Disruption of platelet-derived chemokine heteromers prevents neutrophil extravasation in acute lung injury

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Online Data Supplement

Methods

Detailed description of ALI model

30 min before euthanasia, 5 µl FITC-Ly-6G (Gr1) (eBioscience) and 100 µl Fluorescein isothiocyanate–Dextran (30 mg/ml FITC-Dextran; 70 kDa, Sigma-Aldrich) were applied by tail vein injection to label intravasal neutrophils and to assess plasma leakage, respectively. The trachea was dissected and cannulated (Portex FineBore Polythene Tubing, 0.28 mm inner diameter (ID)/0.61 mm outer diameter (OD), Smiths Medical International, Keene, NH). 5x 0.5 ml PBS was injected and withdrawn. Thereafter, the ribcage was opened by a midline incision and the pulmonary vasculature was rinsed with 15 ml ice-cold PBS with 0.5 mM EDTA after cutting the inferior cava vein to facilitate exsanguination. The lungs were removed, minced and digested with liberase (1:20; 25 mg Liberase RI/ml aqua, Roche Mannheim Germany). Digested lungs were passed through a cell strainer (70 µm; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and the resulting single cell suspension was centrifuged for 5 min at 300 g. The pellets were resuspended in 1 ml hank's balanced salt solution with 0.3 mmol / l EDTA and 0.1 % BSA. The bronchoalveolar lavage (BAL) fluid was centrifuged for 5 min at 300 g.

Flow cytometry: Cell pellets were labeled with PerCP-Cy5.5 anti-mouse Ly-6G, PE anti-mouse CD115, APC-Cy7 anti-mouse CD45 und APC anti-Mouse F4/80 (all eBioscience). Neutrophils were identified by their typical appearance in the forward scatter-side scatter and as $CD45^{+}$ $CD115^{-}$ and PerCP- $Gr1^{+}$ cells. Within the lung, FITC-Gr1 antibody was used to distinguish between interstitial neutrophils ($CD45^{+}$, $CD115^{-}$, PerCP- $Gr1^{+}$, FITC-$Gr1^{-}$) and intravasal neutrophils ($CD45^{+}$, $CD115^{-}$, PerCP- $Gr1^{+}$, FITC-$Gr1^{+}$). All studies were performed on a FACS Canto II (Becton Dickinson, San Jose, CA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Lung Permeability: FITC-Dextran (70 kDa, Sigma-Aldrich) was used to assess vascular leakage. 100 µl FITC-Dextran (30 mg/ml) were administered by tail vein injection 30 min prior to euthanasia and dye extravasation was used to assess change in vascular permeability. The fluorescence of 100 µl BAL supernatant ($Fluo_{BAL}$) and of 50 µl serum ($Fluo_{Serum}$) was measured and clearance volume was expressed in microlitre $V_{Perm} = [(Fluo_{BAL} / 100\ µl) * BAL\ volume] / (Fluo_{Serum} / 50\ µl)$.

Protein concentration of the BAL. The protein content of the BAL supernatants was assessed using the Bio-Rad Protein Assay based on the method of Bradford (Bio-Rad Laboratories Germany). Measurement of absorbance at 595 nm was performed with a microplate reader (infinite 200, Tecan Group Switzerland).

Characterization of the peptide inhibitor MKEY.

The peptide inhibitor MKEY (CT2009ca) with the sequence NH_2-CKEYFYTSSKSSNLAVVFVTRC-COOH (Mw 2532.95) was produced at American Peptide Company Inc. (Sunnyvale, CA) using standard Fmoc-based solid phase synthesis methods and cyclized and re-purified to >90 % purity using standard reverse-phase high performance liquid chromatography (HPLC). For the determination of pharmacokinetics, MKEY was administered intraperitoneally in C57Bl/6 mice. Blood and lung samples were taken at various time points and the concentration of MKEY was determined by HPLC using a Shimadzu LC -20 and a Symmetrix ODS-AQ column (2.1*50mm, 5 µm pore size) at 277 nm with a linear gradient of acetonitrile in 0.1% formic acid.

