Targeting of Cathepsin S Reduces Cystic Fibrosis-like Lung Disease


This manuscript has recently been accepted for publication in the *European Respiratory Journal*. It is published here in its accepted form prior to copyediting and typesetting by our production team. After these production processes are complete and the authors have approved the resulting proofs, the article will move to the latest issue of the ERJ online.

Copyright ©ERS 2019
Targeting of Cathepsin S Reduces Cystic Fibrosis-like Lung Disease

Donna M. Small1*, Ryan R. Brown1*, Declan F. Doherty1, Anthony Abladey1, Zhe Zhou-Suckow2, Rebecca J. Delaney1, Lauren Kerrigan1, Caoifa M. Dougan1, Keren S. Borensztajn3, Leslie Holsinger4, Robert Booth4, Christopher J. Scott5, Guillermo López-Campos6, J. Stuart Elborn6,7, Marcus A. Mall2,8,9, Sinéad Weldon1, Clifford C. Taggart1

1Airway Innate Immunity Research (AiiR) Group, Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen’s University Belfast, 97 Lisburn Road, Belfast, BT9 7BL, Northern Ireland.

2Department of Translational Pulmonology, Translational Lung Research Center Heidelberg (TLRC), German Center for Lung Research (DZL), University of Heidelberg, Heidelberg, Germany.

3INSERM UMRS_933; Université Pierre et Marie Curie, Hôpital Trousseau, Paris 75012, France.

4ViroBay Inc., Menlo Park, CA, United States.

5Centre for Cancer Research and Cell Biology, Queen’s University Belfast, Northern Ireland.

6Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen’s University Belfast, 97 Lisburn Road, Belfast, BT9 7BL, Northern Ireland.

7Respiratory Medicine, Imperial College and Royal Brompton Hospital, London, United Kingdom.

8Department of Pediatric Pulmonology and Immunology, Charité - Universitätsmedizin Berlin, Berlin, Germany.

9Berlin Institute of Health (BIH), Berlin, Germany.
* These authors contributed equally to the study

**Corresponding author:** Dr Sinéad Weldon, Airway Innate Immunity Research (AiiR) Group, Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, 97 Lisburn Road, Belfast, BT9 7BL, Northern Ireland. Tel: 00442890976382; Fax: 00442890632697; Email: s.weldon@qub.ac.uk.

**Take Home Message** – Cathepsin S is involved in inflammation, mucus production and lung tissue damage in a model of CF-like lung disease.
Abstract

Cathepsin S (CatS) is upregulated in the lungs of patients with cystic fibrosis (CF). However, its role in CF lung disease pathogenesis remains unclear.

In this study β-epithelial Na\(^+\) channel-overexpressing transgenic (βENaC-Tg) mice, a model of CF-like lung disease, were crossed with cathepsin S null (CatS\(^{-/-}\)) mice or treated with the CatS inhibitor VBY-999. Levels of active CatS were elevated in the lungs of βENaC-Tg mice compared to wild-type (WT) littermates. CatS\(^{-/-}\) βENaC-Tg mice exhibited decreased pulmonary inflammation, mucus obstruction and structural lung damage compared to βENaC-Tg mice. Pharmacological inhibition of CatS resulted in a significant decrease in pulmonary inflammation, lung damage and mucus plugging in the lungs of βENaC-Tg mice. In addition, instillation of CatS into the lungs of WT mice resulted in inflammation, lung remodelling and upregulation of mucin expression. Inhibition of the CatS target, protease-activated receptor-2 (PAR-2), in βENaC-Tg mice resulted in a reduction in airway inflammation and mucin expression indicating a role for this receptor in CatS-induced lung pathology.

Our data indicate an important role for CatS in the pathogenesis of CF-like lung disease mediated in part by PAR-2 and highlights CatS as a therapeutic target.
**Introduction**

Cathepsin S (CatS) is expressed by professional and non-professional antigen presenting cells and is a crucial component in MHC class II antigen processing and presentation [1]. CatS is a potent elastinolytic and collagenolytic cysteine protease with the ability to maintain its enzymatic activity in more neutral pH environments [2, 3]. Previous work has demonstrated that levels of CatS are elevated in lungs of patients with CF lung disease [4–7] and chronic obstructive pulmonary disease (COPD) [8] and that CatS correlates significantly with a decline in lung function and increased pulmonary neutrophilic infiltration into the CF lung [7]. Furthermore, CatS possesses the ability to cleave and inactivate host defence proteins such as lactoferrin, β-defensins, LL-37, SLPI and surfactant protein A [4, 9–12], which may increase susceptibility to infection in patients with these muco-obstructive lung diseases.

However, the role of CatS in CF lung disease pathobiology has yet to be fully elucidated. In this study, we employed a mouse model of CF-like lung disease to study the functionality of CatS *in vivo*. Overexpression of the β subunit of the epithelial Na⁺ channel (ENaC) results in airway surface dehydration and impaired mucus clearance producing chronic lung disease characterized by airway mucus obstruction, inflammation and structural lung damage in mice [13]. Therefore, the βENaC-Tg mouse model shares many characteristics with human CF and is a useful *in vivo* model to study the role and therapeutic potential of CatS in CF-like lung disease [14].

