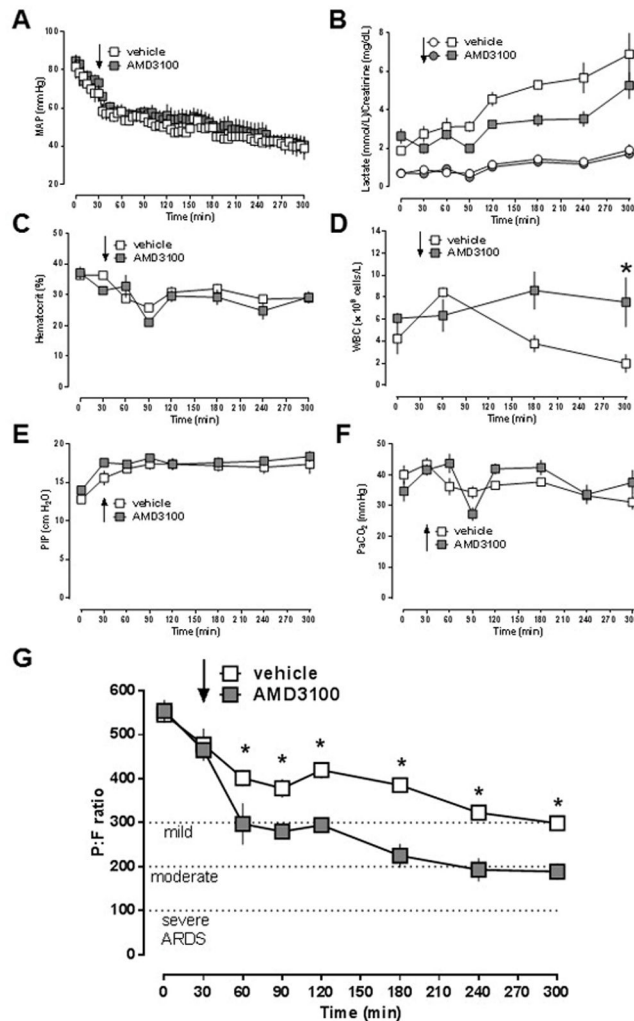
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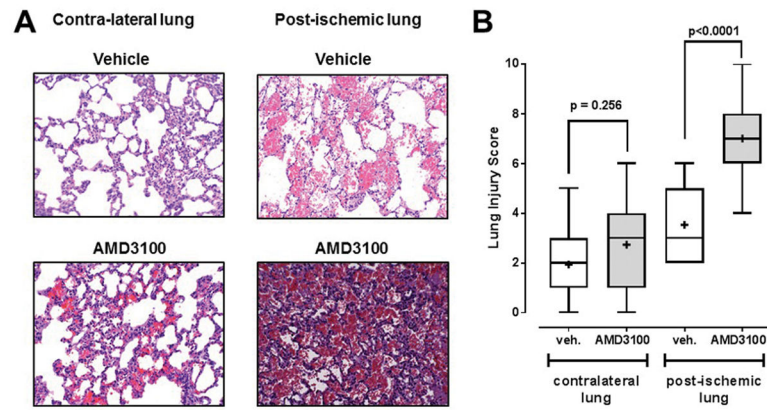
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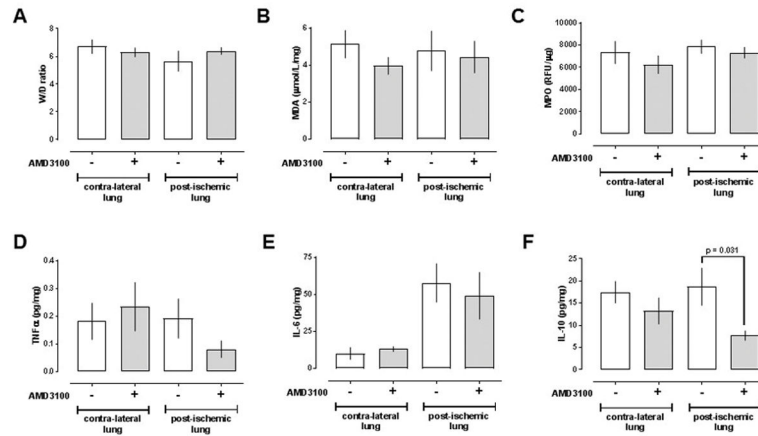


