Cathepsin K in Lymphangioleiomyomatosis: LAM Cell-Fibroblast Interactions Enhance Protease Activity by Extracellular Acidification.

Short Title: Cathepsin K in Lymphangioleiomyomatosis

Arundhati Dongre[1], Debbie Clements[1], Andrew J Fisher[2], Simon R Johnson[1].

1. Division of Respiratory Medicine and Respiratory Research Unit, University of Nottingham
2. Institute of Transplantation, Newcastle upon Tyne Hospitals NHS FT and the Institute of Cellular Medicine, Newcastle University

Address where research was performed: Division of Respiratory Medicine, University of Nottingham, D Floor, South Block, Queens Medical Centre, NG7 2UH, UK.

Corresponding author: Arundhati Dongre, Division of Respiratory Medicine, University of Nottingham, D Floor, South Block, Queens Medical Centre, NG7 2UH, UK.

Tel: +44 (0)115 82 31063

Fax: +44 (0)115 82 31059

E-mail: arundhati.dongre@nottingham.ac.uk

Number of text pages: 27; Number of tables: 0; Number of figures: 9

Ethical approval for the use of LAM lung tissue was given by the University of Nottingham Research Ethics Committee, and written, informed consent was obtained from all patients. This study was funded by LAM

Action (lamaction.org) grant number LAM002 and the University of Nottingham postgraduate studentship award number 072. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Lymphangioleiomyomatosis (LAM) is a rare disease in which clonal 'LAM' cells infiltrate the lungs and lymphatics. In association with recruited fibroblasts, LAM cells form nodules adjacent to lung cysts. It is assumed LAM nodule derived proteases lead to cyst formation although, this is uncertain. We profiled protease gene expression in whole lung tissue and observed cathepsin K was 40 fold over-expressed in LAM compared with control lungs ($p \leq 0.0001$). Immunohistochemistry confirmed cathepsin K protein in LAM nodules but not control lungs. Cathepsin K gene expression, protein and protease activity was detected in LAM associated fibroblasts but not the LAM cell line 621-101. In lung nodules, cathepsin K immuno-reactivity was predominantly co-localised with LAM associated fibroblasts. In vitro, extra-cellular cathepsin K activity was minimal at pH 7.5 but significantly enhanced in fibroblast cultures at pH 7 and 6. 621-101 cells reduced extracellular pH by 0.5 units over 24 hours. Acidification was dependent upon 621-101 cell mTOR activity and net hydrogen ion transporters, particularly sodium/bicarbonate co-transporters and carbonic anhydrases which were also expressed in LAM lung tissue. In LAM cell/fibroblast co-cultures, acidification paralleled cathepsin K activity and both were inhibited by sodium bicarbonate co-transporter ($p \leq 0.0001$) and carbonic anhydrase inhibitors ($p=0.0021$). Our findings suggest cathepsin K activity is dependent on LAM cell/fibroblast interactions and inhibitors of extracellular acidification may be potential therapies for LAM.

Introduction

Lymphangioleiomyomatosis (LAM) is a lung and lymphatic disease which may eventually lead to respiratory failure. In LAM, the lung parenchyma is progressively replaced by cysts surrounded by heterogeneous groups of cells[1]. These groups of cells, termed LAM nodules, contain LAM cells, which are clonal and have inactivating mutations in either *TSC1* or more often *TSC2*. The protein products of *TSC1* and *TSC2*, hamartin and tuberin respectively, form a heterodimer which, by inactivating the small GTPase Rheb, in turn suppresses the activity of the mechanistic target of rapamycin (mTOR)[2]. In LAM cells, constitutive activation of mTOR leads to abnormal proliferation, migration, inhibition of autophagy and metabolic dependence on glycolysis[3-6]. LAM cells express markers of both smooth muscle, including α-smooth muscle actin (α-SMA) and melanocyte lineages with microphthalmia transcription factor (MITF), glycoprotein 100 (gp100) and PNL2[7]. This mixed phenotype is characteristic of the perivascular epithelioid cell (PEC) group of neoplasms[8]. Genetic, and more recently, histologic studies have shown that LAM nodules also contain a significant population of recruited wild type cells[9] including fibroblasts[10], mast cells[11] and other inflammatory cells[12].

The mechanism of cyst formation is not well understood although lung cysts are thought to arise as a result of LAM nodule derived matrix degrading proteases[2]. The expression of various protease families has been described in LAM. The matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases with roles in many biological processes including extra-cellular matrix turnover, inflammation, angiogenesis, metastasis, regulation of growth factor and chemokine activity[13]. LAM lung nodules express MMPs -1, -2 and -14[14-16], MMP-2 is over expressed by *TSC2* knockout cells[17], and we and others have shown that women with LAM have higher levels of MMP-2 and -9 in serum and MMP-9 in urine than healthy women[18-20]. However a recent study of MMP inhibition using doxycycline did not reduce decline in lung function despite suppression of MMP-9, suggesting other proteases are responsible for lung destruction[20]. The serine protease, plasmin is increased in LAM lung whilst its inhibitor, plasminogen activator inhibitor (PAI-1), is reduced suggesting activation of this protease axis[21]. Cathepsin K is a cysteine protease which is

expressed in LAM lung nodules and other PEC neoplasms[22, 23]. Unlike the MMPs and plasmin, cathepsin K is not present in normal lung tissue, but is classically expressed by osteoclasts as a bone remodelling protease[24] and by tumour stromal fibroblasts[25]. Cathepsin K requires low pH for its activation. Inside the cell this generally occurs in lysosomes whereas in tumour stroma, cathepsin K activation is dependent upon acidification of the extra-cellular space by membrane transporters including carbonic anhydrases (CA), vacuolar-type H^+-ATPases (V-ATPases) and sodium bicarbonate co-transporters[26-28].

Here we have investigated the expression of cathepsin K and the mechanism of cathepsin k activation by extra-cellular acidification using *in vitro* models of LAM and LAM lung tissue.

Methods

Patients and tissue

Women with LAM receiving clinical care at the UK LAM Centre, are enrolled in a comprehensive cohort study. Informed consent was obtained for the use of tissue taken as part of clinical care, including diagnostic biopsy or diseased LAM lung removed at the time of lung transplantation to be used for cell and tissue culture. LAM lung tissue removed at the time of transplantation was received from UK transplant centres and the National Disease Research Interchange (USA). The study has approval from the Nottingham research ethics committee (Ref. 13/EM/0264) and written informed consent was obtained from all patients.

