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Michael C. McKelvey[1], Anthony A. Abladey[1], Donna M. Small[1], Declan F. Doherty[1], Richard Williams[2], Aaron Scott[3], C. Arnold Spek[4], Keren S. Borensztajn[5#], Cecilia M. O'Kane[6], Daniel F. McAuley[6], Clifford C. Taggart[1*], Sinéad Weldon[1]

[1]Airway Innate Immunity Research (AiiR) Group, Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Northern Ireland, U.K. [2]Patrick G Johnston Centre for Cancer Research, Queen's University Belfast, Northern Ireland, U.K. [3]Centre for Translational Inflammation Research, University of Birmingham, England, U.K. [4]Center of Experimental and Molecular Medicine, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands. [5]INSERM UMRS_933, Université Pierre et Marie Curie, Hôpital Trousseau, Paris 75012, France. [6]Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Northern Ireland, U.K.

[#]In memory of Dr Keren Borensztajn

Corresponding author: *Clifford C. Taggart, Airway Innate Immunity Research (AiiR) Group, Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, U.K. Telephone: 00442890976383; Email: c.taggart@qub.ac.uk.

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Abstract

Rationale: Although the cysteine protease cathepsin S has been implicated in the pathogenesis of a number of inflammatory lung diseases, its role has not been examined in the context of acute respiratory distress syndrome, a condition which still lacks specific and effective pharmacological treatments.

Objectives: Characterize the status of cathepsin S in acute lung inflammation and examine the role of cathepsin S in disease pathogenesis.

Methods: Human and mouse model bronchoalveolar lavage fluid samples were analyzed for the presence and activity of cathepsin S and its endogenous inhibitors. Recombinant cathepsin S was instilled directly into the lungs of mice. The effects of cathepsin S knockout and pharmacological inhibition were examined in two models of acute lung injury. Protease-activated receptor-1 antagonism was used to test a possible mechanism for cathepsin S-mediated inflammation.

Measurements and Main Results: Pulmonary cathepsin S levels and activity were elevated in acute respiratory distress syndrome, a phenotype possibly exacerbated by the loss of the endogenous antiprotease, cystatin SN. Direct cathepsin S instillation into the lungs induced key pathologies of acute respiratory distress syndrome including neutrophilia and alveolar leakage. Conversely, in murine models of acute lung injury, genetic knockdown and prophylactic or therapeutic inhibition of cathepsin S reduced neutrophil recruitment and protein leakage. Cathepsin S may partly mediate its pathogenic effects via protease-activated receptor-1, as antagonism of this receptor abrogated cathepsin S-induced airway inflammation.

Conclusions: Cathepsin S contributes to acute lung injury and may represent a novel therapeutic target for acute respiratory distress syndrome.

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Introduction

Acute respiratory distress syndrome (ARDS) is characterised by the flooding of the alveoli with protein- and leukocyte-rich oedema as a result of a direct injury to the lung, such as pneumonia or acid aspiration, or a systemic inflammatory response causing indirect lung injury, such as in sepsis (1). With a mortality rate between 30-50 % and no specific pharmacological therapies available, novel therapeutic approaches are required to improve outcomes in patients with ARDS (2, 3).

Neutrophils are the first leukocytes recruited to sites of injury and inflammation in response to chemotactic factors released by activated macrophages and epithelial and endothelial cells (4–6). Despite being the first line of defence against pathogens, uncontrolled neutrophil recruitment and activation can lead to bystander tissue damage and additional loss of lung function (7, 8). Bronchoalveolar lavage fluid (BALF) from patients with ARDS is chemotactic for human neutrophils, with a potential role for the chemokines CXCL8, CCL2 and CCL7 (9, 10). Neutrophil counts in BALF from patients with ARDS positively correlated with disease severity and poor outcome (8, 11–13). In addition, a number of animal models of acute lung injury have demonstrated a neutrophil-dependent pathogenesis (14, 15). With an important role for neutrophils in at least a subset of patients with ARDS, neutrophil products such as the serine protease neutrophil elastase (NE) have been investigated as potential therapeutic targets. Samples from patients with ARDS have elevated NE proteolytic activity (16, 17) and the potential role for pathogenic proteolysis has been investigated in ARDS. However, NE inhibitor therapy has not consistently proven effective and other protease targets warrant investigation (18, 19).

