Supplementary methods section

A novel anti-apoptotic role for alpha-1 antitrypsin in the prevention of pulmonary emphysema

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Chemicals and Reagents. Primary antibodies are listed in **Supplemental Table 1.** Recombinant active caspase-3, recombinant active caspase-8, and A1AT purified from human plasma were from Calbiochem (EMD Biosciences, San Diego, CA). All other reagents were from Sigma-Aldrich, St. Louis, MO, unless otherwise specified.

Animal studies were approved by the Animal Care and Use Committee of Johns Hopkins University and University of Colorado Health Sciences Center and performed on male C57Bl/6 mice *n*= 4-6 mice /group (3 month-old, Jackson Laboratory Bar Harbor, ME) or Sprague-Daley rats (*n* = 4-6/ group). Experiments were performed in the same shipment lot.

AAV administration. For experiments involving direct lung administration, mice were anesthetized and trachea instilled with $5x10^{10}$ particles of cAAV (UF11-AAV5) or hA1AT-AAV (AAV5-CB-AAT) in 25 μl 0.9% NaCl. For i.m. injection (hind leg), 9.6 $x10^{10}$ cAAV (UF11-622) or 1.2 $x10^{10}$ of A1AT-AAV (pTR2-CB-AAT) were injected in 40 μl H_2O in experiments involving SU5416. For experiments involving VEGFR-antibodies, mice received 9.6 $x10^{10}$ UF11-622 or 9.6 $x10^{10}$ A1AT-AAV.

Viral constructs: All experiments were performed with recombinant AAV, rAAV-CB-AAT (22) which consists of AAV serotype 2 inverted terminal repeats flanking

an expression cassette that drives hA1AT expression from a hybrid cytomegalovirus enhancer/beta actin promoter. For i.m. experiments we utilized the vector pseudotyped into AAV serotype 1 capsids (23). For i.t. experiments we utilized the vector pseudotyped into AAV serotype 5 (23, 24). All preparations were titrated using DNA dot-blot hybridization (23).

Administration of agents inducing airspace enlargement. SU5416 ({3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-indolin 2-one}, SUGEN Inc. (South San Francisco, CA) in a diluent of 0.5% carboxymethylcellulose sodium, 0.9% sodium chloride, 0.4% polysorbate 80, 0.9% benzyl alcohol (CMC) or CMC alone was administered 20 mg/kg, s.c. once in mice and rats (14).

VEGFR-specific antibodies DC101 and MF1 (Imclone, 800 μg/mouse/injection) or dialyzed rat IgG were administered i.p. three times a week for the duration of the experiment (4 weeks).

A1AT augmentation in rat. Sprague-Dawley rats were divided into three groups: (a) control (CMC) group (n=6) +cell culture-grade bovine serum albumin (BSA) (30 mg/kg i.v. three times a week); (b) SU5416 (20 mg/kg s.c.) +BSA (30 mg/kg i.v. three times a week) (n=6); and (c) SU5416 + hA1AT(30 mg/kg i.v. three times a week) (n=6).

Morphometric analysis was performed on coded slides as described (25, 26). Briefly, the left lung was inflated with formalin in low-melt agarose under constant pressure, fixed in 10% buffered formalin and then paraffin embedded, while the right lung was processed for biochemical studies. Morphometry was performed on coded slides as described (14), utilizing the image processing software ImagePro (Media Cybernetics, Silver Spring, MD) and MetaMorph (Molecular Devices, Downington, PA). Measurements of mean alveolar diameter, alveolar perimeter and mean linear intercept were recorded and compared among groups utilizing a statistical software package as described below. In selected rat studies, the alveolar surface-volume ratio was measured by examining random 10 microscopic fields per animal, using Weibel's multipurpose test grid and formula to estimate surface to volume ratios (38).