Analysis of CCL5-CXCL4 heteromers in bronchoalveolar lavage fluids from acute respiratory distress syndrome/acute lung injury patients

All patient investigations were approved by the local ethics committee and written informed consent was obtained from all participants or their next-of-kin. Bronchoalveolar lavage (BAL)

fluids were obtained from 9 acute respiratory distress syndrome (ARDS)/acute lung injury (ALI) patients according to the criteria of the American-European Consensus Conference on ARDS/ALI (1) admitted to the Intensive Care Unit of the Department of Internal Medicine, Pneumology, and Intensive Care Medicine at the University of Giessen Lung Center, Germany. All patients required mechanical ventilation. BALF was obtained by flexible fibreoptic bronchoscopy within 24 h following diagnosis. One segment of the lingula or the right middle lobe was lavaged with a total volume of 200 ml of sterile 0.9 % NaCl in 10 aliquots with a fluid recovery ranging between 50 and 70 %. The fractions of the BALF were pooled, filtered through sterile gauze and centrifuged at 200g (10 minutes, 4 °C) to remove cells and membraneous debris. Staining and counting of cells within the BAL fluid cell pellet were performed by routine methods (May–Gruenwald–Giemsa staining). Table E3 documents the demographic and baseline clinical characteristics of the patient cohort.

Detection of CCL5–CXCL4 heteromers

The detection of heteromeric CCL5-CXCL4 was performed by enzyme-linked immunosorbent assay. Microtiterplates (Costar) were coated overnight at 4°C with anti-mouse CXCL4 (2 µg/mL, R&D Systems), blocked with 1 % BSA in PBS and incubated overnight with human bronchioalveolar lavage fluids or supernatant of lung homogenates from mice that underwent lung injury. Bound heteromers were detected after incubation with biotinylated anti-mouse CCL5 antibodies (10 ng/mL, 2 h, R&D Systems), streptavidin-peroxidase conjugate (20 min, R&D Systems) and tetramethylbenzidine substrate (Pierce). After stopping the colorimetric reaction with 5 % sulfuric acid, absorbance was measured at 450 and 540 nm detection and reference wave lengths, respectively.

References

1. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, Legall JR, Morris A, Spragg RG and the Consensus Committee. The American-European Consensus Conference on ARDS. *Am J Resp Crit Care Med* 1994;149: 818-824.

Table E1: Lung injury scoring system

	Score per field		
parameter	**0**	**1**	**2**
A. alveolar neutrophils	**none**	**1-5**	**>5**
B. interstitial neutrophils	**none**	**1-5**	**>5**
C. hyaline membranes	**none**	**1**	**>1**
D. proteinaceous debris filling the airspaces	**none**	**1**	**>1**
E. alveolar septal thickening	**<2x**	**2x-4x**	**>4x**

Score = [(20xA) + (14xB) + (7xC) + (7xD) + (2xE)] / (number of fieldsx100)

Table E2: Differential leukocyte counts and platelet counts in mice receiving anti-platelet serum or anti-neutrophil antibodies. All values are given in count/ml venous blood.

	WBC	neutrophils	monocytes	lymphocytes	platelets
control	8.2×10^6 +/- 1.1×10^6	2.6×10^6 +/- 0.2×10^6	6.0×10^5 +/- 1.1×10^5	4.5×10^6 +/- 1.4×10^6	50.9×10^8 +/- 8.0×10^8
platelet depletion	7.9×10^6 +/- 0.8×10^6	2.9×10^6 +/- 0.4×10^6	5.6×10^5 +/- 0.8×10^5	4.1×10^6 +/- 0.9×10^6	1.9×10^8 +/- 0.2×10^8
neutrophil depletion	5.9×10^6 +/- 1.3×10^6	0.1×10^6 +/- 0.1×10^6	4.9×10^5 +/- 1.1×10^5	4.3×10^6 +/- 1.2×10^6	60.1×10^8 +/- 10.1×10^8

Table E3. Baseline demographic and clinical data of the ARDS patients

Patient characteristics	
Number of patients	9
Age	42 ± 16 years
Gender, male/female	4/5
Pa_{O2}/Fi_{O2}	117 ± 29.3 mmHg
Predisposing events (n, %)	
Pneumonia	4 (44.4)
Sepsis syndrome	4 (44.4)
Polytransfusion	1 (11.2)

Data are presented as n or mean values ± SD. Pa_{O2}, partial pressure of oxygen in arterial blood; Fi_{O2}, fraction of inspired oxygen.

