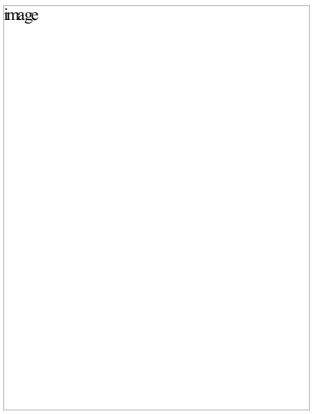

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Preservation with alpha-1-antitrypsin improves primary graft function of murine lung transplants

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Abstract

Background

Vascular damage and primary graft dysfunction increases with prolonged preservation times of transplanted donor lungs. Hence, storage and conservation of donated lungs in protein-free, dextran-containing electrolyte solutions like Perfadex® is limited to about six hours.

Objectives

We hypothesized that transplanted lungs are protected against neutrophil-mediated proteolytic damage by adding alpha-1-antitrypsin (AAT), a highly abundant human plasma proteinase inhibitor, to Perfadex®.

Methods

A realistic clinically oriented murine model of lung transplantation was used to simulate the ischemia reperfusion process. Lung grafts were stored at 4°C in Perfadex® solution supplemented with AAT or an AAT mutant devoid of elastase-inhibiting activity for 18 hours. We examined wild type and proteinase 3/neutrophil elastase (PR3/NE) double deficient mice as graft recipients. Gas exchange function and infiltrating neutrophils of the transplanted lung, as well as protein content and neutrophil numbers in the bronchoalveolar lavage fluid were determined.

Results

AAT as a supplement to Perfadex® reduced the extent of primary graft dysfunction and early neutrophil responses after extended storage for 18 hours at 4 degrees and 4 hours of reperfusion in the recipients. Double knockout recipients that lack elastase-like activities in neutrophils were also protected from early reperfusion injury, but not lung grafts that were perfused with a reactive center mutant of AAT devoid of elastase-inhibiting activity.

Conclusions

PR3 and NE, the principal targets of AAT, are major triggers of post-ischemic reperfusion damage. Their effective inhibition in the graft and recipient is a promising strategy for organ usage after storage for more than 6 hours.

Introduction

Lung grafts of donors are generally considered suitable for transplantation after static cold storage in Perfadex for a time period of up to six hours. This short preservation time limits the

availability of donor lungs and reduces the area of an organ sharing geographic region. Prolongation of cold ischemia times increases the extent of metabolic and structural changes in the transplant in the absence of cellular and blood plasma and subsequently, the intensity of the early innate immune response in the transplanted organ after the onset of reperfusion.

Irreversible damage to transplanted lung tissue after cold ischemic storage is primarily caused by the reintroduction of blood plasma and cellular blood components at body temperature after transplantation (1), in particular by infiltrating neutrophils (2, 3) and increases with the duration of oxygen deprivation (1, 4). As activated neutrophils release large amounts of elastase-related serine proteases, some perioperative treatment options for the recipient have been explored experimentally in animal models using protease inhibitors like bovine aprotinin (5, 6), Lex032 (7, 8), and AAT (9-12) in the past. In these studies, intravenous or intraperitoneal administration of inhibitors has been used to further raise the already high plasma concentrations of protease inhibitors in the circulation (13). We suggested that some inhibitors applied in these studies did not display the appropriate inhibitory profile, or flooded the inhibitor-depleted graft after ex vivo perfusion and in vivo reperfusion too late to efficiently protect the transplanted organ against infiltrating activated neutrophils.

Beneficial effects of protease inhibitors, in particular of alpha-1-antitrypsin (AAT), have been noticed in certain models of ischemia-reperfusion injury (7, 9-11) at warm body temperatures. The experimental conditions chosen in these examples, however, do not appropriately mimic the challenges of *ex vivo* organ storage in a cold cell- and protein-free electrolyte solution like Perfadex (14). Positive results from these previous studies cannot be extrapolated to reperfusion injury after preservation of organs in the cold.

