Supporting Information

Jonigk et al. 10.1073/pnas.1309648110

SI Materials and Methods

Mice. For the experiments, neutrophil elastase (NE) ($^{-/-}$) mice B6.129 × 1-Elane^{tm1sds}/J pair (female × male) were purchased from The Jackson Laboratory. NE-deficient B6.129 × 1-Elane^{tm1sds}/J mice have a C57BL/6J WT background. These mice are viable, fertile, and phenotypically normal in the absence of inflammatory stress, and do not express the targeted gene in bone marrow myeloid cells. NE-deficient mice have increased susceptibility to sepsis, morbidity, and mortality following intraperitoneal injection of Gram-negative (e.g., *Klebsiella pneumoniae* and *Escherichia coli*), but not Gram-positive (e.g., *Staphylococcus aureus*) bacteria. Despite this susceptibility, mutant mice are not at increased risk to spontaneous infection. Although neutrophil, T-cell, and macrophage migration/recruitment to the site of infection is unaffected in homozygous mutant mice, neutrophils show impaired bactericidal activity.

Female WT (C57BL/6J) and NE-deficient mice, 6–8 wk of age, were used in the experiments. Mice were housed in a temperature- and moisture-controlled room with a 12-h/12-h light-dark cycle and free access to food and water. All mice used for experiments had a body weight between 24 and 26 g. Animal experiments were performed according to ethics permit 33.9–42502-04–09/1766. Mice were lightly anesthetized using isoflurane (3%, vol/vol) and then challenged intranasally, drop-wise externally on the nares with the test substances.

Model of Acute Lung Inflammation. The study used an α1-antitrypsin (AAT) pretreatment model, whereby mice were pretreated with AAT for 24 h before the LPS challenge. The mice were divided into four groups, each group comprising 12–15 animals. Day 1: group 1: clinical grade water, 40 μL to each nostril; group 2: AAT (Prolastin) at a dose of 60 mg/kg (2,000 μg per mouse), final volume amounted to 80 μL with 40 μL administered to each nostril. Day 2: group 1–0.9% NaCl (saline), 40 μL to each nostril; group 2: LPS; group 3: LPS; and group 4: saline. Day 3: all groups were killed using pentobarbital intraperitoneally.

In each group the trachea of six mice was cannulated and the lungs were lavaged with 1.6 mL cold saline (2 × 800 μL). Bronchoalveolar lavage (BAL) fluid was centrifuged at 300 × *g* for 10 min to pellet the cells. The supernatant was collected and filtered through 0.22-μm membranes and stored at −80 °C for protein and cytokine analysis. Cells in the BAL pellet were counted on a hemocytometer, and cytologic examination was performed on cytospin preparations fixed and stained using Diff Quick (American Scientific Products). Differential counts were based on counts of 300 cells using standard morphologic criteria to classify the cells as eosinophils, lymphocytes, or other mononuclear leukocytes (e.g., alveolar macrophages, monocytes). Counts were performed by a single observer who was blinded to the study group. After being killed, lungs from another six animals were removed, inflated with buffered formalin via the main bronchi, and fixed in formalin overnight before being embedded in paraffin.

Bronchoalveolar Lavage. The BAL was centrifuged for 10 min and the supernatants frozen at −80 °C. Protein concentration in BAL samples was determined and the cell pellet was assessed for total and differential cell counts.

Materials. *E. coli* LPS at 300 μg/kg, (Sigma-Aldrich), clinical grade human AAT (Prolastin; Grifols), Aralast (Baxter), or recombinant AAT Fc (rAAT) as described in Lee et al. (1) were used. Prolastin and a recombinant, noninhibitory form of rAAT were administered intranasally at a dose of 60 mg/kg (2,000 μg per mouse) and 1.5 mg/kg (50 μg per mouse), respectively.

BAL Elastase Activity Determination. BAL samples were transferred to 96-well black fluorescence plates and incubated with chromogenic elastase substrate (Elastase V Substrate; Calbiochem) diluted in 0.1 M Tris buffer, pH 8.0, containing 0.5 M NaCl and 0.1 mM Ca^{2+} at a final concentration of 70 μM. After 1 h of incubation at 37 °C, the fluorescent product was determined at an excitation λ of 390 nm and emission λ of 460 nm. Under these conditions, generation of fluorescent product was linear with respect to the enzyme concentration. Porcine pancreatic elastase (Sigma-Aldrich) was used at 2–250 nM for control. Results were expressed as arbitrary fluorescence units.

