

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

3 **Short Title: Cathepsin K in Lymphangioleiomyomatosis**

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26

27 **Abstract**

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

## 44 Introduction

45

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

58

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

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

76

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

79

## 80 **Methods**

81

### 82 Patients and tissue

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

90

### 91 Cell isolation and culture

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

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

103

#### 104 Cell and tissue models

105 Co-cultures were established either in 12-well Boyden chamber Transwells or as direct contact co-cultures.  
106 In the Transwell system LAF and 621-101 cells were incorporated in a 10:1 ratio. Polycarbonate membrane  
107 Transwell inserts (0.4 $\mu$ m pore size, Corning Life Sciences, SLS, Nottingham UK) were equilibrated for one  
108 hour at 37°C and 5% CO<sub>2</sub> prior to adding cells. LAF were seeded at 5x10<sup>5</sup> cells per ml in the lower chamber  
109 and 5x10<sup>4</sup> 621-101 cells (500 $\mu$ l) in the upper chamber. Mono-cultures of both cell types maintained the  
110 same cell number as co-cultures. Direct contact LAF and 621-101 co-cultures were set up using a total of  
111 5x10<sup>4</sup> cells in a 1:1 ratio. A mixture of cells was resuspended in serum-free DME-F12 and then cultured in  
112 tissue culture treated plastic. Mono-cultures of both cell types were set up using 5x10<sup>4</sup> cells per well. For  
113 pH measurement, 5x10<sup>4</sup> 621-101 cells, *TSC2*<sup>-/-</sup> MEFs (rapamycin or vehicle treated) or *TSC2*<sup>+/+</sup> MEFs were  
114 cultured in 24-well tissue culture plates.

115

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

122

123 MTT assay

124 An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was performed to  
125 assess cell viability after treatment with low pH media or inhibitors of mTOR or membrane transporters and  
126 proton pumps. LAF, 621-101 cells or *TSC2*<sup>+/+</sup> and *TSC2*<sup>-/-</sup> MEF cultured in unbuffered medium were treated  
127 with a sterile 0.5mg/ml MTT (Sigma) solution for 4 hours at 37°C and 5% CO<sub>2</sub>. Remaining MTT solution was  
128 discarded after 4 hours and the resulting formazan crystals were dissolved in propan-2-ol. Samples were  
129 then transferred to a 96-well plate and absorbance was read at 570nm with a background subtraction of  
130 690nm.

131

132 Quantification of gene expression

133 Total RNA was extracted from 10<sup>6</sup> LAF, NHLF or 621-101 cells cultured in 6-well tissue culture plates for 24  
134 hours using GenElute Mammalian Total RNA Miniprep Kit (Sigma, Dorset, UK). RNA from treated and  
135 untreated tissue explants was extracted by first homogenising the tissue using an IKA-ultra-turrax® T25  
136 homogeniser (IKA, Oxford, UK) followed by shearing, centrifuging and filtering to remove tissue debris.  
137 Contaminating genomic DNA was removed using On-Column DNase I digest set (Sigma, Dorset, UK). cDNA  
138 was synthesised using Superscript III First-Strand Synthesis System (Invitrogen, Life Technologies Ltd,  
139 Paisley, UK) with random hexamer primers as per the manufacturer's instructions. Relative gene expression  
140 of MMPs -1, -2, -9, -12, -13, -14, tissue inhibitor of metalloproteinases (TIMPs) 1-3, cathepsins B,D,H,K,L,S,  
141 urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), PAI-1, ADAM metallopeptidase domain  
142 17 (ADAM17), calpains (CAPNs) 1-2 was determined by amplifying cDNA via quantitative real-time PCR  
143 using the Brilliant III SYBR Green QPCR master mix (Agilent Technologies, Cheshire, UK). Pre-designed and  
144 validated KiCq Start SYBR Green Primers (Sigma, Dorset, UK) were used. Primers were selected on the basis  
145 of their rank and exon locations. Reactions were performed in triplicate. Expression levels of target genes  
146 were determined relative to a housekeeping gene  $\beta$ -actin using the comparative CT ( $2^{-\Delta CT}$ ) method<sup>31</sup>.