To develop a new clinically appropriate lung preservation protocol and to initiate a proof of concept study for lung transplant patients, we used an orthotopic lung transplantation model in mice and stored the transplant in AAT containing Perfadex for an extended time period of

18 hours in the cold. The goal of this preclinical study was to test whether vascular leakage and immediate neutrophil-mediated inflammation after reperfusion can be prevented or mitigated by adding AAT to the perfusion solution during cold ischemia and whether the anticipated improvement of graft function was mediated by the direct anti-protease activity of AAT or by other anti-inflammatory, tissue protective and immune modulatory properties of AAT. Our results demonstrate that AAT acts on neutrophil-derived elastase and proteinase 3 and primarily prevents immediate reperfusion damage to transplanted lung tissue by protease inhibition.

Methods

Mice

Pathogen-free C57BL/6J mice were obtained from Charles River. *Ela2*$^{-/-}$*Prtn3*$^{-/-}$ mice were established by Pfister *et al.* (15). They were backcrossed for more than 10 generations with C57BL/6J mice. All animals were housed in rooms maintained at constant temperature and humidity with a 12-hour light cycle, and were allowed food and water ad libitum.

Study approval

All animal experiments were conducted under strict governmental and international guidelines and were approved by the local government for the administrative region of Upper Bavaria (Project 55.2–1-54-2532-120-2015).

Orthotopic lung transplant model

Orthotopic lung transplantations were performed as described with minor modifications (16). C57BL/6J male mice were used as donors, and C57BL/6J and *Ela2*$^{-/-}$*Prtn3*$^{-/-}$ male mice were used as recipients (all 8-12 weeks old). Briefly, donors were anesthetized with an i.p. injection of ketamine/xylazine. The pulmonary artery (PA), bronchus and pulmonary vein (PV) were carefully separated one from the other with blunted forceps, prior to cuffing with,

respectively, 24G, 20G and 22G cuffs. The left lung graft was perfused i.v. with 3 mL of Perfadex (5% dextran 40 (Mw 40 000), Na^+ 138 mmol, K^+ 6 mmol, $Mg2^+$ 0.8 mmol, Cl^- 142 mmol, SO_4^{2-} 0.8 mmol, $H_2PO_4^-$ plus HPO_4^{2-} 0.8 mmol, Glucose 5 mmol). Then it was perfused with Perfadex supplemented with albumin, AAT^{wt} or AAT^{mt} (all 1 mg/mL). To keep the solution inside the vasculature, the PA and PV were clamped with Biemer micro vessel clips (Bbraun) and stored for 18 hours at 4°C before implantation. The recipient mouse was anesthetized with a mixture of medetomidine (1 mg/kg), midazolam (0.05 mg/kg) and fentanyl (0.02 mg/kg), intubated and connected to a small animal ventilator (Harvard apparatus), at a respiratory rate of 120 bpm and a tidal volume of 300 µL. The chest was opened on the left side between ribs 3 and 4 and the native left lung retracted with a clamp. The hilar structures were carefully separated one from the other with blunted forceps. The donor lung graft, prepared 18 hours earlier, was perfused i.v. with Perfadex to rinse away the storage solution. After arrest of the blood and air flow towards the left lung, the cuffed graft PA, bronchus and PV were inserted into the recipient counterparts, and the connection between donor and recipient structures was secured with 9-0 sutures. The native left lung was removed and the incision in the chest was closed with a 6-0 suture, after removing all potential air bubbles from the chest. Antagonist was administrated and the animal was extubated when it showed signs of spontaneous breathing. After the operation, the recipient mice were allowed to recover at 30 °C and received buprenorphine (0.1 mg/kg).