Cell isolation and culture

Fibroblast-like cells, now termed LAM-Associated Fibroblasts (LAFs), were obtained from collagenase digested fresh LAM lung tissue, cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DME-F12, Life Technologies Ltd, Paisley, UK) and were used between passages 3 and 6. LAF do not have TSC mutations, express full-length tuberin protein and suppressible mTOR activity in the absence of serum

consistent with wild-type cells as previously described[10]. 621-101 cells were derived from the renal angiomyolipoma of a patient with sporadic LAM, have inactivation of both alleles of *TSC2*, express oestrogen receptor α and β[29] and were a gift from Lisa Henske (Harvard). These cells were maintained in DME-F12 with 10% FCS. $TSC2^{-/-}$ and $TSC2^{+/+}$ murine embryonic fibroblasts (MEF) were a gift from David Kwaitkowski and were derived as described in Onda et al[30]. Normal Human Lung Fibroblasts (NHLFs) from female premenopausal donors were purchased from Lonza (Slough, UK) and Promocell (Heidelberg, Germany) and were maintained in DME-F12 with 10% FCS.

Cell and tissue models

Co-cultures were established either in 12-well Boyden chamber Transwells or as direct contact co-cultures. In the Transwell system LAF and 621-101 cells were incorporated in a 10:1 ratio. Polycarbonate membrane Transwell inserts (0.4µm pore size, Corning Life Sciences, SLS, Nottingham UK) were equilibrated for one hour at 37°C and 5% CO_2 prior to adding cells. LAF were seeded at $5x10^5$ cells per ml in the lower chamber and $5x10^4$ 621-101 cells (500µl) in the upper chamber. Mono-cultures of both cell types maintained the same cell number as co-cultures. Direct contact LAF and 621-101 co-cultures were set up using a total of $5x10^4$ cells in a 1:1 ratio. A mixture of cells was resuspended in serum-free DME-F12 and then cultured in tissue culture treated plastic. Mono-cultures of both cell types were set up using $5x10^4$ cells per well. For pH measurement, $5x10^4$ 621-101 cells, $TSC2^{-/-}$ MEFs (rapamycin or vehicle treated) or $TSC2^{+/+}$ MEFs were cultured in 24-well tissue culture plates.

Fresh ex-vivo LAM lung tissue obtained from transplant lungs was washed thoroughly in Dulbecco's Phosphate Buffered Saline (DPBS, Sigma, Dorset, UK) and Dulbecco's Modified Eagle Medium containing Penicillin/Streptomycin/ Amphotericin B (Sigma, Dorset, UK). Tissue from different areas in the lung parenchyma was cut into 3mm cubes and placed in 24-well tissue culture plates. Tissue was equilibrated overnight in serum-free DME-F12 after which it was treated with vehicle or 10nM Rapamycin or 10nM Oestrogen or both in serum-free DME-F12 for 48 hours.

MTT assay

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was performed to assess cell viability after treatment with low pH media or inhibitors of mTOR or membrane transporters and proton pumps. LAF, 621-101 cells or *TSC2*$^{+/+}$ and *TSC2*$^{-/-}$ MEF cultured in unbuffered medium were treated with a sterile 0.5mg/ml MTT (Sigma) solution for 4 hours at 37°C and 5% CO_2. Remaining MTT solution was discarded after 4 hours and the resulting formazan crystals were dissolved in propan-2-ol. Samples were then transferred to a 96-well plate and absorbance was read at 570nm with a background subtraction of 690nm.

Quantification of gene expression

Total RNA was extracted from 10^6 LAF, NHLF or 621-101 cells cultured in 6-well tissue culture plates for 24 hours using GenElute Mammalian Total RNA Miniprep Kit (Sigma, Dorset, UK). RNA from treated and untreated tissue explants was extracted by first homogenising the tissue using an IKA-ultra-turrax® T25 homogeniser (IKA, Oxford, UK) followed by shearing, centrifuging and filtering to remove tissue debris. Contaminating genomic DNA was removed using On-Column DNase I digest set (Sigma, Dorset, UK). cDNA was synthesised using Superscript III First-Strand Synthesis System (Invitrogen, Life Technologies Ltd, Paisley, UK) with random hexamer primers as per the manufacturer's instructions. Relative gene expression of MMPs -1, -2, -9, -12, -13, -14, tissue inhibitor of metalloproteinases (TIMPs) 1-3, cathepsins B,D,H,K,L,S, urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), PAI-1, ADAM metallopeptidase domain 17 (ADAM17), calpains (CAPNs) 1-2 was determined by amplifying cDNA via quantitative real-time PCR using the Brilliant III SYBR Green QPCR master mix (Agilent Technologies, Cheshire, UK). Pre-designed and validated KiCq Start SYBR Green Primers (Sigma, Dorset, UK) were used. Primers were selected on the basis of their rank and exon locations. Reactions were performed in triplicate. Expression levels of target genes were determined relative to a housekeeping gene β-actin using the comparative CT ($2^{-\Delta CT}$) method[31].

147

148 Immunohistochemistry and Immunofluorescence

149 Immunohistochemistry (IHC) was performed on formalin-fixed paraffin embedded (FFPE) sections. After
150 deparaffinization, antigen retrieval, where required, was carried out in sodium citrate buffer solution, pH
151 6.0, for 20 minutes in a steamer. Sections were then blocked with 3% hydrogen peroxide (Sigma, Dorset,
152 UK) followed by 2.5% horse serum (Vector Laboratories, Peterborough, UK) before incubation with primary
153 antibody at 4°C overnight. Sections were washed in PBS with 0.05% Tween 20 (PBS-T) then incubated with
154 secondary antibody for one hour at room temperature. Chromogenic detection was carried out using
155 ImmPact DAB (Vector Laboratories, Peterborough, UK). For double chromogenic IHC, following ImmPact
156 DAB incubation, sections were blocked, incubated with the second primary antibody, washed in PBS-T, and
157 then incubated with secondary antibody as described above. Chromogenic detection for second antibody
158 was performed using Vector Blue Alkaline Phosphatase Substrate (Vector Laboratories, Peterborough, UK).
159 Levamisole was added to block endogenous alkaline phosphatases. Sections were counterstained with
160 Mayer's Haematoxylin (Sigma, Dorset, UK) and mounted using Vectamount (Vector Laboratories). For
161 double fluorescent IHC FFPE sections were sequentially incubated with primary antibodies against both
162 antigens followed by washing and then incubation with both fluorophore conjugated secondary antibodies
163 (pre-adsorbed against the other species), counterstaining with 4',6-diamidino-2-phenylindole (DAPI) and
164 mounted in Fluorescent Mounting Medium (Dako UK Ltd, Ely, UK).

