**Supplemental Digital Content 2**

**SUPPLEMENTAL MATERIALS AND METHODS**

**Clinical study**

This was an ancillary study of PROGRESS,[1](#_ENREF_1) an observational, prospective cohort study enrolling consecutive patients with community-acquired pneumonia as approved by central and local ethics committees (ethics committee of the University of Jena, registration number 2403-10/08). The PROGRESS cohort was recruited with the aim to identify biomarkers for progression of community-acquired pneumonia.[1](#_ENREF_1) Since the majority of PROGRESS patients recover quickly and without further complications, the cohort comprised two distinct subsets of patients. The first subset consisted of patients dying within 28 days of enrolment or showing deterioration from uncomplicated community-acquired pneumonia to severe community-acquired pneumonia, from severe community-acquired pneumonia to pneumogenic sepsis and septic shock, or from uncomplicated community-acquired pneumonia to pneumogenic sepsis and septic shock. The second subset comprised patients without further deterioration with a similar age and sex distribution. Data and laboratory samples were obtained as controls from healthy volunteers, as approved by the local ethics committee. Healthy controls were chosen to best match the distribution of age and gender in the PROGRESS study cohort. Written informed consent was obtained from each patient and volunteer, or each patient’s legal representative.

PROGRESS samples were collected between 2009 and 2013 according to the same protocol, control samples in May 2012. Case/control status was defined prospectively, i.e. there was no systematic time-shift between collected cases and controls. C5a measurements of all samples were performed in a single run according to the same protocol.

Samples of patients with community-acquired pneumonia (≥18 years) enrolled within 48 h of hospitalization were provided. Community-acquired pneumonia was confirmed by a new pulmonary infiltrate on chest radiograph and more than one of the following signs and symptoms of lower respiratory tract infection: 1) Fever, 2) cough, 3) purulent sputum, 4) shortness of breath or need for respiratory support, or 5) crackling or rales on auscultation, dullness to percussion, or bronchial breathing.

Serum C5a/C5a-desArg concentrations were quantified by enzyme-linked immunosorbent assay according to manufacturer’s instructions (HK349; Hycult, Uden, Netherlands). Briefly, serum and standard samples were transferred to microtiter stripes pre-coated with anti-C5a antibodies. Plate-bound C5a was detected by a biotinylated tracer antibody. Streptavidin-peroxidase conjugate and substrate tetramethylbenzidine were used for signal amplification. The enzyme reaction was stopped by addition of oxalic acid and absorbance at 450 nm was measured (BioTek Synergy 2 plate reader).

**Mice study**

Animal procedures were approved by the animal ethics committee of Charité – Universitätsmedizin Berlin and local governmental authorities (Landesamt für Gesundheit und Soziales Berlin). Animal housing and experimental procedures complied with the Federation of European Laboratory Animal Science Associations guidelines and recommendations for the care and use of laboratory animals.

For all experiments, female C57Bl/6N mice (8 to 11 weeks old, weighing 18 to 23 g; Charles River, Sulzfeld, Germany) were used. The infection model was established in female mice for better comparability of different studies and for practical reasons of housing and handling. The mice were randomly assigned to experimental groups as illustrated in Fig. S2 in Supplemental Digital Content 1. Charles River performs routine health monitoring and animals are tested at regular intervals for a wide variety of pathogens and opportunistic agents. Mice were given at least 7 days to acclimatize to the housing facility after arrival. Animals were housed under specific pathogen-free conditions with a 12 h light/dark cycle and free access to food and water, and monitored daily for health and wellbeing prior to the experiment. No adverse events were documented. All experimental procedures were carried out at the in-house animal laboratory. All experimental data and biological samples are stored and available for future studies to improve power calculation, thereby refining study planning and reducing animal experiments in the future.

**Pneumococcal pneumonia and NOX-D19 treatment**

*Streptococcus pneumoniae* (*S. pneumonia*e, serotype 3, strain NCTC7978) was grown to mid-log phase. Mice were anesthetized by intraperitoneal ketamine (80 mg/kg) and xylazine (25 mg/kg) and transnasally inoculated with 5x106 colony-forming units of *S. pneumoniae* in 20 µl sterile phosphate-buffered saline.[2](#_ENREF_2) Sham-infected control mice received 20 µl of sterile phosphate-buffered saline. Infections were performed between 8 and 10 a.m.

In the first pneumonia model (for experimental design and groups see Fig. S2A, Supplemental Digital Content 1), at time of infection (0 h) and 24 h post infection mice were intraperitoneally treated with anti-C5a l-aptamer NOX-D19 (20 mg/kg in 220 µl 5% glucose) or solvent (5% glucose) (n=13 each group). The dosage was based on previous studies determining the pharmacokinetic profile of NOX-D19 (unpublished data) and NOX-D20,[3](#_ENREF_3) a very close relative of NOX-D19. Twenty-four hours (all groups) or 48 h post infection (only *S. pneumoniae*-infected groups), mice were anesthetized (160 mg/kg ketamine and 75 mg/kg xylazine) and exsanguinated.