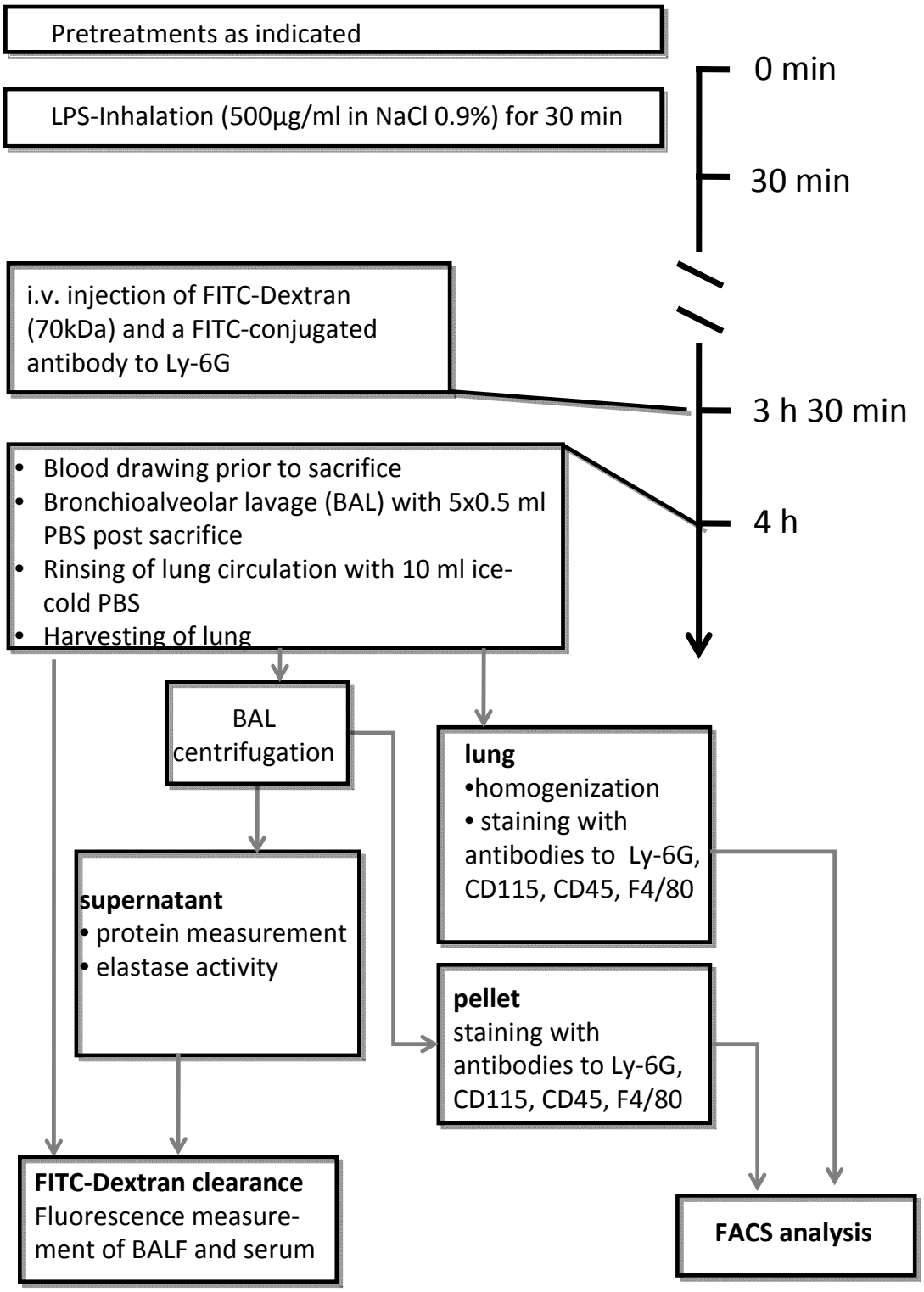

Figure E1: Experimental outline.