Lung analyses in recipients

The mice were sacrificed 4 hours after lung transplantation for primary graft dysfunction assessment. The recipient mouse was anesthetized with a mixture of medetomidine (1 mg/kg), midazolam (0.05 mg/kg) and fentanyl (0.02 mg/kg) 4 hours after lung transplantation. After intubation (300 µl room air/120 strokes/min), a midline abdominal incision was performed, the diaphragm incised and the chest opened to expose the lung and heart block. The right bronchus and pulmonary vein were clamped for 5 min (75 µl room air; 120

strokes/min) and the oxygenation function of the transplanted left lobe was assessed by collecting blood from the left ventricle of the heart and directly measuring the pO_2 [%] in a blood gas analyzer (ABL80 FLEX CO-OX analyzer, Radiometer). Further, with the right bronchus still clamped, a bronchoalveolar lavage of the transplanted left lung was performed by instilling three times 200 µL cold PBS into the trachea. The bronchoalveolar lavage fluid was obtained by centrifugation (400 g, 20 min, 4°C) and protein concentration was measured with the Pierce™ BCA Protein Assay Kit (catalog no. 23225, Thermo Fisher). 30.000 cells from the BAL cell pellet were used for cytospin (200 x g, 6 min, 4°C) and stained with May Grünwald (1424, Merck) and Giemsa (Merck, 9204) staining solution to perform a differential cell count. The lower part of the lung was kept for protein analysis (snap freezing, storage at -80 °C) and the leftover part was used for histology (inflation with and storage in 4% PFA).

Recombinant protein expression and purification

The cDNA modification of the human AAT^{mt} (A355D/I356P/P357D/M358S) was introduced by amplifying the cDNA of human AAT with the forward primer DJ3608 (5'-TCGAGGACCCTGACTCATCG-3') and the reverse primer DJ3609 (5'-ATCGATGAGTCAGGGTCC-3') (17). The PCR product was digested with Acc I and Abs I restriction enzymes (NEB) and cloned into the respective sites of the previously modified wild type AAT pTT5 plasmid. Recombinant clones were identified by sequencing (Eurofins). The cDNA constructs of AAT^{wt} (human M1(V213)) and AAT^{mt} were transiently expressed in HEK293 cells as described (18). Harvesting of supernatants and purification of recombinant AAT variants were performed as described (17).

Western blot analysis

Please see online supplement.

Assay for inhibitory activity measurement

The enzymatic activity of the serine proteases, NE and PR3, was determined using thiobenzylester substrates and Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB). Free thiobenzylester groups react with DTNB and form yellow 2-nitro-5-thiobenzoate ions. The rate of hydrolysis was measured at 405 nm. Inhibitory activities of 0.5 µM AAT^{wt} and AAT^{mt} were tested in an activity assay using 0.1 µM recombinant mouse and human NE and PR3. The remaining activity of the proteases was determined using thiobenzyl ester substrate (Boc-Ala-Pro-Nva-chloro-SBzl, 4008235.0010, Bachem) at 1 mM substrate concentration and 0.5 mM DTNB in activity assay buffer (50 mM Tris, 150 mM NaCl, 0.01% Triton X-100, pH 8.0).

Tissue preparation

Please see online supplement.

Hematoxylin and eosin staining

Please see online supplement.

Immunohistochemistry

Please see online supplement.

Statistics

Results are reported as mean ± SEM. A Mann-Whitney U-test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, and www.graphpad.com. P values of less than or equal to 0.05 were considered to be statistically significant.

Results

Treatment of lung grafts with AAT

We determined the levels of AAT 4 hours after lung transplantation in transplanted mice whose graft had been stored in AAT (1 mg/ml in Perfadex) by Western blotting (Figure 1A and B). We found that this procedure achieved a sustained local deposition of AAT in the transplanted lungs in the absence of plasma proteins (Figure 1B).

AAT treatment during storage improves primary graft function

Primary graft dysfunction after prolonged cold storage of the left mouse lung in Perfadex was observed using the mouse orthotopic left lung transplantation model (Figure 1B). Cellular infiltrates in the transplanted left lung lobe and neutrophils in the bronchoalveolar lavage fluid were clearly noticed. After interrupting the blood flow from the non-transplanted right lung, the oxygen pressure in the left heart ventricle decreased indicating graft dysfunction after prolonged ischemic storage in the cold. To prevent primary graft dysfunction after prolonged storage, we explored the potential beneficial effect of the protease inhibitor AAT by perfusing and storing the lung graft in AAT-containing Perfadex at 4°C. We directly compared the AAT with albumin as two similar proteinaceous supplements at the same concentration in our mouse transplantation model (Figure 2). As envisaged, we found a highly significant protective effect for AAT on graft preservation both at the histopathological (Figure 2A) and

