Therapeutic targeting of the IL-6 trans-signalling/mTORC1 axis in pulmonary emphysema

Saleela M. Ruwanpura¹,², Louise McLeod¹,², Lovisa F. Dousha³, Huei J. Seow³, Sultan Alhayyani¹,², Michelle D. Tate¹,², Virginie Deswaert¹,², Gavin D. Brooks¹,², Steven Bozinovski³,⁴, Martin McDonald⁵, Christoph Garbers⁶, Paul T. King¹,⁵, Philip G. Bardin¹,⁵, Ross Vlahos³,⁴, Stefan Rose-John⁶, Gary P. Anderson³ and Brendan J. Jenkins¹,²*

¹Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research, 27-31 Wright Street, Clayton, Victoria, 3168, Australia.
²Department of Molecular Translational Science, Faculty of Medicine, Nursing and Health Sciences, Monash University, Clayton, Victoria, 3800, Australia.
³Lung Health Research Centre, Department of Pharmacology and Therapeutics, The University of Melbourne, Parkville, Victoria, 3050, Australia.
⁴School of Health and Biomedical Sciences, RMIT University, Bundoora, Victoria, 3083, Australia.
⁵Monash Lung and Sleep, Monash Medical Centre, Victoria, 3168, Australia.
⁶Institute of Biochemistry, Christian-Albrechts-University, Olshausenstrasse 40, D-24098 Kiel, Germany.

*To whom correspondence should be addressed: Brendan J. Jenkins, Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research, 27-31 Wright Street, Clayton, Victoria, 3168, Australia.

Tel +61 3 8572 2740; E-mail Brendan.Jenkins@hudson.org.au
Author contributions: S.M.R designed and performed experiments, analysed data, prepared the figures and co-wrote the manuscript. L.M. performed mouse experiments, cell culture, immunohistochemistry and molecular experiments. L.F.D. and H.J.S. performed smoke and lung function studies. M.D.T. and V.D. performed cell culture and flow cytometry analyses, respectively, and S.A. and G.D.B. assisted with immunohistochemistry. P.B. and P.T.K. provided human lung biopsies, and M.M. provided human sera. G.P.A. assisted with the experimental design of smoke-related studies and editing of the manuscript. R.V. and S.B. analyzed data, and assisted with experimental design of smoke-related studies and editing of the manuscript. S.R-J. provided mouse strains and novel reagents to target IL-6 trans-signalling, and assisted with the experimental design and editing of the manuscript. B.J.J. conceived, designed and supervised the research, and co-wrote the manuscript.

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Running title: IL-6 trans-signalling/mTORC1 axis in emphysema
Scientific Knowledge on the Subject

Emphysema is a major pathological component of COPD, and there is an urgent need for new intervention strategies against this disease. The potent immunomodulatory cytokine IL-6 is a prominent biomarker for emphysema. However, IL-6 is highly pleiotropic, and targeting IL-6 has been problematic since both pathogenic and beneficial (homeostatic) actions are blocked concurrently by conventional anti-IL-6 antibodies. It has thus proven difficult to exploit IL-6 as a therapeutic modality in human emphysema.

What this Study Adds to the Field

Our work addresses a new area of IL-6 biology that exploits understanding differential modes of IL-6 signalling, and their applicability to promoting the molecular pathogenesis of emphysema. Specifically, IL-6 utilizes two distinct signalling modes via the gp130 signal-transducing receptor subunit to regulate many pathophysiological responses, namely classical signalling through the membrane-bound IL-6 receptor (IL-6R) subunit, and trans-signalling via a naturally-occurring soluble IL-6R. In a novel combinatorial approach comparing the lungs of emphysematous patients and mouse models for spontaneous (gp130F/F) and experimentally-induced (cigarette smoke exposure) emphysema, we have elucidated the specific contribution of IL-6 trans-signalling to emphysema. The knowledge generated by our
findings is crucial for the future design and clinical application of novel therapeutics against emphysema.