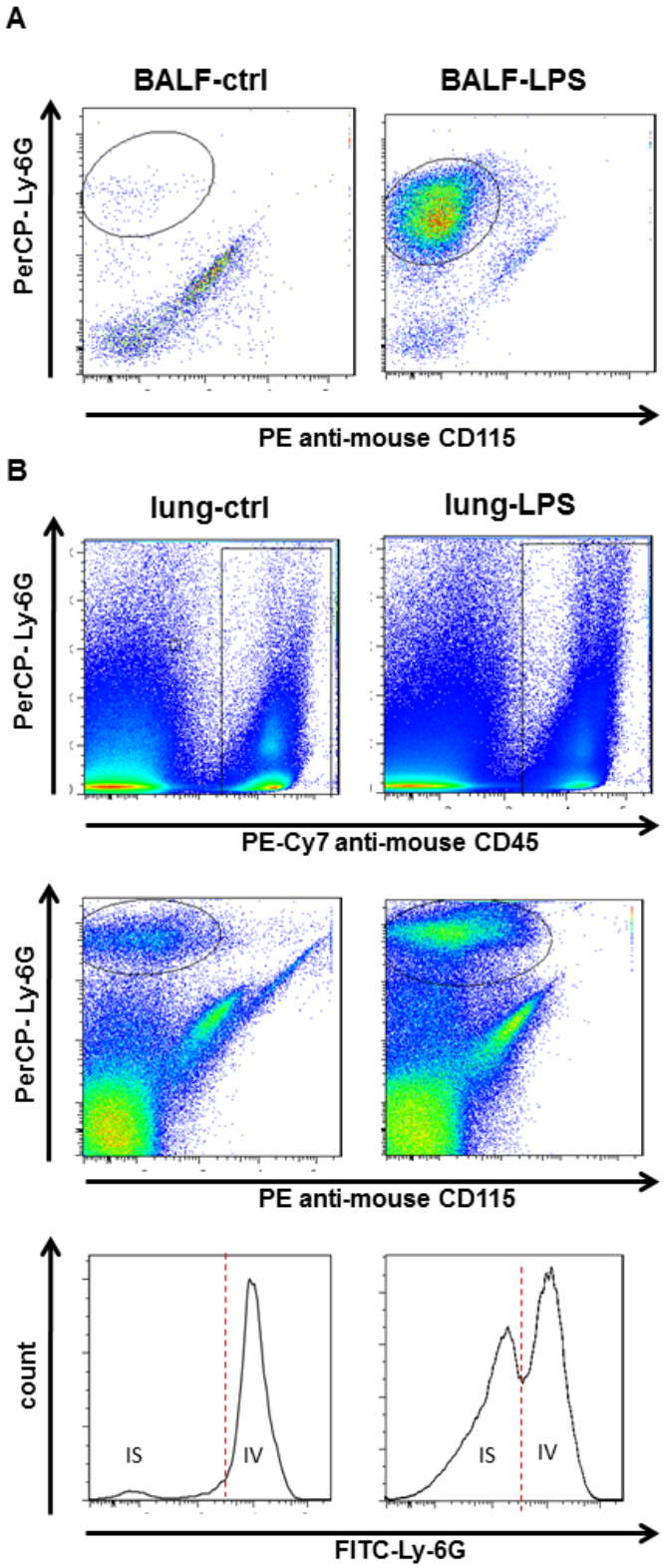

Figure E2: Gating strategy. A: Neutrophils in the BAL of PBS or LPS treated mice are identified as $CD45^{+}CD115^{-}Ly6G^{+}$. **B:** Intravascular (IV) neutrophils are discriminated from interstitial (IS) neutrophils by being positive for a FITC conjugated antibody to Ly6G that was injected i.v. 30 minutes before mice were sacrificed.