Immunohistochemistry. Slides were immunostained (following 10 min antigen retrieval in Dako Buffer) with A1AT antibody (Santa Cruz; 1:200), followed DAB staining or, in the case of colocalization studies, followed by Alexa Fluor 488-labeled secondary antibody (Molecular Probes). Cell-type markers were identified with antibody against SPC (1:200, Chemicon, for alveolar type II epithelial cells), CD34 (Zymed), and smooth muscle actin (1:25, Sigma), followed by Texas-red conjugated secondary antibody and DAPI (Molecular Probes) counter-staining of alveolar cell nuclei.

Apoptosis. Apoptosis of alveolar cells was detected by complementary methods, including on inflated lung sections, which enabled us to focus on the alveolar tissue, rather than on large airways and vessels (14). Active caspase-3 IHC on mouse lung was performed utilizing a Catalyzed Signal Amplification kit (DAKO) (14). Measurements of active caspase-3 expression were performed on 10-15 images/slide captured by independent observers blinded to the experiment. Rat IHC was performed on paraffin-embedded rat lung tissue sections utilizing anti-active caspase-3 antibodies (**Supplementary table 1**). Negative controls for nonspecific binding included normal rat serum. The caspase-3 activity was measured in lung tissue lysates using a colorimetric assay (Clontech Laboratories, Palo Alto, CA) which was subsequently replaced by a more sensitive fluorometric assay Apo-ONE (Promega, Madison, WI) according to the manufacturer's instructions. The fluorescence (excitation 499 nm, emission 521 nm) was measured by phosphoimager (Amersham, Biosciences, Inc., Piscataway, NJ). The assay calculates the rate of specific caspase-3 substrate cleavage normalized by total protein concentration and expressed relative to the units of recombinant active caspase-3 utilized as positive control. The *in situ* labeling of apoptotic DNA on mouse lung was performed using a terminal deoxyribonucleotide transferase apoptosis detection kit (Oncogene Research Products (La Jolla, CA). Apoptotic cells exhibited intense nuclear green fluorescence at 465-495 nm. Co-staining with DAPI (330-380 nm) confirmed the nuclear localization of TUNEL staining and provided a

denominator for nuclear counts per captured field. Measurements were performed as described for caspase-3 IHC.

Measurements of oxidative stress included detection of nitrotyrosine and 4-hydroxynonenol (25) markers and of catalase activity (Cayman Chemical). We measured nitrotyrosine and 4-hydroxynonenol as markers of modification of proteins and lipids, respectively, by hydroperoxide adducts with the intention of capturing levels of injurious ROS. Detection was performed by IHC on paraffin-embedded sections as previously described (14). Catalase activity was measured with a Catalase Assay Kit (Cayman Chemical, Ann Arbor, MI), following the manufacturer's instructions. Tissue was homogenized in cold buffer (50 mM potassium phosphate, pH 7.0, 1mM EDTA/ g tissue). The absorbance (540 nm) was converted to μM using formaldehyde standards. Bovine liver catalase was used as a positive control. Results were expressed μM/min/mg protein.

Overexpression of mouse A1AT. Mouse A1AT (isoform 1) cDNA was generated by RT-PCR using liver RNA from C57BL/6 mouse. This cDNA was inserted into rAAV-CB-AAT vector plasmid to replace the hA1AT cDNA, thus creating rAAV-CB-mA1AT1 plasmid, which was transfected into 293 cells and medium containing mA1AT tested by immunoblot.

Rabbit anti-mouse A1AT antiserum was raised against all isoforms of mouse A1AT, by synthesizing and injecting a short peptide (15 aa) representing a conserved surface region of mA1AT into rabbits.