### Figure 1. AMD3100 treatment promotes development of ARDS after lung ischemia-reperfusion injury

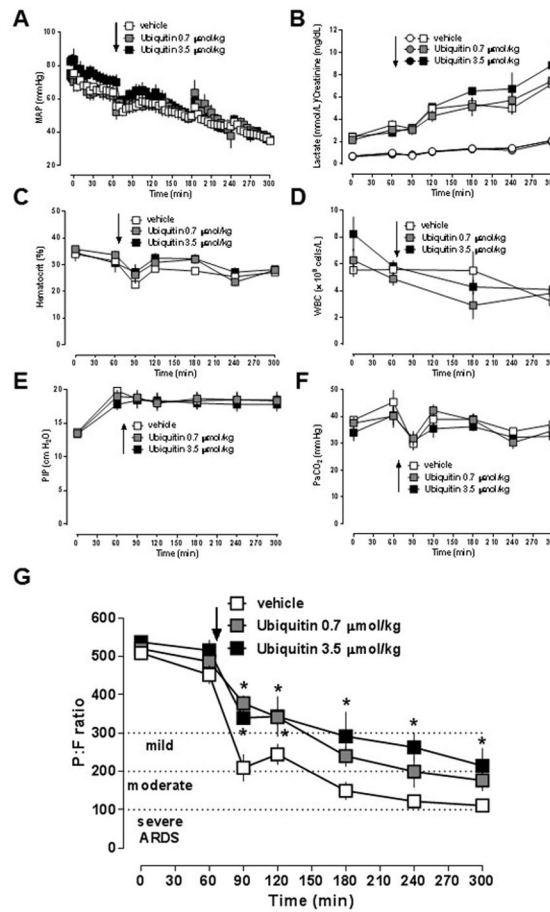
Animals underwent lung ischemia for 30 min, followed by reperfusion until  $t = 300$  min. Arrows indicate the time point of drug injection. Open symbols: vehicle treatment ( $n = 5$ ). Grey symbols: AMD3100 ( $0.7 \mu\text{mol/kg}$ ) treatment ( $n = 5$ ). Data are mean  $\pm$  SEM. **A.** Mean arterial blood pressure (MAP, mmHg). **B.** Blood lactate (mmol/L, squares) and creatinine (mg/dL, circles) concentrations. **C.** Hematocrit values (%). **D.** White blood cell count (WBC,  $\times 10^9$  cells/L). **E.** Peak inspiratory pressure (PIP, cm H<sub>2</sub>O). **F.** Partial pressure of carbon dioxide in arterial blood (PaCO<sub>2</sub>, mmHg). **G.** PaO<sub>2</sub>/FiO<sub>2</sub> ratio. \*:  $p < 0.05$  vs. vehicle (2-way ANOVA with Sidak's multiple comparisons test).



**Figure 2. AMD3100 treatment increases histological lung injury in the post-ischemic lung**  
**A.** Representative images from H&E stained lung sections from animals after vehicle (top) and AMD3100 (bottom) treatment, as in Fig. 1. **B.** Lung injury scores (LIS) from animals in Fig. 1. Boxes extend from the 25<sup>th</sup> to 75<sup>th</sup> percentile, the horizontal line shows the median. Error bars show the range of data (min/max). LIS were analyzed with the Mann-Whitney U test. The level of statistical significance is indicated.