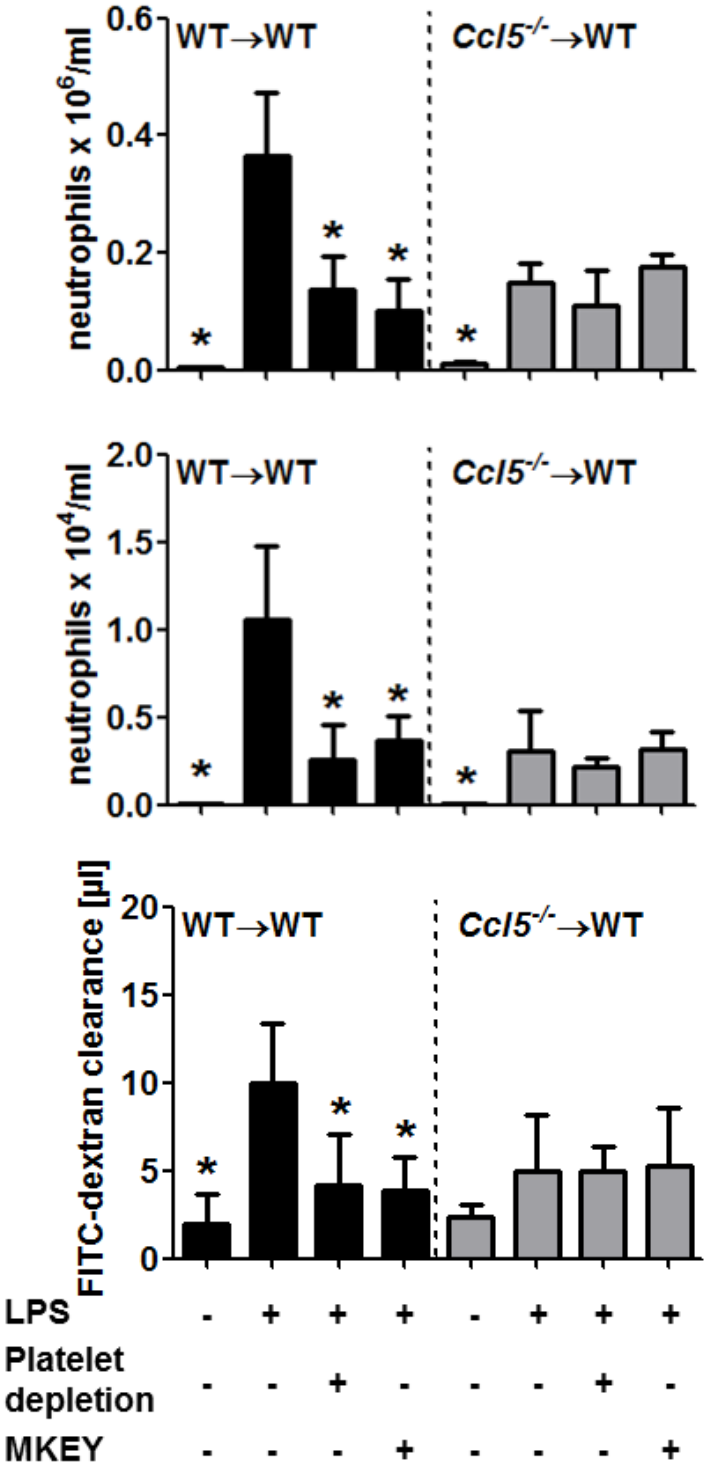

Figure E3: Bone marrow-derived CCL5 is crucially involved in LS-mediated ALI. Lethally irradiated C57Bl/6 wild type (WT) mice were reconstituted with bone marrow from WT (WT→WT) or *Ccl5*$^{-/-}$ (*Ccl5*$^{-/-}$→WT) mice. Mice were challenged with LPS via inhalation and sacrificed 4 h later. Platelets were depleted by application of anti-platelet serum (50 µl, i.p.) or treated with MKEY (50 µg). Quantification of interstitial (top) and alveolar neutrophils (middle) as well as FITC-dextran clearance (bottom). n=7 for each bar. Statistical significance was tested using one way ANOVA with Dunnett post-hoc test. * indicates significant difference compared to LPS-treated animals in respective group.