"This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org"
ABSTRACT

Rationale: The potent immunomodulatory cytokine interleukin (IL)-6 is consistently upregulated in human lungs with emphysema, and in mouse emphysema models; however, the mechanism(s) by which IL-6 promotes emphysema remains obscure. IL-6 signals using two distinct modes, classical signalling via its membrane-bound IL-6 receptor (mIL-6R), and trans-signalling via a naturally-occurring soluble IL-6R (sIL-6R).

Objectives: To identify whether IL-6 trans-signalling and/or classical signalling contribute to the pathogenesis of emphysema.

Methods: We utilized the gp130F/F genetic mouse model for spontaneous emphysema, and cigarette smoke-induced emphysema models. Emphysema in mice was quantified by various methods including in vivo lung function and stereology, and TUNEL assay was employed to assess alveolar cell apoptosis. In mouse and human lung tissues, the expression level and location of IL-6 signalling-related genes and proteins were measured, and the levels of IL-6 and related proteins in sera from emphysematous mice and patients were also assessed.

Measurements and Main Results: Lung tissues from emphysema patients, as well as from spontaneous and cigarette smoke-induced emphysema mouse models, were characterized by excessive production of sIL-6R. Genetic blockade of IL-6 trans-signalling in emphysema mouse models, and therapy with the IL-6 trans-signalling antagonist sgp130Fc, ameliorated emphysema by suppressing augmented alveolar type II cell apoptosis. Furthermore, IL-6 trans-signalling-driven emphysematous changes in the lung correlated with mammalian target of rapamycin complex 1 (mTORC1) hyper-activation, and treatment of emphysema mouse models with the mTORC1 inhibitor rapamycin attenuated emphysematous changes.

Conclusions: Collectively, our data reveal that specific targeting of IL-6 trans-signalling may represent a novel treatment strategy for emphysema.

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1, mouse models, trans-signalling
INTRODUCTION

Pulmonary emphysema is the major component of Chronic Obstructive Pulmonary Disease (COPD), a condition predicted to be the third-leading cause of death worldwide by 2020 (1). The primary cause of emphysema is cigarette smoke (CS), which triggers a deregulated immune response in the lung that drives the destructive loss of alveolar cells and airspace enlargement (2). Despite these observations, the molecular basis by which key modulators of the immune system promote emphysema remains unclear. In this regard, increased expression of the immunomodulatory cytokine interleukin(IL)-6 is a common feature of emphysema, and increased IL-6 expression and IL6 gene polymorphisms correlate with rapid lung function decline and increased disease risk in smokers (3-6). Furthermore, elevated IL-6 expression in various mouse emphysema models has been linked to disease pathogenesis (7-11).

IL-6 utilizes two distinct signalling modes, both dependent upon the essential and ubiquitously-expressed gp130 cytokine receptor signal-transducing β-subunit, to regulate many pathophysiological responses. IL-6 classical signalling occurs via its membrane-bound (m) IL-6 receptor (mIL-6R) (12), and IL-6 trans-signalling (IL-6TS) via a naturally-occurring soluble (s) IL-6R (13) produced either by alternative splicing of IL6R mRNA or proteolytic cleavage of mIL-6R, the latter involving metalloproteases ADAM10 and/or ADAM17 (14). Notably, IL-6TS enables a large array of mIL-6R cell types to respond to IL-6 (12), thus increasing the overall IL-6 signalling spectrum. The predominant signalling pathway of either mode is JAK/STAT3, with other pathways including PI3K/AKT, mTORC1, and ERK/MAPK (15, 16).

The broad array of cell types responsive to IL-6TS underpins its role as the likely pathogenic IL-6 signalling mode in various inflammatory conditions such as arthritis, asthma, Crohn’s disease, and sepsis (17-22), as well as numerous cancers (23-25). However, despite these observations, the causal involvement of specific IL-6 signalling modes in emphysema is
unknown. We have previously reported the spontaneous development of IL-6-driven alveolar cell apoptosis leading to emphysema in $gp130^{F/F}$ mice (11), which have been engineered to display IL-6/gp130-dependent hyper-activation of the JAK-mediated STAT and mTORC1 pathways, in the absence of SHP2-mediated ERK MAPK and PI3K/AKT signalling. Here, we reveal in both $gp130^{F/F}$ and CS-induced emphysema models that the specific blockade of IL-6TS with the antagonist sgp130Fc (26) suppresses alveolar type II (ATII) cell apoptosis and disease pathogenesis. Furthermore, we uncover a novel role for mTORC1 hyper-activation in facilitating IL-6TS-driven emphysematous changes in the lung. Notably, the IL-6TS/mTORC1 axis was also augmented in patients with emphysema. Collectively, our observations provide the rationale for the clinical evaluation of IL-6TS as a new target for the treatment of emphysema.
METHODS