165

166 Immunofluorescent (IF) detection of proteins was carried out in cultured cells grown on 8-well Nunc Lab-
167 Tek II Chamber Slide System (Fisher Scientific, Loughborough, UK) in DME-F12 with 10% FCS for 24h hours.
168 The cells were then fixed in 4% formaldehyde overnight at 4°C, washed in PBS, then permeabilised in 0.15%
169 Triton x100 in PBS for 10 minutes at room temperature. Samples were blocked in 10% goat serum,
170 incubated with primary antibodies overnight at 4°C followed by incubation with fluorophore conjugated
171 secondary antibodies for one hour at room temperature in the dark. Samples were incubated with DAPI
172 and mounted in Fluorescent Mounting Medium (Dako UK Ltd, Ely, UK).

Primary antibodies used were: mouse anti-Cathepsin K (3F9, ab37259), 1:2000 (IHC), 1:100 (IF) (Abcam, Cambridge, UK); rabbit anti-Cathepsin K (11239-1-AP), 1:500 (IHC-F), 1:1000 (IHC) (Proteintech, Manchester, UK); anti-α-Smooth Muscle Actin (1A4, A2547), 1:10,000; anti-Fibroblast Surface Protein (1B10, F4771), 1:50 (Sigma, Dorset, UK); anti-Melanoma Associated Antigen PNL2(MSK082) 1:50 (Zytomed, Berlin, Germany); rabbit anti-Carbonic Anhydrase IX (ab15086), 1:500; rabbit anti-SLC4A4 (ab187511), 1:2000 (Abcam, Cambridge, UK). Secondary antibodies used were: Vector ImmPress HRP anti-Mouse and anti-Rabbit (Vector Laboratories, Peterborough, UK), Alexa Flour 488 goat anti-mouse IgG antibody, Alexa Fluor goat anti-rabbit IgG antibody (Fisher Scientific, Loughborough, UK), anti-Mouse IgM peroxidase conjugate (Sigma, Dorset, UK).

Cathepsin K activity assays

Intracellular Cathepsin K activity was recorded in live cells using the Magic Red substrate (ImmunoChemistry Technologies, 2B Scientific, Bicester, UK). Cultured cells were grown on 8-well Nunc Lab-Tek II Chamber Slide System in DME-F12 serum free for 24 hours. Cells were then treated with unbuffered Dulbecco's Modified Eagle's Medium (DMEM) medium, pH 6.5 for 2 hours after which Magic Red substrate was added to the media at a 1:26 ratio in the presence and absence of Cathepsin K inhibitor L006235 (100nM), and cysteine protease inhibitor E64 (10μM) (Tocris, Abingdon, UK). The cells were then incubated for 16 hours at 37°C and 5% CO_2. Cells were washed in PBS and nuclei labelled using 0.5% v/v Hoechst stain for 10 minutes at 37°C and 5% CO_2. Samples were then mounted using PBS.

Extra-cellular cathepsin K activity was measured using a Fluorometric Cathepsin K Activity Assay Kit (Abcam, Cambridge, UK). Indirect contact co-cultures and monocultures were run as described. Cells were cultured in 12-well plates and Transwell inserts in unbuffered DMEM supplemented with 0.584 gm/L L-glutamine and 0.004 gm/L folic acid at pH 6.0, 7.0 and 7.5 for 48 or 96 hours. Media were then harvested, clarified and concentrated five-fold using Vivaspin 2 Centrifugal Concentrators (Sartorius, SLS, Nottingham, UK). Ac-

LR-AFC substrate (200µM) was added to each concentrated media sample with cathepsin K reaction buffer. Samples were incubated in a black-walled 96-well plate at 37°C for 16-18 hours in the dark and fluorescence was read at a 400-nm excitation and 505-nm emission.

pH measurement

Unbuffered media were prepared by mixing 1 volume of 10x DMEM (Sigma, Dorset, UK) with 9 volumes of sterile deionised water and was supplemented with 0.584 gm/L L-glutamine and 0.004 gm/L folic acid (Sigma, Dorset, UK). Where required, starting pH was adjusted using 2M sodium hydroxide solution. pH was then measured over 24 or 48 hours using an Oakton Waterproof pH Spear Pocket pH Tester (Cole-Parmer, London, UK).

Membrane transporters and proton pump inhibitors

Inhibitors used were: Carbonic Anhydrases, S4 (IX and XII inhibitor) 100µM (Tocris, Abingdon, UK) and Acetazolamide (universal) 1mM (Sigma, Dorset, UK); Sodium H^+ exchanger, BIX (Tocris, Abingdon, UK) 100nM; Sodium HCO_3^- co-transporter, S0859 (Sigma, Dorset, UK) 50µM; Vacuolar-type H^+ATPase, Concanamycin A (Santa Cruz, Insight Biotechnology, Middlesex, UK) 100nM; mTOR, Rapamycin (Calbiochem, Merck Millipore, Watford, UK) 10nM.

Statistical Analyses

Statistical analysis was performed using Graphpad Prism version 6 software (Graphpad, La Jolla, USA). Paired experiments were analysed by t-test and multiple comparisons by two-way ANOVA with Dunnett's or Bonferroni's correction with a P value of <0.05 regarded as significant.

Results

Cathepsin K is overexpressed in LAM lung tissue

mRNA was extracted from whole lung tissue of six patients with sporadic LAM. Normal human total lung RNA was obtained from Ambion (ThermoFisher Scientific, Paisley, UK). Quantitative RT-PCR was performed for candidate proteases and protease inhibitors *MMPs -1, -2, -3, -9, -13, -14*, *TIMPs 1, 2, 3*, *cathepsins B, C, D, K, L, S*, *uPA*, *uPAR* and *PAI1*. To determine that the method was appropriate, we first compared expression of the LAM specific genes premelanosome protein (*PMEL*), Melan-A (*MLANA*) and vascular endothelial growth factor D (*VEGF-D*) in control and LAM lungs. Transcripts for *PMEL*, *MLANA* and *VEGF-D* were 297, 267 and 2.47 fold more abundant in LAM than control lung tissue (supplemental figure S1). mRNA was detected for all proteases examined. Transcript expression was variable between individual donors. The most strongly expressed protease transcript in LAM lung was cathepsin K which was increased 40 fold compared with normal tissue (control mean 0.00092 95% C.I. 0.177. LAM mean 0.375, 95% C.I. 0.136 $p \leq 0.0001$). *Cathepsins B* and *D* and *TIMP3* were significantly reduced in LAM, other proteases were unchanged (figure 1a).

We next incubated fresh LAM lung tissue in culture with rapamycin, oestrogen or vehicle for 48 hours with *cathepsin K* expression measured by quantitative RT-PCR. Six tissue explants obtained from different areas of the lung parenchyma of two donors were assessed. *Cathepsin K* gene expression was detected in all tissue explants. Rapamycin (10nM) reduced *cathepsin K* gene expression to around one quarter of vehicle control treated levels ($p<0.001$). Oestrogen (10nM) had no significant effect upon *cathepsin K* levels (figure 1b).