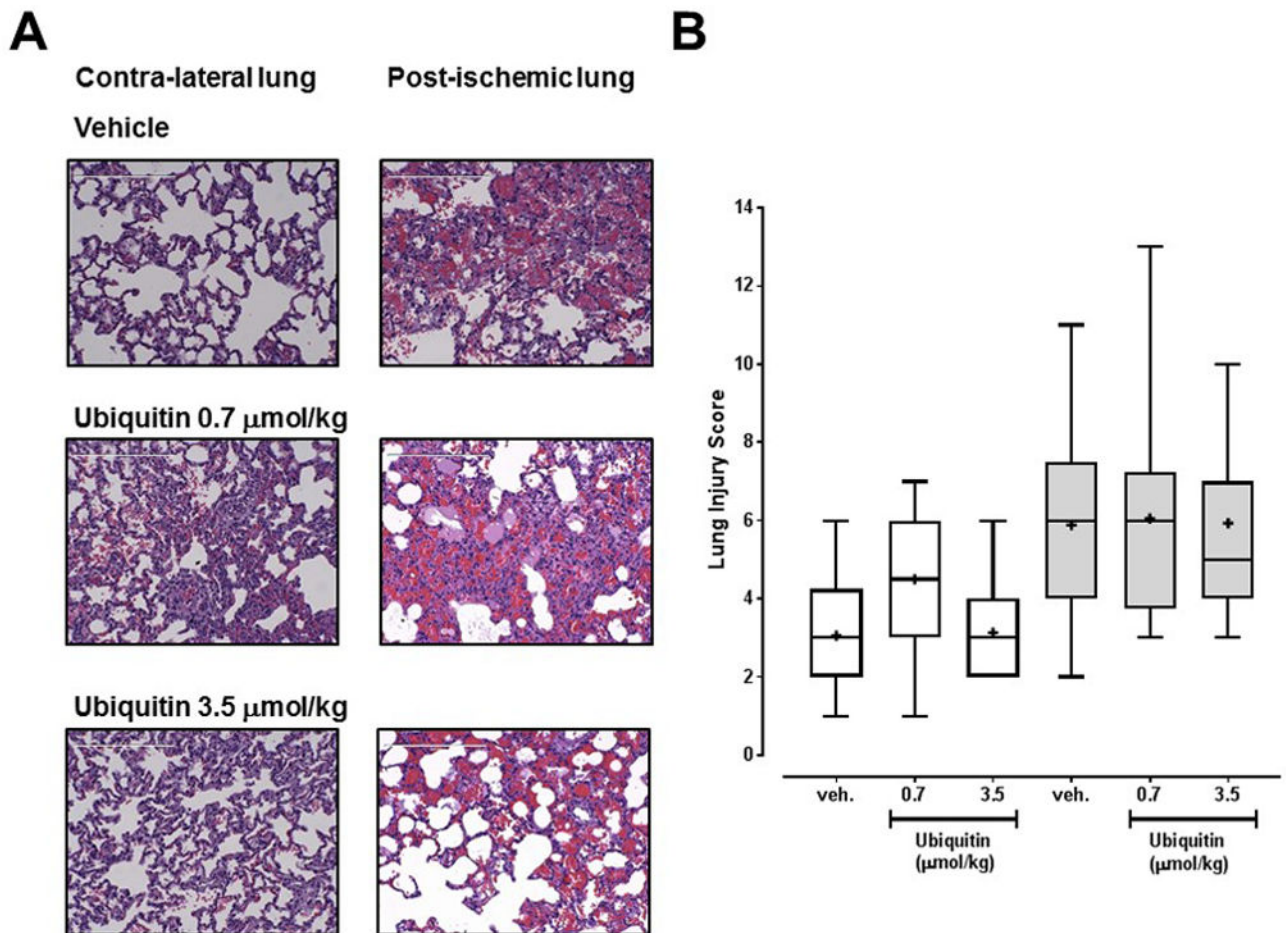


**Figure 3. Effects of AMD3100 treatment after lung ischemia-reperfusion injury on lung wet to dry weight ratios and surrogate markers of tissue injury and inflammation**  
 Data are mean ± SEM. Statistically significant differences between groups are labeled. **A.** Wet weight to dry weight (W/D) ratios. **B.** malondialdehyde (MDA, µmol/L/mg). **C.** Myeloperoxidase (MPO, relative fluorescence units (RFU)/µg). **D.–F.** TNF-α (pg/mg, **D.**), IL-6 (pg/mg, **E.**) and IL-10 (IL-10, ng/mg **F.**) concentrations in lung homogenates. Groups were compared with unpaired Student’s t-test. The level of statistical significance is indicated.