A sandwich **ELISA** assay detected hA1AT (22), and **western blotting** was performed as described (27). The ELISA was performed by coating a flat bottom, high binding extra, 96-well plate (Immulon 4, Dynatech,) with 100uL of 1° antibody (goat-anti-human α1-Antitrypsin, Sigma) 1:200 dilution in Voller's Buffer (Na_2CO_3 (106.0g/mol), $NaHCO_3$ (84.01g/mol), NaN_3 (65.01 g/mol), dH_2O;pH to 9.6). The plate was incubated overnight at 4˚C, followed by washes with PBS-Tween. Standard α1AT (Sigma) and samples (150uL/well) were added and incubate 60min at 37°C. After washing, we utilized BSA as blocking solution, followed by a secondary antibody (rabbit anti-human α1-Antitrypsin, Sigma) incubation for 60min at 37°C. A tertiary antibody (goat anti-rabbit IgG conjugated w/ peroxidase, Sigma) was incubated for 60min at 37°C, followed by reaction with TMB Peroxidase which was stopped after 1 minute with 1M H_3PO4. The absorbance was then read at 450nm. Briefly, for western blotting, lung tissue lysates were loaded in equal protein amounts (10 μg, unless otherwise noted) determined by Bradford assay (Pierce Biotechnology, Inc., Rockford, IL). Proteins were separated by SDS-PAGE (Novex, San Diego, CA), followed by immunoblotting as previously described (45). The chemilluminescent signals were quantified by densitometry (ImageQuant, Amersham) and normalized by actin.

Statistical analysis was performed with SPSS software (SPSS Inc.) using unpaired Student t-test or One-way ANOVA with Student-Newman-Keuls *post hoc* test. Statistical difference was accepted at $p < 0.05$.

Supplementary Figure E1. Specificity of A1AT antibodies tested by western blotting. (**a**)**.** Lane 1: purified human (h) A1AT (50 ng). Lane 2: supernatant with recombinant mouse (m) A1AT (250 ng total protein). The antibodies tested were against human A1AT rabbit polyclonal from Dako 1.62 μg/ml, sheep polyclonal from Enzyme Research 0.4 μg/ml, goat polyclonal from Santa Cruz 0.4 μg/ml and 4 μg/ml, respectively , and rabbit serum against mouse A1AT 1:1000. (**b**) Comparison of the A1AT primary structure in human versus rodents. R=rat, M=mouse, G=guinea pigs.

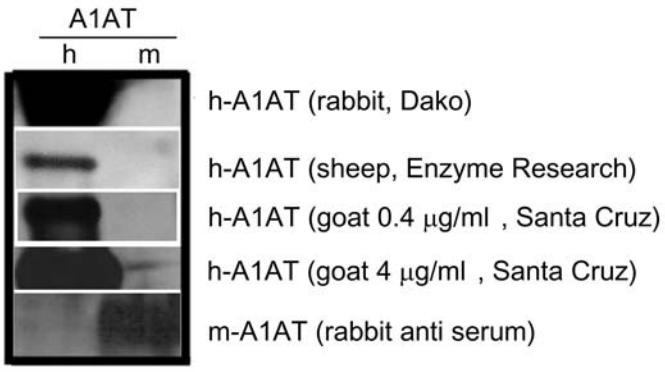
Supplementary Figure E2. a-d. Protective effect of hA1AT overexpression on the alveolar space destruction triggered by VEGFR inhibition in rats. (**a-c**) Representative lung morphology from (**a**) control (Ctl) animals treated with bovine serum albumin, (**b**) rats treated with SU5416 + albumin for 3 weeks (extended panel to highlight enlarged airspaces), and (**c**) rats co-treated with SU5416 and hA1AT (30 mg/kg, i.v. thrice weekly for 3 weeks). (**d**) Mean linear intercept (MLI) and (**e**) surface-volume ratio of lungs at week 3 in Ctl, SU5416-treated (VEGFR-inh), and hA1AT co-treated rats (*$p<0.05$ vs. Ctl and vs. VEGFR-inh + hA1AT; n= 6/group). **(f) Protective effects of A1AT on VEGFR-blockade-triggered lung apoptosis in rat.** Representative IHC of active caspase-3 (arrows) in Ctl (bovine serum albumin), SU5416-treated (VEGFR-inh + bovine serum albumin), and SU5416 + hA1AT (i.v. instillation of purified protein)–treated rat lung.

