**Supplemental Digital Content 1**

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**Figure S1. Overview of the role of C5a in the inflammatory response.**

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**Figure S2. Experimental design and groups for *in vivo* study of pneumococcal pneumonia (A) and combined severe pneumococcal pneumonia and mechanical ventilation (B). A,** Mice were transnasally infected with *S. pneumoniae* (5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline (20 µl) and intraperitoneally (i.p.) treated with the anti-C5a l-aptamer NOX-D19 or solvent at time of infection (0 h) and 24 h post infection. Twenty-four hours (all groups) or 48 h post infection (only *S. pneumoniae*-infected groups), mice were anesthetized and different analyses performed. **B,** Mice were transnasally infected with *S. pneumoniae* (5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline (20 µl) and intraperitoneally (i.p.) treated with NOX-D19 or solvent 23 h post infection. Twenty-four hours after infection, mice were anesthetized, mechanical ventilation (MV) was conducted for 6 hours, and different analyses performed. ’Non-ventilated mice’ (control) were also sacrificed 30 h post infection after 5 min of mechanical ventilation.



**Figure S3. Treatment with anti-C5a l-aptamer NOX-D19 led to a lower clinical disease severity.** A subset of animals (18 mice) 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 (S. pn.)*-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.,[[1]](#footnote-1) as detailed in Table S1 in Supplemental Digital Content 3. Values are given as mean and SD (n=6 each group). \*\**p*<0.01 vs. sham-infected, solvent-treated group, #*p*<0.05 vs. *S. pneumoniae*-infected, solvent-treated group (multiple Mann-Whitney *U*-Tests with Bonferroni correction).

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**Figure S4. Treatment with anti-C5a l-aptamer NOX-D19 led to a higher number of alveolar polymorphonuclear cells (PMN), but did not affect the frequency of alveolar leukocyte subsets.** Mice were transnasally infected with *S. pneumoniae* (*S. pn.*; 5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline, and intraperitoneally treated with NOX-D19 or solvent at time of infection (0 h) and 24 h post infection. Twenty-four hours or 48 h after infection, leukocytes in bronchoalveolar lavage fluid (BALF) were differentially quantified. **A-B,** Treatment with NOX-D19 led to a higher number of polymorphonuclear cells (PMN) in bronchoalveolar lavage fluid (**A**), while numbers of alveolar macrophages (AM) and lymphocytes (LYM) (**A**) as well as frequencies of leukocyte subsets (**B**) were not altered by NOX-D19 treatment. Values are given as mean and SD. In **A** n=13 (sham, *S. pn.*/NOX-D19 24 h) or n=12 (*S. pn.*/solvent 24 h) or n=10 (*S. pn.*/solvent 48 h) or n=11 (*S. pn.*/NOX-D19 48 h); in **B** (PMN, AM) n=13 (sham, *S. pn.*/NOX-D19 24 h) or n=12 (*S. pn.*/solvent 24 h) or n=10 (*S. pn.*/solvent 48 h) or n=11 (*S. pn.*/NOX-D19 48 h); in **B** (LYM) n=12 (sham) or n=11 (*S. pn.*/solvent 24 h, *S. pn.*/NOX-D19 48 h) or n=13 (*S. pn.*/NOX-D19 24 h) or n=10 (*S. pn*./solvent 48 h). \**p*<0.05, \*\*\*\**p*<0.0001 vs. sham-infected, solvent-treated group, #*p*<0.05 vs. *S. pneumoniae*-infected, solvent-treated group at the respective time point (one-way ANOVA and Sidak’s multiple comparisons test).



**Figure S5. Treatment with anti-C5a l-aptamer NOX-D19 did not affect pulmonary inflammatory mediators.** Mice were transnasally infected with *S. pneumoniae* (*S. pn.*; 5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline, and intraperitoneally treated with NOX-D19 or solvent at time of infection (0 h) and 24 h post infection. Twenty-four hours or 48 h after infection, cytokines in bronchoalveolar lavage fluid (BALF) were quantified. Cytokine concentrations in bronchoalveolar lavage fluid were not significantly altered by NOX-D19 treatment. Values are given as mean and SD; n=12 (sham) or n=11 (*S. pn.*/solvent 24 h, *S. pn.*/NOX-D19 48 h) or n=13 (*S. pn.*/NOX-D19 24 h) or n=10 (*S. pn.*/solvent 48 h). \*\**p*<0.01, \*\*\**p*<0.001, \*\*\*\**p*<0.0001 vs. sham-infected, solvent-treated group (one-way ANOVA and Sidak’s multiple comparisons test). Abbreviations: CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; G-CSF, granulocyte colony-stimulating factor; IL, interleukin.