functional level (Figure 2B and E). The addition of AAT resulted in an almost 40% higher oxygenation of the blood (Figure 2B) in the transplanted left lung. The protein and neutrophil content of the bronchoalveolar lavage fluid (Figure 2C and D) and the neutrophil infiltration of the transplanted lung (Figure 2E) were much lower in the AAT-treated lungs compared with albumin-treated lungs after 4 hours of reperfusion.

Non-inhibitory AAT has no effect on graft preservation

To explore the potentially pleiotropic effects of AAT in graft protection, we decided to separate the anti-proteolytic functions of the reactive center loop from other AAT-associated modulatory functions on the remaining surface of the AAT molecule. To eliminate trypsin-, chymotrypsin- or elastase-targeting specificity we substituted two residues in the reactive center loop by aspartate residues at the P4 and P2 position and the two residues at P3 and P1 by a proline and serine, respectively (Figure 3A). Changing the P4 Ala-355 and the P2 Pro-357 to a negatively charged Asp eliminates the hydrophobic nature of the reactive center loop and reduces potential interactions of AAT with phospholipid bilayers (19). Shifting the Pro-357 to position 356 (P3) and replacing the methionine-358 conservatively by a serine alters the canonical inhibitory conformation and ß-pleated strand extension of the reactive center loop (P4 to P3') (20) and its specificity which are both required for the inhibition of serine proteases.

In accordance with our predictions, murine and human neutrophil elastase (NE) and proteinase 3 (PR3) were not inhibited by this AAT reactive center loop mutant (AAT^{mt}) (Figure 3B) produced in HEK293 cells (18) while the purified recombinant wild type AAT (AAT^{wt}) inhibited human and mouse NE and PR3 irreversibly. The sequence modifications of AAT introduced into the reactive center loop were intentionally designed to abolish its cleavability by various mammalian proteases as assessed with the programs Prosper, Cascleave2.0 and PeptideCutter. In the lung transplantation experiments with AAT^{mt} in direct

comparison to albumin, acute lung inflammation as judged from histology sections (Figure 3C), the oxygenation of blood in the transplanted left lobe (Figure 3D), and the protein content in the bronchoalveolar lavage fluid (Figure 3E and F) was indistinguishable between the two treatment groups.

PR3/NE double deficient mice demonstrate enhanced primary graft function

To further corroborate the detrimental role of neutrophil serine proteases in primary graft dysfunction, we examined wild type and PR3/NE double deficient mice as graft recipients which had the same genetic background (C57BL/6J) as the donor mice. The lung tissue of the transplanted lobe in the double knockout mice showed a healthier alveoli structure than that of wild type mice after 4 hours of reperfusion as assessed by hematoxylin and eosin staining (Figure 4A). Gas exchange function of the transplanted lobe was clearly preserved after 4 hours of exposure to blood of PR3 and NE deficient mice, whereas the lung graft in wild type recipient mice performed poorly (Figure 4B). The mean partial pressure of oxygen was 80% in the arterial blood of the left ventricle of knockout mice compared to 50% in wild type recipients after clamping the arterial blood flow from the non-transplanted lung lobes. The degree of protein exudation (Figure 4C) and cellular content of neutrophils in the bronchoalveolar lavage fluid (Figure 4D) were not significantly different between the knockout and the wild type recipient group.

Discussion

Cold storage of human lung grafts for more than six hours is known to increase the risk of early graft dysfunction after transplantation. Likewise we found that the left lung of mice stored for 18 hours in a colloid-based electrolyte solution (Perfadex) displayed primary graft dysfunction after transplantation. When we, however, perfused and stored the lung graft in

Perfadex containing AAT (1 mg/ml), we were able to significantly reduce the immediate inflammatory response after reperfusion of the transplanted lung. We moreover demonstrated that the protective effect of AAT was mediated by the inhibitory function of AAT targeting the major serine proteases of neutrophils, neutrophil elastase and proteinase 3.