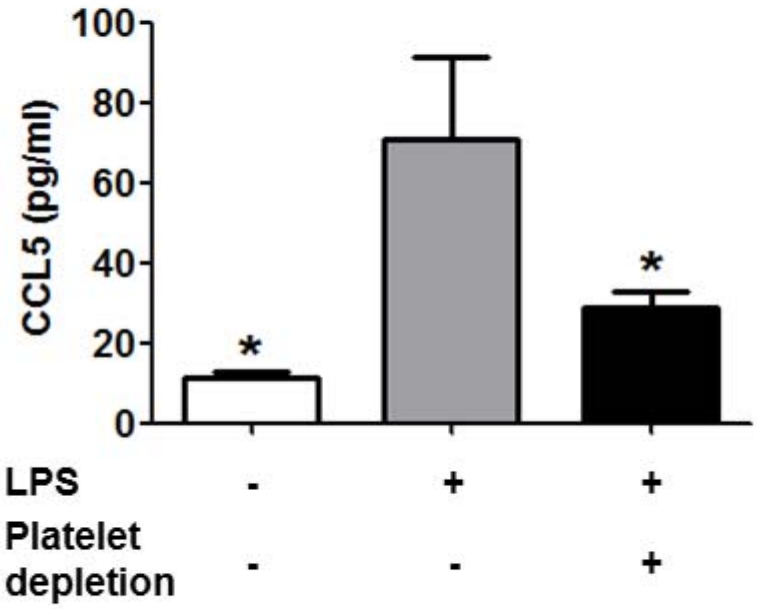

Figure E4: CCL5 is derived from platelets. Quantification of CCL5 in supernatants of homogenates of lungs from mice exposed to LPS and having received anti-platelet serum. n=4-5 for each bar. Statistical significance was tested using Kruskal-Wallis test with Dunn post-hoc test. * indicates significant difference compared to LPS-treated animals in respective group.