**Figure 4. Ubiquitin treatment delays and attenuates development of ARDS after lung ischemia-reperfusion injury**

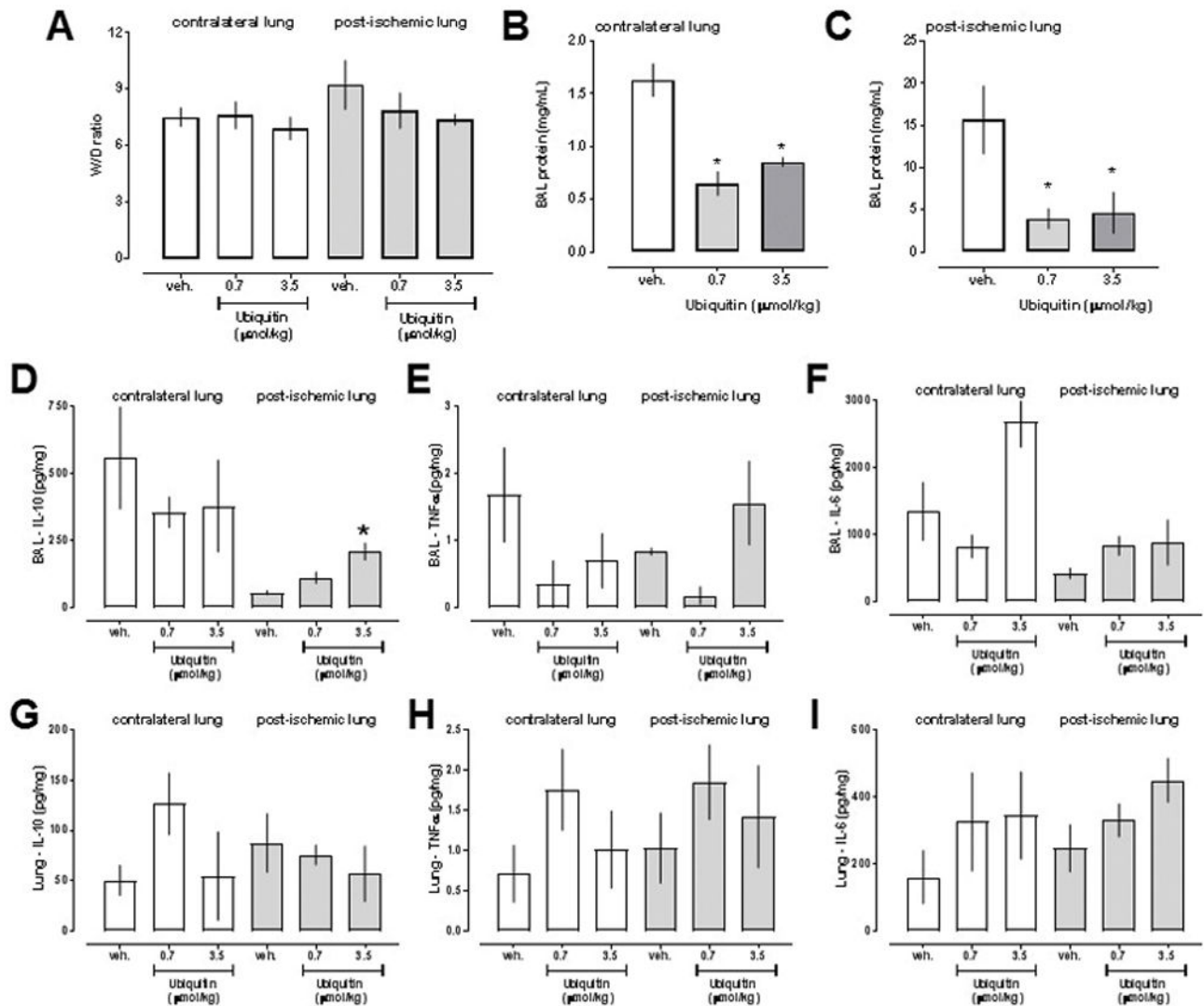
Animals underwent lung ischemia for 60 min, followed by reperfusion until  $t = 300$  min. Arrows indicate the time point of drug injection. Open symbols: vehicle treatment ( $n = 6$ ). Grey symbols: Ubiquitin ( $0.7 \mu\text{mol/kg}$ ) treatment ( $n = 6$ ). Black symbols: Ubiquitin ( $3.5 \mu\text{mol/kg}$ ) treatment ( $n = 5$ ). Data are mean  $\pm$  SEM. **A.** Mean arterial blood pressure (MAP, mmHg). **B.** Blood lactate (mmol/L, squares) and creatinine (mg/dL, circles) concentrations. **C.** Hematocrit values (%). **D.** White blood cell count (WBC,  $\times 10^9$  cells/L). **E.** Peak inspiratory pressure (PIP, cm H<sub>2</sub>O). **F.** Partial pressure of carbon dioxide in arterial blood (PaCO<sub>2</sub>, mmHg). **G.** PaO<sub>2</sub>/FiO<sub>2</sub> ratio. \*:  $p < 0.05$  vs. vehicle (2-way ANOVA with Sidak's multiple comparisons test).



**Figure 5. Lung histology after ischemia reperfusion injury and ubiquitin treatment**

**A.** Representative images from H&E stained lung sections from animals after vehicle (top), 0.7  $\mu\text{mol/kg}$  ubiquitin (center) and 3.5  $\mu\text{mol/kg}$  ubiquitin (bottom) treatment, as in Fig. 1. **B.** Lung injury scores (LIS) from animals in Fig. 1. Boxes extend from the 25<sup>th</sup> to 75<sup>th</sup> percentile, the horizontal line shows the median. Error bars show the range of data (min/max).





**Figure 6. Effects of ubiquitin treatment after lung ischemia-reperfusion injury on lung wet to dry weight ratios and surrogate markers of tissue injury and inflammation**  
 Data are mean  $\pm$  SEM. **A.** Wet weight to dry weight (W/D) ratios. **B.** Protein concentrations (mg/mL) in bronchoalveolar lavage (BAL) fluid from contralateral lungs. **C.** Protein concentrations (mg/mL) in bronchoalveolar lavage (BAL) fluid from post-ischemic lungs. **D.–F.** IL-10 (pg/mg, **D.**), TNF $\alpha$  (pg/mg, **E.**) and IL-6 (pg/mg, **F.**) levels in BAL fluid. **G.–H.** IL-10 (pg/mg, **D.**), TNF $\alpha$  (pg/mg, **E.**) and IL-6 (pg/mg, **F.**) levels in lung homogenates. \*:  $p < 0.05$  vs. vehicle treatment (1-way ANOVA with Sidak's multiple comparisons test).