mTORC1 hyperactivation in lymphangioleiomyomatosis leads to *ACE2* upregulation in type II pneumocytes: implications for COVID-19

To the Editor:

Respiratory failure is among the gravest outcomes of coronavirus disease 2019 (COVID-19), and most of the severe cases are associated with significant lung comorbidities [1]. Lymphangioleiomyomatosis (LAM) is a progressive cystic lung disease of women caused by mutations in tuberous sclerosis genes, resulting in aberrant hyperactivation of the mTOR complex 1 (mTORC1) signalling network in LAM cells, which are of mesenchymal origin [2]. The risk of COVID-19 in LAM is unknown.

We profiled the explant lungs of five women with LAM (46 659 cells) by single cell RNA sequencing (scRNA-seq) using 10× genomics Chromium platform. Low quality cells, defined as cells in which fewer than 200 different genes were detected or with mitochondrial gene percentage higher than 10%, were filtered out. Raw data was normalised, and log transformed as previously described [3]. Batch effects of datasets from different platforms were removed, and datasets were integrated using Seurat [4]. Major cell types identified in LAM using the algorithm SingleR [5] (figure 1a) included epithelial cells (type I and type II pneumocytes, ciliated cells), immune cells (T cells, B cells, macrophages, dendritic cells, natural killer cells) and endothelial cells (lymphatic endothelial cells and blood endothelial cells). The cell type annotation was further confirmed by plotting marker genes manually. Re-clustering of pneumocytes showed that *ACE2* was primarily expressed in type II pneumocytes, while *TMPRSS2* was expressed in all epithelial cell types (figure 1a), consistent with prior data [6–8]. The LAM lungs were compared with existing scRNA-seq datasets (GSE122960) of healthy lungs (n=8) [9], derived from donors who had died from stroke, haemorrhage, brain injury or gunshot. One healthy control with an Asian genetic background was excluded because of reports that Asian individuals have higher expression of *ACE2* than other populations [10].

The percentage of type II pneumocytes expressing *ACE2* in LAM lungs (2.6%) was significantly higher than in healthy lungs (0.9%; p=0.03, Mann–Whitney U-test) (figure 1a). Compared with healthy lungs, *ACE2* expression was also higher in LAM-associated type I (1.5% *versus* 0.7%; p=0.14) and ciliated cells (1.1% *versus* 0.5%; p=0.39). *TMPRRS2* expression was also significantly higher in LAM-associated type II pneumocytes (56% *versus* 45%; p=0.048) (figure 1a) and ciliated cells (33% *versus* 22%; p=0.14) compared to healthy lung. Significant upregulation of *ACE2* was not observed in single cell datasets from other lung conditions compared to healthy lungs, including pulmonary fibrosis (GSE122960) (n=4) [9] and lungs with cancer (n=2) [8] and infectious diseases (n=6) [8], with the exception of one lung from an HIV-positive and *Mycobacterium tuberculosis*-positive patient (figure 1a) [8]. These observations indicate that increased pneumocyte expression of *ACE2* and *TMPRSS2* is specific to LAM and not seen in other lung conditions, and suggest that the mTORC1-hyperactive LAM cells may induce this effect.

We further investigated whether *ACE2*+/*TMPRSS2*+ cells in LAM are primed for infection beyond the expression of these receptors for virus entry. Gordon *et al.* [11] identified 332 human proteins physically interacting with severe acute respiratory syndrome coronavirus (SARS-CoV-2) proteins in HEK293T cell expression experiments. We calculated a COVID-19 module score based on the average expression of these