Cathepsin S (CTSS) is a lysosomal and extracellular cysteine protease that is abundantly expressed in antigen presenting cells, including macrophages and dendritic cells, as well as airway epithelial cells, neutrophils and B cells (20–22). The localization of CTSS, coupled with broad substrate specificity, suggests an important role for this protease in the immune response (23, 24). CTSS upregulation in response to inflammatory stimuli may have a direct influence on immune cell responses, particularly those involved in antigen presentation through the major histocompatibility complex (MHC) class II. Cleavage of the invariant chain (li), a type II transmembrane glycoprotein, by CTSS is an integral part of exogenous antigen presentation through MHC class II complexes (21). The aberrant expression and activity of CTSS has been implicated in the pathogenesis of a number of conditions including cardiovascular disease, cancer, rheumatoid arthritis and a number of pulmonary diseases (25, 26).

CTSS, along with cathepsins B and L are upregulated in the lungs of patients with cystic fibrosis (CF) (21, 27, 28). Small *et al.* demonstrated that CTSS contributes to neutrophilic pulmonary inflammation and mucus plugging in CF-like lung disease, mediated at least in part through activation of protease-activated receptor (PAR)-2 (29). CTSS has also been shown to be upregulated in the lungs of patients with chronic obstructive pulmonary disease and in response to cigarette smoke *in vivo* (30–32). However, the status and role of CTSS in ARDS has not been evaluated in detail. In this study, we demonstrate elevated CTSS levels and activity in the lungs of patients with ARDS and that this increase in activity coincides with the loss of the potent CTSS inhibitor, cystatin SN. In addition, elevated CTSS activity was implicated in neutrophil recruitment to the lungs, a process mediated at least in part via activation of PAR-1. Therefore, these results

suggest that CTSS plays a role in neutrophil recruitment to the acutely inflamed lung, making it a potential therapeutic target for ARDS.

Results

Cathepsin S activity in patients with ARDS and in models of acute lung injury

The status of CTSS in ARDS was determined by assessing CTSS protein levels and activity in BALF samples from patients with ARDS, healthy volunteers who received nebulised lipopolysaccharide (LPS) and healthy control volunteers. CTSS levels and activity were significantly increased in patients with ARDS (***Figure 1a,b***), a finding that was verified by Western blot (***Figure 1c***). Mature CTSS (approximately 25 kDa) along with bands analogous to the precursor form of CTSS (approximately 37 kDa) were detected. This finding translated into a murine model of LPS-induced acute lung injury, in which CTSS activity was significantly increased in BALF from LPS-instilled mice compared to controls (***Figure 1d***). This finding was accompanied by increased levels of both precursor and mature CTSS protein in murine BALF when analysed by western blot (***Figure 1e***). These data provide evidence for the presence of elevated pulmonary CTSS activity in patients and *in vivo* models of ARDS.

The cysteine protease-antiprotease imbalance in ARDS

As elevated CTSS activity was detected in patients with ARDS, we assessed the protease-antiprotease hypothesis in ARDS as an explanation for this observation. Dysregulation of the canonical extracellular cathepsin inhibitor cystatin C (23, 33) was considered the most likely cause of elevated CTSS activity. Although recent work identified a strong association between mortality and elevated plasma cystatin C measured early in the course of ARDS (34), its status in the lungs

of ARDS patients is unknown. We found that the ratio of BALF CTSS:cystatin C was unchanged between healthy and ARDS (data not shown) and therefore we turned our attention to other extracellular cystatins. Relatively little is known about the status of these antiproteases in the inflamed lung, especially the so-called 'salivary' or SD-type cystatins (35). A preliminary screen of BALF samples from healthy volunteers and patients with ARDS for cystatins S, SA, SN and D revealed that these SD-type cystatins were not detectable in samples from patients with ARDS compared to healthy controls ***(see Figure E1 in the online data supplement***).