We assume that the AAT of the preservation solution diffuses into the perivascular and interstitial space during perfusion and storage of the donor transplant and is, therefore, optimally deployed in advance to protect the lung tissue against the influx of adhering and extravasating neutrophils around the capillaries and venules. During reperfusion the anti-protease shield of AAT against elastase and other serine proteases of neutrophils appears to represent the most pivotal mechanism of lung preservation in this clinical setting. However, multiple alternative mechanisms of tissue protection and anti-inflammation by AAT unrelated to its anti-protease functions have been reported (21-28).

To clarify the major therapeutic mechanism of AAT in immediate graft protection, we decided to separate the anti-proteolytic functions of the reactive center loop from other potential functions on the remaining surface of the AAT molecule. The inhibitory function of AAT primarily targets neutrophil elastase and proteinase 3 and to a much lesser extent other proteases like cathepsin G and trypsin-like enzymes (13, 29-32). Serine proteases occur in the oxidizing extra- and pericellular environment or within the secretory route including storage granules and are active at neutral pH after regulated secretion. Inhibition of proteases in the cytosol after membrane binding (19), endocytosis (33) and translocation to the cytosol (34, 35) was, therefore, unlikely. Inhibition of caspases (21, 36), and calpain I (37) in the cytosol have been inferred to be responsible for additional anti-inflammatory properties of the wild type AAT. Our results strongly suggest that other, non-protease mediated functions of AAT do not significantly contribute to organ protection during the early phase of reperfusion after transplantation surgery.

In agreement with the findings using the non-inhibitory AAT variant we found that PR3/NE double deficient mice exhibited improved primary graft functions compared to wild type mice. The murine homologs of human PR3 and NE are exclusively expressed in the myelomonocyte lineage and constitutively stored in primary granules of neutrophils (31). Deficiency of these two proteases in the germline does not have a biological or developmental impact on peripheral tissues and organs and is not causing any phenotype under normal housing and living conditions (15). Hence these mice were optimally suited to clarify the role of neutrophil serine proteases after initiating reperfusion of the lung graft (Figure 4). Taken together, these findings in knockout mice were consistent with recent observations in macaques after prolonged inhibition of cathepsin C, which resulted in the depletion of serine proteases in neutrophils (38). Under these experimental pharmacological conditions, neutrophil migration and recruitment to the lungs and bronchi was not altered in response to tracheal LPS instillation.

The preservation procedure assessed in this preclinical study corresponded very closely to the graft handling procedure in a routine thoracosurgical setting. AAT at a final concentration of 1 mg/ml added to Perfadex solution is a safe and easily acceptable modification of the most widely used preservation solution, as this natural human plasma protein has already been approved for long term substitution therapy in emphysema patients with constitutively low levels of AAT in their blood. In the absence of other plasma proteins, flushing and perfusion with 1 mg/ml (15 µM) AAT in Perfadex solution suffices to deliver this inhibitor to endothelial cells and the interstitial lung space after lung removal from the brain-dead donor. Without competing proteins, endocytosis and transcytosis of AAT from the Perfadex solution by the lung endothelium (33, 39) is presumably more efficient than its uptake from whole blood even then, when additional AAT is infused intravenously prior to the operation.

Ex vivo perfusion of the lung graft with AAT has clearly a great advantage over its systemic delivery in the donor and recipient, not only because this protocol complies with current ethical standards. With this procedure, we, moreover, achieved maximum tissue levels of AAT in advance, before the first neutrophils together with soluble plasma components from the recipient reach the transplanted lungs after connecting the lungs to the patient's circulation. Treatment of the graft recipient with AAT before and after surgery is clearly a complementary therapeutic option, as shown in the previous study (12). Patients with severe COPD show increased elastase activity markers in the circulation (40) and may therefore benefit in particular. Safety and tolerability of high dose AAT infusions in end-stage lung disease patients, however, is a serious concern and remains to be clarified. Our experiments with an AAT reactive center loop mutant and with NE and PR3 deficient mice proved our suggestion that the protease inhibiting effects of AAT on neutrophils in the early phase of blood reperfusion were essential and sufficient to protect the graft from immediate inflammatory damage after extended storage times. Other selective inhibitors of neutrophil elastases may be equally effective, but target specificity and very low toxicity of the inhibitors are crucial for drug safety.