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).



173

174 Primary antibodies used were: mouse anti-Cathepsin K (3F9, ab37259), 1:2000 (IHC), 1:100 (IF) (Abcam,  
175 Cambridge, UK); rabbit anti-Cathepsin K (11239-1-AP), 1:500 (IHC-F), 1:1000 (IHC) (Proteintech,  
176 Manchester, UK); anti- $\alpha$ -Smooth Muscle Actin (1A4, A2547), 1:10,000; anti-Fibroblast Surface Protein  
177 (1B10, F4771), 1:50 (Sigma, Dorset, UK); anti-Melanoma Associated Antigen PNL2(MSK082) 1:50 (Zytomed,  
178 Berlin, Germany); rabbit anti-Carbonic Anhydrase IX (ab15086), 1:500; rabbit anti-SLC4A4 (ab187511),  
179 1:2000 (Abcam, Cambridge, UK). Secondary antibodies used were: Vector ImmPress HRP anti-Mouse and  
180 anti-Rabbit (Vector Laboratories, Peterborough, UK), Alexa Flour 488 goat anti-mouse IgG antibody, Alexa  
181 Fluor goat anti-rabbit IgG antibody (Fisher Scientific, Loughborough, UK), anti-Mouse IgM peroxidase  
182 conjugate (Sigma, Dorset, UK).

183

#### 184 Cathepsin K activity assays

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

193

194 Extra-cellular cathepsin K activity was measured using a Fluorometric Cathepsin K Activity Assay Kit (Abcam,  
195 Cambridge, UK). Indirect contact co-cultures and monocultures were run as described. Cells were cultured  
196 in 12-well plates and Transwell inserts in unbuffered DMEM supplemented with 0.584 gm/L L-glutamine  
197 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  
198 and concentrated five-fold using Vivaspin 2 Centrifugal Concentrators (Sartorius, SLS, Nottingham, UK). Ac-

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

202

#### 203 pH measurement

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

209

#### 210 Membrane transporters and proton pump inhibitors

211 Inhibitors used were: Carbonic Anhydrases, S4 (IX and XII inhibitor) 100 $\mu$ M (Tocris, Abingdon, UK) and  
212 Acetazolamide (universal) 1mM (Sigma, Dorset, UK); Sodium H<sup>+</sup> exchanger, BIX (Tocris, Abingdon, UK)  
213 100nM; Sodium HCO<sub>3</sub><sup>-</sup> co-transporter, S0859 (Sigma, Dorset, UK) 50 $\mu$ M; Vacuolar-type H<sup>+</sup>ATPase,  
214 Concanamycin A (Santa Cruz, Insight Biotechnology, Middlesex, UK) 100nM; mTOR, Rapamycin  
215 (Calbiochem, Merck Millipore, Watford, UK) 10nM.

216

#### 217 Statistical Analyses

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

221

## 222 **Results**

223

### 224 **Cathepsin K is overexpressed in LAM lung tissue**

225 mRNA was extracted from whole lung tissue of six patients with sporadic LAM. Normal human total lung  
226 RNA was obtained from Ambion (ThermoFisher Scientific, Paisley, UK). Quantitative RT-PCR was performed  
227 for candidate proteases and protease inhibitors *MMPs -1, -2, -3, -9, -13, -14, TIMPs 1, 2, 3, cathepsins B, C,*  
228 *D, K, L, S, uPA, uPAR* and *PAI1*. To determine that the method was appropriate, we first compared  
229 expression of the LAM specific genes premelanosome protein (*PMEL*), Melan-A (*MLANA*) and vascular  
230 endothelial growth factor D (*VEGF-D*) in control and LAM lungs. Transcripts for *PMEL*, *MLANA* and *VEGF-D*  
231 were 297, 267 and 2.47 fold more abundant in LAM than control lung tissue (supplemental figure S1).  
232 mRNA was detected for all proteases examined. Transcript expression was variable between individual  
233 donors. The most strongly expressed protease transcript in LAM lung was cathepsin K which was increased  
234 40 fold compared with normal tissue (control mean 0.00092 95% C.I. 0.177. LAM mean 0.375, 95% C.I.  
235 0.136  $p \leq 0.0001$ ). *Cathepsins B* and *D* and *TIMP3* were significantly reduced in LAM, other proteases were  
236 unchanged (figure 1a).

