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_{iapp} 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-NH_2 (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 (L_m) 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 Schiffs reagent (Merck, Nottingham, UK) for 15 min. Slides were briefly dipped into distilled water and de-hydrated in two changed 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(−ΔΔCt) 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

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Supplemental Figures

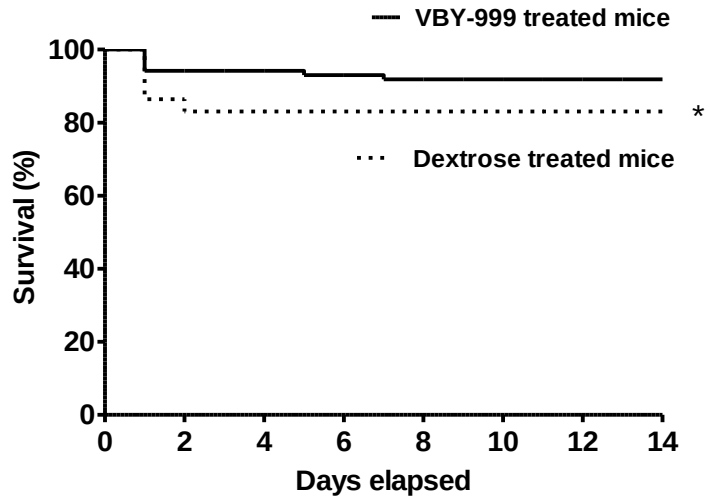

Figure S1. Inhibition of CatS increases the survival of βENaC-Tg mice.

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.

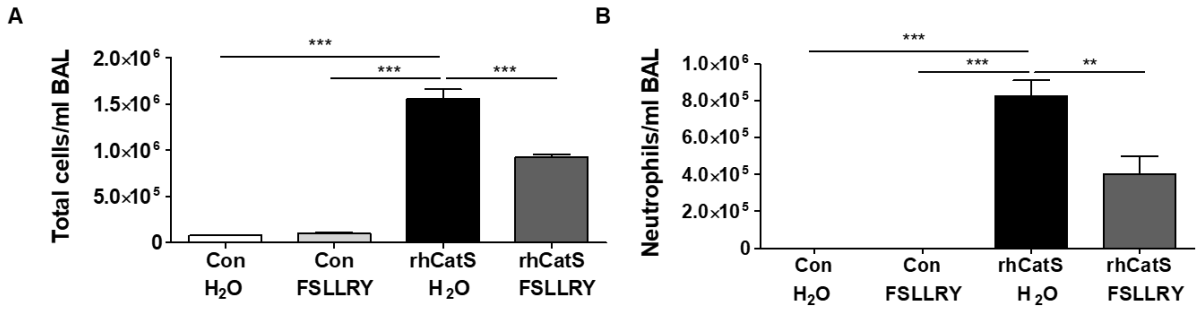

Figure S2. PAR-2 inhibition reduces CatS-induced airway inflammation in WT mice.

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.