Mice and treatments
The $gp130^{F/F}$ and $gp130^{F/F}:Il6^{-/-}$ mice have been previously reported (11), and $gp130^{F/F}:sgp130Fc^{tg/tg}$ mice were generated using $sgp130Fc$ transgenic mice (27). $Gp130^{F/F}$ mice aged 3 months were intraperitoneally (i.p) administered with either sgp130Fc (once weekly, low dose of 0.5mg/kg) (21), PBS (once weekly), rapamycin (3 times a week, 4mg/kg) (36) or vehicle (0.8% DMSO, 5.2% PEG-400, 5.2% Tween-20, 3 times a week), over 12 weeks. For acute and chronic CS models (details are provided in the online data supplement), mice were concurrently administered with sgp130Fc (0.5mg/kg) or PBS once over 4 days and once weekly over 12 weeks, respectively. Rapamycin (4mg/kg) or vehicle was administered either twice over 4 days (acute CS) or 3 times a week over 12 weeks (chronic CS). All mice were housed under specific pathogen-free conditions. Experiments were approved by the Monash University Animal Ethics Monash Medical Centre “A” Committee.

Human lung biopsy and serum samples
Details are provided in an online data supplement.

RNA isolation and gene expression analysis
Details are provided in an online data supplement.

Protein extraction, ELISA and immunoblotting
Details are provided in an online data supplement.

Immunohistochemistry and immunofluorescence
Details are provided in an online data supplement.
Stereology and mean linear intercept (MLI) analyses

Mouse lungs were perfusion fixed by instillation of 1.5% glutaraldehyde/1.5% formaldehyde in 0.04M phosphate-buffered saline, following which tissue blocks were prepared by processing and embedding in glycol methacrylate, as previously described (11). Lung stereology was performed on methylene blue-stained lung tissue sections using computer-assisted newCAST software (version 2.14; Visiopharm, Hørsholm, Denmark (7, 11). Airspace enlargement was quantified by the MLI technique on H&E-stained lung sections (7).

Lung function analyses

The assessment of lung function on anesthetized mice was performed using the flexiVent system (SCIREQ, Montreal, Canada) (7, 11).

Statistical analyses

Statistical analyses were performed using GraphPad Prism for Windows version 6.0. D'Agostino and Pearson omnibus K2 normality tests were performed for all data. Paired t-tests were used to analyze normally distributed data among 2 sample groups, and Mann-Whitney tests for non-normally distributed data or smaller data sets. One-way analysis of variance (ANOVA) was used to assess differences between 3 or more groups for normally distributed data, and Kruskal-Wallis tests for non-normally distributed data or smaller data sets, along with appropriate post-tests. Linear regression was performed for correlations between given markers in moderate emphysema patient groups only. Data are expressed as the mean ± standard error of the mean (SEM), and $P < 0.05$ was considered statistically significant.
RESULTS

Augmented expression of IL-6TS components in gp130\textsuperscript{F/F} emphysematous mouse lungs

To delineate the mode of IL-6 signalling associated with IL-6-driven emphysema, we initially assessed whether key IL-6TS components were deregulated in emphysematous lungs of 6 month old (mo) gp130\textsuperscript{F/F} mice. While gene expression levels for \textit{Il6r\alpha} and \textit{Adam10} were normal, \textit{Il6} and \textit{Adam17} were significantly increased in gp130\textsuperscript{F/F} mouse lungs (Fig. 1A). IL-6 protein levels were also significantly increased in the lungs and bronchoalveolar lavage fluid (BALF) of gp130\textsuperscript{F/F} compared to gp130\textsuperscript{+/+} control wild-type mice (Fig. 1B, Fig. E1A). Since sgp130 protein levels were unchanged, the high sIL-6R levels in lungs and BALF of gp130\textsuperscript{F/F} mice (Fig. 1B, Fig. E1A) suggest augmented IL-6TS and its possible role in the emphysema phenotype of gp130\textsuperscript{F/F} mice.