A subset of animals (n=18) was subjected to assessment of specific murine pneumonia symptoms (clinical signs) at time of sacrifice (24 h post infection, sham-infected group; 48 h post infection, *S. pneumoniae*-infected groups). One mouse of the *S. pneumoniae*-infected, solvent-treated group met the euthanasia criteria and was sacrificed 36 h after infection. The parameters incorporated into the score included appearance of the fur and eyes, behavior/degree of activity, and breathing rate, and were rated on a scale of 0 (absent) to 1 (present) and 2 (severe) based on the clinical scoring system published by Berger et al.,[4](#_ENREF_4) as detailed in Table S1, Supplemental Digital Content 3.

In the second model of pneumonia combined with mechanical ventilation (for experimental design and groups see Fig. S2B, Supplemental Digital Content 1), mice received a single intraperitoneal injection of NOX-D19 (20 mg/kg in 220 µl 5% glucose) or solvent (5% glucose) 23 h post infection and were subjected to mechanical ventilation for 6 h starting 24 h after infection, as described in detail below, when severe pneumonia had developed (n=11 each group). In both models of pneumonia, mice were randomly assigned to the different groups by simple randomization.

**Mechanical ventilation**

Twenty-four hours after infection, mice were subjected to mechanical ventilation as previously described.[5](#_ENREF_5),[6](#_ENREF_6) Briefly, mice were anesthetized with intraperitoneal injections of fentanyl (0.05 mg/kg), midazolam (5 mg/kg), and medetomidine (0.5 mg/kg). Repetitively, fentanyl (0.016 mg/kg), midazolam (1.6 mg/kg), and medetomidine (0.16 mg/kg) were supplied via an intraperitoneal catheter, when required, to guarantee adequate anesthesia during the experiment. Body temperature was maintained at 37°C by a body temperature-controlled heating pad. Mice were tracheotomized, intubated, and ventilated with tidal volume of 12 ml/kg, respiratory rate of 120 per minute, fraction of inspiratory oxygen (FiO2) of 0.75 and 2 cm H2O positive endexpiratory pressure.

A tidal volume of 12 ml/kg was applied as we have observed that this tidal volume causes a subtle but well characterized extent of ventilator-induced lung injury in the available, rather short time frame of 6 h, which makes it possible to evaluate potential interventions. Further, mice with pneumonia subjected to this regime of mechanical ventilation develop severe lung injury. This reflects the scenario in patients with severe pneumonia subjected to mechanical ventilation in whom even a small tidal volume induces high lung strain due to a significantly reduced functional residual capacity. In this condition, adjuvant therapies to reduce lung permeability should ideally be effective.

A carotid artery catheter was placed for blood pressure monitoring and infusion of a balanced electrolyte solution (Jonosteril; Fresenius Kabi, Bad Homburg Germany) containing 150 *M* trometamolhydrochloride (350 μl/h). Mice were ventilated by using a special rodent ventilation system, which continuously recorded airway pressure, respiratory rate, and tidal volume (flexiVent; Scireq, Montreal, QC, Canada). After preparation, a recruitment maneuver was performed (increasing airway pressure to 35 cm H2O), and mice were ventilated for 6 h with the ventilator settings outlined above. A second recruitment maneuver was performed 5 min before termination of the experiment. All mice survived the protocol. At termination of the experiment, mice were sacrificed by exsanguination via the carotid artery catheter. “Nonventilated mice” that served as controls were subjected to identical preparation procedures 30 h after infection and were euthanized after 5 min of mechanical ventilation (n=5 each group).

**Quantification of C5a in bronchoalveolar lavage fluid and plasma of mice**

C5a/C5a-desArg concentrations in bronchoalveolar lavage fluid and plasma of *S. pneumoniae*-infected and control mice were quantified by surface plasmon resonance measurements (Biacore 2000 instrument; GE Healthcare, Munich, Germany). Anti-C5a antibody (clone MAB21501; R&D Systems, Wiesbaden, Germany) was immobilized by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) covalent coupling chemistry. Association and dissociation of C5a from plasma, bronchoalveolar lavage fluid and standard samples (recombinant mouse C5a, R&D Systems) to the immobilized antibody were recorded for 180s each at a flow of 20 μl/min. The initial slope (RU/s) of the association binding event correlates with the total concentration of C5a and C5a-desArg in the sample with c(C5a) = slope (RU/s) / association constant *ka* (1/Ms). Data analysis was performed by BIAevaluation 3.1.1 software (GE Healthcare).