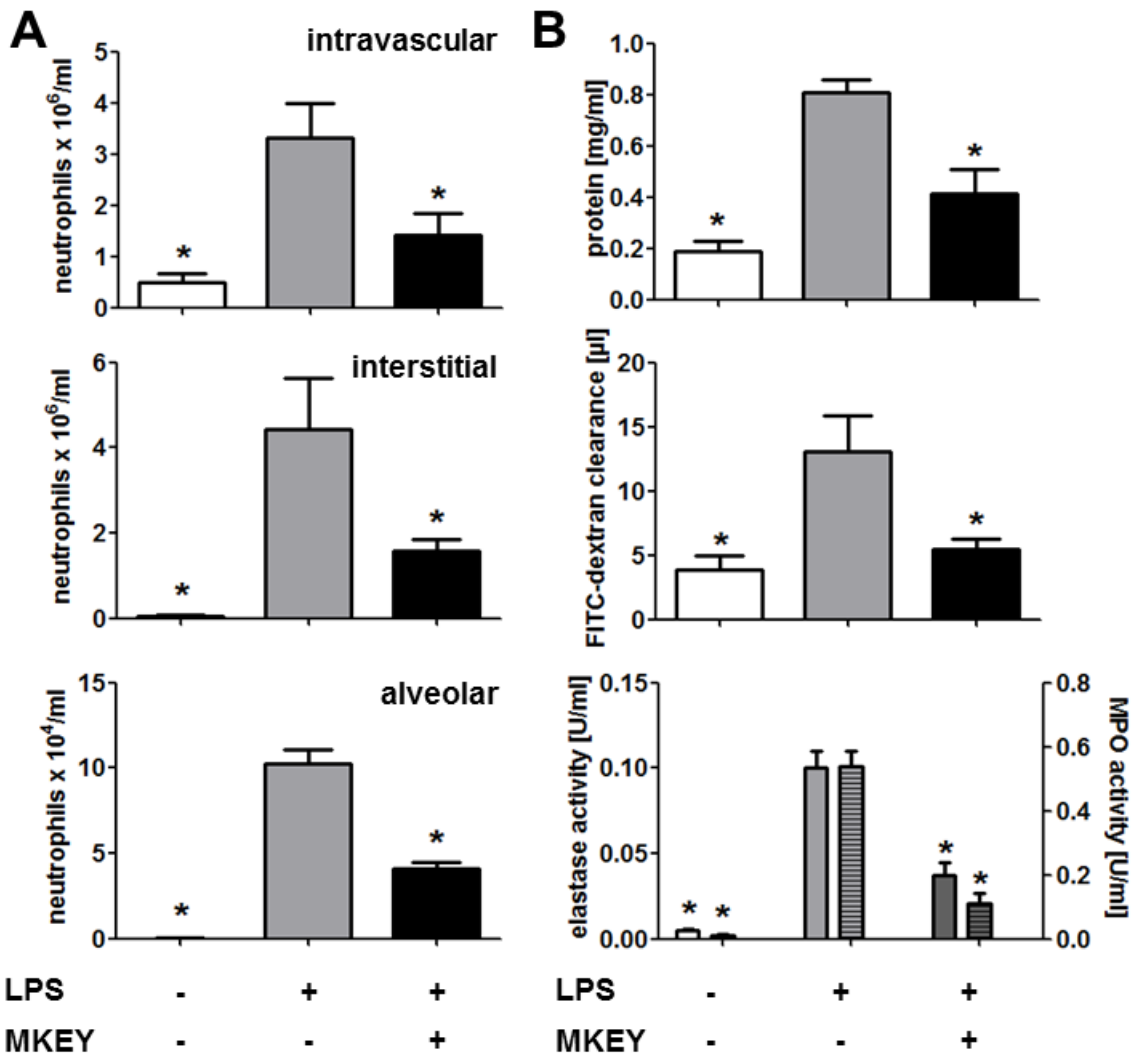

Figure E5: Delayed MKEY application protects from LPS-induced ALI. Mice were treated with MKEY (50 µg) 2 hours after LPS inhalation. 4 hours following LPS inhalation, mice were sacrificed. **A:** Displayed are intravascular (top), interstitial (middle), and alveolar neutrophil counts (bottom). **B:** Protein concentration (top), FITC-dextran clearance (middle), and elastase (bottom, uniform bars) and MPO activity (bottom, hatched bars) in BAL fluids. n=8-10 for each bar. Statistical significance was tested using one way ANOVA with Dunnett post-hoc test. * indicates significant difference compared to mice receiving LPS.

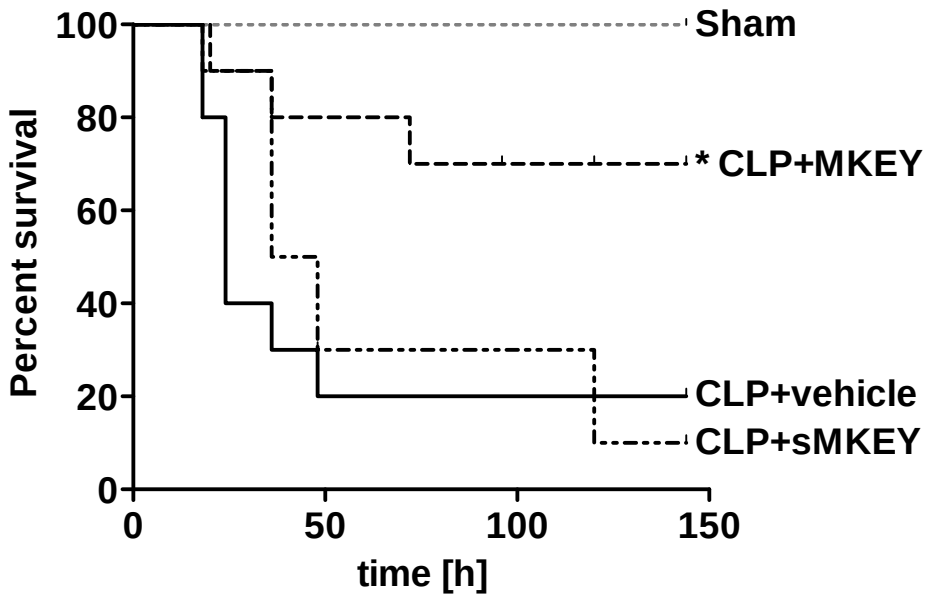

Figure E6: MKEY improves survival after cecal ligation and puncture. C57Bl6/J mice underwent cecal ligation and puncture (CLP) or sham operation. Mice with CLP were treated with MKEY (50µg i.p., every 12h), sMKEY (50µg i.p., every 12h), or vehicle and the number of surviving mice was quantified in regular intervals. Statistical significance was tested using Gehan-Breslow-Wilcoxon test. * indicates significant difference compared to mice receiving vehicle.