Following demonstration that elevated CatS levels and activity were a feature of the βENaC-Tg lung, we crossed βENaC-Tg mice onto a CatS⁻/⁻ background and assessed airway
inflammation, mucus obstruction and lung damage. To validate observations in this model, we also investigated the effects of therapeutic targeting of CatS via a small molecule inhibitor in the diseased lung. In addition, we instilled CatS directly into the lungs of wild-type (WT) mice to investigate effects on inflammatory cell recruitment, mucin production and lung remodelling. To further understand how CatS functions in the lung, we assessed the role of the protease-activated receptor-2 (PAR-2), a target for CatS [15–20], in mediating the effects of CatS in βENaC-Tg mice.

Methods

Full details are available in the online supplement (Supplemental Methods).

Abbreviated Methods

All experimentation was carried out in accordance with the Animal (Scientific Procedures) Act 1986 and current guidelines approved by the Queen’s University Ethical Review Committee. All mice used in this study were from a C57Bl/6 background. This breeding program generated 4 genotypes of mice for investigation; WT, CatS−/−, βENaC-Tg and CatS−/− βENaC-Tg mice. Offspring were genotyped by PCR as previously described [13, 21, 22] and mice were used from the newborn stage. Newborn βENaC-Tg mice and WT littermates were dosed once daily for 14 days via a subcutaneous injection of the CatS inhibitor VBY-999 (100 mg/kg; Virobay Inc., Menlo Park, California, USA), the selective PAR-2 peptide antagonist FSLLRY-NH2 (4 mg/kg; R&D Systems, Abingdon, UK), or their respective vehicle controls, dextrose (Dex) solution and sterile water. WT mice were intratracheally (IT) instilled with recombinant human CatS (rhCatS, 5 μg, Merck Millipore, Nottingham, UK). Bronchoalveolar lavage (BAL) fluid was collected for analysis of inflammatory markers and cell recruitment as
previously described [13, 23]. Histological staining and morphometric assessments of airspace enlargement, alveolar wall destruction and airway mucus content were conducted as previously described [24–26].

Results

Upregulation of CatS in the lungs of βENaC-Tg mice

In order to validate the βENaC-Tg mouse model as a suitable model to test the effects of targeting CatS, we determined the status of CatS levels and activity in βENaC-Tg mice. We observed increased levels of CatS protein (Figure 1A) and CatS activity in BAL fluid (Figure 1B) from βENaC-Tg mice compared to the healthy WT counterparts. In addition, we observed increased CatS staining in the βENaC-Tg mouse lungs, which was primarily localised to macrophages, with some epithelial staining also present (Figure 1C). These findings demonstrate that, like human CF, increased levels of active CatS are a feature of the βENaC-Tg mouse model.

Genetic ablation of CatS results in decreased pulmonary inflammation in βENaC-Tg mice

In order to investigate the role of CatS in the pathogenesis of CF-like lung disease, we first assessed pulmonary inflammation in CatS−/−βENaC-Tg, βENaC-Tg and control mice (CatS−/− and WT littermates). There was no significant change in BAL fluid total cell numbers in CatS−/− compared to WT control mice (Figure 2A). As expected, βENaC-Tg mice demonstrated significantly increased cell infiltration compared to WT and CatS−/− mice (Figure 2A). CatS−/− βENaC-Tg mice BAL total cell counts were significantly decreased compared to βENaC-Tg
mice (Figure 2A) evidenced by a reduction in both macrophage (Figure 2B) and neutrophil cell populations (Figure 2C). BAL fluid total protein concentrations were significantly higher in βENaC-Tg mice compared to control mice (Figure 2D). However, total protein content was significantly reduced in CatS⁻/⁻βENaC-Tg mice compared to βENaC-Tg mice suggesting a reduction in the severity of lung damage and inflammation (Figure 2D). In addition, we observed significantly decreased levels of the chemokine KC (Figure 2E) in the BAL fluid of CatS⁻/⁻βENaC-Tg mice compared to βENaC-Tg mice.

**Genetic ablation of CatS results in decreased structural lung damage and mucus obstruction in the lungs of βENaC-Tg mice**