Transgenic over-expression of the IL-6TS antagonist sgp130Fc in gp130\textsuperscript{F/F} mice prevents the onset of emphysema

To define a causative role for IL-6TS in the pathogenesis of emphysema in gp130\textsuperscript{F/F} mice, we crossed gp130\textsuperscript{F/F} mice with sgp130Fc transgenic mice (27) to generate gp130\textsuperscript{F/F:sgp130Fc}\textsuperscript{tg/tg} mice over-expressing sgp130Fc, a fusion protein of recombinant sgp130 and the Fc region of human IgG1 which acts as a potent specific inhibitor of IL-6TS-driven pathologies (21, 24). Histological evaluation of 6mo gp130\textsuperscript{F/F:sgp130Fc}\textsuperscript{tg/tg} mice revealed normal alveolar lung architecture compared to enlargement of the distal air spaces and destruction of alveoli, measured as alveolar mean linear intercept (MLI), that were observed for gp130\textsuperscript{F/F} mice (Fig. 1C, Fig. E1B). Consistent with these histological observations, the elevated static compliance and lung volume, due to destruction of elastic fibres in emphysematous lungs, that are a feature of gp130\textsuperscript{F/F} mice (11) were also normal in gp130\textsuperscript{F/F:sgp130Fc}\textsuperscript{tg/tg} mice (Fig. 1D, E). In further support of these findings, stereology indicated that the volume fractions of air space...
and alveolar septal tissue, and the surface density within lung parenchyma, were similar between \(gp130^{+/+}\), \(gp130^{+/+}:sgp130Fc^{tg/tg}\) and \(gp130^{F/F}:sgp130Fc^{tg/tg}\) mice, while these parameters were significantly elevated in \(gp130^{F/F}\) mice (Table E1). The serum and lung expression levels of the acute phase response (APR) protein serum amyloid A, which is regulated by IL-6 classical signalling, remained elevated and unchanged in \(gp130^{F/F}:sgp130Fc^{tg/tg}\) compared to \(gp130^{F/F}\) mice (Fig. E1C), thus indicating that IL-6 classical signalling was unaffected in \(gp130^{F/F}:sgp130Fc^{tg/tg}\) mice. Furthermore, the absence of emphysema in \(gp130^{F/F}:sgp130Fc^{tg/tg}\) mice was associated with a significant reduction in the lung expression levels of IL-6 and sIL-6R back to those observed in \(gp130^{+/+}:sgp130Fc^{tg/tg}\) and \(gp130^{+/+}\) mice (Fig. E1D, E). Collectively, these data reveal that IL-6TS drives the development of emphysema in \(gp130^{F/F}\) mice.