Reported as the most potent SD-type cystatin, altered expression of cystatin SN has been reported in lung fibrosis, pneumonitis and allergic rhinitis (36–38). Expression of cystatin SN is thought to be highly localised to the oral and nasal epithelium, along with the epithelium of the upper respiratory tract (39, 40). Furthermore, there is evidence that cystatin SN may be differentially regulated by inflammatory mediators (40). Cystatin SN levels were significantly reduced in BALF from patients with ARDS and in LPS volunteers compared to healthy controls (***Figure 2a,c***). Consequently, a CTSS:cystatin SN ratio in favour of CTSS was identified in ARDS and in the human LPS model (***Figure 2b***). Cystatin SN has been reported to inhibit cathepsin B and papain (41), but has not previously been reported as a CTSS inhibitor. The ability of cystatin SN to inhibit CTSS was assessed, and the results indicate that cystatin SN is a potent, tight-binding, reversible inhibitor of CTSS *in vitro* with a K_i in the nanomolar range (***Figure 2d***).

As elevated CTSS activity and a deficiency of cysteine antiproteases were characteristic features of ARDS, we investigated the effects of introducing exogenous cystatin SN into the murine LPS

model. Cystatin SN treatment significantly decreased LPS-induced total cell and neutrophil recruitment to the lung (***Figure 2e,f***).

The pro-inflammatory role of pulmonary cathepsin S *in vivo*

To characterize the effects of active pulmonary CTSS *in vivo,* recombinant CTSS or buffer control was administered via intratracheal instillation into the lungs of mice. Intratracheal instillation of CTSS produced a dose-dependent inflammatory response, resulting in total cell and neutrophil infiltration into the lungs (***Figure 3a,b***) in agreement with previous work (29). Alveolar leakage (as measured by BALF protein) was significantly increased in mice that received CTSS compared to controls (***Figure 3c***). The pro-inflammatory cytokines IL-6 and KC were also increased in a dose-dependent manner in CTSS-instilled mice (***Figure 3d,e***). These data showed that active, pulmonary CTSS recapitulated hallmarks of ARDS *in vivo*.

Having established that CTSS was elevated and active in a well-established mouse model of acute lung injury, and that direct instillation of CTSS resulted in acute lung inflammation, the role of CTSS in the pathogenesis of LPS-induced pulmonary inflammation was investigated using CTSS knockout ($CTSS^{-/-}$) mice. Total inflammatory cell and neutrophil infiltration into the lungs were significantly reduced in $CTSS^{-/-}$ mice compared to wild-type (WT) mice receiving LPS (***Figure 4a,b***). These findings were accompanied by a significant decrease in BALF total protein and the neutrophil chemoattractant KC in $CTSS^{-/-}$ mice (***Figure 4c,d***). These data provide further evidence to support the hypothesis that CTSS plays an important role in mediating LPS-induced acute lung injury *in vivo*.

Pharmacological targeting of cathepsin S *in vivo*

We next investigated the therapeutic potential of a small molecule reversible inhibitor of CTSS (I.6) (42) on LPS-induced pulmonary inflammation. Similar to $CTSS^{-/-}$ mice, significant reductions in total cell and neutrophil counts were observed in mice pre-treated with I.6 compared to vehicle control (***Figure 5a,b***). Furthermore, prophylactic CTSS inhibition reduced BALF total protein and KC levels (***Figure 5c,d***).

Since ARDS has diverse aetiologies including both direct and indirect injuries, we investigated whether CTSS inhibition would also alter measures of inflammation in an indirect model of ARDS; the caecal ligation and puncture (CLP) model of polymicrobial sepsis-induced ARDS. Treatment with I.6 significantly reduced total cell and neutrophil counts in peritoneal lavage fluid (PLF) (***Figure 6a-d***). Within the lung, I.6 treatment had no significant effect on the number of cells, however, there were significant changes in the cellular composition of BALF with a decrease in the percentage of neutrophils and a concomitant increase in monocytic cells in the I.6-treated group (***Figure 6e-h***). These changes were accompanied by reductions in the inflammatory cytokines KC and IL-6 (***Figure 6i,j***). Overall, these results indicate that prophylactic pharmacological inhibition of CTSS with a small molecule inhibitor protects against inflammation in direct and indirect lung injury models of ARDS.