Mean linear intercept length (Lₘ) is a measurement of distal airspace enlargement. Airway sections stained with haematoxylin and eosin (H&E, representative images Figure 3A) demonstrated no difference in Lₘ between CatS⁻/⁻ and WT mice (Figure 3B). As expected, a significant increase in Lₘ was evident in the lungs of βENaC-Tg mice compared to the control mice (Figure 3B). In comparison to the βENaC-Tg mice, Lₘ was significantly reduced in CatS⁻/⁻ βENaC-Tg mice (Figure 3B), suggesting that increased CatS in the lungs of βENaC-Tg mice may play a role in distal airspace enlargement. In addition, the level of alveolar septal destruction, as assessed by the measurement of destructive index (DI), was significantly reduced in CatS⁻/⁻ βENaC-Tg mice compared to βENaC-Tg mice with no difference in DI measurements observed between WT and CatS⁻/⁻ control mice (Figure 3C). Given the levels of airspace enlargement and damage observed, these findings indicate that the augmented CatS levels found in βENaC-Tg may play an important role in mediating lung tissue damage and remodelling in CF-like lung disease. Histological staining and quantification of airway mucus content demonstrated a significant reduction in airway mucus obstruction in CatS⁻/⁻
βENaC-Tg mice compared to βENaC-Tg mice (Figure 3D-E). Overall, these findings suggest that CatS is implicated in the pathogenesis of chronic inflammation, lung tissue damage and the development of mucus plugging in CF-like lung disease.

**Pharmacological inhibition of CatS reduces airway inflammation in βENaC-Tg mice**

CatS was targeted using a small molecule inhibitor, VBY-999 [27], in βENaC-Tg mice. Inhibition of CatS alters invariant chain (Ii) processing and results in the accumulation of the 10 kDa intermediate (p10) [1, 22]. To determine whether VBY-999 was successfully targeting CatS in the lung, we assessed the accumulation of the Ii p10 fragment by Western blotting. The lungs of βENaC-Tg mice treated with VBY-999 exhibited an accumulation of Ii p10 levels compared to vehicle (Dex) treated βENaC-Tg mice (Figure 4A). Initially, we observed that βENaC-Tg mice treated with VBY-999 from birth for a period of 14 days demonstrated a significant increase in survival compared to Dex treated mice (Supplemental Figure S1). βENaC-Tg mice treated with VBY-999 had significantly lower BAL total inflammatory cellular infiltrate compared to vehicle treated βENaC-Tg mice (Figure 4B). There was no effect on total cell numbers in the control mice regardless of treatments. VBY-999 treatment significantly decreased the number of BAL macrophages (Figure 4C) and neutrophils (Figure 4D) in βENaC-Tg mice. In addition, we observed significantly decreased levels of KC (Figure 4E) in the BAL fluid of VBY-999 treated βENaC-Tg mice compared to vehicle treated βENaC-Tg mice. This data suggests that inhibition of CatS suppresses pulmonary inflammation in this mouse model of CF-like lung disease.
Inhibition of pulmonary CatS reduces the level of structural lung damage and lung protein content in βENaC-Tg mice

Protein levels were assessed by BCA assay and VBY-999 treated βENaC-Tg mice had lower levels of total protein in BAL fluid compared to vehicle treated βENaC-Tg mice (Figure 5A). Lm and DI were measured using H&E stained lung sections from each group (Figure 5B) as markers of airspace enlargement and alveolar destruction, respectively. There was a significant decrease in Lm in βENaC-Tg mice treated with VBY-999 compared to vehicle (Figure 5C). Additionally, a significant decline in alveolar wall destruction was observed in VBY-999 treated βENaC-Tg mice compared to vehicle treated βENaC-Tg mice (Figure 5D). These data demonstrate that inhibition of CatS resulted in a reduction in airway damage in CF-like lung disease.

Inhibition of CatS results in reduced airway mucus content in βENaC-Tg mice

Histological analyses demonstrated that VBY-999 treated βENaC-Tg mice presented with a significant decrease in airway mucus content compared to Dex treated βENaC-Tg mice (Figure 6A-B). Given our previous findings of altered mucus content in CatS−/−βENaC-Tg mice (Figure 3D-E), we investigated the expression of the mucins Muc5ac and Muc5b and the goblet cell marker Gob5 (Figure 6C-E). Treatment of βENaC-Tg mice with VBY-999 from birth significantly reduced the expression of Muc5ac (Figure 6C) and Gob5 (Figure 6E) expression compared to Dex treated βENaC-Tg mice. In contrast, inhibition of CatS had no effect on the expression of Muc5b (Figure 6D).
Effect of CatS pulmonary instillation on lung inflammation and mucin production

Direct instillation of rhCatS into the lungs of WT mice induced significant recruitment of inflammatory cells (Figure 7A) largely attributed to the influx in macrophages (Figure 7B) and neutrophils (Figure 7C). Although there was a significant increase in the expression of Muc5ac (Figure 7D) and Gob5 (Figure 7E) in the lungs of mice that received CatS, Muc5B expression was unchanged (Figure 7F). In addition, rhCatS administration was associated with goblet cell hyperplasia (Figure 7G-I). KC levels in BAL fluid (Figure 7J) were significantly increased following rhCatS instillation and evidence of lung damage was also observed with significantly increased levels of the elastin breakdown product, desmosine, detectable in the lungs of CatS treated mice (Figure 7K).