**IL-6TS promotes apoptosis-driven emphysema in \(gp130^{F/F}\) mice**

Since apoptosis is a key process associated with the development of human emphysema and several disease models, including IL-6/gp130-driven emphysema in \(gp130^{F/F}\) mice (11, 28, 29), we compared the extent of apoptotic TUNEL-stained cells in the lungs of 6mo mice. The number of TUNEL-stained alveolar cells in \(gp130^{F/F}:sgp130Fc^{tg/tg}\) mice was significantly reduced compared to \(gp130^{F/F}\) mice, and was comparable to \(gp130^{+/+}\) and \(gp130^{+/+}:sgp130Fc^{tg/tg}\) control mice (Fig. 2A) (7, 11). The alveolar epithelium covers >99% of the internal surface area of the lung and is mainly composed of ATI and ATII cells, the latter of which uniquely synthesize and secrete surfactants, including the biomarker surfactant protein C (SPC) which prevents small airway closure and alveolar collapse, thereby maintaining normal lung structure (30, 31). Notably, the number of SPC-positive cells in the lungs of \(gp130^{F/F}\) mice was significantly reduced compared to \(gp130^{+/+}\) mice, whereas SPC-positive cell numbers remained elevated in the lungs of \(gp130^{F/F}:sgp130Fc^{tg/tg}\) mice (Fig. 2B).
Dual immunofluorescence staining with cleaved caspase-3 (marker of apoptosis) and SPC further confirmed that there was a significant increase in the number of apoptotic SPC-positive ATII cells in $gp130^{+/-}$ mice compared to $gp130^{+/-}.sgp130F_{c}^{tg/tg}$ and $gp130^{+/-}.sgp130F_{c}^{tg/tg}$ mice (Fig. 2C), while augmented apoptosis was not detected for ATI (podoplanin-positive) and endothelial (CD31-positive) cells (Fig. E2). Collectively, these data suggest that IL-6TS promotes type II cells to undergo apoptosis during emphysema.

**Therapeutic blockade of IL-6TS with sgp130Fc prevents alveolar cell death and emphysema in $gp130^{F/F}$ mice**

To determine whether IL-6TS can serve as a *bona fide* therapeutic target for emphysema, we next explored whether suppression of IL-6TS upon administration with sgp130Fc would prevent emphysema development in $gp130^{F/F}$ mice from 3 months of age onwards. At the completion of 12 weeks treatment with sgp130Fc, the lungs of $gp130^{F/F}$ mice were comparable to those of untreated $gp130^{+/-}$ mice, with fully-preserved lung morphology (Fig. 3A), static compliance (Fig. 3B), lung volume (Fig. 3C), stereological parameters (Table E1) and the number of apoptotic cells (Fig. 3D). Taken together these data demonstrate that therapeutic targeting of IL-6TS can prevent emphysema development in $gp130^{F/F}$ mice.

**Acute CS exposure of wild-type mice augments expression of IL-6TS components which is associated with elevated alveolar cell apoptosis**

Since CS is the major trigger of emphysema in humans, we next assessed whether our mechanistic findings for spontaneous IL-6TS-driven emphysema are relevant to CS-induced lung disease. Upon an initial exposure of 4-6 week old $gp130^{+/-}$ mice to an acute (4 day) CS model, mRNA levels for *Il6* (11) and *Adam17* were significantly increased, while those for *Il6ra* and *Adam10* remained normal, compared to non-CS-exposed control mice (Fig. 4A).
Acute CS exposure also significantly augmented IL-6 and sIL-6R, but not sgp130, protein levels (Fig. 4B). By contrast, the lungs of gp130\(^{++}\):sgp130Fc\(^{++}\) mice exposed to CS showed no changes in Il6 and Adam17 mRNA levels (Fig. E3A), nor sIL-6R and sgp130 levels (IL-6 levels remained undetectable) (Fig. E3B). The response of gp130\(^{++}\) mouse lungs to CS exposure was confirmed by CS-induced increases in whole lung mRNA levels of inflammatory mediators Cxcl2, Tnfa and Ccl2 (32, 33), which were ameliorated in either CS-exposed gp130\(^{++}\):sgp130Fc\(^{++}\) mice or CS-exposed gp130\(^{++}\) mice concurrently administered once with sgp130Fc (Fig. E3C). Moreover, while acute CS exposure of gp130\(^{++}\) mice also augmented numbers of TUNEL-positive apoptotic alveolar cells (Fig. 4C) (11), no such increases were observed in acute CS-exposed gp130\(^{++}\):sgp130Fc\(^{++}\) mice and CS-exposed gp130\(^{++}\) mice treated with sgp130Fc (Fig. 4C).