237

238 We next incubated fresh LAM lung tissue in culture with rapamycin, oestrogen or vehicle for 48 hours with  
239 *cathepsin K* expression measured by quantitative RT-PCR. Six tissue explants obtained from different areas  
240 of the lung parenchyma of two donors were assessed. *Cathepsin K* gene expression was detected in all  
241 tissue explants. Rapamycin (10nM) reduced *cathepsin K* gene expression to around one quarter of vehicle  
242 control treated levels ( $p < 0.001$ ). Oestrogen (10nM) had no significant effect upon *cathepsin K* levels (figure  
243 1b).

244

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

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

252

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

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

258

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

264

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

270

### 271 **Cathepsin K activity is pH dependent**

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

276 and above over 24 hours assessed by MTT reduction (supplemental figure S2). At pH 7.0 and 6.0, LAF  
277 cathepsin K activity was 1.7 and 2.2 fold higher ( $p=0.044$  and  $0.0017$  respectively) than at pH 7.5, and  
278 almost 3 fold higher ( $p\leq 0.0001$ ) in co-cultures. Cathepsin K activity in 621-101 cell supernatants was low at  
279 all pH values (figure 6a).

280

### 281 **TSC2<sup>-/-</sup> cells acidify the extracellular pH as a consequence of mTOR dysregulation**

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

287 To determine whether extracellular acidification was a consequence of mTOR dysregulation, we examined  
288 MEFs lacking TSC2, a negative regulator of mTOR and their genotypic TSC2<sup>+/+</sup> counterparts. MEFs and 621-  
289 101 cells were grown in unbuffered tissue culture medium at initial pH values of 7.5, 7.0 and 6.0. Cell  
290 viability of both TSC2<sup>+/+</sup> and TSC2<sup>-/-</sup> MEFs was unimpaired at low pH over 24 hours (supplemental figure S3).  
291 TSC2<sup>+/+</sup> MEFs had no significant affect upon extra-cellular pH over 24 hours. TSC2<sup>-/-</sup> MEFs and 621-101 cells  
292 reduced the culture medium pH by 0.35 and 0.51 pH units from a starting pH of 7.5 ( $p=0.0067$  and  
293  $p=0.0004$  respectively) (figure 6c). Treatment of TSC2<sup>-/-</sup> MEFs and 621-101 cells with rapamycin completely  
294 abrogated the change in pH over 24 hours. Similar findings were observed at starting pH values of 7.0 and  
295 6.0 (data not shown).

296

### 297 **Expression of H<sup>+</sup> ion transporters in LAM**

298 To determine the mechanism of extra-cellular acidification by 621-101 cells we profiled candidate  
299 membrane transporter expression in 621-101 cells using quantitative real time PCR. Carbonic anhydrases  
300 (CA), II, IX and XII, monocarboxylate transporters (MCT) 1 and 4, sodium bicarbonate (Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup>) co-

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

310

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

312 We then used pharmacological inhibitors of these membrane transporters to determine if we could inhibit  
313 extra-cellular acidification by 621-101 cells. In unbuffered media, treated with vehicle control, 621-101 cells  
314 reduced extra-cellular pH by 0.75 pH units over 24 hours. Inhibition of V-ATPases, CAs, Na<sup>+</sup>/H<sup>+</sup> exchanger  
315 and the Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> co-transporter blocked extra-cellular acidification increasingly strongly. Interestingly the  
316 mTOR inhibitor rapamycin was more potent than any of the membrane transporter inhibitors and  
317 completely abolished extra-cellular acidification (figure 7d).