CatS induced lung inflammation and mucin expression is mediated in part via PAR-2

CatS has previously been shown to activate the receptor PAR-2 leading to symptoms of inflammation, itch and pain and has been associated with conditions such as atopic dermatitis and colitis as well as endothelial dysfunction associated with lupus nephritis and diabetes diabetes [15–20]. However, a role for PAR-2 in CatS-mediated lung inflammation or mucin expression in vivo has not been described to date. Treatment of WT mice with a PAR-2 antagonist (FSLLRY-NH₂) reduced the infiltration of inflammatory cells into the lungs of rhCatS-treated mice (Supplemental Figure E2). Therefore, the impact of a PAR-2 antagonist in the βENaC-Tg mouse lung was evaluated. Similar to genetic and pharmacological inhibition of CatS, blockade of PAR-2 in βENaC-Tg mice lead to a significant reduction in lung inflammation with a decline in total inflammatory cell lung infiltrate (Figure 8A), macrophages (Figure 8B) and neutrophils (Figure 8C) in the lungs of βENaC-Tg mice compared to vehicle (H₂O) treated βENaC-Tg mice. In addition, BAL fluid KC levels were
significantly decreased in PAR-2 antagonist treated βENaC-Tg mice compared to vehicle treated βENaC-Tg mice (Figure 8D). The expression of Muc5ac and Gob5 (Figure 8E-F) were decreased in the lungs of PAR-2 antagonist treated βENaC-Tg mice compared to vehicle treated βENaC-Tg mice. However, there was no reduction in the level of airspace enlargement or alveolar destruction as measured by \( L_m \) and DI respectively (data not shown). Overall, these data indicate a significant role for PAR-2 in CatS-mediated lung inflammation and mucus production in the lungs of βENaC-Tg mice.

**Discussion**

CatS is elevated in a number of muco-inflammatory conditions like CF lung disease and COPD and is viewed as a potential therapeutic target for various diseases (recently reviewed in [28, 29]). We have previously shown that in the CF lung, increased CatS activity is associated with a decline in lung function [7]. In addition, pulmonary CatS levels and activity significantly correlated with neutrophilic infiltration in the CF lung [7], substantiating the hypothesis that CatS may play a role in pulmonary neutrophil recruitment [30], which is associated with CF lung disease. In the current study, we show that elevated CatS is also a key feature of the pulmonary disease of βENaC-Tg mice, a model of CF-like lung disease. The βENaC-Tg mouse provides a relevant model for investigating CF lung disease pathogenesis, particularly the interactions between ion transport, airway surface liquid and mucociliary clearance [13, 14]. However, because cystic fibrosis transmembrane conductance regulator (CFTR) function is normal in the βENaC-Tg mouse lung [13], this model does not allow the study of pathophysiological changes associated directly with mutant CFTR, such as cellular dysfunction and mucus composition. Knockdown of CatS by pharmacological (VBY-999
inhibitor) or genetic methods (CatS\textsuperscript{−/−}) in the βENaC-Tg model resulted in a significant decline in both lung macrophage and neutrophil cell populations providing further evidence of a role for CatS in mediating pulmonary inflammation [30–32].

Previous work in βENaC-Tg mice demonstrated that genetic knockdown of NE and MMP-12 significantly reduced structural lung damage, but had no effect on mucus obstruction and pulmonary mortality [33, 34]. In this study, pharmacological inhibition and genetic ablation of CatS led to not only reduced airway structural damage and pulmonary inflammation but also ameliorated airway mucus plugging and improved survival rates of neonatal mice. These data indicate the involvement of CatS in inflammatory cell recruitment, lung tissue damage and the development of mucus plugging. In agreement, we have also shown that direct administration of CatS to the lungs of WT mice induced neutrophil recruitment and increased KC levels in the lungs of these mice. Pulmonary CatS instillation resulted in significantly increased expression of the mucin Muc5ac and Gob5, a marker associated with goblet cell hyperplasia [35, 36], as well as the number of goblet cells and mucus producing cells, although no effect on Muc5b expression was observed. These results indicate a role for CatS mediated lung inflammation in the CF lung and suggest that CatS may contribute to airway mucus plugging via goblet cell hyperplasia and increased mucus production.

These effects may be due, in part, to the activation of PAR-2 by CatS which has previously been demonstrated in other organ systems outside of the lung [15–20]. Research to date suggests that PAR-2 activation is associated with inflammation, leukocyte recruitment, and mucin expression [37–40]. In this study, we have shown that antagonism of PAR-2 lead to a reduction in the inflammatory cell infiltrate in the lungs of mice exposed to CatS as well as in
the βENaC-Tg lung. In addition, reductions in BAL fluid KC levels and the expression of Muc5ac and Gob5 were also observed in PAR-2 antagonist treated βENaC-Tg mice. In agreement with previous reports, our data suggest that CatS may exert its effects on pulmonary inflammation and mucus plugging, at least in part, via PAR-2. In contrast, while knockdown or inhibition of CatS reduced lung tissue destruction in the βENaC-Tg mice, we did not observe a reduction in lung damage (Lm/DI) in PAR-2 antagonist treated βENaC-Tg mice, suggesting that CatS may be acting via other pathways to elicit lung damage. For example, we have shown that direct instillation of CatS into the WT mouse lung can rapidly induce (within 24 hr) desmosine levels in BAL fluid indicating that the effects of CatS may also be mediated directly on lung tissue as well as via PAR-2.