Assay for Elastase Activity in the Presence of rAAT-Fc, in Vitro. rAAT-Fc was tested for capacity to inhibit pancreatic elastase (ES 3.4.21.36; Sigma-Aldrich). Elastase inhibitory activity was assessed spectrophotometrically (Tecan Software Competence Center). In brief, rAAT was incubated with pancreatic elastase at a molar ratio from 1.2:1–12:1 for 5 min at room temperature in 0.1 M Tris buffer, pH 8. After incubation, elastase substrate *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma) was added and absorbance was measured at 405 nm for 60 s. The absorbance values were corrected with blanks for buffer plus substrate.

Mouse Lung Specimen Processing. Lungs were harvested, inflated with buffered formalin to the main bronchi, and fixed in formalin overnight before being embedded in paraffin (1). Subsequently, coronal 1-μm sections of each lung were prepared, stained with H&E, and evaluated histologically: the degree and extension of morphologically apparent lung injury was assessed and scored on the basis of the grade of congestion, interstitial edema with thickening of the alveolar septae, neutrophilic infiltration of the interstitium, and intraalveolar accumulation of neutrophils: no injury (score 0), injury limited up to 25% of the explant (score 1), injury limited up to 50% (score 2), injury limited up to 75% (score 3), and diffuse injury encompassing more than 75% of the lung (score 4). The scoring was performed by two reviewers blinded to the experimental groups.

Mouse Lung Tissues. After being killed, both lungs were harvested, inflated with 4% (wt/vol) (buffered) formalin via the bronchi, and immersed in formalin for 36 h. The lungs were then sectioned and placed in tissue cassettes. These cassettes were subsequently immersed in formalin for 20 h. After that, specimens were dehydrated in ethanol, transferred into xylene, and embedded in paraffin wax. Serial sections were cut from the resulting blocks for histopathological evaluation and mRNA extraction: after assessing the quality of the lung samples using standard H&E/trichrome stains, complementary (5- to 10-μm-thick) sections were used for whole-tissue analysis.

Human Lung Tissue Laser-Assisted Microdissection. Anatomic structures were sampled from at least three locations in both lobes of the lung explants, using a no-touch technique. Approximately 1,500 cells were harvested from each specimen. Five-micrometer tissue sections were deparaffinised and stained with hemalum. The Cell Cut Plus system (MMI Molecular Machines & Industries) was used for laser-assisted microdissection. The microdissected areas were subsequently suspended in a proteinase K digestion buffer by placing it directly in the adhesive cap. A High Capacity

cDNA Reverse Transcription Kit (Applied Biosystems) was used to generate cDNA, and the cDNA was preamplified and analyzed by TaqMan 7500 Real-Time PCR system, Applied Biosystems.

Mouse and Human Tissue Real-Time PCR and Gene-Expression Analysis. RNA was isolated using phenol-chloroform extraction and precipitation following our established procedure. cDNA was subsequently synthesized by means of the High Capacity cDNA reverse transcription kit (Applied Biosystems), following the manufacturer's protocol. Next, 6.25 mL of cDNA were mixed with 12.5 mL TaqMan PreAmp master mix (Applied Biosystems) and 6.25 mL assay pool in a 200-mL tube [3 mL of each individual primer to be used in the RT-PCR filled up to a total reaction volume of 300 mL with 1× TE buffer (5 mL 100 mM Tris buffer, pH 8.1 with 100 mL 0.5 M EDTA and 45 mL Ampuwa)]. The tube was incubated in a thermocycler (one cycle of 95 °C for 10 min, 14 cycles of 95 °C for 20 s and 60 °C for 4 min). Specific primers were designed and purchased from Applied Biosystems: IL-8 (amplicon length: 81; assay location: 215), TNF-α (amplicon length: 80; assay location: 451), X-box binding protein-1 (XBP1) (amplicon length: 60; assay location: 499); DNA damage-inducible transcript 3 (DDIT3) (amplicon length: 78; assay location: 104), activating transcription factor 4 (ATF4) (amplicon length: 68; assay location: 1104).

The preamplified cDNA was analyzed by real-time PCR (TaqMan 7500 Real-Time PCR system; Applied Biosystems), as previously described in detail (2, 3). Quantification was performed in reactions containing preamplified cDNA, TaqMan Gene Expression Master Mix, and the individual TaqMan Gene Expression Assay (both from Applied Biosystems). A panel of genes was selected and analyzed, following the manufacturer's protocol. For negative controls, the cDNA was replaced by water. The 45 cycles were used for real-time PCR assay. The cycle threshold (Ct) values were calculated by normalization to the mean expression of three endogenous controls (POLR2A, β-GUS, and GAPDH) and converted into $2^{-\Delta CT}$ values with Excel 8.0 (Microsoft) before being statistically analyzed using the Mann-Whitney *U* test and Prism 5.0.

