Supplementary methodology

Monocyte NLRP3 inflammasome and interleukin-1β activation modulated by alpha-1 antitrypsin therapy in deficient individuals.

Debananda Gogoi, Howard Yu, Michelle Casey, Rory Baird, Azeez Yusuf, Luke Forde, Michael E. O'Brien, Jesse R. West, Tammy Flagg, Noel G. McElvaney, Edward Eden, Christian Muller, Mark L. Brantly, Patrick Geraghty, and Emer P. Reeves.

Chemicals and reagents:

All chemicals and reagents were of the highest purity available and were purchased from Sigma-Aldrich, Dublin, Ireland unless specified otherwise.

Patient Genotyping and Phenotyping

AATD patient genotyping was performed on a LightCycler 480 (Roche) with primers for the Z mutation [1] and phenotyping was performed using a Sebia isoelectrofocusing kit with the HYDRASYS system [2].

Murine studies

Serpina1a-e knockout mice [3], on a C57BL/6J background, were maintained in a specific pathogen-free facility at SUNY Downstate Health Sciences University. Both male and female mice, 8-week-old, were used for the experiments. Blood was collected from the animals and monocytes were isolated using the EasySep™ Mouse Monocyte Isolation Kit (Stemcell™ Technologies) according to manufacturer's instructions. All animal experiments were performed with approval from SUNY Downstate Health Sciences University's Institutional Animal Care and Use Committee. This study was performed in strict accordance with the

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recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and Institutional Animal Care and Use Committee (IACUC) guidelines.

Plasma isolation and analysis

Blood was collected in EDTA tubes and placed immediately on ice. Blood tubes were centrifuged at 875 xg for 10 min (4°C) and the upper layer (plasma) was collected. C3d levels were detected in plasma using a Human complement C3 ELISA kit (Cusabio) according to manufacturer's protocol. IL-1β and TNF-α was detected in plasma and cell free supernatants using Human IL-1β DuoSet ELISA kit (R&D Systems) or Human TNF-α DuoSet ELISA kit (R&D Systems) according to manufacturer's protocol.

Monocytes isolation and culture

HC and AATD monocytes were cell sorted using the EasySep™ Human CD14 Positive Selection Kit II (Stemcell™ Technologies) according to manufacturer's instructions. Briefly, up to 25 ml blood was mixed with 0.9% (w/v) saline and layered over Lymphoprep™ for gradient density isolation of peripheral blood mononuclear cells. Monocytes were then isolated, washed and sorted using the Human CD14 Positive Selection Kit II with EasySep magnets (Stemcell™ Technologies). For AAT augmentation therapy studies, we have previously reported that weekly intravenous augmentation therapy 2 days post treatment of ZZ-AATD patients results in serum AAT concentrations similar to healthy control serum levels but also significantly greater than day 0 pre-treatment and 7 days post-treatment [4]. As neutrophil [4-8] and monocyte function [9] is modulated by peak serum levels of AAT on day 2, we chose to obtain blood donations for monocyte isolation from consenting AATD patients, 2 days post augmentation therapy. Resulting monocytes were cultured in RPMI 1640 (Corning) supplemented with 10% (w/v) Fetal Bovine Serum and 0.2% (v/v) Primocin (Invivogen) [10].

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Cells were then challenged with C3d with or without receptor inhibitors or inhibitors of cell signalling pathways for indicated times (supplementary table 1). At the end of each treatment protocol, cell supernatants were collected and stored at -80°C for subsequent cytokine analysis, and whole cells processed for indicated assays.

Supplementary Table 1: List of inhibitors used in this study

Inhibitor	Target receptor/protein	Product Supplier	Reference
Clone mAb 107	CR3	Merck Life Sciences	# MABF2085
BAY 11-7082	NF-κβ	Enzo Life Sciences	#15124578
LY 294002	PI3 kinase	Abcam	#Ab120243
Isobavachalcone	Akt	Abcam	#Ab141168
MCC950	NLRP3	Fischer Scientific	#15967265

qPCR analysis

Total RNA was extracted from HC or AATD monocytes using the PureLink™ RNA Mini Kit (Invitrogen™) according to manufacturer's instructions. RNA concentrations and purity were assessed with a NanoDrop™ 8000 Spectrophotometer (Thermo Scientific™). RT-qPCR was performed using the Luna® Universal One-Step RT-qPCR Kit (New England Biolabs) with specific primers targeting NLRP3, IL-1β, GRP78, ATF6 and CHOP (supplementary Table 2). Analysis was performed on a LightCycler480 Detection System (Roche) and gene expression was calculated with the 2-ΔΔCt method using GAPDH as the housekeeping gene [10].