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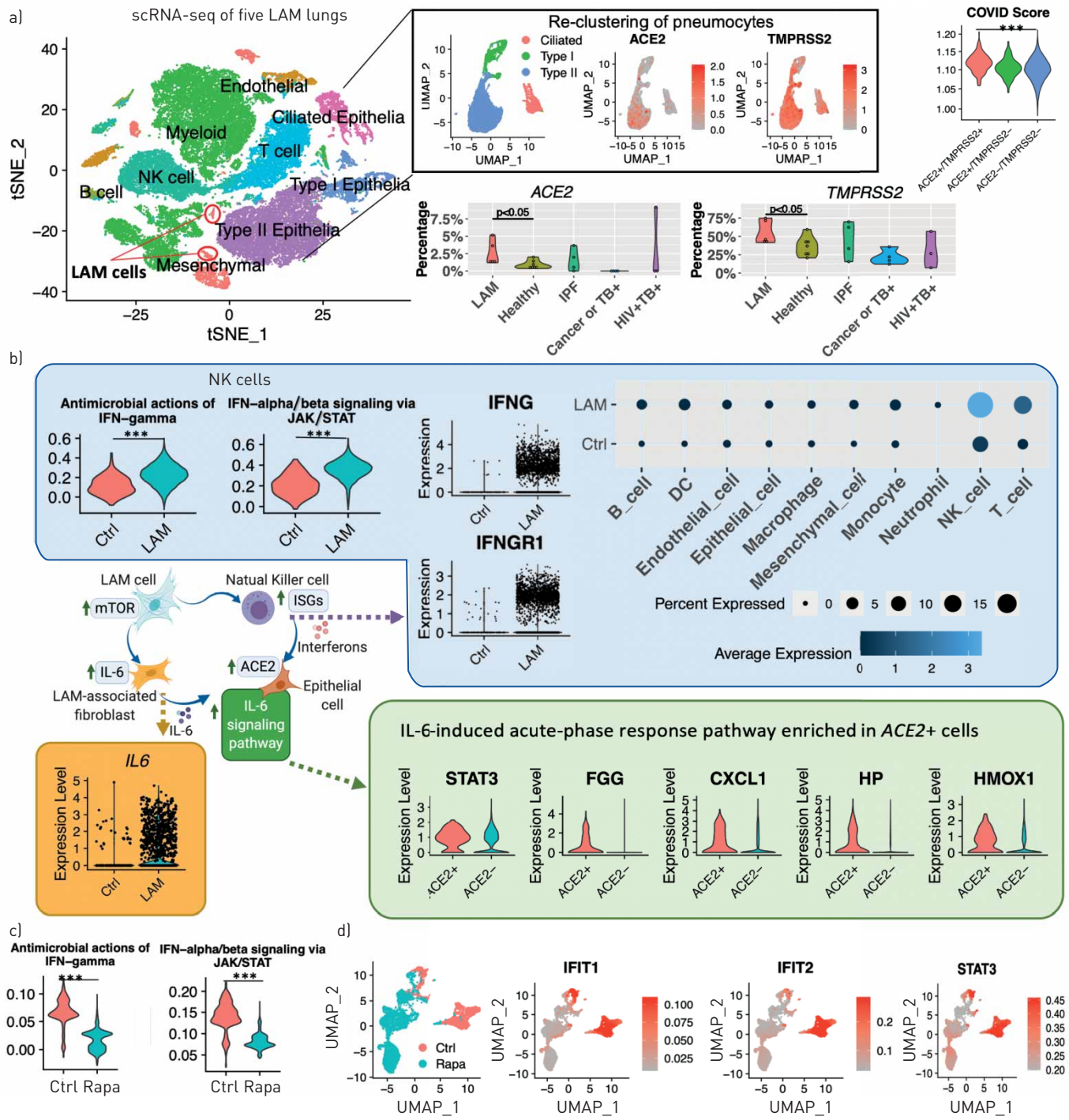