The lung transplantation procedure performed here with isogenic donors and recipients has several limitations. In comparison to human allogenic lung grafts, the transplanted lungs are taken from a young and healthy isogenic donor without pre-existing illness, and no immunosuppressive therapy is used. The microsurgical procedure is time-consuming and challenging even for very skilled persons after a long exercise period. The inevitable blood loss of recipient mice cannot be compensated by mouse blood transfusions. Hence, the hemodynamic and circulatory status of the transplanted mice during the postsurgical phase is suboptimal. The blood pressure in the left pulmonary artery could not be continuously monitored and measured after transplanation within the four hour period, as this is technically not feasible in transplanted mice. Although it is unlikely, initial blood flow to the AAT-

pretreated lung grafts could have been lower than in untreated donor lungs leading to reduced edema formation. Despite these challenging experimental conditions which affected all experimental groups, we suppose that the effects of AAT are primarily restricted to the transplanted graft and do not change the cardiac output and systemic blood supply to the transplanted lung.

Local deposition of AAT in the graft cannot stop or significantly delay the ischemia-induced metabolic and structural changes of the graft. Cellular instability and breakdown is expected to increase even with storage in AAT beyond the six-hour-period. Residential neutrophils, however, retained in the graft, are also affected by this process and presumably release a limited amount of preformed proinflammatory mediators including serine and matrix metalloproteases. In contrast to matrix metalloproteases, neutrophil serine proteases are fully processed and stored in acidic primary granules, but are immediately active in a neutral (pH 7) environment in the absence of AAT. As the PR3/NE deficient recipient mice also showed improved graft function, we arrived at the conclusion that infiltrating neutrophils and macrophages of the recipient executed the major tissue damage during reperfusion at body temperature, and that this immediate attack by leukocytes could be counterbalanced by a local protection shield against proteases.

How long the local effects of AAT are maintained in the transplanted lung and favorablly affect a later immune response, remains to be clarified. Negative side effects of AAT in lung transplantation are conceivable in view of a delayed neutrophil response which might favor bacterial lung infections during or directly after surgery. In a previous clinical attempt to reduce primary lung dysfunction with aprotinin, a bovine inhibitor, this study had to be stopped prematurely as renal toxicity of aprotinin was discerned (41). The organ conservation procedure, which we propose here on the basis of an appropriately designed small animal study with human AAT, appears to be relatively safe, ethically acceptable

clinically translatable and it is well supported by long-standing clinical research on AAT. It may be applicable to organ transplantation surgery in general.

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Authorship Contributions

J.G. designed and performed experiments, including the pO_2 measurement and sample collection for the mouse experiments, analyzed the data and helped with manuscript writing. N.F.S. designed and performed the lung transplantation experiments and wrote the TVA. C.M. performed immunohistochemical stainings and analyzed the corresponding data. B.K. identified and characterized the mutant AAT variant. O.E. discussed the project and unmet clinical need of the project. A.O.Y. supervised the microsurgery procedure and wrote the TVA. D.E.J. designed, discussed and oversaw the study and wrote the manuscript. All authors contributed to manuscript revisions.

Conflict of Interest Disclosures

The authors have declared that no conflict of interest exists.