Cathepsin K protein was examined using immunohistochemistry in six lung biopsy and seven transplant tissues from women with LAM. LAM nodules were identified using immunostaining for α-SMA, the melanoma marker PNL2 and fibroblast surface protein (FSP). LAM nodules were located adjacent to lung cysts (figure 3). Cathepsin K was expressed within LAM nodules in all cases but was not present in surrounding uninvolved lung tissue from patients with LAM or in control patients (figure 3). Cathepsin K

expression was particularly strong in the spindle-like cells within nodules that were also immuno-positive for FSP and α-SMA (figure 2).

Association of cathepsin K with fibroblast-like cells in LAM nodules

LAM nodules are heterogeneous structures with a complex mixture of cell types. To determine which cell types are responsible for cathepsin K expression we first examined expression of the *CTSK* transcript by RT-PCR in normal human lung fibroblasts, LAF and 621-101 cells. *CTSK* transcript was present in both normal lung fibroblasts and LAF but was not significantly expressed by 621-101 cells (figure 4a).

Using immunofluorescence, NHLF and LAF but not 621-101 cells were positive for cathepsin K protein which was concentrated in intra-cytoplasmic granules (figure 4b). To determine the presence of intra-cellular cathepsin activity, we then used Magic Red, a substrate that generates red fluorescence when processed by cathepsins. Red fluorescence was detected in LAFs which was partially inhibited by the cathepsin K inhibitor L006235 and completely inhibited by the broad-spectrum cysteine protease inhibitor E64 (figure 4c).

To determine if LAF are the predominant source of cathepsin K in LAM lung tissue we co-immunostained using differential immunostaining with both chromogenic and fluorescent labels. Using both systems we observed strong co-localisation of cathepsin K and FSP in LAM nodules consistent with expression of cathepsin K by LAM lung fibroblasts. A lower level of cathepsin K staining could also be detected by immunofluorescence in FSP negative cells (figure 5).

Cathepsin K activity is pH dependent

We examined cathepsin K activity *in vitro* using four separate LAF primary cultures and 621-101 cells both separately as mono-cultures and combined in co-cultures. At physiological pH, cathepsin K activity was not significantly elevated above baseline values in any cell type or culture condition. As cathepsin K requires low pH for its activity, cell cultures were also studied at pH 7.0 and 6.0. Cell viability was unimpaired at pH 6

and above over 24 hours assessed by MTT reduction (supplemental figure S2). At pH 7.0 and 6.0, LAF cathepsin K activity was 1.7 and 2.2 fold higher ($p=0.044$ and 0.0017 respectively) than at pH 7.5, and almost 3 fold higher ($p\leq0.0001$) in co-cultures. Cathepsin K activity in 621-101 cell supernatants was low at all pH values (figure 6a).

$TSC2^{-/-}$ cells acidify the extracellular pH as a consequence of mTOR dysregualtion

As LAF derived cathepsin K requires low pH for its proteolytic activity, we set out to determine if cells within LAM nodules could acidify tissue culture medium *in vitro*. 621-101 cells and LAFs were grown in unbuffered tissue culture medium at initial pH values of 7.5, 7.0 and 6.0. LAFs had no significant effect on culture medium pH over 24 hours. 621-101 cell culture medium fell by around half of one pH unit over 24 hours independent of the starting pH value (figure 6b).

To determine whether extracellular acidification was a consequence of mTOR dysregulation, we examined MEFs lacking TSC2, a negative regulator of mTOR and their genotypic $TSC2^{+/+}$ counterparts. MEFs and 621-101 cells were grown in unbuffered tissue culture medium at initial pH values of 7.5, 7.0 and 6.0. Cell viability of both $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs was unimpaired at low pH over 24 hours (supplemental figure S3). $TSC2^{+/+}$ MEFs had no significant affect upon extra-cellular pH over 24 hours. $TSC2^{-/-}$ MEFs and 621-101 cells reduced the culture medium pH by 0.35 and 0.51 pH units from a starting pH of 7.5 ($p=0.0067$ and $p=0.0004$ respectively) (figure 6c). Treatment of $TSC2^{-/-}$ MEFs and 621-101 cells with rapamycin completely abrogated the change in pH over 24 hours. Similar findings were observed at starting pH values of 7.0 and 6.0 (data not shown).

Expression of H^+ ion transporters in LAM

To determine the mechanism of extra-cellular acidification by 621-101 cells we profiled candidate membrane transporter expression in 621-101 cells using quantitative real time PCR. Carbonic anhydrases *(CA), II, IX* and *XII*, monocarboxylate transporters *(MCT) 1* and *4*, sodium bicarbonate (Na^+/HCO_3^-) co-

transporters, members of the NBC family *(NBC) 1 / SLC4A4* and *3 / SLC4A7*, sodium H^+ (Na^+/H^+) exchanger, member of the NHE family *(NHE) 1 / SLC9A1* and vacuolar-type H^+-ATPases *(V-ATPases) ATP6V1B2* and *ATP6V0A4* were all expressed in 621-101 cells (figure 7a). When 621-101 cells were incubated with rapamycin, oestrogen or LAF conditioned medium, *CA IX* gene expression was reduced in the presence of rapamycin although no other changes were significant. In LAM and control lung tissue, gene expression for *CA II, XII, MCT1, 4, NHE1, SLC4A4, SLC4A7, ATP6V1B2* and *ATP6V0A4* was similar (figure 7b). We then examined the expression of the two most strongly expressed transporter proteins, CA IX and the Na^+/HCO_3^- co-transporter, SLC4A4, in lung tissue. Both CA IX and SLC4A4 were strongly expressed in LAM nodules. SLC4A4, but not CA IX was present in control lung tissue (figure 7c).

Inhibition of membrane transporters affects 621-101 cell extra-cellular pH and cathepsin K activity

We then used pharmacological inhibitors of these membrane transporters to determine if we could inhibit extra-cellular acidification by 621-101 cells. In unbuffered media, treated with vehicle control, 621-101 cells reduced extra-cellular pH by 0.75 pH units over 24 hours. Inhibition of V-ATPases, CAs, Na^+/H^+ exchanger and the Na^+/HCO_3^- co-transporter blocked extra-cellular acidification increasingly strongly. Interestingly the mTOR inhibitor rapamycin was more potent than any of the membrane transporter inhibitors and completely abolished extra-cellular acidification (figure 7d).