**Lung permeability**

Human serum albumin (1 mg/75 µl) was injected intravenously 60 min (pneumonia alone model) or 90 min (combined model of pneumonia and mechanical ventilation) before lung preparation. Lungs were flushed with sterile 0.9% sodium chloride via the pulmonary artery, and bronchoalveolar lavage was performed twice using 800 µl phosphate-buffered saline with protease inhibitor cocktail in the pneumonia model. In the combined pneumonia/ventilation model, bronchoalveolar lavage was performed after ligation of the left stem bronchus with two times 400 μl phosphate-buffered saline. In both models from each bronchoalveolar lavage fluid portion, 250 μl were pooled and the human serum albumin concentration in bronchoalveolar lavage fluid and plasma was determined by enzyme-linked immunosorbent assay. The ratio of the human serum albumin concentration in bronchoalveolar lavage fluid and plasma was calculated[5](#_ENREF_5),[7](#_ENREF_7) and defined as permeability index.

**Leukocyte and cytokine quantification in bronchoalveolar lavage fluid and blood**

Leukocytes in bronchoalveolar lavage fluid were differentially quantified by fluorescence-activated cell sorting analysis (FACS Calibur; BD Biosciences, Heidelberg, Germany) using forward- vs. side-scatter characteristics and staining with CD45 PerCP (clone 30-F11), GR-1 PE (clone RB6-8C5), CD3 FITC (clone 145-2C11) (all BD Biosciences) and F4-80 (clone BM8) APC (Invitrogen). Blood leukocytes were differentially quantified by flow cytometry using TruCount-Tubes (BD Biosciences) according to cellular forward-/side-scatter characteristics and CD45, Gr-1 and CD3 expression.

Cytokines in bronchoalveolar lavage fluid and plasma were quantified using a multiplex assay (BioRad, Hercules, CA, USA).

**Bacterial burden**

Serial dilutions of bronchoalveolar lavage fluid, blood and spleen homogenate were plated on blood agar and incubated at 37°C under 5% CO2 for 24 h to count colony-forming units.

**Aspartate transaminase and blood urea nitrogen**

Aspartate transaminase (AST) and blood urea nitrogen (BUN) were quantified 48 h post infection by routine laboratory tests.

**Histological analysis of liver tissue**

Liver tissue was removed and immediately immersion-fixed in 4% buffered formalin, embedded in paraffin and cut into 2-µm-thick sections. For analysis of apoptosis, liver sections were stained for caspase 3a and fibrin, and counterstained with hemalaun. The tissue sections were analyzed and scored (0, no signal; 1, signal) by an independent investigator blinded to the study groups (n=8 per group).

**Lung histopathology, digital image analysis and immunohistochemistry**

Formalin-fixed lungs (n=18) were embedded in paraffin, cut in three evenly distributed sections of 2 µm, and stained with hematoxylin and eosin after dewaxing in xylene and rehydration in graded ethanols. Sections were scored for defined inflammation parameters as described with minor modifications,[8](#_ENREF_8) including total lung area affected, distribution of lung lesions, suppurative, granulomatous and necrotizing character of pneumonia, interstitial and bronchoalveolar inflammation, hemorrhage, perivascular inflammatory infiltrate and necrosis, pleuritis, steatitis and lymphadenitis. For automated digital image analysis, hematoxylin and eosin stained lung sections were digitized using the Aperio CS2 slide Scanner (Leica Biosystems Imaging Ins., CA, USA) and analyzed using the Aperio nuclear v9 Algorithm (Leica Biosystems Imaging Ins., CA, USA) to establish the total number of nuclei per section. For immunohistochemical detection of *S. pneumoniae*, microwave heating (600W) in 10 mM citric acid (pH 6.0) for 12 min was used for antigen retrieval, followed by incubation with a purified rabbit antibody polyclonal to *S. pneumoniae* (1:2,000, kindly provided by S. Hammerschmidt) at 4° C overnight. An irrelevant immune-purified rabbit antibody at the same dilution served as negative control. Slides were incubated with a secondary, alkaline phosphatase-conjugated goat anti-rabbit antibody (1:500, AP-1000; Vector, Burlingame, CA, USA) for 30 min at room temperature. Triamino-tritolyl-methanechloride (Neufuchsin) was used as phosphatase substrate for color development and hematoxylin for counterstaining. Slides were dehydrated through graded ethanols, cleared in xylene and coverslipped as previously described.[9](#_ENREF_9) Immunohistochemically stained slides were graded microscopically for bacterial loads.