FIGURE 1 Expression of ACE2 and TMPRSS2 by epithelial cells is upregulated in lymphangioleiomyomatosis (LAM) lungs, which is linked with upregulation of interferon (IFN) pathways and IFN-γ expression in natural killer (NK) cells as well as increased interleukin (IL)-6 expression in LAM-associated fibroblasts. a) Increased expression of *ACE2* and *TMPRSS2* in LAM. UMAP plot shows major cell types identified in five LAM lung specimens. Re-clustering of pneumocytes showed that *ACE2* was expressed primarily in type II pneumocytes, while *TMPRSS2* was expressed by all subtypes. Scale bar colour intensity represents normalised expression. Significantly higher percentage of type II pneumocytes expressed *ACE2* and *TMPRSS2* in LAM lungs compared to healthy human lungs (Mann–Whitney U-test). Violin plot showing highest coronavirus disease 2019 (COVID-19) score in *ACE2+/TMPRSS2+* cells. b) Interferon secretion by LAM NK cells may cooperate with IL-6 to upregulate *ACE2* expression in LAM epithelial cells. In the IL-6-induced acute phase response pathway, master transcription factor STAT3 and four downstream genes were significantly upregulated in *ACE2+* epithelial cells. LAM-associated fibroblasts showed upregulation of *IL6* expression compared to fibroblasts derived from healthy lungs. Interferon pathways, as well as key genes (*IFNG* and *IFNGR1*), were upregulated in NK cells in LAM. Interferon gamma expression is significantly upregulated in LAM-associated NK cells compared to that of healthy lungs. Dot size represents fraction of cells within that type expressing interferon gamma (*IFNG*). Dot colour intensity represents average expression within that cell type. c) Rapamycin treatment downregulated interferon pathways. Violin plot shows that both type I (IFN-α/β signalling *via* JAK/STAT) and type II (antimicrobial actions of IFN-γ pathway) interferon pathways were downregulated by rapamycin treatment of a primary culture of a TSC2-mutant angiomyolipoma. d) Rapamycin treatment downregulated interferon-induced genes in a primary culture of a TSC2-mutant angiomyolipoma. UMAP of cells in control group (red) and cells in rapamycin treatment group (cyan). Scale bar colour intensity represents imputed expressions. ***: p<0.001.

332 genes using Seurat [4] in LAM pneumocytes. The significant upregulation of COVID-19 module score was seen in *ACE2+/TMPRSS2+* cells compared to *ACE2+/TMPRSS2−* cells (Mann–Whitney U-test, $p=5.1\times10^{-5}$) and *ACE2−/TMPRSS2−* cells (Mann–Whitney U-test; $p=1.1\times10^{-14}$) (figure 1a). Consistent with this, the percentage of cells expressing each of these 332 individual genes was highest in the *ACE2+/TMPRSS2+* cells (Mann–Whitney U-test, $p=2.2\times10^{-16}$; *ACE2+/TMPRSS2+ versus ACE2+/TMPRSS2−*) and lowest in the *ACE2−TMPRSS2−* cells (Mann–Whitney U-test, $p=2.2\times10^{-16}$; *ACE2+/TMPRSS2+ versus ACE2−/TMPRSS2−*) (data not shown due to limited space). These analyses demonstrate that *ACE2* expression in LAM correlates with increased expression of genes whose protein products interact with, and are hypothesised to enable virus replication.

To identify pathways co-regulated with the upregulation of *ACE2* in LAM, we performed pathway analysis, comparing expression of *ACE2+ versus ACE2−* epithelial cells in the LAM dataset. Both hypoxia and IL-6-induced acute-phase response pathways were upregulated in the LAM *ACE2+* pneumocytes. The master transcription factor of the IL-6 pathway, *STAT3*, was upregulated in LAM *ACE2+* pneumocytes, along with four genes directly or indirectly regulated by *STAT3*: *FGG*, *CXCL1*, *HP* and *HMOX1* (figure 1b), suggesting IL-6 might be driving these effects. Consistent with this, we found increased IL-6 in LAM-associated fibroblasts compared to mesenchymal cells derived from healthy lungs (figure 1b), supporting the concept that IL-6 drives *ACE2* expression in LAM.

ACE2 is an interferon-stimulated gene in human airway epithelial cells [8]. We used lists of genes involved in interferon (IFN)-α/β signalling *via* JAK/STAT (type I) and IFN-γ (type II) signalling pathways (MetaCore from Clarivate Analytics) to calculate cell type-specific interferon pathway activity scores in healthy lungs compared with LAM lungs [4]. In the LAM lungs, both type I and type II interferon pathways were upregulated in natural killer (NK), but not in T cells, B cells or macrophages (figure 1b). Many key regulators in the interferon pathways were significantly upregulated in LAM NK cells, including *INFG* and *INFGR1* (figure 1b). In addition, IFN-γ expression was upregulated in LAM-associated NK cells compared to healthy lungs (figure 1b). These analyses suggest that interferon secretion by LAM NK cells into the tumour microenvironment may cooperate with IL-6 to upregulate *ACE2* expression in LAM epithelial cells (figure 1b).