Human Neutrophil Adhesion to Endothelial Cells. Human lung microvascular endothelial cells (HMVEC-L) were derived from lung tissues (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), and were grown in medium with 5% (wt/vol) FCS. EGM2-MV medium was supplemented with 5% (wt/vol) FCS at 37 °C in 5% CO_2. After pretreatment with Prolastin or rAAT, aliquots of calcein-labeled neutrophils (100,000 cells per well) were added to wells of HMVEC-L, either alone and in combination with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; 100 nM). The plates were incubated at 37 °C for 25 min under static conditions. Nonadherent neutrophils were aspirated and the adherent cells were analyzed with a fluorescence spectrophotometer (Tecan Software Competence Center) using an excitation λ of 485 nm and emission λ of 520 nm.

Mouse Islet Cell Analysis. Primary islets were isolated as previously described (4). Ninety islets were cultured in triplicate, preincubated overnight with either AAT (Aralast) or rAAT and then LPS was added at 10 ng/mL After 72 h, the cells were dispersed for FACS analysis.

Production of Recombinant Human AAT-Fc–Fused Protein. rAAT was derived from a stable CHO cell clone expressing AAT-Fc with the cDNA sequence of human AAT and the corresponding sequence of human Fc of IgG1 at the C terminus, as described in Lee et al. (1). The serum-free supernatant from the CHO cell line was harvested, filtered and added to a slurry of Protein A beads (Bio-Rad) for affinity purification. The Protein A bound recombinant AAT-Fc slurry was washed thoroughly with PBS, and AAT-Fc was eluted using mild acid citrate buffer. The eluted AAT-Fc was immediately neutralized, dialyzed against PBS, and lyophilized. Reducing 10% SDS/PAGE revealed a molecular weight of AAT-Fc of ~70,000, which represents mature AAT sequence plus the single-chain Fc. The calculated molecular weight of mature human AAT with 394 amino acids is 44,296 and the molecular weight of recombinant IgG1 Fc with 628 amino acids is 70,628. Based on spontaneous dimerization of the AAT-Fc construct, the molecular weight of Fc AAT fused to Fc of IgG1 is 141,526. The molarity of Prolastin was based on a molecular weight of 44,296. Before use, the lyophilized AAT-Fc was reconstituted in water.

Preparation of Mouse Bone Marrow Neutrophils. Femurs and tibias were obtained from 6- to 8-wk-old mice. To prevent dry-out the bones were immediately placed into calcium-magnesium–free HBSS supplemented with 20 mM Hepes (pH 7.4) and 0.5% FCS. The ends of the bones were cut and the bone marrow flushed into a 50-mL conical tube with HBSS solution (see above) using a short 25-G needle and a 10-mL syringe. The suspension was pelleted at 400 × *g* for 5 min and resuspended in 0.2% NaCl. After lysing for 45 s, osmolarity was restored with 1.2% (wt/vol) NaCl. The cell suspension was then layered on 62% layer Percoll gradient (Santa Cruz Biotechnology), diluted in HBSS (100% Percoll: nine parts Percoll and one part 10× HBSS), and centrifuged (1,000 × g, 30 min at room temperature) without braking. The neutrophil pellets were collected and washed twice with HBSS solution, resuspended in assay medium, and counted. We obtained 10–15 million neutrophils per mouse, with a 90% purity.

Preparation of Human Blood Neutrophils. Neutrophils were isolated from the peripheral blood of healthy volunteers using Polymorphprep (Axis-Shield PoC), according to the manufacturer's recommendations. Neutrophils were harvested as the lower cellular band above the red cell pellet and washed by centrifugation with PBS. Residual erythrocytes were removed by hypotonic lysis using ice-cold 0.2% NaCl (wt/vol) for 30 s, followed by addition of an equal volume of 1.6% (wt/vol) NaCl to restore isotonicity. Purified neutrophils were washed, and then resuspended in RPMI-1640 containing glutamax and 0.1% BSA (RPMI-BSA). The neutrophil purity was typically 90% and cell viability exceeded 95% according to Trypan blue staining results from Cellometer Auto T4, Instrument-Nexcelom Biosciences (Peqlab). Neutrophils ($2.5–3 \times 10^6$ cells per mL) were plated into 12-well plates precoated with 10% (wt/vol) FCS. Cells were allowed to settle for 30 min before the experiment.