318

319 To recapitulate the LAM nodule environment, we next examined if 621-101 cells were capable of acidifying  
320 their environment in the presence of LAF and whether this resulted in activation of LAF derived cathepsin K.  
321 621-101 / LAF co-cultures acidified the extra-cellular space, which was associated with cathepsin K activity  
322 in the co-cultures (figure 8). Membrane transporter inhibitors blocked extra-cellular acidification to the  
323 same degree as seen in 621-101 monocultures. Inhibition of pH change was also associated with reduced  
324 cathepsin K activity. Importantly, the Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> co-transporter inhibitor was the strongest inhibitor of both  
325 acidification and cathepsin K activity, reducing activity by almost 75%. Inhibitors with more modest effects  
326 on acidification had had lesser effects on cathepsin K activity. Again, rapamycin was the strongest inhibitor

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

329

### 330 **Discussion**

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

337

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

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

354

355 The requirement for low pH to activate cathepsin K is well described<sup>36, 37</sup>. Monocyte-derived macrophages  
356 acidify their pericellular environment via vacuolar-type H<sup>+</sup>-ATPases thus enabling them to maintain  
357 cathepsin K in its active form<sup>38</sup>. Here we have shown that acidic conditions may exist within a LAM nodule  
358 and that this extra-cellular acidification is a consequence of mTOR dysregulation, likely to result both in the  
359 expression of membrane transporters including, carbonic anhydrases, monocarboxylate transporters and  
360 Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> co-transporters and mTOR dysregulation causing the Warburg effect, a metabolic dependence  
361 on aerobic glycolysis (figure 9). In 621-101 cell / LAF co-cultures, the transporters acidify the extra-cellular  
362 space to resulting in activation of cathepsin K, whilst their inhibition, particularly the Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> co-  
363 transporters, block both extra-cellular acidification and protease activation. Strikingly, rapamycin  
364 completely and rapidly, abrogated acidification in culture despite only suppressing the transcription of *CA*  
365 *IX*, suggesting that the part of the effect may have been on 621-101 cell metabolism rather than exclusively  
366 on the transporters themselves.

367

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

376



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

382

383 Taken together, our findings suggest that LAM cell / LAF interactions within LAM nodules promote disease  
384 progression by protease activation in a similar manner to tumour cell / cancer associated fibroblast  
385 interactions in cancer. These similarities are consistent with the idea that LAM is a low grade neoplastic  
386 disease, with a stroma similar to cancer<sup>46</sup>. Moreover, mTOR inhibitors which reduce lung function decline in  
387 LAM<sup>39, 47, 48</sup>, may exert some of their protective action in the lung upon LAM cell related extracellular pH,  
388 cathepsin K activation and expression. The expression of transporters including the  $\text{Na}^+/\text{HCO}_3^-$  co-  
389 transporter are downstream of mTORC1 / hypoxia inducible factor  $1\alpha$ <sup>49, 50</sup>. Further studies are required to  
390 understand, how mTOR activation and the expression of membrane transporters are related and whether  
391 inhibition of these transporters or cathepsin K activity will be of benefit in addition to mTOR inhibitors for  
392 LAM.

393

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399

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533

534 **Main Figure Legends**

535

536 **Figure 1**

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

544

545 **Figure 2**

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

549

550 **Figure 3**

551 **Cathepsin K expression within LAM lung nodules.** Immunohistochemical staining for cathepsin K (CTSK),  $\alpha$   
552 smooth muscle actin ( $\alpha$ -SMA), fibroblast specific protein (FSP) and melanoma marker antibody (PNL2) in  
553 two patients with LAM and normal lung tissue. Magnification x10, scale bar 200 $\mu$ m.

554

555 **Figure 4**

556 **Expression and activity of cathepsin K in cultured cells.** A: Quantitative real-time PCR for cathepsin K in  
557 LAM associated fibroblasts (LAF, n=4), normal human lung fibroblasts (NHLE, n=3) and 621-101 cells.  
558 \* $p < 0.05$ . B: Immunofluorescent detection of cathepsin K protein in two LAF, NHLE 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≤0.001. 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<sup>-/-</sup> and TSC2<sup>+/+</sup> MEF  
581 were cultured in unbuffered media for 24 hours at pH 7.5. TSC2<sup>+/+</sup> MEF and, TSC2<sup>-/-</sup> MEF and 621-101 cells  
582 treated with 10nM rapamycin did not affect pH whereas untreated TSC2<sup>-/-</sup> MEF and 621-101 cells  
583 significantly acidified media. \*\*p≤0.01, \*\*\*p≤0.001.