Next, to establish whether CTSS inhibition could also effectively reduce inflammation when administered at a later time-point, a therapeutic dosing strategy was tested in the LPS model. In this study, the CTSS inhibitor I.6 was administered two hours post-LPS and significant reductions in BALF total and neutrophil cell counts, protein and KC levels were observed (***Figure 7***).

The role of protease-activated receptor-1 in CTSS-induced inflammation

Our group (29) and others (43) have previously highlighted a role for PAR-2 in CTSS-mediated signalling. However, bacterial cysteine proteases and several human non-cysteine proteases have also been shown to activate PAR-1 (44), which has previously been implicated in acute lung inflammation (45, 46). To explore whether PAR-1 plays a role in CTSS-induced inflammation *in vitro*, human macrophage-like cells derived from THP-1 monocytes were treated with recombinant CTSS. As had been observed during *in vivo* CTSS instillations, CTSS induced the release of neutrophilic cytokines including IL-8 and CXCL1 from these cells (***Figure 8a,b***). However, when the synthetic PAR-1 antagonist SCH-530358 was added to cells concomitantly with CTSS, these cytokine responses were significantly decreased. To consolidate this finding *in vivo*, mice were treated with SCH-530358 30 min before intratracheal CTSS instillation. Administration of SCH-530358 significantly reduced CTSS-induced BALF total cell and neutrophil infiltration as well as total protein and KC levels (***Figure 8c-f***).

To investigate whether the reduced LPS-induced inflammation observed in our previous studies where CTSS was knocked down could be due to diminished PAR-1 activation, PAR-1 knockout (PAR-$1^{-/-}$) mice were treated with I.6 using the same prophylactic dosing strategy previously used in WT mice (***Figure 7***). In PAR-$1^{-/-}$ mice that received LPS the protective effects of I.6 were lost (***Figure 9***). In this model, CTSS inhibitor treatment had no significant effect on total cell or neutrophils counts, suggesting that CTSS-mediated PAR-1 activation is an important part of neutrophil recruitment in this model (***Figure 9a,b***). PAR-$1^{-/-}$ mice treated with I.6 also did not

show decreased BALF protein or IL-6 (***Figure 9c,d***). Taken together, these findings suggest an important role for PAR-1 in CTSS-mediated pathology in ARDS-like disease.

Discussion

In this study, we have demonstrated that CTSS is elevated in the lungs of patients with ARDS and in *in vivo* models of ARDS. Furthermore, a quantitative imbalance between CTSS and a newly identified CTSS inhibitor, cystatin SN, in patients with ARDS was identified. Active CTSS instilled into the lungs produced typical symptoms of ARDS in mice, namely pulmonary neutrophilia, alveolar-capillary leakage and increased levels of potent neutrophil chemoattractants. We also show that PAR-1 antagonism significantly abrogated CTSS-induced inflammation *in vitro* and *in vivo*. Targeting of CTSS limited neutrophilic inflammation in both direct and indirect murine models of ARDS. The protective effects of CTSS inhibition were not replicated in PAR-$1^{-/-}$ mice, suggesting that the pathogenic effects of CTSS may be mediated, at least in part, through PAR-1. To our knowledge, this is the first study to comprehensively investigate CTSS in the acutely inflamed lung.

Proteases play key roles in pulmonary health and disease, fulfilling basic homeostatic roles and regulating regeneration and repair processes within the healthy lung (47). Previous studies have reported that an imbalance between proteases and their physiological inhibitors can lead to the destruction of lung parenchyma and leakage of protein-rich fluid into alveolar spaces and interstitium, which is critical in the instigation and propagation of ARDS (48). In the context of ARDS, the deficiency of endogenous protease inhibitors, such as cystatin SN, may lead to a protease-antiprotease imbalance that favours inflammatory and injurious proteolytic activity. As

mice do not express any of the SD-type cystatins, cystatin SN downregulation was not a feature of the murine model of ARDS and as such could not be examined *in vivo*. However, treating mice with recombinant cystatin SN did show some protective effects, particularly in limiting LPS-induced neutrophil recruitment, suggesting that the loss of cystatin SN in human ARDS may accentuate the neutrophilic response. The causes of cystatin SN deficiency in human ARDS are unknown, although IL-17A, an important cytokine in ARDS (49), has been shown to repress cystatin SN expression in neutrophil-infiltrated nasal polyps, suggesting that a pro-inflammatory environment may downregulate this antiprotease (40).