Fc Receptor Blocking. Neutrophils constitutively express the IgA Fc receptor FcαRI (CD89) as well as IgG Fc receptors FcγRIIa (CD32) and FcγRIIIb (CD16) (5, 6). Isolated human neutrophils ($2.5–3 \times 10^6$ cells per mL) were preincubated with various amounts (from 2.5% to 10%, wt/vol) of azide-free Fc blocker (Innovex Biosciences) for 30 min and exposed to LPS or AAT separately and in combination for a determined time. In another set of experiments, neutrophils were incubated in medium containing 1% or 10% (wt/vol) of FCS, which is known to block Fc receptors (7). As expected, neutrophil preincubation with an Fc blocker or serum resulted in increased IL-8 release (8, 9), which provides indirect proof that Fc receptors are efficiently cross-linked. In an additional set of experiments we also used human IgG Fc fragment protein (Abcam) as a control protein for Fc-rAAT. Neutrophils were preincubated for 30 min with various concentrations of Fc fragment protein (2–200 nM), and when treated with LPS for 10 h. Neutrophil supernatants were collected for IL-8 analysis using ELISA; the detection limit of the assays was 10 pg/mL.

ELISA IL-1 Receptor Antagonist. Freshly isolated human blood neutrophils (2.5×10^6 cells/mL) from three donors were incubated for 10 h with Prolastin or rAAT alone and with LPS (10 ng/mL). Cell

culture supernatants were collected and analyzed for IL-1 receptor antagonist (IL-1Ra) concentrations using the RayBio ELISA kit (RayBiotech). The lowest detectable level of IL-1Ra is 0.1 ng/mL.

Mouse Islet Cell Analysis. Primary islets were isolated as previously described (4). Ninety islets were cultured in triplicate, preincubated overnight with either AAT (Aralast) or rAAT, and then LPS was added at 10 ng/mL. After 72 h, the cells were dispersed for FACS analysis. After washing with FACS buffer (PBS containing 1% BSA, 0.1% sodium azide, and 2 mM EDTA, pH 7.4), the cells (1×10^6 per sample) were incubated with Fc RII/III blocker (BioLegend). Cells were then placed on ice for 30 min with the following fluorochrome-conjugated mAb (each from BioLegend): anti-CD11b-PerCP, anti–Toll-like receptor (TLR2)-APC, anti–TLR4-PE, anti–MHCII-APC-Cy7, and anti–CD45-FITC. Cells were washed twice with FACS buffer and analyzed by flow cytometry using BD FACSCanto II flow cytometers (BD Biosciences). $CD45^+$ $CD11b^+$ cells were analyzed for surface TLR2, TLR4, and MHC II levels. Data were analyzed by FlowJo software (Tree Star).

1. Lee S, et al. (2013) Effect of recombinant α1-antitrypsin Fc-fused (AAT-Fc)protein on the inhibition of inflammatory cytokine production and streptozotocin-induced diabetes. *Mol Med* 19:65–71.
2. Theophile K, Jonigk D, Kreipe H, Bock O (2008) Amplification of mRNA from laser-microdissected single or clustered cells in formalin-fixed and paraffin-embedded tissues for application in quantitative real-time PCR. *Diagn Mol Pathol* 17(2):101–106.
3. Jonigk D, et al. (2011) Plexiform lesions in pulmonary arterial hypertension composition, architecture, and microenvironment. *Am J Pathol* 179(1):167–179.
4. Ozeri E, Mizrahi M, Shahaf G, Lewis EC (2012) α-1 Antitrypsin promotes semimature, IL-10-producing and readily migrating tolerogenic dendritic cells. *J Immunol* 189(1): 146–153.
5. Ravetch JV, Bolland S (2001) IgG Fc receptors. *Annu Rev Immunol* 19:275–290.
6. van Egmond M, et al. (2001) IgA and the IgA Fc receptor. *Trends Immunol* 22(4): 205–211.
7. Fleit HB, Wright SD, Unkeless JC (1982) Human neutrophil Fc γ receptor distribution and structure. *Proc Natl Acad Sci USA* 79(10):3275–3279.
8. Gounni AS, et al. (2001) Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc ε RI): Role in asthma. *FASEB J* 15(6):940–949.
9. Hussain A, et al. (2009) Chimeric IgG4 PR3-ANCA induces selective inflammatory responses from neutrophils through engagement of Fcgamma receptors. *Immunology* 128(2):236–244.