**Inhibition of IL-6TS with sgp130Fc suppresses alveolar cell apoptosis and emphysematous changes induced by chronic CS exposure of wild-type mice**

To determine the relevance of IL-6TS to CS-induced emphysema development, we next utilized a chronic (12 week) CS-induced emphysema model. Over 12 weeks, we observed significant increases in numerous parameters indicative of emphysema, namely static compliance, lung volume and MLI, in CS-exposed gp130\(^{++}\) mice compared to their age-matched non-CS-exposed counterparts (Fig. 4D-F). By contrast, chronic CS-exposed mice displayed significant reductions in all of these emphysematous parameters upon inhibition of IL-6TS with sgp130Fc (Fig. 4D-F). Importantly, these findings correlated with our data demonstrating that chronic CS exposure increased TUNEL-positive cell numbers in the lungs of gp130\(^{++}\) mice compared to non-CS-exposed mice, and this was significantly reduced (~40%) in response to sgp130Fc treatment (Fig. 4G).
Collectively, these data in CS-exposed wild-type mice reveal that therapeutic and genetic blockade of IL-6TS suppresses augmented expression of inflammatory mediators and alveolar cell death, leading to amelioration of emphysema, therefore validating the relevance of IL-6TS to the pathogenesis of CS-induced emphysema.

**IL-6TS in the lungs of emphysematous gp130^{F/F} mice is associated with hyper-activation of the mTORC1 pathway**

Our data here indicating that suppression of IL-6TS in mouse emphysema models prevents emphysema implies that IL-6TS-driven emphysematous changes in the lung are a consequence of increased activation of a gp130-dependent signalling pathway. We have previously eliminated a role for IL-6-driven hyper-activation of STAT3 and STAT1 in promoting emphysema in gp130^{F/F} mice (7). In addition, such a pathway is unlikely to be either SHP2-mediated ERK MAPK or PI3K/AKT, since gp130/SHP2-mediated activation of these pathways has been abolished in gp130^{F/F} mice (34). Another candidate pathway is rapamycin-sensitive mTORC1, which is a large multi-protein complex comprising the serine/threonine kinase mTOR subunit that is activated in response to diverse stimuli, such as cytokines (35). Hyper-activation of mTORC1 is observed in many diseases, including pulmonary fibrosis, which highlights its ability to regulate various cellular processes such as proliferation and apoptosis (35, 36).

We observed a significant increase in the number of cells expressing phosphorylated ribosomal S6 kinase (P-rpS6), a key mTORC1 substrate (35), in the lungs of gp130^{F/F} mice compared to emphysema-free gp130^{+/+} and gp130^{F/F}:Il6^{-/-} mice, the latter deficient in IL-6 (11) (Fig. 5A). Dual immunofluorescence staining confirmed that P-rpS6 expression co-localized to SPC-positive ATII cells (Fig. E4A), but not CD45-positive inflammatory cells (Fig. E4B). Immunoblot analyses also indicated that P-rpS6 protein levels, as well as P-mTOR (ser2448)
levels, were significantly reduced in \( gp130^{F/F};Il6^{-/-} \) compared to \( gp130^{F/F} \) lungs (Fig. 5B, Fig. E4C). Furthermore, we demonstrated activation of the IL-6TS/mTORC1 axis in cultured \( gp130^{F/F} \) primary ATII cells stimulated with the potent IL-6TS agonist Hyper-IL-6 (12) (Fig. E5). Moreover, both the sgp130Fc-mediated genetic and therapeutic blockade of IL-6TS in \( gp130^{F/F} \) mice significantly reduced P-rpS6-positive cell numbers in the lung compared to their respective controls (Fig. 5C, D, Fig. E6A, B). Therefore, these data support the notion that IL-6TS augments activation of the mTORC1 pathway in the lungs of emphysematous \( gp130^{F/F} \) mice.

The therapeutic blockade of the mTORC1 pathway in rapamycin-treated \( gp130^{F/F} \) mice suppresses the development of emphysema

To specifically delineate a role for increased mTORC1 activity in IL-6TS-driven emphysema, we explored whether mTORC1 pathway blockade with the specific mTORC1 inhibitor, rapamycin, could alleviate emphysema development in \( gp130^{F/F} \) and CS-induced emphysema models. Examination of lung morphology, static compliance, lung volumes and various stereological parameters revealed that emphysematous changes (Fig. 5E-G, Table E1), and also TUNEL- and P-rpS6-positive cell numbers (Fig. 5H, I, Fig. E6C, D), were significantly reduced in the lungs of \( gp130^{F/F} \) mice following 12 weeks of rapamycin treatment compared to vehicle-treated \( gp130^{F/F} \) control mice.