Our finding that dysregulated CTSS activity is a feature of the ARDS lung and the lungs of ARDS models led us to investigate whether pulmonary instillation of CTSS was damaging and produced traits of ARDS *in vivo*. Indeed, a significant increase in neutrophil recruitment and protein levels, along with elevated cytokine levels were observed following CTSS instillation, in agreement with previous findings (29, 50), indicating that CTSS can induce typical features of acute lung inflammation (51). CTSS has previously been shown to activate PAR-2 (43), thereby upregulating expression of pro-inflammatory cytokines and inducing pain and itch responses (50, 52, 53). However, there is no evidence linking CTSS to PAR-1 activation in the existing literature. The role of PAR-1 in experimental models of ARDS has previously been highlighted, with PAR-1 signalling reported to influence key features of ARDS including neutrophil recruitment, alveolar-capillary leakage and fibrosis in LPS-, acute infection- and bleomycin-induced murine lung injury models (45, 46, 54). Based on these observations, we hypothesized that PAR-1 plays a role in modulating the immune response during CTSS-induced acute lung inflammation. The data from this study showed that CTSS-induced lung inflammation was attenuated by a specific PAR-1

antagonist and that PAR-1$^{-/-}$ mice received no additional benefit from treatment with a CTSS inhibitor, unlike their WT counterparts. Although we show clear evidence of CTSS-induced activation of PAR-1, it is not clear if that activation step occurs directly, or indirectly via another protease, as has been shown in previous studies (55, 56).

The use of synthetic CTSS inhibitors in our studies demonstrated that both prophylactic and therapeutic inhibition had beneficial effects on key readouts of injury and inflammation in preclinical models of ARDS. The case for the use of such inhibitors is strengthened by the discovery that endogenous cysteine protease inhibitors are lost in ARDS. Although we explored a PAR-1 mediated pathway of CTSS-induced inflammation, more work is required to understand other pathways modulated by CTSS to account for the residual inflammation that is present following PAR-1 antagonism (***Figure 8***). Furthermore, it is not yet clear which LPS-mediated signalling pathways are affected by CTSS inhibition. Even a relatively simple model of ARDS, such as the intratracheal LPS model, activates numerous pathways (57) and future work should explore which of these pathways are affected by CTSS inhibition, resulting in an abrogated phenotype.

A number of pre-clinical studies have demonstrated a beneficial role for the inhibition of CTSS in various inflammatory diseases (25, 26, 58). The use of protease inhibitors in the treatment of pulmonary disease is a promising therapeutic strategy primarily aimed at attenuating lung tissue destruction. For instance, recent evidence has shown that α_1-antitrypsin augmentation therapy slows the progression of emphysema in patients with α_1-antitrypsin-deficiency (59). The present work demonstrates that CTSS also has roles in the setting of acute lung injury, such as that seen in

292 ARDS. Given the availability of clinical grade CTSS inhibitors, and the evidence from this study
293 indicating a role for CTSS in ARDS disease pathogenesis, CTSS inhibitors may offer a novel
294 therapeutic approach for prevention and management of excessive neutrophilic inflammation
295 associated with ARDS.

Methods

Human samples

Cathepsin S and cystatins were evaluated in BALF samples obtained from several clinical trials. Samples from patients within 48 h of ARDS onset were collected as part of the Hydroxymethylglutaryl-CoA reductase inhibition with simvastatin in Acute lung injury to Reduce Pulmonary dysfunction (HARP) study (ISRCTN70127774) (60). BALF samples were collected from healthy volunteers 6 h after receiving 50 μg nebulised LPS (*Escherichia coli* serotype 026:B6, Sigma-Aldrich, Dorset, UK) as part of NCT01659307 (the effect of Aspirin on REducing iNflammation in human in vivo model of Acute lung injury (ARENA)) (61). Ethical approval for the use of samples from the HARP and ARENA studies as control samples was granted by the local institution and the local research ethics committee (06/NIR02/77, 12/NI/0082, respectively). Samples were collected from healthy volunteers who did not receive LPS under the Office for Research Ethics Committees Northern Ireland ethical approval study number 08/NIR02/46 (62).