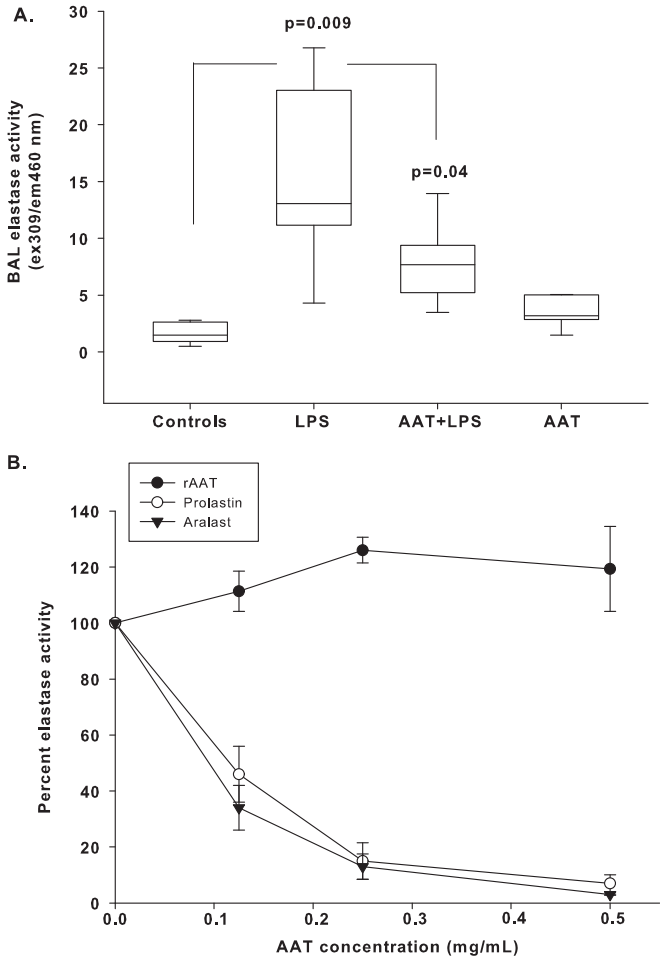

Fig. S1. BAL fluid elastase activity. (*A*) Mean ± SD of BAL elastase activity in WT mice 24 h after intranasal LPS (7.2 μg per mouse). Another group of WT mice before LPS were treated with Prolastin before receiving LPS 24 h later (2 mg per mouse). Mean ± SD of six animals. (*B*) In vitro elastase activity of rAAT. Pancreatic elastase was incubated with rAAT, Prolastin or Aralast for 5 min at 37 °C (*SI Materials and Methods*). Each point represents the mean of two independent measurements.