In summary, our investigations show that CatS is elevated in an in vivo model of CF-like lung disease and may promote pulmonary cell infiltration, mucus obstruction and structural lung damage associated with chronic lung diseases such as CF. These results suggest that CatS may play a vital role in CF-like lung disease pathogenesis, which ultimately leads to the loss of tissue structural integrity and lung function. As previously mentioned, CatS possesses the ability to cleave and inactivate host antimicrobial proteins [4, 9–12], which may increase susceptibility to infection in patients with muco-obstructive lung diseases such as CF and COPD. However, further work is needed to investigate the impact of elevated CatS activity and the effects of pulmonary CatS inhibition on Pseudomonas infection in the context of chronic lung disease. Our results indicate that CatS may be a promising therapeutic target to halt the progression of airway inflammation, mucus obstruction and pulmonary damage associated with CF lung disease.
Acknowledgements

Supported by Cystic Fibrosis Foundation (TAGGAR12/0, WELDON15G0, WELDON18G0), EU FP7 Grant (CFMATTERS; grant agreement number 603038), Engineering and Physical Sciences Research Council (studentships to AA and LK), QUB start-up funds (SW), Medical Research Council Confidence in Concept Programme (SW, CCT), Department for the Economy (CCT) and German Federal Ministry of Education and Research (82DZL004A1 to MAM).
References


**Figure Legends**

**Figure 1. Levels and activity of CatS are upregulated in the lungs of βENaC-Tg mice.**

(A) Western blot of CatS in BAL fluid from βENaC-Tg and WT mice (n = 4 per group). (B) CatS activity in BAL fluid from WT and βENaC-Tg mice (n = 10 per group) was determined using FR-AMC substrate (pH 7.5). Results expressed as change (Δ) in relative fluorescence units (ΔRFU) over time. *** P < 0.001. (C) Immunohistochemical staining for CatS on lung sections from WT and βENaC-Tg mice. Red arrows denote macrophage CatS staining, yellow arrows indicate epithelial CatS staining.

**Figure 2. Genetic ablation of CatS reduces airway inflammation in βENaC-Tg mice.**

βENaC-Tg mice were crossed with CatS−/− mice to produce CatS−/−βENaC-Tg mice. BAL fluid was collected from WT, CatS−/−, βENaC-Tg and CatS−/−βENaC-Tg mice. BAL (A) total cell, (B) macrophage, (C) neutrophil counts and (D) total protein concentration were quantified (n = 7-10 per group). (E) BAL fluid levels of KC were assessed by ELISA (n = 5-7 per group). * P < 0.05, ** P < 0.01, *** P < 0.001.

**Figure 3. Lack of CatS in βENaC-Tg mice reduces airway mucus obstruction and lung tissue damage.**

(A) Representative images of lung sections from in WT, CatS−/−, βENaC-Tg and CatS−/−βENaC-Tg mice stained with haematoxylin and eosin (H&E) used to assess airway damage. Scale bar = 100 μm. (B) Mean linear intercept length (Lm) and (C) destructive index (DI) measurements were assessed from the H&E stained lung sections. (D) Representative images of lung sections stained with AB-PAS. Scale bar = 100 μm. (E) Airway mucus quantification
expressed as the percentage of the total airway containing mucus. n = 7-10 per group; * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 4. Pharmacological targeting of CatS leads to a reduction in pulmonary inflammation in βENaC-Tg mice.

WT and βENaC-Tg mice were treated daily with the CatS inhibitor VBY-999 (VBY; 100 mg/kg) or dextrose vehicle control (Dex) for 14 days. (A) Western blot of p10 in lung homogenate from Dex and VBY-999 treated βENaC-Tg mice (n = 4 per group). BAL (B) total cell, (C) macrophage and (D) neutrophil counts were quantified (n = 10-15 per group). (E) BAL fluid levels of KC were quantified by ELISA (n = 9-15 per group). * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 5. CatS inhibition reduces airway tissue damage in βENaC-Tg mice.

WT and βENaC-Tg mice were treated daily with the CatS inhibitor VBY-999 (VBY, 100 mg/kg) or dextrose vehicle control (Dex) for 14 days. (A) BAL fluid protein levels were quantified by BCA assay (n = 7-8 per group). (B) Representative images of lung sections stained with haematoxylin and eosin (H&E) used to assess airway damage. Scale bar = 100 µm. (C) Mean linear intercept length (Lm) and (D) destructive index measurements were assessed from the H&E stained lung sections (n = 6-7 per group). * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 6. Pharmacological targeting of CatS reduces airway mucus plugging and mucin gene expression in βENaC-Tg mice.