**Figure S6. Treatment with anti-C5a l-aptamer NOX-D19 did not affect numbers and frequency of blood leukocyte subsets.** Mice were transnasally infected with *S. pneumoniae* (*S. pn.*; 5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline, and intraperitoneally treated with NOX-D19 or solvent at time of infection (0 h) and 24 h post infection. Twenty-four hours or 48 h after infection, blood leukocytes were differentially quantified. **A-B,** Leukocyte subsets were not significantly altered by NOD-D19 treatment. Values are given as mean and SD. In **A-B** n=11 (sham, *S. pn.*/solvent 24 h) or n=12 (*S. pn.*/NOX-D19 24 h) or n=10 (*S. pn.* 48 h). \**p*<0.01, \*\**p*<0.01, \*\*\*\**p*<0.0001 vs. sham-infected, solvent-treated group (one-way ANOVA and Sidak’s multiple comparisons test).



**Figure S7. Treatment with anti-C5a l-aptamer NOX-D19 led to lower systemic granulocyte colony-stimulating factor (G-CSF) concentrations, while further systemic inflammatory mediators were not affected.** Mice were transnasally infected with *S. pneumoniae* (*S. pn.*; 5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline, and intraperitoneally treated with NOX-D19 or solvent at time of infection (0 h) and 24 h post infection. Twenty-four hours or 48 h after infection, cytokines in blood were quantified. Granulocyte colony-stimulating factor concentrations were lower 48 h post infection in the NOX-D19- compared to the solvent-treated group, while further measured cytokines remained largely unaffected by NOX-D19 treatment. Values are given as mean and SD. G-CSF: n=9 (sham, *S. pn.*/NOX-D19 48 h) or n=11 (*S. pn.*/solvent 24 h) or n=12 (*S. pn.*/NOX-D19 24 h) or n=7 (*S. pn.*/solvent 48 h); IL-6, CXCL-1, CXCL2: n=10 (sham) or n=11 (*S. pn.*/solvent 24 h) or n=12 (*S. pn.*/NOX-D19 24 h) or n=8 (*S. pn.*/solvent 48 h) or n=9 (*S. pn.*/NOX-D19 48 h); IL-1β: n=10 (sham) or n=11 (*S. pn.*/solvent 24 h) or n=12 (*S. pn.*/NOX-D19 24 h) or n=7 (*S. pn.*/solvent 48 h) or n=8 (*S. pn.*/NOX-D19 48 h); IL-10: n=9 (sham, *S. pn.*/NOX-D19 48 h) or n=11 (*S. pn.*/solvent 24 h) or n=12 (*S. pn.*/NOX-D19 24 h) or n=8 (*S. pn.*/solvent 48 h); CCL3: n=11 (sham, *S. pn.*/solvent 24 h) or n=12 (*S. pn.*/NOX-D19 24 h) or n=8 (*S. pn.*/solvent 48 h) or n=9 (*S. pn.*/NOX-D19 48 h). \*\*\**p*<0.001, \*\*\*\**p*<0.0001 vs. sham-infected, solvent-treated group, ###*p*<0.001 vs. *S. pneumoniae*-infected, solvent-treated group at the respective time point (one-way ANOVA and Sidak’s multiple comparisons test).



**Figure S8. Immunohistochemistry (IHC) scoring frequency for caspase and fibrin.** Mice were transnasally infected with *S. pneumoniae* (*S. pn.*; 5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline, and intraperitoneally treated with NOX-D19 or solvent at time of infection (0 h) and 24 h post infection. Twenty-four h or 48 h after infection, liver sections were stained for caspase 3A and fibrin and counterstained with hemalaun (n=8 each group). Tissue sections were analyzed and scored (0, no signal; 1, signal) by an independent investigator blinded to the study groups. Data are represented as stacked bars demonstrating the caspase (**A**) or fibrin (**B**) immunohistochemistry grading (signal/no signal) per group.