Animals

All experimentation was carried out in accordance with the Animal (Scientific Procedures) Act 1986 and current guidelines approved by the Queen's University Belfast Ethical Review Committee and the University of Birmingham Animal Welfare and Ethical Review Body.

Full details of the animals used in this study and the in vivo experiments conducted can be found in the **Supplementary Methods**.

Protein analysis

ELISAs were performed as per the manufacturer's instructions: murine IL-6 and KC (R&D Systems, Abingdon, UK); human cystatin SN (RayBiotech, Georgia, USA); human total CTSS, IL-8 and CXCL1 (R&D Systems, Abingdon, UK). Samples below the lower limit of detection of an assay were arbitrarily assigned a value of half the lower limit of detection, in order to minimise the difficulties associated with statistical analysis of zero values, as previously described (63). Total protein concentrations were determined using the BCA method (Pierce BCA Assay, Thermo Scientific) as per the manufacturer's instructions.

THP-1 experiments

Full details can be found in the **Supplementary Methods**. Briefly, THP-1 monocytes differentiated into macrophage-like cells by incubation with phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, Dorset, UK) were stimulated with 1 µg/mL recombinant human CTSS (Merck-Millipore, Hertfordshire, UK) for 24 h in the presence or absence of the PAR-1 antagonist SCH-530348 (Axon Medchem, Groningen, Netherlands) at a concentration of 10 µM.

Calculating K_i and IC_{50} for CTSS inhibitors

Full details can be found in the **Supplementary Methods**. Briefly, a range of concentrations of recombinant cystatin SN (R&D Systems, Abingdon, UK) were incubated with recombinant CTSS (Merck-Millipore, Hertfordshire, UK) and proteolytic degradation of Z-FR-AMC fluorogenic substrate (Enzo Life Sciences, Exeter, UK) was measured using a BioTek Synergy HT plate reader (BioTek, Swindon, UK). ΔRFU was then converted into µM AMC released by calibration with an AMC standard curve and the rate of product formation (v) was calculated. The reciprocal of this

unit of velocity (1/v) was plotted against the concentration of inhibitor used ([i]), forming a Dixon plot (64, 65) from which K_i and IC_{50} were determined.

SDS-PAGE and Western blotting

BALF samples were separated on 15 % SDS-PAGE gels and transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). Membranes were blocked with 5 % non-fat milk in PBS-Tween20 (0.05 %) and incubated with anti-CTSS (AF1183, R&D Systems), anti-cystatin SN (AF1285, R&D Systems), anti-cystatin S (AF1296, R&D Systems), anti-cystatin D (AF1202, R&D Systems), anti-cystatin SA (MAB1201, R&D Systems) antibodies overnight at 4 °C. Binding was detected using the appropriate horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (PerkinElmer, Coventry, UK) using the Syngene G:Box and GeneSnap software (SynGene UK, Cambridge).

Statistics

All data were analysed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA). Data are presented as mean ± standard error of the mean (SEM). Means were compared by unpaired two-tailed t test, two-tailed Mann Whitney test or two way ANOVA with Sidak's multiple comparisons test as indicated in the figure legends. $P < 0.05$ was accepted to indicate statistical significance; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, $****P < 0.0001$. Data points are biological replicates taken from distinct samples.

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564 **Figure Legends**

565

566 **Figure 1. Cathepsin S is elevated in the lungs of patients with ARDS and in models of**
567 **ARDS. a** Cathepsin S (CTSS) levels and **b** activity were analysed in bronchoalveolar lavage fluid
568 (BALF) from healthy volunteers (*n* = 15), healthy volunteers who received 50 µg nebulised
569 lipopolysaccharide (LPS) (*n* = 13) and patients with ARDS (*n* = 38). CTSS levels were quantified
570 by ELISA. CTSS activity was detected by fluorometric activity assay and results are expressed as
571 the change (Δ) in relative fluorescence units (ΔRFU) over time. *** $P < 0.001$, **** $P < 0.0001$
572 (two-tailed Mann-Whitney test). **c** Western blot detection of CTSS in BALF from healthy
573 volunteers (*n* =3), healthy volunteers who received LPS (*n* = 4) and ARDS patients (*n* = 4). In a
574 murine model of endotoxin-induced acute lung injury, mice received 1 mg/kg LPS or saline (Ctrl)
575 by intratracheal instillation and BALF was collected 16 h post-LPS administration. **d** BALF CTSS
576 activity (*n* = 8 per group) was detected by fluorometric activity assay. *** $P < 0.001$ (unpaired
577 two-tailed t test). **e** Western blot detection of CTSS in BALF (*n* = 5 per group).