Table E1.

Primary Antibody	Source	Dilution	Application	Figure
alpha 1 antitrypsin goat polyclonal	Santa Cruz Biotechnology, Inc., Santa Cruz, CA	1:10000	IB Mouse lung	1d
		1:200	IHC mouse lung	1c
human alpha 1 antitrypsin rabbit polyclonal	Dako, Carpinteria, CA	1:1000	IHC mouse lung	1b
human alpha 1 antitrypsin goat polyclonal	Sigma	1:200	ELISA (primary)	1a
human alpha 1 antitrypsin rabbit polyclonal	Sigma	1:1000	ELISA (secondary)	1a
active caspase-3 rabbit polyclonal	Cell Signaling Technology,	1:200	IHC mouse lung	3
active caspase-3 rabbit polyclonal	Abcam, Cambridge, MA	1:600	IHC mouse lung	4
active caspase-3	PharMingen, San Diego, CA	1:500	IHC rat lung	S2
4-Hydroxy-2-nonenal rabbit polyclonal	Alpha Diagnostic, San Antonio, TX	1:1000	IHC mouse lung	5
Nitrotyrosine mouse monoclonal	Abcam ,Cambridge, MA	1: 1000	IHC mouse lung	5

FIGURE E1

a.



b.

(H). gi|28966|emb|CAA25838.1| alpha 1-antitrypsin [Homo sapiens]

(R). gi|51036655|ref|NP_071964.2| serine protease inhibitor alpha 1 [Rattus norvegicus]
Length=411
Score = 563 bits (1452), Expect = 3e-159, Method: Composition-based stats.
Identities = 289/417 (69%), Positives = 354/417 (84%), Gaps = 6/417 (1%)

(M). gi|21322148|gb|AAM47489.1| DOM2 [Mus musculus]
Length=413
Score = 521 bits (1341), Expect = 3e-146, Method: Composition-based stats.
Identities = 270/419 (64%), Positives = 343/419 (81%), Gaps = 7/419 (1%)

(G). gi|112498|pir||B39088 alpha-1-antiproteinase F precursor - guinea pig (fragment)
Length=388
Score = 450 bits (1157), Expect = 6e-125, Method: Composition-based stats.
Identities = 231/370 (62%), Positives = 280/370 (75%), Gaps = 15/370 (4%)

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H. MPSSVSWGILLLAGLCCLVPVSLAEDPQGDAAQKTDTSHHDQDHPTFNKITPNLAEFAFS  60
R. .AP.I.R.L.........A.SF....------..E....QQ..S-..YR..SS...D....  54
M. .TP.I...L...........SF....-----V.E....QK..S-.ASHE.AT..GD..I.  54
G.                                          Q.PRS..H..H.  50

H. LYRQLAHQSNSTNIFFSPVSIATAFAMLSLGTKADTHDEILEGLNFNLTEIPEAQIHEGF  120
R. ...E.V....TS......M..T.........S.G..RKQ.....E....Q....D..KA.  114
M. ...E.V....TS.....................S.G...TQ.....Q....QTS..D..KS.  114
G. M..V.TQ...TS............L..V...A.G...TQ..W..E......A..D..D..  110

H. QELLRTLNQPDSQLQLTTGNGLFLSEGLKLVDKFLEDVKKLYHSEAFTVNFGDTEEAKKQ  180
R. HH..Q...R...E...N......VNKN....E....E..NN......S...A.S.....V  174
M. .H..Q...R...E...S......VNND....E....EA.NH.QA.V.S...AES.....V  174
G. .N..H...R.H.EHE.........DQN...KE..S....T...A...P T..SNPK..E..  170