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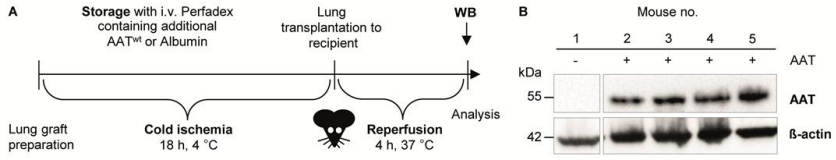
Figure legends

Figure 1. Storage of lung grafts in AAT containing Perfadex results in high AAT levels after 4 hours of reperfusion. (A) Experimental setup for perfusion, cold ischemic storage, transplantation and reperfusion. Donor lungs were perfused with AAT^{wt} or albumin in Perfadex and stored in the respective solutions at 4°C for 18 h (cold ischemia). Thereafter, the left lung was orthotopically transplanted to C57BL/6J recipient mice. The transplanted animals were kept alive for 4 h (reperfusion) before Western blot analysis (B) Western blot of total lung tissue lysates of transplanted left lungs of four C57BL/6J recipient mice after 4 h of reperfusion using an AAT-specific monoclonal antibody. Immunodetection of β-actin served as a loading control (lower panel). The AAT-specific antibody did not cross-react with endogenous mouse proteins (left panel).

Figure 2. AAT reduces primary graft dysfunction. Left lungs from C57BL/6J donor mice were stored either in Perfadex supplemented with wild type AAT (AAT^{wt}) or albumin at 4°C for 18 hours (cold ischemia). Four hours after orthotopic transplantation, C57BL/6J recipient mice were sacrificed and the outcome of the two treatment groups labelled as AAT^{wt} or albumin (n=4-5 per group) were compared. (A) H&E staining showing the tissue morphology of a transplanted left lung (scale bars, 200 µm). (B) Partial oxygen pressure (pO_2) of the oxygenated blood in the left heart ventricle was determined after clamping the right bronchus for five minutes. The oxygen exchange function of the transplanted lung alone was assessed during mechanically controlled ventilation. (C) Total protein concentration in the bronchoalveolar lavage fluid and (D) bronchoalveolar lavage neutrophil count in the indicated transplanted mice. (E) Immunohistochemistry (scale bars, 200 µm) and quantification of neutrophils in the transplanted left lungs from 10 randomly chosen visual microscopic fields. Data are represented as mean ± SEM and compared with a Mann-Whitney test (**p=0.0159, *p<0.05).

Figure 3. A non-inhibitory reactive center loop variant of AAT (AAT^{mt}) added to the preservation solution is ineffective. (A) Design of a non-inhibitory reactive center loop variant (mutant AAT, AAT^{mt}) by replacing the residues at P4 and P2 with aspartates and P1 with a more polar residue and shifting the P2 proline to the P3 position. While hydrophobic intramolecular interactions of the extended loop were conserved, the flexibility and conformation of the loop was altered by the proline at P3 (B) Protease activities of human (h) and murine (m) neutrophil elastase (NE) and proteinase 3 (PR3) alone or in the presence of AAT^{wt} or AAT^{mt}. Data represent three independent experiments (*n*=3, ± SEM). (c to f) Left donor lungs from C57BL/6J mice were stored in either AAT^{mt} or albumin supplemented Perfadex at 4°C for 18 hours (cold ischemia). C57BL/6J recipient mice were sacrificed 4 h after orthotopic transplantation. The two groups are labelled as albumin and AAT^{mt} (*n*=4-5 per group). (C) H&E staining showing the tissue morphology of a transplanted left lung (scale bars, 200 µm). (D) Partial pressure of oxygen (pO_2) measured in the blood collected from the left ventricles of ventilated mice, five minutes after clamping the right bronchus. (E) Total protein concentration in the bronchoalveolar lavage fluid and (F) neutrophil content of the lavage fluid in the two treatment groups. Data are represented as mean ± SEM.