To recapitulate the LAM nodule environment, we next examined if 621-101 cells were capable of acidifying their environment in the presence of LAF and whether this resulted in activation of LAF derived cathepsin K. 621-101 / LAF co-cultures acidified the extra-cellular space, which was associated with cathepsin K activity in the co-cultures (figure 8). Membrane transporter inhibitors blocked extra-cellular acidification to the same degree as seen in 621-101 monocultures. Inhibition of pH change was also associated with reduced cathepsin K activity. Importantly, the Na^+/HCO_3^- co-transporter inhibitor was the strongest inhibitor of both acidification and cathepsin K activity, reducing activity by almost 75%. Inhibitors with more modest effects on acidification had had lesser effects on cathepsin K activity. Again, rapamycin was the strongest inhibitor

of acidification and reduced cathepsin K activity by around 50%. The inhibitors used did not affect cell viability (supplemental figure S4).

Discussion

Here we have shown that cathepsin K expression in LAM is mainly dependent upon the presence of fibroblasts within LAM nodules. *In vitro* LAF derived cathepsin K is only active below pH 7.0 and importantly, LAM-derived 621-101 cells, in common with other $TSC2^{-/-}$ cell lines, express net hydrogen ion exporters which acidify their local environment to the extent that cathepsin K is activated. Our findings show that cell-cell interactions within the LAM nodule stroma can generate the conditions in which proteolytic lung damage may occur.

Cathepsin K has a primary role as a bone remodelling protease expressed by osteoclasts and dependent for its extra-cellular activity upon the low pH in bone resorbing lacunae generated by carbonic anhydrases, V-ATPases, Na^+/H^+ exchangers and chloride bicarbonate exchangers[24, 32]. Cathepsin K is a potent collagenase and elastase, but also selectively processes ELR chemokines which enhances their chemotactic activity[33], suggesting a potential role in inflammatory cell chemotaxis. Unlike the metalloproteinases and serine proteases previously described in LAM, cathepsin K is not present in normal lung tissue but is expressed strongly by tumour stromal fibroblasts[25]. Expression of cathepsin K in LAM and other PEComas was first described by Chilosi and colleagues who also suggested cathepsin k expression may be mTOR dependent[22]. Here we show that by suppressing mTOR activity in LAM lung tissue with rapamycin; cathepsin K gene expression was significantly reduced. In the osteoclast, cathepsin K expression is dependent on MITF, a helix-loop-helix transcription factor which regulates melanocyte development, cyclin dependent kinase and anti-apoptotic gene expression[34]. MITF binds three consensus sites in the cathepsin K promoter as a heterodimer with various partners including TFE3 and is partially mTOR dependent[35]. Moreover, mTOR inhibition in human osteoclasts reduced cathepsin K protein expression and bone resorption[35]: raising the

possibility that inhibition of mTOR and cathepsin K may have synergistic effects on inhibition of lung destruction in LAM.

The requirement for low pH to activate cathepsin K is well described[36, 37]. Monocyte-derived macrophages acidify their pericellular environment via vacuolar-type H^+-ATPases thus enabling them to maintain cathepsin K in its active form[38]. Here we have shown that acidic conditions may exist within a LAM nodule and that this extra-cellular acidification is a consequence of mTOR dysregulation, likely to result both in the expression of membrane transporters including, carbonic anhydrases, monocarboxylate transporters and Na^+/HCO_3^- co-transporters and mTOR dysregulation causing the Warburg effect, a metabolic dependence on aerobic glycolysis (figure 9). In 621-101 cell / LAF co-cultures, the transporters acidify the extra-cellular space to resulting in activation of cathepsin K, whilst their inhibition, particularly the Na^+/HCO_3^- co-transporters, block both extra-cellular acidification and protease activation. Strikingly, rapamycin completely and rapidly, abrogated acidification in culture despite only suppressing the transcription of *CA IX*, suggesting that the part of the effect may have been on 621-101 cell metabolism rather than exclusively on the transporters themselves.

Inhibition of the mTOR pathway is the only proven treatment for LAM but does not arrest lung destruction in all cases[39]. Our findings suggest that part of the beneficial effect of mTOR inhibition may be suppression of Warburg metabolism and extracellular acidification, a phenomenon also observed in lymphoma models[40]. Small molecule inhibitors of carbonic anhydrases and sodium bicarbonate co-transporters have been successfully used in pre-clinical cancer models[41-44] and may have synergistic benefits with mTOR inhibition in LAM to reduce destructive protease activation. In addition, direct inhibition of cathepsin K has been shown to reduce bone loss in osteoporosis[45]. Combinations of these therapeutic approaches may be superior to mTOR inhibition alone in reducing lung destruction in LAM.

Whilst the 621-101 cell and LAF in culture may not completely recapitulate the LAM nodule environment, we have been careful to show that in addition to our *in vitro* studies that the elements necessary to synthesise and activate cathepsin K are present in human lung tissue. Whilst we have not directly shown that there is an acidic environment in human LAM lung nodules, the presence of these components and the existence of an analogous situation in human cancers, suggest this is likely to be the case.

Taken together, our findings suggest that LAM cell / LAF interactions within LAM nodules promote disease progression by protease activation in a similar manner to tumour cell / cancer associated fibroblast interactions in cancer. These similarities are consistent with the idea that LAM is a low grade neoplastic disease, with a stroma similar to cancer[46]. Moreover, mTOR inhibitors which reduce lung function decline in LAM[39, 47, 48], may exert some of their protective action in the lung upon LAM cell related extracellular pH, cathepsin K activation and expression. The expression of transporters including the Na^+/HCO_3^- co-transporter are downstream of mTORC1 / hypoxia inducible factor 1α[49, 50]. Further studies are required to understand, how mTOR activation and the expression of membrane transporters are related and whether inhibition of these transporters or cathepsin K activity will be of benefit in addition to mTOR inhibitors for LAM.

Acknowledgements

The authors would like to thank the Institute of Transplantation, Newcastle upon Tyne Hospitals, the Transplant Unit, Harefield Hospital, the National Disease Research Interchange (NDRI) and the LAM Treatment Alliance for providing access to tissue samples, Professors Elizabeth Henske and David Kwaitkowski (Harvard Medical School, Boston, MA) for 621–101 cells and MEFs.

400 **References**

401 [1] Johnson SR: Lymphangioleiomyomatosis. Eur Respir J 2006, 27:1056-65.

402 [2] Henske EP, McCormack FX: Lymphangioleiomyomatosis — a wolf in sheep's clothing. The Journal of
403 Clinical Investigation 2012, 122:3807-16.

404 [3] Goncharova EA, Goncharov DA, Eszterhas A, Hunter DS, Glassberg MK, Yeung RS, Walker CL, Noonan D,
405 Kwiatkowski DJ, Chou MM, Panettieri RA, Jr., Krymskaya VP: Tuberin regulates p70 S6 kinase activation and
406 ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary
407 lymphangioleiomyomatosis (LAM). J Biol Chem 2002, 277:30958-67.