WT and βENaC-Tg mice were treated daily with the CatS inhibitor VBY-999 (VBY, 100 mg/kg) or dextrose vehicle control (Dex) for 14 days. Representative images of (A) WT and βENaC-
Tg mice treated with either dextrose or VBY-999 lung sections stained with AB-PAS to assess airway mucus content, scale bar = 100 µm. (B) Airway mucus quantification in WT and βENaC-Tg mice treated with Dex or VBY-999. Results are expressed as the percentage of the total airway area containing mucus (n = 9-13 per group). Expression of (C) Muc5ac, (D) Muc5b and (E) Gob5 in lung tissue were analysed by qPCR (n = 6-14 per group). * P < 0.05, ** P < 0.01, *** P < 0.001.

**Figure 7.** CatS instillation induces inflammation and mucus in the lungs.

WT mice received a single intratracheal instillation of recombinant human CatS (rhCatS, 5 µg) or sodium acetate buffer control (Con). After 24 hr, BAL fluid was collected and (A) total cells, (B) macrophage and (C) neutrophil cell counts were quantified. Expression of (D) Muc5ac, (E) Gob5 and (F) Muc5b in lung tissue were analysed by qPCR. (G) Representative images of lung sections stained with Alcian Blue-Periodic Acid Schiff (AB-PAS). Scale bar = 50 µm. (H) Goblet and (I) mucus producing cells were quantified from AB-PAS stained lung sections. Counts are expressed as cells/mm basement membrane (BM). BAL fluid (J) KC and (K) desmosine levels were measured by ELISA. n = 4-9 per group; * P < 0.05, ** P < 0.01, *** P < 0.001.

**Figure 8.** PAR-2 inhibition reduces airway inflammation in βENaC-Tg mice.

WT and βENaC-Tg mice were treated daily with the PAR-2 peptide antagonist FSLLRY-NH₂ (4 mg/kg) or sterile water vehicle control (H₂O) for 14 days. BAL (A) total cells, (B) macrophage and (C) neutrophil counts and (D) KC levels were quantified (n = 6-11 per group). Expression of (E) Muc5ac and (F) Gob5 in lung tissue were analysed by qPCR (n = 9 per group). * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 1

A

WT | βENaC-Tg
---|---
25 kDa

B

<table>
<thead>
<tr>
<th>Cathepsin S activity (ΔRFU)</th>
<th>WT</th>
<th>βENaC-Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1000</td>
<td><strong>3000</strong></td>
</tr>
</tbody>
</table>

***

C

WT | βENaC-Tg
---|---

[Images of WT and βENaC-Tg samples with arrows indicating differences]
Figure 3

A. WT and CatS^−/−

βENaC-Tg
CatS^−/−βENaC-Tg

B. Lumen diameter (μm)

WT CatS^−/− βENaC-Tg CatS^−/−βENaC-Tg

C. Destructive Index (%)

WT CatS^−/− βENaC-Tg CatS^−/−βENaC-Tg

D. WT and CatS^−/−

βENaC-Tg
CatS^−/−βENaC-Tg

E. Airway mucus content (%)

WT CatS^−/− βENaC-Tg CatS^−/−βENaC-Tg

*** *** **

*** *** ***

*** *** *

*** *** ***

*** *** ***
Online Supplement

Supplemental Methods

Mice

All mice were housed in specific pathogen free (SPF) facilities where housing and experimentation was carried out in accordance with the Animal (Scientific Procedures) Act 1986 and current guidelines approved by the Queen’s University Ethical Review Committee. The animals were maintained on a 12 hr cycle of light followed by 12 hr cycle of darkness with free access to chow and water. βENaC-Tg mice were generated as previously described [1] and backcrossed on to the C57Bl/6 background [2]. Cathepsin S (CatS)−/− mice were generated as previously described [3] and were intercrossed with βENaC-Tg mice for five generations to generate the double mutant CatS−/−βENaC-Tg mice. The above-mentioned breeding programs generated four genotypes of mice for investigation; βENaC-Tg mice, WT littermates, CatS−/− mice and CatS−/−βENaC-Tg mice. Offspring from all the breeding programs were genotyped by PCR and mice were studied at day 14. For CatS instillation experiments, C57Bl/6 mice were purchased from Charles River Laboratories (UK).

In vivo targeting of cathepsin S and PAR-2

VBY-999 was provided by Virobay Inc, Menlo Park, California, USA. VBY-999 is a selective and reversible inhibitor of CatS with an inhibition constant \( K_{\text{app}} \) of 290 pM on the purified human CatS enzyme and 690 pM on mouse CatS [4]. VBY-999 demonstrates over 3,000-fold selectivity versus the other related cathepsins such as K, L, B, and F and has no detectable activity against other cysteine, serine, and aspartyl proteases tested [4]. FSLLRY-NH2 is a selective PAR-2 peptide antagonist (R&D Systems, Abingdon, UK) prepared in sterile distilled
water. Newborn βENaC-Tg mice and WT littermates were dosed once daily for 14 days via a subcutaneous injection of either VBY (100 mg/kg), FSLLRY-NH2 (4 mg/kg), dextrose solution or sterile water. Experiments were terminated 16 hr after the last subcutaneous treatment.