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Supplementary Table 2: Primers used for qPCR analysis.

Primer	Sequence	Tm used (°C)
IL-1β Forward	5'-TCGCCAGTGAAATGATGGCT-3'	60
IL-1β Reverse	5'-TGGAAGGAGCACTTCATCTGTT-3'	60
IL-1β Forward	5'-ATCGCTTCCTCTCGCAACAA-3'	60
IL-1β Reverse	5'-TCCAGGTTTTCATCATCTTCAGC-3'	59
NLRP3 Forward	5'- GCC GAA GTG GGG TTC AGA TAA -3'	60
NLRP3 Reverse	5'- GCA GTC GTG TGT AGC GTT TG -3'	60
GRP78 Forward	TaqMan assay ID: Hs00607129_gH	58
GRP78 Reverse	TaqMan assay ID: Hs00607129_gH	58
ATF6 Forward	TaqMan assay ID: Hs00232586_m1	58
ATF6 Reverse	TaqMan assay ID: Hs00232586_m1	58
CHOP Forward	TaqMan assay ID: Hs00358796_g1	60
CHOP Reverse	TaqMan assay ID: Hs00358796_g1	60
NFκβ Forward	5'-CCGCTTAGGAGGGAGAGCC-3'	60
NFκβ Reverse	5'-TGCCATTCTGAAGCCGGG-3'	60
GAPDH Forward	5'- CAT GAG AAG TAT GAC AAC AGC CT -3'	60
GAPDH Reverse	5'- AGT CCT TCC ACG ATA CCA AAG T -3'	60

Western blot analysis

HC and AATD monocytes were lysed in RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, 0.1% (w/v) SDS and 0.01% (w/v) sodium azide at a pH of 7.4) containing 1% (v/v) Triton X-100 and proteases inhibitor cocktail (ThermoFisher Scientific (#A32959)) for 1h at 4°C. Protein amounts were measured using a BCA Protein Assay Kit (Pierce®), and absorbance was recorded at 540 nm using a SpectraMax M3 microplate Reader (Molecular Devices). Protein samples (10 µg) were loaded on 10% (w/v) acrylamide gels and transferred to PVDF membranes by Western blotting (Roche). Primary antibodies (supplementary table 3) were incubated overnight at 4°C. Corresponding secondary antibodies (supplementary table 3) were then incubated for 1h at room temperature. Proteins were detected with Immobilon Western Chemiluminescent Substrate (Merck Millipore) using a ChemiDoc™ Imaging System (Bio-Rad). Densitometry quantification by ImageJ software (NIH) [10].

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Supplementary Table 3: Antibodies used for Western blot analysis.

Primary antibodies	**Species**	**Supplier**	**Catalogue #**
NLRP3	Rabbit	Cell Signalling Technology	#13158
IL-1β	Rabbit	Cell Signalling Technology	#12703
Phospho PI3 kinase p85	Rabbit	Cell Signalling Technology	#4228
PI3K p85	Rabbit	Cell Signalling Technology	#4292
pAkt	Rabbit	Cell Signalling Technology	#4060
Akt	Rabbit	Cell Signalling Technology	#9272
IKβα	Rabbit	Cell Signalling Technology	#2859
GRP78	Mouse	Enzo Life Sciences	#ADI-SPA-826-D
GRP78	Rabbit	Cell Signalling Technology	#5554
ATF6	Mouse	Novus Biologicals	# NBP1-40256SS
ATF6	Rabbit	Abcam	#ab11909
ASC/TMS1	Rabbit	Cell Signalling Technology	#13833
β-Actin	Mouse	Santa Cruz Technology	#sc-47778
β-Actin	Rabbit	Cell Signalling Technology	#8457
Secondary antibodies	**Species**	**Supplier**	**Catalogue #**
Rabbit-HRP conjugate	Rabbit	Cell Signaling Technology	#7074
Mouse-HRP conjugate	Horse	Cell Signaling Technology	#7076