408 [4] Goncharova E, Goncharov D, Noonan D, Walker C, Krymskaya V: Pulmonary LAM: TSC2 Regulates Cell
409 Migration. Am J Respir Crit Care Med 2004, 169:224 (Abstract).

410 [5] Parkhitko A, Myachina F, Morrison TA, Hindi KM, Auricchio N, Karbowniczek M, Wu JJ, Finkel T,
411 Kwiatkowski DJ, Yu JJ, Henske EP: Tumorigenesis in tuberous sclerosis complex is autophagy and
412 p62/sequestosome 1 (SQSTM1)-dependent. Proceedings of the National Academy of Sciences 2011,
413 108:12455-60.

414 [6] Kang SA, Pacold ME, Cervantes CL, Lim D, Lou HJ, Ottina K, Gray NS, Turk BE, Yaffe MB, Sabatini DM:
415 mTORC1 Phosphorylation Sites Encode Their Sensitivity to Starvation and Rapamycin. Science 2013, 341.

416 [7] Finlay G: The LAM cell: what is it, where does it come from, and why does it grow? Am J Physiol Lung
417 Cell Mol Physiol 2004, 286:L690-3.

418 [8] Pea M, Martignoni G, Bonetti F, Zamboni G, Colombari C, Manfrin E, Lestani M, Mombello A, Mariuzzi
419 GM: Tumors characterized by the presence of HMB45-positive perivascular epithelioid cell (PEC) - A novel
420 entity in surgical pathology. Electronic Journal of Pathology & Histology 1997, 3:28-40.

421 [9] Badri KR, Gao L, Hyjek E, Schuger N, Schuger L, Qin W, Chekaluk Y, Kwiatkowski DJ, Zhe X: Exonic
422 Mutations of TSC2/TSC1 Are Common but Not Seen in All Sporadic Pulmonary Lymphangioleiomyomatosis.
423 American Journal of Respiratory and Critical Care Medicine 2013, 187:663-5.

424 [10] Clements D, Dongre A, Krymskyaya V, Johnson S: Wild Type Mesenchymal Cells Contribute to the Lung
425 Pathology of Lymphangioleiomyomatosis. PLoS ONE 2015.

426 [11] Inoue Y, King TE, Jr., Barker E, Daniloff E, Newman LS: Basic Fibroblast Growth Factor and Its Receptors
427 in Idiopathic Pulmonary Fibrosis and Lymphangioleiomyomatosis. Am J Respir Crit Care Med 2002, 166:765-
428 73.
429 [12] Atochina-Vasserman EN, Guo C-J, Abramova E, Golden TN, Sims M, James ML, Beers MF, Gow AJ,
430 Krymskaya VP: Surfactant Dysfunction and Lung Inflammation in the Female Mouse Model of
431 Lymphangioleiomyomatosis. American Journal of Respiratory Cell and Molecular Biology 2014, 53:96-104.
432 [13] Greenlee KJ, Werb Z, Kheradmand F: Matrix metalloproteinases in lung: multiple, multifarious, and
433 multifaceted. Physiol Rev 2007, 87:69-98.
434 [14] Hayashi T, Fleming MV, Stetler-Stevenson WG, Liotta LA, Moss J, Ferrans VJ, Travis WD:
435 Immunohistochemical study of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in
436 pulmonary lymphangioleiomyomatosis (LAM). Hum Pathol 1997, 28:1071-8.
437 [15] Matsui K, Takeda K, Yu ZX, Travis WD, Moss J, Ferrans VJ: Role for activation of matrix
438 metalloproteinases in the pathogenesis of pulmonary lymphangioleiomyomatosis. Arch Pathol Lab Med
439 2000, 124:267-75.
440 [16] Ferri N, Carragher NO, Raines EW: Role of Discoidin Domain Receptors 1 and 2 in Human Smooth
441 Muscle Cell-Mediated Collagen Remodeling: Potential Implications in Atherosclerosis and
442 Lymphangioleiomyomatosis. Am J Pathol 2004, 164:1575-85.
443 [17] Lee P-S, Tsang SW, Moses MA, Trayes-Gibson Z, Hsiao L-L, Jensen R, Squillace R, Kwiatkowski DJ:
444 Rapamycin-insensitive Up-regulation of MMP2 and Other Genes in TSC2-deficient LAM-like Cells. Am J
445 Respir Cell Mol Biol 2009:2009-0050OC.
446 [18] Chang W, Cane JL, Blakey J, Kumaran M, Pointon K, Johnson SR: Clinical utility of diagnostic guidelines
447 and putative biomarkers in lymphangioleiomyomatosis. Respiratory Research 2012, 13:34.
448 [19] Odajima N, Betsuyaku T, Nasuhara Y, Inoue H, Seyama K, Nishimura M: Matrix metalloproteinases in
449 blood from patients with LAM. Respiratory Medicine 2009, 103:124-9.
450 [20] Chang WYC, Cane JL, Kumaran M, Lewis S, Tattersfield AE, Johnson SR: A two year randomised placebo
451 controlled trial of doxycycline for lymphangioleiomyomatosis. Eur Resp J 2013, In press.

[21] Zhe X, Yang Y, Schuger L: Imbalanced Plasminogen System in Lymphangioleiomyomatosis: Potential Role of Serum Response Factor. Am J Respir Cell Mol Biol 2004:2004-0289OC.

[22] Chilosi M, Pea M, Martignoni G, Brunelli M, Gobbo S, Poletti V, Bonetti F: Cathepsin-k expression in pulmonary lymphangioleiomyomatosis. Mod Pathol 2009, 22:161-6.

[23] Martignoni G, Pea M, Reghellin D, Gobbo S, Zamboni G, Chilosi M, Bonetti F: Molecular Pathology of Lymphangioleiomyomatosis and Other Perivascular Epithelioid Cell Tumors. Archives of Pathology & Laboratory Medicine 2010, 134:33-40.

[24] Zhao Q, Jia Y, Xiao Y: Cathepsin K: A therapeutic target for bone diseases. Biochemical and Biophysical Research Communications 2009, 380:721-3.

[25] Montgomery N, Hill A, McFarlane S, Neisen J, O'Grady A, Conlon S, Jirstrom K, Kay E, Waugh D: CD44 enhances invasion of basal-like breast cancer cells by upregulating serine protease and collagen-degrading enzymatic expression and activity. Breast Cancer Research 2012, 14:R84.

[26] Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, Turk D: Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 2012, 1824:68-88.

[27] McDonald PC, Winum J-Y, Supuran CT, Dedhar S: Recent Developments in Targeting Carbonic Anhydrase IX for Cancer Therapeutics. Oncotarget 2012, 3:84-97.

[28] Romero MF, Chen A-P, Parker MD, Boron WF: The SLC4 family of bicarbonate transporters. Molecular Aspects of Medicine 2013, 34:159-82.