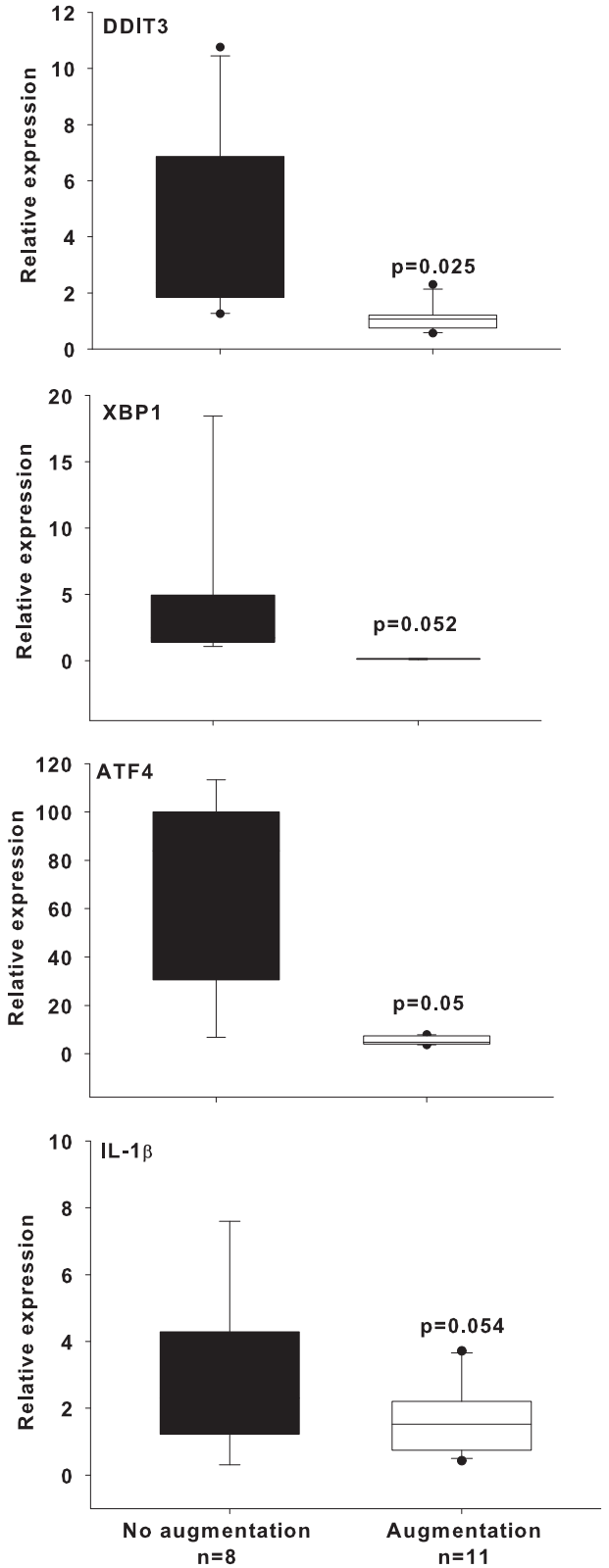

Fig. S2. Gene expression in lung explants from AAT deficiency patients. Mean ± SD relative mRNA levels for genes in lung explants from ZZ AAT deficiency-related emphysema patients treated with Prolastin augmentation therapy for a period ranging from 3 to 20 y, compared with samples from matched, but untreated ZZ patients (see *Materials and Methods* for microdissection, RNA extraction, and RT-PCR).

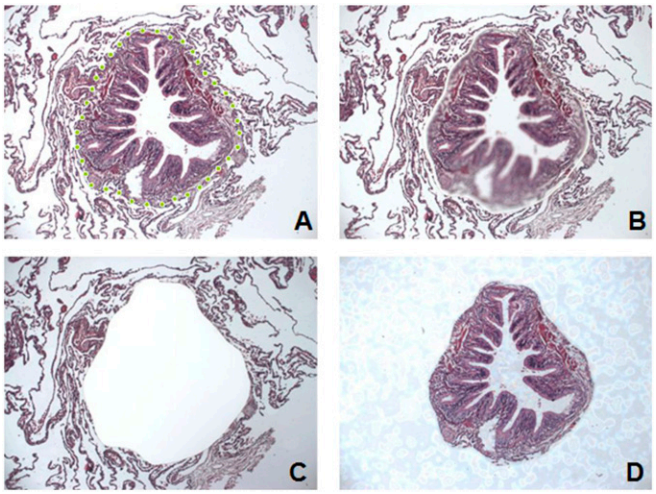

Fig. S3. Histological examinations. A small bronchus from the surrounding lung parenchyma was isolated by laser-assisted microdissection (*Materials and Methods*). (*A*) Border of excision is indicated by the green dotted line. (*B*) Laser cut is visible. (*C*) Microdissected area. (*D*) Isolated bronchus. H&E was used to clarify the cellular composition. Hemalum staining was used for all samples from which mRNA was extracted. Original magnification 100×.

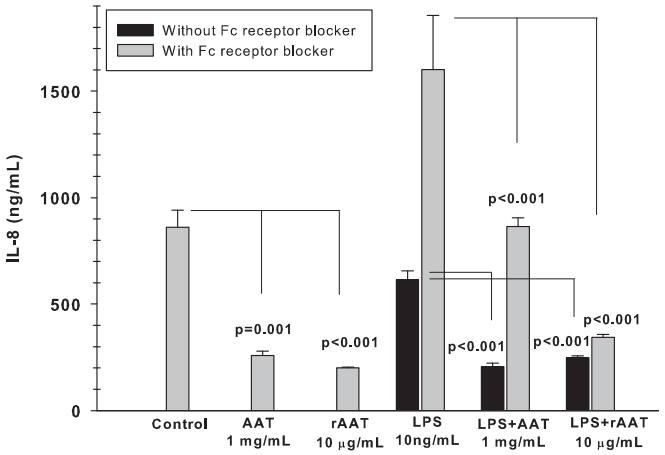

Fig. S4. IL-8 release from human neutrophils. Freshly isolated human neutrophils (2×10^6 cells per mL) were preincubated for 30 min with a constant amount (5%) of NaN_3-free Fc blocker (Innovex Bioscience). The cells were then stimulated with LPS, AAT (Prolastin), or rAAT separately, or LPS in combination with AAT (Prolastin) or rAAT for 17 h at the concentrations indicated under the horizontal axis. Mean ± SE of three separate experiments.