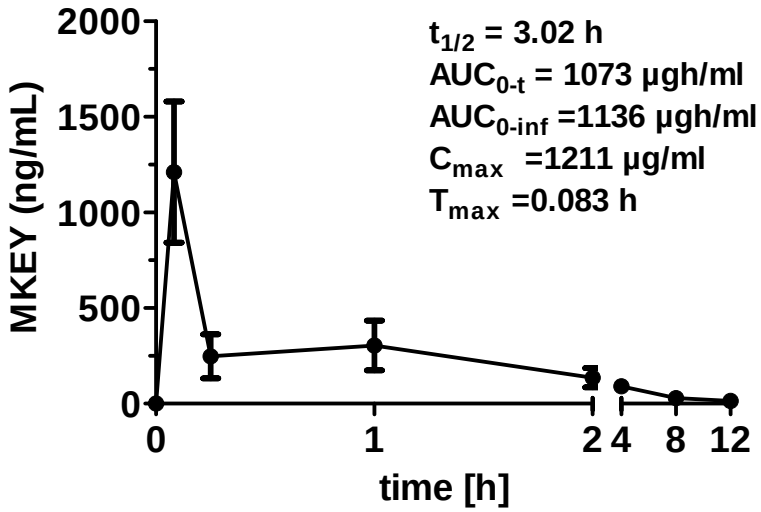

Figure E7: Pharmacokinetic data of MKEY. A single dose of MKEY (50 µg) was injected i.p. and MKEY plasma levels were measured at indicated time points. n = 3 for each time point.

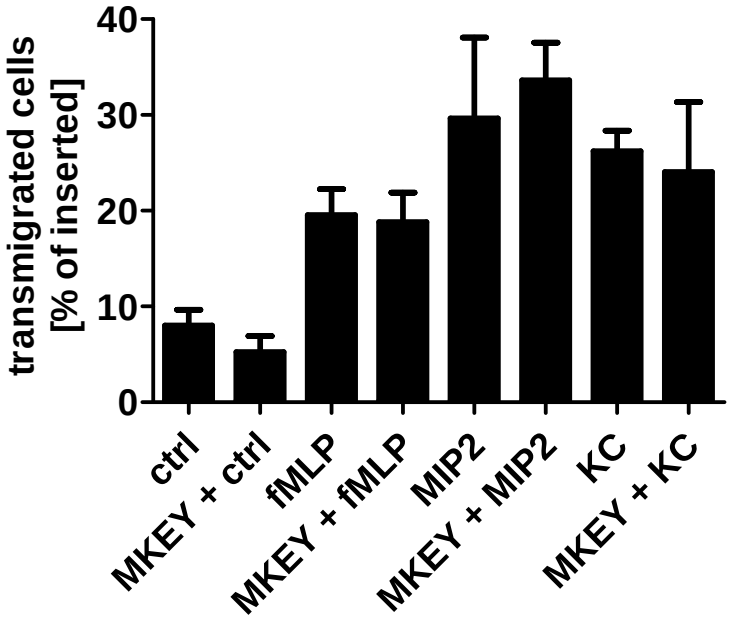

Figure E8: MKEY does not affect transmigration in vitro. Neutrophils were seeded onto a transwell filter (5 µm pore size) and transmigration was initiated by addition of fMLP, MIP2, or KC (100 ng/ml each) or vehicle control (ctrl) to the lower well. Assays were performed in presence or absence of MKEY (10 µM). After 4 hours the number of transmigrated neutrophils was quantified. n = 4 for each bar.