H. INDYVEKGTQGKIVDLVKELDRDTVFALVNYIFFKGKWERPFEVKDTEEEDFHVDQVTTV  240
R. ................M.Q..E................K...NPEH.RDA.....KS...  234
M. ...F..........EA.....Q......A...L.....KK..DPEN...AE....KS...  234
G. ..A..............D.SA...L........R....K..D..H.T Q...L..MN...  230


H. KVPMMKRLGMFNIQHCKKLSSWVLLMKYLGNATAIFFLPDEGKLQHLENELTHDIITKFL  300
R. .....N.....DMHY.ST......M.D.........L...D..M....QT..K.L.SR..  294
M. .....TLS..LDVH..ST........D.V...S.V.L..ED..M....QT.SKEL.S.I.  294
G. N......Q..YKAF..STIQ.....LD.E..V.TL.L...K..M....  ET..PEL.F..A 290


H. ENEDRRSASLHLPKLSITGTYDLKSVLGQLGITKVFSNGADLSGVTEEAPLKLSKAVHKA  360
R. L.RQT...I.YF.....S...N..TL.SS....R..N.D.....I..D......Q.....  354
M. L.RH..LVQI.I.R...S.D.N..TLMSP....RI.N.......I...N...........  354
G. RKTE.MF.NV.......S......E...H....N...GA.....I..D M...I------- 350

H. VLTIDEKGTEAAGAMFLEAIPMSIPPEVKFNKPFVFLMIEQNTKSPLFMGKVVNPTQ  417
R. ...L..R.......TVV..V...L..Q...DH..I.MIV.SE.Q....V...ID..R  411
M. .......T.....A.TVF..V...M..ILR.DH..L.IIF.EH.Q..I.V....D..H.  413
G. --------......TE..IT.H.V.QDLF.....L..I.DHS.DT... V...MD..K.  388
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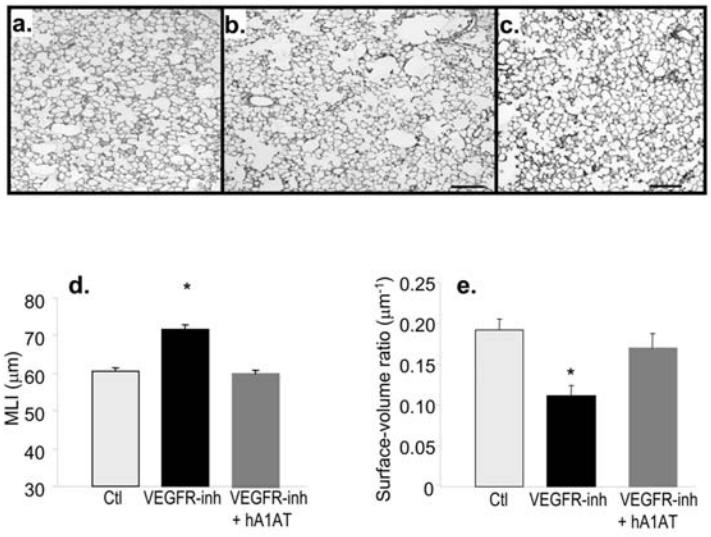
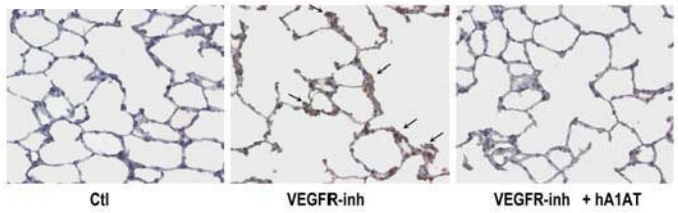
Figure E2


a.
b.
c.
d.
*
MLI (μm)
80
70
60
50
40
30
Ctl
VEGFR-inh
VEGFR-inh + hA1AT
e.
Surface-volume ratio (μm⁻¹)
0.25
0.20
0.15
0.10
0.05
0
*
Ctl
VEGFR-inh
VEGFR-inh + hA1AT


f.
Ctl
VEGFR-inh
VEGFR-inh + hA1AT