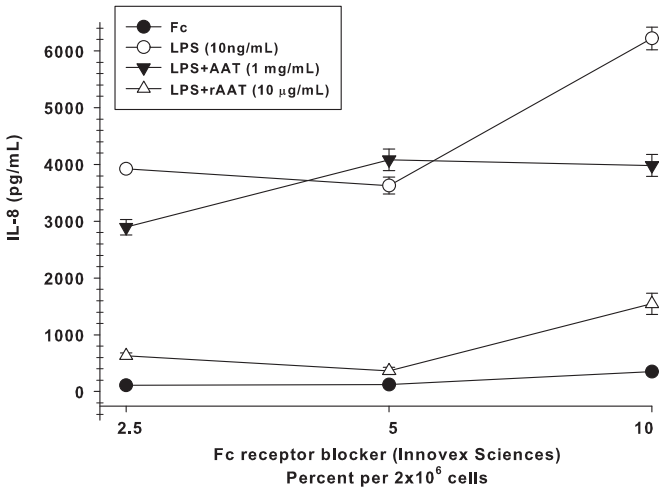

Fig. S5. IL-8 release from human neutrophils pretreated with increasing concentrations of Fc blocker. Human neutrophils were incubated for 6 h with increasing concentrations of NaN3-free Fc Blocker (%) as indicated under the horizontal axis with and without addition of LPS (10 ng/mL) and AAT (Prolastin, 1 mg/mL) or rAAT (10 μg/mL) separately and in a combination as indicated by the symbols. IL-8 was measured in the culture supernatants by specific ELISA. Mean ± SE of two separate experiments.

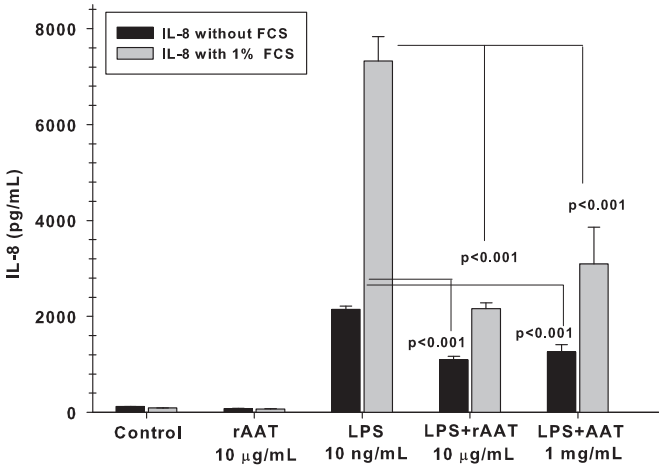

Fig. S6. IL-8 release from human neutrophils in the presence of 1% serum. Mean supernatant IL-8 levels ± SE of three separate donors. Freshly isolated human neutrophils were cultured in medium supplemented with 1% FCS and then stimulated with LPS (10 ng/mL) and AAT (Prolastin, 1 mg/mL) or rAAT (10 μg/mL) separately and in combination for 17 h.

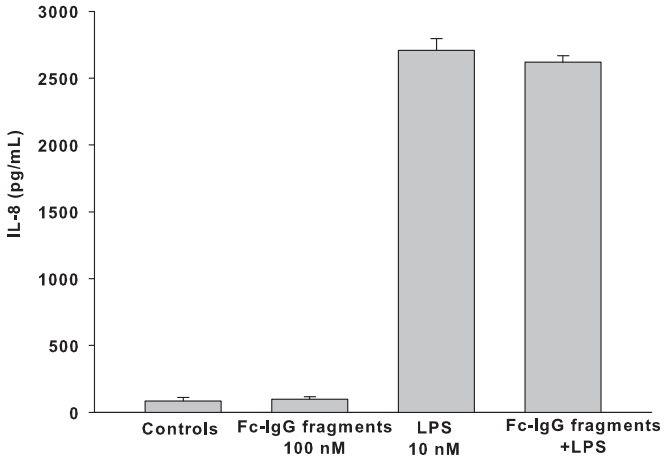

Fig. S7. LPS-induced IL-8 release from human neutrophils pretreated with Fc IgG1 fragments. Mean ± SE IL-8 levels of two independent experiments performed in quadruplicate. Freshly isolated human blood neutrophils (2.5×10^6 cells per mL) from two donors were incubated with Fc IgG1 fragments (100 nM) and with LPS (10 ng/mL) separately or in combination. After 10 h, cell-culture supernatants were collected for IL-8 determination.