mTORC1, which is hyperactive in LAM, promotes protein synthesis and cellular growth, and is believed to be used by SARS-CoV-2 to favour replication [12]. LARP1, an mTOR-regulated translational repressor, is among the 332 virus-interacting proteins identified by Gordon *et al.* [11], and Appelberg *et al.* [13] identified crosstalk between SARS-CoV-2 and mTOR/HIF-1 signalling. Therefore, we investigated the effect of rapamycin (an allosteric mTORC1 inhibitor) on gene expression in these pathways in primary cells, derived from a dissociated renal angiomyolipoma tumour carrying a *TSC2* mutation obtained at the time of surgery. Angiomyolipomas are benign tumours that share common genetic mutations with LAM and occur in half of women with LAM. Primary cultures were treated with 20 nM rapamycin or DMSO for 24 h and subjected to single cell profiling. To overcome the challenge of inflated zeroes in single cell data, we used MAGIC [14] to impute gene expression in each cell. Using the same pathway gene lists described above (MetaCore from Clarivate Analytics), we observed that rapamycin treatment had major effects in downregulating both type I and type II interferon pathways (Mann–Whitney U-test, $p<2.2\times10^{-16}$ for all comparisons) (figure 1c), including many interferon-stimulated genes (*e.g.* IFIT, interferon induced protein with tetratricopeptide repeats family) (Mann–Whitney U-test $p<0.001$ for all genes) (figure 1d). IL-6-induced acute-phase response genes that were enriched in *ACE2+* cells were also downregulated by rapamycin treatment (Mann–Whitney U-test, $p<0.001$) (figure 1d). The expression of the 332 SARS-CoV-2-interacting host proteins also showed downregulation by rapamycin treatment in general (data not shown due to limited space). These analyses suggest that mTORC1 inhibition may have benefit in SARS-CoV-2 infection by decreasing the interferon and IL-6 pathways that promote the expression of ACE2.

In conclusion, we observed an increased percentage of type II pneumocytes expressing *ACE2* in LAM lungs, associated with upregulation of the type I and type II interferon pathways in LAM-associated NK cells, and increased *IL6* expression in LAM-associated fibroblasts, suggesting that both mechanisms drive increased *ACE2* expression. Similar mTORC1-dependent mechanisms may contribute to *ACE2* expression in other settings. An advantage of using LAM as a model system is the consistent activation of mTORC1 within LAM cells. While other tumours often have elevated mTORC1 activity, it is rarely present as a single, defining genetic feature, making it much more difficult to pinpoint these types of non-cell autonomous mechanisms. How mTORC1 signalling within tumour cells impacts *ACE2* expression in neighbouring epithelial cells will require additional single-cell datasets using genetically defined specimens, and/or mouse models in which mTORC1 is specifically perturbed. Alterations in mTORC1 signalling may also be present in non-malignant disorders, such as idiopathic pulmonary fibrosis (IPF). We analysed a published single cell dataset of four IPF samples, and did not find a significant increase in *ACE2* expression in type II pneumocytes compared to healthy donors.

Notably, using cultured primary cells from a LAM-related tumour, angiomyolipoma, we discovered that rapamycin treatment downregulated the interferon pathways and also downregulated a set of previously identified putative SARS-CoV-2-interacting proteins. From a clinical perspective, the upregulation of *ACE2/TMPRSS* expression in LAM lung epithelial cells suggests that women with LAM may be at increased risk of severe COVID-19 infection. Although all of the LAM specimens studied here are from lung transplantation tissue representing advanced disease, since our analyses link mTORC1 activity to the expression of ACE2 and TMPRSS2, we speculate that this would also be true in mild disease. Future studies will be needed to confirm this. The downregulation of IL-6 and interferon signalling in response to rapamycin suggests that rapamycin treatment may have benefit both in limiting initial infection upon virus exposure and progression of COVID-19 to pneumonia both in general and in women with LAM. This is particularly important for patients already taking rapamycin for LAM or other diseases. Further analyses including clinical trials will be necessary to assess the potential benefit of rapamycin for both aspects of COVID-19.

Yan Tang[1], David J. Kwiatkowski[1,2] and Elizabeth P. Henske[1,2]
[1]Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA. [2]D.J. Kwiatkowski and E.P. Henske contributed equally to this article as lead authors and supervised the work.

Correspondence: Elizabeth P. Henske, 20 Shattuck Street, Division of Pulmonary Medicine and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA. E-mail: ehenske@bwh.harvard.edu

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