578

579 **Figure 2. Cystatin SN inhibits cathepsin S and is lost in the lungs of patients with ARDS. a**
580 Cystatin SN levels were quantified in bronchoalveolar lavage fluid (BALF) from healthy
581 volunteers (*n* = 10), healthy volunteers who received 50 µg nebulised lipopolysaccharide (LPS) (*n*
582 = 9) and patients with ARDS (*n* = 13) by ELISA. **b** The protease-antiprotease imbalance was
583 expressed as a ratio of cathepsin S (CTSS) to cystatin SN. * $P < 0.05$, *** $P < 0.001$, ****
584 $P < 0.0001$ (two-tailed Mann-Whitney test). **c** Cystatin SN in BALF from healthy volunteers (*n* =
585 2), healthy volunteers who received 50 µg nebulised LPS (*n* = 2) and patients with ARDS (*n* = 8)
586 was detected by Western blot. **d** The inhibitory activity of cystatin SN against active CTSS was
587 assessed by incubating recombinant CTSS with increasing concentrations of recombinant cystatin

SN and quantifying activity with varying concentrations of 7-amino-4-methylcoumarin (AMC)-conjugated substrate. The turnover of substrate over time was quantified by calibrating the change in fluorescence with a standard curve of free AMC. A Dixon plot was generated to allow the calculation of a theoretical value for the inhibition constant K_i (representative plot shown, K_i calculated from $n = 4$ individual experiments) and half-maximal inhibitory concentration (IC_{50}, $n = 3$). To test the anti-inflammatory activity of cystatin SN *in vivo*, mice received an intratracheal instillation of 1 mg/kg LPS (n = 5-7 per group) or saline (n = 4-5 per group) and were left to recover for 15 min before receiving a subcutaneous injection of recombinant cystatin SN (cys SN; 0.5 mg/kg). After 16 h, BALF was collected, and **e** total cell and **f** neutrophil counts were quantified. *** $P < 0.001$ (unpaired two-tailed t test).

Figure 3. Intratracheal instillation of cathepsin S induces pulmonary inflammation. Mice received sodium acetate (Ctrl, $n = 3$), 1 µg ($n = 4$) or 5 µg of recombinant cathepsin S (CTSS, $n = 5$) via intratracheal instillation. After 24 h, bronchoalveolar lavage fluid (BALF) was collected for analysis and **a** total cell and **b** neutrophil counts were quantified. BALF **c** total protein concentration was quantified by BCA, **d** IL-6 and **e** KC levels were measured by ELISA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (**a,c-e** unpaired two-tailed t test and **b** two-tailed Mann-Whitney test).

Figure 4. Genetic cathepsin S knockdown protects mice from LPS-induced acute lung inflammation. WT ($n = 6$ per group) and cathepsin S $(CTSS)^{-/-}$ ($n = 5$ per group) mice received 1 mg/kg lipopolysaccharide (LPS) or saline (Ctrl) via intratracheal instillation. After 16 h, bronchoalveolar lavage fluid (BALF) was collected for analysis and **a** total cell and **b** neutrophil

counts were quantified. BALF **c** total protein and **d** KC levels were quantified by BCA and ELISA, respectively. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ (**a,c,d** unpaired two-tailed t test and **b** two-tailed Mann-Whitney test).

Figure 5. Prophylactic inhibition of cathepsin S is protective in the murine model of LPS-induced acute lung injury. Mice were treated with the cathepsin S inhibitor I.6 (100 mg/kg) or vehicle via intraperitoneal injection 24 h before receiving 1 mg/kg lipopolysaccharide (LPS) or saline vehicle via intratracheal instillation (n = 7 per group). Fifteen minutes later, mice received a second injection of I.6 and bronchoalveolar lavage fluid (BALF) was collected for analysis 16 h later and **a** total cell and **b** neutrophil counts were quantified. BALF **c** total protein and **d** KC concentrations were measured by BCA and ELISA, respectively. * $P < 0.05$, ** $P < 0.01$ (**a-c** unpaired two-tailed t test, **d** two-tailed Mann-Whitney test).