[29] Yu J, Astrinidis A, Howard S, Henske EP: Estradiol and tamoxifen stimulate LAM-associated angiomyolipoma cell growth and activate both genomic and nongenomic signaling pathways. Am J Physiol Lung Cell Mol Physiol 2004, 286:L694-700.

[30] Onda H, Lueck A, Marks PW, Warren HB, Kwiatkowski DJ: Tsc2+/– mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. The Journal of Clinical Investigation 1999, 104:687-95.

[31] Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif) 2001, 25:402-8.

[32] Riihonen R, Supuran CT, Parkkila S, Pastorekova S, Vaananen HK, Laitala-Leinonen T: Membrane-bound carbonic anhydrases in osteoclasts. Bone 2007, 40:1021-31.

[33] Repnik U, Starr AE, Overall CM, Turk B: Cysteine Cathepsins Activate ELR Chemokines and Inactivate Non-ELR Chemokines. Journal of Biological Chemistry 2015, 290:13800-11.

[34] Levy C, Khaled M, Fisher DE: MITF: master regulator of melanocyte development and melanoma oncogene. Trends in molecular medicine 2006, 12:406-14.

[35] Kneissel M, Luong-Nguyen N-H, Baptist M, Cortesi R, Zumstein-Mecker S, Kossida S, O'Reilly T, Lane H, Susa M: Everolimus suppresses cancellous bone loss, bone resorption, and cathepsin K expression by osteoclasts. Bone 2004, 35:1144-56.

[36] Bromme D, Okamoto K, Wang BB, Biroc S: Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. Functional expression of human cathepsin O2 in Spodoptera frugiperda and characterization of the enzyme. Journal of Biological Chemistry 1996, 271:2126-32.

[37] Lecaille F, Bromme D, Lalmanach G: Biochemical properties and regulation of cathepsin K activity. Biochimie 2008, 90:208-26.

[38] Punturieri A, Filippov S, Allen E, Caras I, Murray R, Reddy V, Weiss SJ: Regulation of Elastinolytic Cysteine Proteinase Activity in Normal and Cathepsin K–Deficient Human Macrophages. The Journal of Experimental Medicine 2000, 192:789-800.

[39] McCormack FX, Inoue Y, Moss J, Singer LG, Strange C, Nakata K, Barker AF, Chapman JT, Brantly ML, Stocks JM, Brown KK, Lynch JP, Goldberg HJ, Young LR, Kinder BW, Downey GP, Sullivan EJ, Colby TV, McKay RT, Cohen MM, Korbee L, Taveira-DaSilva AM, Lee H-S, Krischer JP, Trapnell BC: Efficacy and Safety of Sirolimus in Lymphangioleiomyomatosis. New England Journal of Medicine 2011, 364:1595-606.

[40] Akhenblit PJ, Hanke NT, Gill A, Persky DO, Howison CM, Pagel MD, Baker AF: Assessing Metabolic Changes in Response to mTOR Inhibition in a Mantle Cell Lymphoma Xenograft Model Using AcidoCEST MRI. Molecular imaging 2016, 15.

503 [41] Kant S, Kumar A, Singh SM: Bicarbonate transport inhibitor SITS modulates pH homeostasis triggering
504 apoptosis of Dalton's lymphoma: implication of novel molecular mechanisms. Molecular and cellular
505 biochemistry 2014, 397:167-78.
506 [42] Pacchiano F, Carta F, McDonald PC, Lou Y, Vullo D, Scozzafava A, Dedhar S, Supuran CT: Ureido-
507 substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in
508 a model of breast cancer metastasis. Journal of medicinal chemistry 2011, 54:1896-902.
509 [43] Touisni N, Maresca A, McDonald PC, Lou Y, Scozzafava A, Dedhar S, Winum JY, Supuran CT: Glycosyl
510 coumarin carbonic anhydrase IX and XII inhibitors strongly attenuate the growth of primary breast tumors.
511 Journal of medicinal chemistry 2011, 54:8271-7.
512 [44] Lou Y, McDonald PC, Oloumi A, Chia S, Ostlund C, Ahmadi A, Kyle A, Auf dem Keller U, Leung S,
513 Huntsman D, Clarke B, Sutherland BW, Waterhouse D, Bally M, Roskelley C, Overall CM, Minchinton A,
514 Pacchiano F, Carta F, Scozzafava A, Touisni N, Winum JY, Supuran CT, Dedhar S: Targeting tumor hypoxia:
515 suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. Cancer Res
516 2011, 71:3364-76.
517 [45] Boonen S, Rosenberg E, Claessens F, Vanderschueren D, Papapoulos S: Inhibition of Cathepsin K for
518 Treatment of Osteoporosis. Current Osteoporosis Reports 2012, 10:73-9.
519 [46] McCormack FX, Travis WD, Colby TV, Henske EP, Moss J: Lymphangioleiomyomatosis. American Journal
520 of Respiratory and Critical Care Medicine 2012, 186:1210-2.
521 [47] Goldberg HJ, Harari S, Cottin V, Rosas IO, Peters E, Biswal S, Cheng Y, Khindri S, Kovarik JM, Ma S,
522 McCormack FX, Henske EP: Everolimus for the treatment of lymphangioleiomyomatosis: a phase II study.
523 European Respiratory Journal 2015, 46:783-94.
524 [48] Bee J, Bhatt R, McCafferty I, Johnson S: Audit, research and guideline update: A 4-year prospective
525 evaluation of protocols to improve clinical outcomes for patients with lymphangioleiomyomatosis in a
526 national clinical centre. Thorax 2015.

527 [49] Kaluz S, Kaluzova M, Liao SY, Lerman M, Stanbridge EJ: Transcriptional control of the tumor- and
528 hypoxia-marker carbonic anhydrase 9: A one transcription factor (HIF-1) show? Biochimica et biophysica
529 acta 2009, 1795:162-72.
530 [50] McIntyre A, Hulikova A, Ledaki I, Snell C, Singleton D, Steers G, Seden P, Jones D, Bridges E, Wigfield S,
531 Li J-L, Russell A, Swietach P, Harris AL: Disrupting Hypoxia-Induced Bicarbonate Transport Acidifies Tumor
532 Cells and Suppresses Tumor Growth. Cancer Research 2016, 76:3744-55.

533

Main Figure Legends

Figure 1

Relative protease gene expression in human LAM and control lungs. A: Whole lung RNA was extracted from 6 patients with LAM and 3 control patients without LAM. Protease gene expression was analysed by quantitative real time PCR. Figure shows mean (± standard deviation) protease gene expression normalised to β-actin. MMP (matrix metalloproteinase), TIMP (tissue inhibitor of metalloproteinase, CTS (cathepsin), CAPN (calpain) B: Lung tissue was incubated for 48 hours with rapamycin (10nM), oestrogen (10nM), oestrogen and rapamycin or vehicle and gene expression measured by quantitative RT-PCR. *p<0.05, **p<0.01, ***p<0.001.