Flow cytometry analysis

In CR3 receptor blocking experiments, isolated monocytes were incubated with or without the CR3 inhibitor, clone mAb 107 (monoclonal antibody, #MABF2085) (1µg/ml) for 1h. Subsequently, C3d (5µg) (Complement Technologies) binding was evaluated using C3d mouse monoclonal IgG1 antibody (1µg/ml) (Abcam) followed by FITC labelled mouse IgG kappa binding protein (m-IgGκ BP) (1µg/ml) (Santa Cruz Biotechnology). In a subset of experiments, C3d binding to AAT was measured with rabbit anti-C3d fluorescein isothiocyanate (FITC)-labelled antibody (Abcam) or a non-specific IgG1 FITC control (IsoAb) (Thermo Fisher Scientific) using a FACScalibur flow cytometer (Becton Dickinson). Binding of C3d to CR3 receptor in the presence of glycosylated human-AAT (h-AAT; Athens Research) or deglycosylated AAT was quantified using rabbit anti-C3d FITC-labelled antibody. Controls for the experiments employed cells alone, or cells incubated with respective mouse/rabbit

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monoclonal IgG1 antibody and/or FITC labelled m-IgGκ BP. Cells were then washed and fluorescence recorded. Ten thousand events per reaction were quantified. Analysis of fluorescence was carried out using FlowJo_v10.8.1 software and data represented as median fluorescent intensity (MFI).

Caspase-1 activity assays

Caspase-1 activity was assessed in HC or AATD monocytes using the Caspase-Glo® 1 Inflammasome Assay (Promega) according to manufacturer's instructions. Briefly, Caspase-Glo® 1 Reagent was added to monocytes to a ratio of 1:1. After 1h, luminescence was recorded using a SpectraMax M3 microplate reader (Molecular Devices).

Intracellular Ca^{2+} assay

Intracellular Ca^{2+} was detected by fluorescence using the Fluo-8 No Wash assay kit (Abcam) following manufacturer's instructions and fluroscence was recorded using a SpectraMax M3 microplate reader (Molecular Devices) [10].

Z-AAT polymer quantification

A sandwich ELISA with plasma samples and monocyte lysates was performed using the 2C1 monoclonal antibody (mAb) against AAT polymers [11]. Plates were coated overnight at room temperature with 50 µL/well of purified 2C1 mAb at 2 µg/mL as per supplier instructions (SRM Monoclonal Antibody (2C1), Invitrogen, #15588852). The following day, plates were washed and incubated with 300 µL/well of blocking solution for 1 h. Standards and samples were diluted in blocking buffer, added to the plate and incubated for 2 h at room temperature. Bound polymers were detected with anti-total AAT 3C11 mAb labelled with horseradish peroxidase incubated for 1h (ZNF197 Monoclonal Antibody (3C11), Invitrogen, #15578932),

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and quantified in a plate reader at 450 nm using a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution. Concentrations of AAT polymers (µg/mL) were determined by interpolation of absorbance values on a standard curve. An AAT polymer standard was prepared by heating purified AAT (0.5 mg/mL) at 55 ºC for 16 h. The proportion of polymers versus the total levels of AAT (%) was determined in all samples together with total polymer concentrations (µg/mL). Measurements were carried out in triplicates and the lower limit of detection of the ELISA was 10 µg/mL.

Statistical analysis

Data were analysed using GraphPad Prism 8.0 for PC. Appropriate descriptive statistics (e.g., mean, standard deviation (SD), median, frequencies, and counts) were used to describe patient characteristics and outcomes of interest. Statistical significance was calculated using a Student's t-test when comparisons were made between two groups when normally distributed. Non-normal data were analysed by the two tailed non parametric Mann-Whitney U test. Linear and multiple regression modelling was employed to examine the significance of any relationship within the clinical data or between the clinical data. Adjustment was performed for covariates that included age and was reported using the standardized ANCOVA analysis. Two tailed non parametric spearman correlation test was used to determine the positive and/or negative correlation between two continuous variables. One-way ANOVA was used to determine statistical significance when comparing three or more groups followed by Tukeys' post-hoc multiple comparison test. A value of $p<0.05$ was considered statistically significant.

Supplementary Reference list

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