584

585 **Figure 7**

586 **Expression of membrane transporters in LAM cells and lung tissue.** A: Quantitative real time PCR for  
587 carbonic anhydrase (*CA II, IX, XII*), monocarboxylate transporter (*MCT 1, 4*), sodium bicarbonate ( $\text{Na}^+/\text{HCO}_3^-$ )  
588 ) co-transporters (*NBC 1 / SLC4A4, 3 / SLC4A7*), sodium  $\text{H}^+$  ( $\text{Na}^+/\text{H}^+$ ) exchanger (*NHE 1 / SLC9A1*) and  
589 vacuolar-type  $\text{H}^+$ -ATPases (*V-ATPases*) *ATP6V1B2* and *ATP6V0A4* in 621-101 cells treated for 24 hours with  
590 either vehicle, rapamycin (10nM), oestrogen (10nM) or LAF conditioned medium (LAF CM). B: Quantitative  
591 real time PCR for *CA II, IX, XII, MCT 1, 4*,  $\text{Na}^+/\text{H}^+$  exchanger, *NHE1*,  $\text{Na}^+/\text{HCO}_3^-$  co-transporters *SLC4A4*,  
592 *SLC4A7* and V-ATPases *ATP6V1B2* and *ATP6V0A4* in six LAM and three control patient derived lung tissues.  
593 C: Immunohistochemical staining of two representative LAM lung tissues showing positive staining in serial  
594 sections for CA IX and SLC4A4 within LAM nodules. Normal lung showed positive staining for SLC4A4 but  
595 not CA IX. Magnification x4, scale bar 500 $\mu\text{m}$  and x40, scale bar 50 $\mu\text{m}$ . D: Pharmacological inhibition of  
596 membrane transporters or mTOR inhibits extra-cellular acidification in 621-101 cell cultures. S4 and  
597 Acetazolamide - carbonic anhydrase inhibitors, BIX -  $\text{Na}^+/\text{H}^+$  exchanger inhibitor, S0895 -  $\text{Na}^+/\text{HCO}_3^-$  co-  
598 transporter inhibitor and Concanamycin A - V-ATPase inhibitor.

599

600 **Figure 8**

601 **Inhibition of membrane transporters blocks extracellular acidification and cathepsin K activity in LAM cell**  
602 **co-cultures.** A: 621-101 / LAM associated fibroblast (LAF) co-cultures were grown in unbuffered media in  
603 the presence of either vehicle control or S4 (carbonic anhydrase IX, XII inhibitor), acetazolamide (non-  
604 specific carbonic anhydrase inhibitor), BIX ( $\text{Na}^+/\text{H}^+$  exchanger inhibitor), S0895 ( $\text{Na}^+/\text{HCO}_3^-$  co-transporter  
605 inhibitor) concanamycin A (V-ATPase inhibitor) or rapamycin (mTOR inhibitor). B: Cathepsin K activity in co-  
606 culture supernatants treated as above showing strong inhibition of both acidification and cathepsin K  
607 activity by inhibition of the  $\text{Na}^+/\text{HCO}_3^-$  co-transporter and mTOR. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

608

609 **Figure 9**

610 **Proposed mechanism of Cathepsin K activation in LAM.** TSC2<sup>-/-</sup> LAM cells express carbonic anhydrases (CA)  
611 II, IX and XII within the cell which, in tandem, catalyse the conversion of CO<sub>2</sub> and water to H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>  
612 ions. Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> co-transporters (NBC) shuttle HCO<sub>3</sub><sup>-</sup> ions into the cell resulting in net H<sup>+</sup> export into the  
613 extracellular space. Cathepsin K containing lysosomes of LAM associated fibroblasts (LAF) translocate to the  
614 cell membrane discharging cathepsin K where it is activated in the low extracellular pH.