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**Figure S9. Treatment with anti-C5a l-aptamer NOX-D19 improved lung mechanics in combined severe pneumonia and mechanical ventilation.** Mice were transnasally infected with *S. pneumoniae* (*S. pn.*; 5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline, and intraperitoneally treated with NOX-D19 or solvent 23 h post infection. One hour later (24 h post infection), mechanical ventilation was performed for 6 h. Mice were sacrificed 30 h after infection. **A,** Mean airway pressure (AWPmean) was recorded every 10 min during mechanical ventilation. Its increase was lower in the NOX-D19-treated group. **B**, The pneumonia-induced increase of mean airway pressure (AWPmean), measured at the end of the experiment (after 6 h of mechanical ventilation) was attenuated by NOX-D19 treatment. Values are given as mean and SD; n=11 (sham) or n=10 (*S. pn.*). \*\**p*<0.01 between indicated groups, #*p*<0.05 vs. *S. pneumoniae*-infected, solvent-treated, ventilated group (one-way ANOVA and Sidak’s multiple comparisons test).

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**Figure S10. Treatment with anti-C5a l-aptamer NOX-D19 did not affect alveolar leukocyte subsets in combined severe pneumonia and mechanical ventilation.** Mice were transnasally infected with *S. pneumoniae* (*S. pn.*; 5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline, and intraperitoneally treated with NOX-D19 or solvent 23 h post infection. One hour later (24 h post infection), mechanical ventilation was performed for 6 h. Ventilated and non-ventilated mice were sacrificed 30 h after infection.Numbers **(A)** and frequencies **(B)** of leukocyte subsets in bronchoalveolar lavage fluid (BALF) were not significantly altered by NOX-D19 treatment. In **A** n=5 (non-ventilated) or n=11 (solvent/ventilated) or n=10 (sham/NOX-D19/ventilated) or n=9 (*S. pn.*/NOX-D19/ventilated); in **B** n=5 (NV) or n=11 (solvent/ventilated) or n=10 (NOX-D19/ventilated). \*\**p*<0.01, \*\*\**p*<0.001, \*\*\*\**p*<0.0001 between indicated groups (one-way ANOVA and Sidak’s multiple comparisons test).

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**Figure S11. Treatment with anti-C5a l-aptamer NOX-D19 led to lower pulmonary CC chemokine ligand 3 (CCL3) concentrations, while further pulmonary inflammatory mediators were not affected in combined severe pneumonia and mechanical ventilation.** Mice were transnasally infected with *S. pneumoniae* (*S. pn.*; 5x106 colony-forming units/mouse) or sham-infected with phosphate-buffered saline, and intraperitoneally treated with NOX-D19 or solvent 23 h post infection. One hour later (24 h post infection), mechanical ventilation was performed for 6 h. Ventilated and non-ventilated mice were sacrificed 30 h after infection.Pneumonia and mechanical ventilation induced high CC chemokine ligand 3 (CCL3) concentrations in bronchoalveolar lavage fluid (BALF), which were lower after NOX-D19 treatment, while further cytokines measured were not significantly altered by NOX-D19 treatment. Values are given as mean and SD. G-CSF, CCL3, IL-10, IL-6: n=5 (non-ventilated) or n=10 (sham/solvent/ventilated) or n=9 (sham/NOX-D19/ventilated) or n=11 (*S. pn.*/ventilated); CXCL1: n=5 (non-ventilated) or n=10 (sham/solvent/ventilated, *S. pn.*/NOX-D19/ventilated) or n=9 (sham/NOX-D19/ventilated) or n=11 (*S. pn.*/solvent/ventilated). \**p*<0.05, \*\**p*<0.01, \*\*\*\**p*<0.0001 between indicated groups, #*p*<0.05 vs. *S. pneumoniae*-infected, solvent-treated, ventilated group (one-way ANOVA and Sidak’s multiple comparisons test). Abbreviations: CXCL, CXC chemokine ligand; G-CSF, granulocyte colony-stimulating factor; IL, interleukin.

1. 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. [↑](#footnote-ref-1)