Figure 6. Cathepsin S inhibitor treatment selectively dampens inflammation in the caecal ligation and puncture mouse model of acute lung injury. Mice were untreated or received an intraperitoneal injection of the cathepsin S inhibitor I.6 (100 mg/kg) 30 min before undergoing caecal ligation and puncture (CLP) surgery. Mice were sacrificed 18 h post-CLP and peritoneal lavage fluid (PLF) and bronchoalveolar lavage fluid (BALF) were collected. PLF **a** total cell, **b** neutrophil and **c** monocyte/macrophage cell counts were quantified. ** $P < 0.01$, *** $P < 0.001$ (n = 6 per group, unpaired two-tailed t test). **d** Neutrophil and monocytic cells were expressed as a percentage of the total PLF cell count. *** $P < 0.001$ (n = 6 per group, two way ANOVA with Sidak's multiple comparisons test). BALF **e** total cell, **f** neutrophil and **g** monocyte/macrophage cell counts were quantified (n = 6 per group, two-tailed Mann Whitney test). **h** Neutrophil and

monocytic cells were expressed as a percentage of the total BALF cell count. ** $P < 0.01$ (two way ANOVA with Sidak's multiple comparisons test). BALF **i** KC and **j** IL-6 were quantified by ELISA ($n = 6$ per group). * $P < 0.05$, ** $P < 0.01$ (unpaired two-tailed t test).

Figure 7. Therapeutic inhibition of cathepsin S is protective in the murine model of LPS-induced acute lung injury. Mice received a single dose of the cathepsin S inhibitor I.6 (100 mg/kg) or vehicle via intraperitoneal injection 2 h after intratracheal lipopolysaccharide (LPS) (1 mg/kg) instillation (n = 8-11 per group). Bronchoalveolar lavage fluid (BALF) **a** total cell and **b** neutrophil counts were quantified. **c** Total protein and **d** KC concentrations in BALF were measured by BCA and ELISA, respectively. * $P < 0.05$, ** $P < 0.01$ (unpaired two-tailed t test).

Figure 8. PAR-1 antagonism reduces cathepsin S-induced inflammation *in vitro* and *in vivo*. **a,b** THP-1 macrophages were treated with 1 μg/mL active CTSS in the presence or absence of the PAR-1 antagonist SCH-530348 (10 μM). Cell supernatants were collected 24h later and levels of IL-8 and CXCL1 were quantified by ELISA. Results are representative of n = 3 independent experiments where each condition was plated in triplicate. **c-f** Mice received 10 mg/kg of the PAR-1 antagonist SCH-530358 (SCH) via intraperitoneal injection 30 min before receiving 5 μg active cathepsin S (CTSS) or vehicle by intratracheal instillation (n = 4-5 per group). Mice were allowed to recover for 24 h before bronchoalveolar lavage fluid (BALF) was collected and total cell and neutrophil counts were quantified. Total protein and KC levels in BALF were quantified by BCA and ELISA, respectively. ** $P<0.01$ *** $P<0.001$, **** $P<0.0001$ (unpaired two-tailed t test).

Figure 9. Cathepsin S inhibition has no significant effect on pulmonary inflammation in PAR-1 knockout mice. PAR-$1^{-/-}$ mice were treated with the cathepsin S inhibitor I.6 (100 mg/kg) or vehicle via intraperitoneal injection 24 h before receiving 1 mg/kg lipopolysaccharide (LPS) (*n* = 8-9 per group) or saline (*n* = 5-6 per group) via intratracheal instillation. Fifteen minutes later, mice received another injection of I.6 and were allowed to recover for 16 h before bronchoalveolar lavage fluid (BALF) was collected for analysis. BALF **a** total cell and **b** neutrophil counts were quantified. **c** Total protein and **d** IL-6 levels in BALF were quantified by BCA and ELISA, respectively.