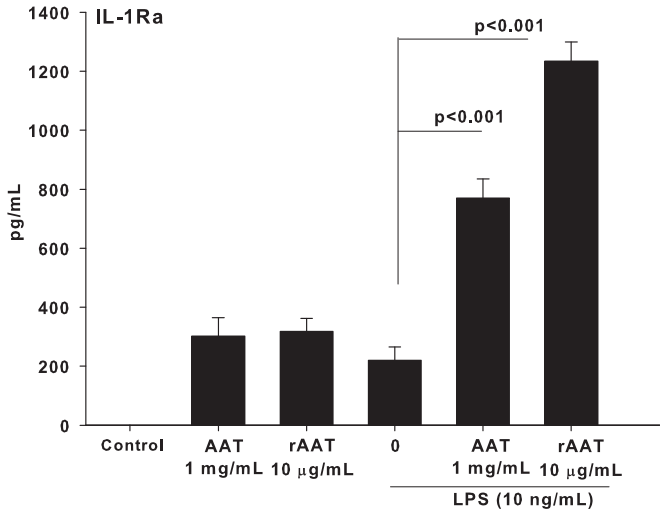

Fig. S8. Release of IL-1Ra from human blood neutrophils. Mean ± SE of supernatant IL -1Ra levels from human blood neutrophils of three separate donors: 2.5×10^6 cells per mL were incubated with and without AAT (Prolastin, 1 mg/mL), rAAT (10 μg/mL), or with LPS (10 ng/mL) for 10 h.

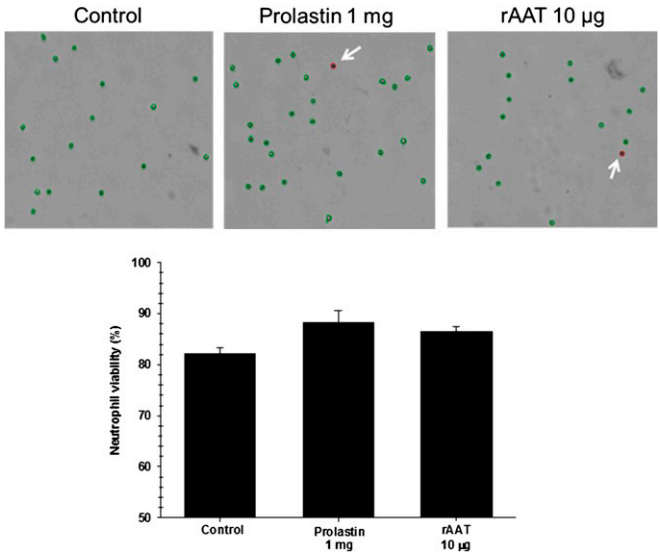

Fig. S9. Effects of Prolastin and rAAT on neutrophil viability. Mean ± SE neutrophil viability exposed to Prolastin (1 mg/mL) or rAAT (10 μg/mL) for 16 h ($n = 3$ donors). Arrows indicate dead cells (red) and living cells (green), respectively.

Table S1. Differential cell counts in BAL fluid

	Respective cells (percentage of 300 cells counted)		
	Macrophages	Lymphocytes	Eosinophils
Mice group	Arithmetic mean ± SD		
Wild-type (C57BL6)			
Controls ($n = 6$)	99.5 ± 0.23	0.1 ± 0.16	0
LPS ($n = 6$)	22.1 ± 6.3†***	2.3 ± 1.3	2.5 ± 1.9†*
Pre-AAT+LPS ($n = 6$)	39.6 ± 14.1‡*	3.1 ± 2.7	0.9 ± 0.6
AAT ($n = 6$)	96.8 ± 2.5	1.6 ± 0.8	0
Knockout (B6.129X1-Ela2 tm1Sds/J)			
Controls ($n = 6$)	77.9 ± 12.9	21.3 ± 13.1	0.5 ± 0.63
LPS ($n = 6$)	42.8 ± 20.3†**	10.7 ± 11.1	0.67 ± 0.82
Pre-AAT+LPS ($n = 8$)	67.9 ± 18.3‡*	16.4 ± 13.9	0.08 ± 0.2
AAT ($n = 6$)	79.0 ± 8.4	13.4 ± 8.1	0.1 ± 0.15

Asterisks indicate the level of significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).
†Significant difference between LPS-challenged and control mice.
‡Significant difference between mice pretreated with AAT prior to LPS-challenge and LPS challenged mice.