Cathepsin S lung instillation

C57Bl/6 female mice used in these experiments were purchased from Charles Rivers Laboratories. Under anaesthesia, recombinant human CatS (rhCatS; 5 μg; Merck Millipore, Nottingham, UK) in sodium acetate buffer (pH 5.5) and buffer alone were intratracheally (IT) instilled with the aid of a blunted 24g IV catheter (BD Biosciences, UK) into the lungs of mice in a final volume of 50 μl. To target PAR-2 in this model, WT mice were treated with the PAR-2 peptide antagonist FSLLRY-NH2 (4 mg/kg) or sterile water vehicle control intraperitoneally 30 min prior to rhCatS or sodium acetate buffer control instillation (IT). Experiments were terminated 24 hr after IT instillation.

Bronchoalveolar lavage fluid collection and differential cell staining

Bronchoalveolar lavage (BAL) fluid was collected as previously described [1, 5]. Briefly, mice were terminally anesthetized with an intra-peritoneal injection of ketamine (Ketaset, Bayer) / xylazine (Rompum, Bayer) cocktail (120 mg/kg and 16 mg/kg, respectively). A blunted 26g needle was inserted through a small incision in the upper trachea and tied in place with 4.0 Mersilk silk sutures (Ethicon, Livingston, UK). BAL fluid was collected, centrifuged at 4 °C and the cell-free BAL fluid was stored at -80°C. Total cells counts were determined on BAL cell pellets by staining with trypan blue stain by counting on a haemocytometer. Differential cell counts were evaluated following cytospin preparations onto coated cytoslides (Shandon / Thermo Scientific, UK). Cells were stained with May-Grünwald Giemsa stains (VWR,
Leicestershire, UK) and visualized on the Leica DM5500B microscope. Images were captured using the image analysis Leica AL software, (version 3.7) under X 40 objective lens. Histological cell counts were conducted on each cytospin with at least 400 cells counted with the aid of the Image J software.

**Immunohistochemical staining for cathepsin S**

Paraffin-embedded tissue sections (6 µm) were de-paraffinized in two changes of Histoclear (Fisher Scientific, Leicestershire, UK) and an ethanol-to-water gradient consisting of absolute ethanol, 95% ethanol and 70% ethanol for 5 min each. The sections underwent antigen-retrieval (microwave: citrate buffer pH6) blocked with 10% horse serum for 1 hr at room temperature, then incubated overnight at 4°C with goat anti-CatS antibody (1:100 dilution) or an equivalent dilution of goat IgG. After washing in PBS-T, sections were incubated with an anti-goat secondary antibody followed by the avidin-biotin complex (ABC) conjugated with horseradish peroxidise (ABC kit, Vector Laboratories, Peterborough, UK) for 30 min at room temperature. CatS was detected using 3,3-diaminobenzidine (DAB) (Dako, Agilent Technologies, Cheshire, UK), followed by counterstaining with Harris haematoxylin solution for 3 min. The sections were washed in running tap water and blued in 0.2% ammonia water. After rinsing in tap water, the sections were de-hydrated through a water-to-ethanol gradient consisting of 70% ethanol, 95% ethanol and absolute ethanol for 5 min each. Following two changes of Histoclear, the slides were finally mounted in DPX mounting media and visualised using a Leica DM5500B microscope. Images were captured using the Leica AL image software (Version 3.7).
Histological and morphometric analyses

After BAL fluid collection, the lungs were inflated with 10% buffered formalin (Sigma-Aldrich, Dorset, UK) to 25 cm of fixative pressure. Lungs were histologically processed, sectioned to 6 μm thickness and stained with haematoxylin and eosin stain for mean linear intercept (chord) length (Lm) which is a measurement of distal airspace enlargement as previously described [6]. Briefly, histologic images from all the lobes were randomly visualised with the Leica DM5500B microscope and images captured using the image analysis Leica AL software (Version 3.7) under X 20 objective lens. With the aid of Image J and line tool, the mean linear intercept length was assessed by dividing the sum of the lengths of all lines in all frames by the number of intercepts between alveolar septi and counting lines, as previously described [6–9]. For each animal, a minimum of 200 intercepts sampled in 5 fields of view from different lobes were measured. Additionally, alveolar septal destruction was determined in the lung sections as destructive indices using a grid of points (42-point grid) overlying images of lung sections and Image J analysis software. Points were classified to lie within destructed (D) or intact (N, normal) alveolar ducts in the lung parenchyma and the destructive index (DI) was calculated by DI = 100 x D / (D + N) as previously described [10, 11]. For each animal, a minimum of 210 points was sampled and classified in 5 fields in different lobes.