**Statistical analysis**

No statistical power calculation was conducted prior to the study, sample size was based on our past experience with pneumococcal pneumonia models in mice and published papers. Four mice were excluded from the study due to technical reasons (1 *S. pneumoniae*/solvent 24 h, 2 *S. pneumoniae*/solvent 48 h, 1 *S. pneumoniae*/NOX-D19 48 h). Further, data were lost for final analysis due to technical errors during preparation or measurements. Exact sample sizes for each group are provided in each figure legend. Data are presented as mean with SD or boxplots depicting median, quartiles, and range excluding outliers (open circles), with N representing the number of animals or human subjects. Lung permeability was defined as primary outcome, all other investigated parameters as secondary outcomes. The difference between means and 95% CI were calculated for the primary outcome. Preselected pairs of groups were compared using one-way ANOVA considering the factors treatment, infection or ventilation and Sidak’s multiple comparisons test for data normally distributed, or two-tailed Mann-Whitney *U*-Tests followed by Bonferroni correction for non-normally distributed data. Analyses were performed using GraphPad Prism 6.05 (San Diego, CA). *P*-values <0.05 were considered significant.

In the PROGRESS study cohort, C5a concentrations were compared between patients with community-acquired pneumonia and controls using two-tailed Mann-Whitney *U*-Test, and the Hodges-Lehmann median difference and its 95% CI were calculated. C5a values were logarithmized to obtain normally distributed values. Time of measurement imposed a small but significant batch effect explaining 14% of the variability. This batch effect was removed by linear mixed model analysis using the R-package “lme4”, function “lmer”. C5a values in selected patient groups were compared using two-tailed *t* tests. Correlation analyses were performed using Spearman’s rank correlation coefficients.

**SUPPLEMENTAL REFERENCES**

1. Ahnert P, Creutz P, Scholz M, Schutte H, Engel C, Hossain H, Chakraborty T, Bauer M, Kiehntopf M, Volker U, Hammerschmidt S, Loeffler M, Suttorp N, group Ps: PROGRESS - prospective observational study on hospitalized community acquired pneumonia. *BMC Pulm Med* 2016; 16:108

2. Witzenrath M, Schmeck B, Doehn JM, Tschernig T, Zahlten J, Loeffler JM, Zemlin M, Muller H, Gutbier B, Schutte H, Hippenstiel S, Fischetti VA, Suttorp N, Rosseau S: Systemic use of the endolysin Cpl-1 rescues mice with fatal pneumococcal pneumonia. *Crit Care Med* 2009; 37:642-9

3. Hoehlig K, Maasch C, Shushakova N, Buchner K, Huber-Lang M, Purschke WG, Vater A, Klussmann S: A novel C5a-neutralizing mirror-image (L-)aptamer prevents organ failure and improves survival in experimental sepsis. *Mol Ther* 2013; 21:2236-46

4. Berger S, Goekeri C, Gupta SK, Vera J, Dietert K, Behrendt U, Lienau J, Wienhold SM, Gruber AD, Suttorp N, Witzenrath M, Nouailles G: Delay in antibiotic therapy results in fatal disease outcome in murine pneumococcal pneumonia. *Crit Care* 2018; 22:287

5. Muller HC, Hellwig K, Rosseau S, Tschernig T, Schmiedl A, Gutbier B, Schmeck B, Hippenstiel S, Peters H, Morawietz L, Suttorp N, Witzenrath M: Simvastatin attenuates ventilator-induced lung injury in mice. *Crit Care* 2010; 14:R143

6. Muller-Redetzky HC, Kummer W, Pfeil U, Hellwig K, Will D, Paddenberg R, Tabeling C, Hippenstiel S, Suttorp N, Witzenrath M: Intermedin stabilized endothelial barrier function and attenuated ventilator-induced lung injury in mice. *PLoS One* 2012; 7:e35832

7. Witzenrath M, Gutbier B, Hocke AC, Schmeck B, Hippenstiel S, Berger K, Mitchell TJ, de los Toyos JR, Rosseau S, Suttorp N, Schutte H: Role of pneumolysin for the development of acute lung injury in pneumococcal pneumonia. *Crit Care Med* 2006; 34:1947-54

8. Reppe K, Radunzel P, Dietert K, Tschernig T, Wolff T, Hammerschmidt S, Gruber AD, Suttorp N, Witzenrath M: Pulmonary immunostimulation with MALP-2 in influenza virus-infected mice increases survival after pneumococcal superinfection. *Infect Immun* 2015; 83:4617-29

9. Dietert K, Gutbier B, Wienhold SM, Reppe K, Jiang X, Yao L, Chaput C, Naujoks J, Brack M, Kupke A, Peteranderl C, Becker S, von Lachner C, Baal N, Slevogt H, Hocke AC, Witzenrath M, Opitz B, Herold S, Hackstein H, Sander LE, Suttorp N, Gruber AD: Spectrum of pathogen- and model-specific histopathologies in mouse models of acute pneumonia. *PLoS One* 2017; 12:e0188251