Figure 2

Expression of cathepsin K in LAM. Immunohistochemical images of three representative LAM lung tissues. α-SMA identifies LAM nodules (black arrowhead) adjacent to cysts which have positive staining for cathepsin K. Magnification x2.5, scale bar 1mm.

Figure 3

Cathepsin K expression within LAM lung nodules. Immunohistochemical staining for cathepsin K (CTSK), α smooth muscle actin (α-SMA), fibroblast specific protein (FSP) and melanoma marker antibody (PNL2) in two patients with LAM and normal lung tissue. Magnification x10, scale bar 200μm.

Figure 4

Expression and activity of cathepsin K in cultured cells. A: Quantitative real-time PCR for cathepsin K in LAM associated fibroblasts (LAF, n=4), normal human lung fibroblasts (NHLF, n=3) and 621-101 cells. *p<0.05. B: Immunofluorescent detection of cathepsin K protein in two LAF, NHLF and 621-101 cell

559 cultures. Magnification x20, scale bar 200µm. C: Intra-cellular cathepsin K activity is visible as red
560 fluorescence in two LAF cultures. Fluorescence is reduced by the cathepsin K specific inhibitor L006235 and
561 completely abrogated by the cysteine protease inhibitor E64. Magnification x20, scale bar 200µm.
562
563 **Figure 5**
564 **Cathepsin K and fibroblast specific protein are co-localised in LAM lung tissue.** A: Immunofluorescent
565 staining in LAM lung tissue from three donors. Individual panels show DAPI staining of nuclei (blue),
566 fibroblast specific protein (FSP, green), cathepsin K (CTSK, red) and the overlay with co-localisation of FSP
567 and CTSK (yellow). Cathepsin K and FSP are strongly co-localised, with only modest cathepsin K expression
568 outside of LAM associated fibroblasts. Images x20 magnification, scale bar 200µm. B: Dual chromogenic
569 immunohistochemistry showing LAM nodules reacting with antibodies against both cathepsin K (blue) and
570 fibroblast surface protein (brown). Left panels are x4 magnification, scale bar 500µm and right are inset
571 area at x40, scale bar 50µm taken from the same three representative donors. All donors showed spindle-
572 shaped cells within nodules reacting with both antibodies.
573
574 **Figure 6**
575 **Cathepsin K activity is pH dependent.** A: 621-101 cells, LAM associated fibroblasts (LAF) and 621-101 / LAF
576 co-cultures were grown in culture at a range of pH values. Cathepsin K activity was low at all pH values in
577 621-101 supernatants but elevated at low pH in LAF cultures. Co-cultures had significantly higher cathepsin
578 K activity than 621-101 cell or LAF cultures at pH 7.0 and 6.0. ** $p<0.01$, *** $p\leq0.001$. B: 621-101 cells and
579 LAF were cultured in unbuffered media for 24 hours at various starting pH values. LAF did not significantly
580 affect pH whereas 621-101 cells acidified the media independent of starting pH. C: $TSC2^{-/-}$ and $TSC2^{+/+}$ MEF
581 were cultured in unbuffered media for 24 hours at pH 7.5. $TSC2^{+/+}$ MEF and, $TSC2^{-/-}$ MEF and 621-101 cells
582 treated with 10nM rapamycin did not affect pH whereas untreated $TSC2^{-/-}$ MEF and 621-101 cells
583 significantly acidified media. ** $p\leq0.01$, *** $p\leq0.001$.
584

Figure 7

Expression of membrane transporters in LAM cells and lung tissue. A: Quantitative real time PCR for carbonic anhydrase *(CA) II, IX, XII*, monocarboxylate transporter *(MCT) 1, 4*, sodium bicarbonate (Na^+/HCO_3^-) co-transporters *(NBC) 1 / SLC4A4, 3 / SLC4A7*, sodium H^+ (Na^+/H^+) exchanger *(NHE) 1 / SLC9A1* and vacuolar-type H^+-ATPases *(V-ATPases) ATP6V1B2* and *ATP6V0A4* in 621-101 cells treated for 24 hours with either vehicle, rapamycin (10nM), oestrogen (10nM) or LAF conditioned medium (LAF CM). B: Quantitative real time PCR for *CA II, IX, XII, MCT 1, 4*, Na^+/H^+ exchanger, *NHE1*, Na^+/HCO_3^- co-transporters *SLC4A4, SLC4A7* and V-ATPases *ATP6V1B2* and *ATP6V0A4* in six LAM and three control patient derived lung tissues. C: Immunohistochemical staining of two representative LAM lung tissues showing positive staining in serial sections for CA IX and SLC4A4 within LAM nodules. Normal lung showed positive staining for SLC4A4 but not CA IX. Magnification x4, scale bar 500µm and x40, scale bar 50µm. D: Pharmacological inhibition of membrane transporters or mTOR inhibits extra-cellular acidification in 621-101 cell cultures. S4 and Acetazolamide - carbonic anhydrase inhibitors, BIX - Na^+/H^+ exchanger inhibitor, S0895 - Na^+/HCO_3^- co-transporter inhibitor and Concanamycin A - V-ATPase inhibitor.

Figure 8

Inhibition of membrane transporters blocks extracellular acidification and cathepsin K activity in LAM cell co-cultures. A: 621-101 / LAM associated fibroblast (LAF) co-cultures were grown in unbuffered media in the presence of either vehicle control or S4 (carbonic anhydrase IX, XII inhibitor), acetazolamide (non-specific carbonic anhydrase inhibitor), BIX (Na^+/H^+ exchanger inhibitor), S0895 (Na^+/HCO_3^- co-transporter inhibitor) concanamycin A (V-ATPase inhibitor) or rapamycin (mTOR inhibitor). B: Cathepsin K activity in co-culture supernatants treated as above showing strong inhibition of both acidification and cathepsin K activity by inhibition of the Na+/HCO3- co-transporter and mTOR. **p<0.01, ***p<0.001.

Figure 9

Proposed mechanism of Cathepsin K activation in LAM. $TSC2^{-/-}$ LAM cells express carbonic anhydrases (CA) II, IX and XII within the cell which, in tandem, catalyse the conversion of CO_2 and water to H^+ and HCO_3^- ions. Na^+/HCO_3^- co-transporters (NBC) shuttle HCO_3^- ions into the cell resulting in net H^+ export into the extracellular space. Cathepsin K containing lysosomes of LAM associated fibroblasts (LAF) translocate to the cell membrane discharging cathepsin K where it is activated in the low extracellular pH.

615