Alcian Blue-Periodic Acid Schiff (AB-PAS) staining

Paraffin-embedded, serially sectioned (6 μm) lung tissue sections were de-paraffinized in two changes of Histoclear (Fisher Scientific, Leicestershire, UK), followed by re-hydration in ethanol-to-water gradient, which consisted of absolute alcohol, 95% alcohol and 70% alcohol for 5 min each. The tissue sections were washed briefly in distilled water and stained
in Alcian blue solution (Sigma-Aldrich) for 30 min. The lung tissue sections were gently rinsed in running tap water for 1 min and placed into distilled water prior to the 5 min incubation in Periodic acid (0.5%, Sigma-Aldrich). The sections were then washed in two quick changes in distilled water and incubated in Schiff's reagent (Merck, Nottingham, UK) for 15 min. Slides were briefly dipped into distilled water and dehydrated in two changes of 95% alcohol and absolute alcohol for 2 min each. Slides were cleared in two changes of Histoclear and finally mounted in DPX mounting media (Sigma-Aldrich). All imaging was carried out on Leica DM5500B microscope and captured using the image analysis software Leica AL software and quantified using ImageJ analysis. Percentage mucus volume was established in the non-inflated left lungs from the same mice used for MLI and DI measurements. Intraluminal airway mucus was measured by assessing the percentage content of AB-PAS positive mucosal material per surface area of the airway lumen.

**Goblet and mucus producing cell quantification**

Goblet cells and mucus producing cells were quantified from AB-PAS stained lung sections imaged at x20 objective. Goblet cells were defined as having a height of more than a third of the epithelial height and lying at least part epithelial. Mucus producing cells include goblet cells and pre-goblet cells. Pre-goblet cells are AB-PAS positive cells not falling into the goblet cell criteria, typically only an apical streak. The length of the basement membrane was measured and counts were expressed as cells/mm basement membrane.
**Cathepsin S activity assay**

CatS activity in BAL fluid from 2 week old WT and βENaC-Tg was determined using the substrate Z-Phe-Arg-7-amido-4-methylcoumarin, Hydrochloride (FR-AMC; Merck) as previously described [12]. Fluorescence (substrate turnover) was determined by excitation at 360 nm and emission at 460 nm in a 96-well microplate reader (Synergy HT using Gen5™ software, BioTek UK, Swindon). Results are expressed as the change (Δ) in relative fluorescence units (ΔRFU) over a 60 min period.

**ELISAs**

BAL fluid KC (R&D Systems) and desmosine (Cusabio, Wuhan, China) levels were quantified as per the manufacturer’s instructions.

**BCA assay**

BAL fluid total protein concentrations were determined using the BCA method (Pierce BCA Assay, Thermo Scientific) as per the manufacturer’s instructions.

**Western Blotting**

Denatured lung lysate or BAL fluid samples were separated on 12% SDS-PAGE gels and transferred onto nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). Membranes were blocked and incubated with anti-CD74 (BD Biosciences, Oxford, UK), anti-CatS (R&D Systems) and γ-tubulin (Sigma-Aldrich) overnight at 4°C. Membranes were washed and incubated with HRP-conjugated secondary antibodies for 1 hr at RT. Following further washing, membranes were developed using chemiluminescent substrate (Western
Lightning, PerkinElmer, Coventry, UK) and viewed using Syngene G:Box and GeneSnap software (Syngene, Cambridge, UK).

**Real-Time RT-PCR**

Total RNA was extracted from mouse lungs using TRI Reagent® (Sigma-Aldrich) as per manufacturer’s instructions. Gene expression was analysed by real-time polymerase chain reaction using inventoried TaqMan® gene expression assays for Muc5ac (Mm01276718_m1), Muc5B (Mm00466391_m1), Gob5 (Mm01320697_m1) and GAPDH (Mm99999915_g1) according to manufacturer’s instructions (Applied Biosystems, Thermo Fisher Scientific). The expression of target genes relative to GAPDH was determined using the $2(-\Delta\Delta Ct)$ method.

**Statistics**

All data were analysed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Data are presented as mean ± SEM. Means were compared by unpaired $t$-test, Mann Whitney test, one-way analysis of variance (ANOVA) or Kruskal-Wallis test as appropriate. $P < 0.05$ was accepted to indicate statistical significance. Survival curves were compared using Kaplan-Meier log rank analysis.
References


Supplemental Figures

**Figure S1. Inhibition of CatS increases the survival of βENaC-Tg mice.**

Kaplan-Meier survival curve of βENaC-Tg mice treated with vehicle (5% dextrose, n = 118) or the CatS inhibitor (VBY-999, 100 mg/kg; n = 103) by subcutaneous injection every day from birth for 14 days. * P = 0.0482, Log-rank (Mantel-Cox) test.

**Figure S2. PAR-2 inhibition reduces CatS-induced airway inflammation in WT mice.**

WT mice were treated with the PAR-2 peptide antagonist FSLLRY-NH2 (4 mg/kg) or sterile water vehicle control (H2O) intraperitoneally 30 min prior to CatS (rhCatS, 5 μg) or sodium acetate buffer control (Con) intratracheal instillation. After 24 hr, BAL fluid was collected and (A) total cells and (B) neutrophil cell counts were quantified. n = 4-7 per group. ** P < 0.01, *** P < 0.001.