Figure 4. Neutrophil elastase and proteinase 3 are major triggers of graft dysfunction. Left lungs were taken from wild type C57BL/6J donor mice, perfused and stored in albumin containing Perfadex at 4°C for 18 hours (cold ischemia). Thereafter, the lungs were orthotopically transplanted either into wild type C57BL/6J (BL/6J) mice or into *Ela2*$^{-/-}$*Prtn3*$^{-/-}$ deficient mice with the same genetic background and sacrificed 4 h later (*n*=4-5 per group). (A) H&E staining showing the tissue morphology of a transplanted left lung (scale bars, 200 µm). (B) Left ventricle arterial blood partial pressure of oxygen (pO_2) in the lung transplanted. (C) Bronchoalveolar lavage fluid protein concentration and (D) bronchoalveolar lavage neutrophil count in the indicated transplanted mice. Data are represented as mean ± SEM and compared with a Mann-Whitney test (*p<0.05).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Storage with i.v. Perfadex containing additional AAT^wt or Albumin
Lung transplantation to recipient
WB
Lung graft preparation
Cold ischemia 18 h, 4 °C
Reperfusion 4 h, 37 °C
Analysis
B
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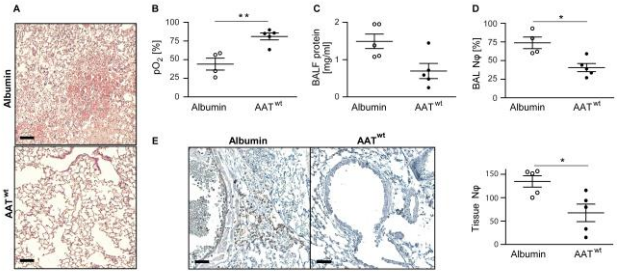
1 2 3 4 5
- + + + +
AAT
kDa
55
AAT
42
ß-actin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Albumin
AAT^wt
B
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Albumin
AAT^wt
C
BALF protein [mg/ml]
D
BAL Nφ [%]
E
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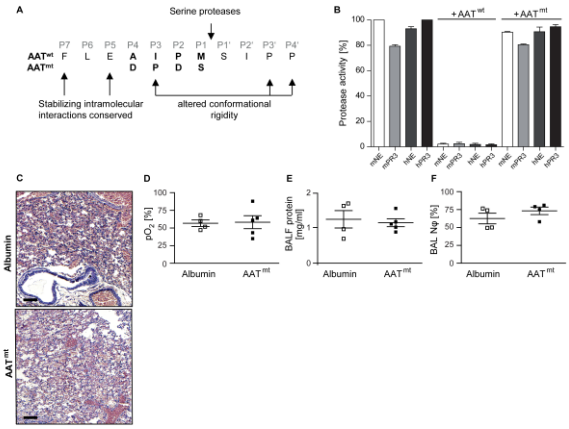
A
Serine proteases
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AAT^mt
Stabilizing intramolecular interactions conserved
altered conformational rigidity
B
+AAT^wt
+AAT^mt
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AAT^mt
D
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E
BALF protein [mg/ml]
F
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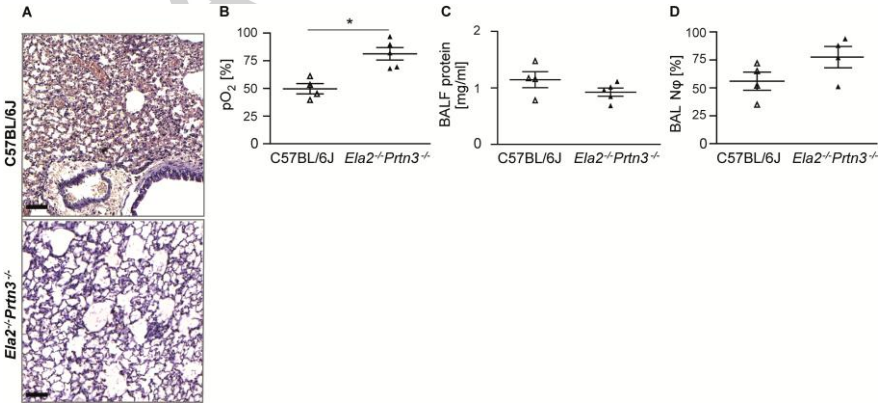
A
C57BL/6J
Ela2-/-Prtn3-/-
B
pO2 [%]
C57BL/6J
Ela2-/-Prtn3-/-
C
BALF protein [mg/ml]
D
BAL Nφ [%]