We also observed increased numbers of P-rpS6-positive cells in the lungs of acute CS-exposed \( gp130^{+/-} \) mice compared to their non-CS-exposed controls (Fig. E7A). However, P-rpS6-positive cell numbers were significantly reduced in CS-exposed \( gp130^{+/-} \) mice concurrently treated with sgp130Fc compared to CS-exposed \( gp130^{+/-} \) mice treated with vehicle (Fig. E7A). Furthermore, the genetic blockade of IL-6TS in \( gp130^{+/-};sgp130Fc^{tg/tg} \) mice prevented any increase in P-rpS6-positive cell numbers in response to CS exposure (Fig.
The numbers of P-rpS6- and TUNEL-positive cells were significantly reduced in acute CS-exposed $gp130^{+/+}$ mice administered with rapamycin compared to their CS-exposed controls that received vehicle (Fig. E7B, C). Moreover, chronic CS exposure increased P-rpS6-positive cell numbers in the lungs of $gp130^{+/+}$ mice compared to their non-CS-exposed counterparts, and this was significantly reduced by $\sim45\%$ in response to sgp130Fc treatment (Fig. 5J, Fig. E7D). Collectively, these data strongly suggest that IL-6TS promotes mTORC1 pathway hyper-activation during emphysema.

**Upregulation of IL-6TS/mTORC1 components correlates with human emphysema**

To translate our emphysematous mouse model findings to human disease, we first assessed the expression of IL-6TS-related genes in human lung tissues from disease-free and emphysema patients as defined by pulmonary lung function tests (gas exchange, FEV1; Table E2). The gene expression level for $IL6$, as well as both IL-6 and sIL-6R protein levels, were significantly increased in lungs of emphysema patients compared to emphysema-free controls (Fig. 6A, B). In addition, $ADAM17$ gene expression was elevated 2-fold in emphysema patients compared to disease-free individuals (Fig. 6A), and ADAM17 protein levels significantly correlated with elevated sIL-6R levels observed in emphysema patients (Fig. 6C, D). Furthermore, we observed a significant positive correlation between sIL-6R levels and the number of cleaved caspase-3-positive cells in the lungs of emphysema patients (Fig. 6E, F), thus further supporting IL-6TS-induced apoptosis in human emphysema.

We next investigated the activation status of the mTORC1 pathway, and immunoblot analyses indicated that P-rpS6 protein levels were significantly increased in lung lysates from emphysema patients (Fig. 6G). Furthermore, regression analyses of IL-6 and P-rpS6 levels showed significant positive correlations with sIL-6R in emphysema patients (Fig. 6H). Notably, circulating levels of IL-6TS components, namely IL-6 and sIL-6R, were also up-
regulated in the serum of emphysema patients compared to healthy individuals (Fig. 6I).
Overall these clinical data are consistent with our in vivo findings and provide translational support for a pathological role for the IL-6TS/mTORC1 axis in human emphysema.
DISCUSSION

Despite the established link between IL-6 and the pathogenesis of emphysema and other lung diseases (1-5, 11), little information is known regarding the identity of the mode of IL-6 signalling and downstream pathways which drive disease pathogenesis. For instance, mouse asthma models and clinical data indicate increased sIL-6R levels in BALF and airway smooth muscle which may be of relevance to airway remodelling (22, 37). In addition, blocking global IL-6 signalling via neutralizing antibodies or genetic ablation of IL-6 during chronic stages of adenosine-mediated lung injury and asthma provide the benefit of halting airway remodelling processes such as fibrosis. However, the above studies only hypothesize involvement of IL-6TS rather than IL-6 classical signalling in driving lung disease (38, 39).

Here, we define for the first time that hyper-activation of the endogenous IL-6TS/mTORC1 axis in the lung augments ATII cell apoptosis during emphysema. Furthermore, our discoveries of augmented expression and activation of IL-6TS and mTORC1 pathway components, respectively, in the lung of patients with emphysema, together with elevated levels of IL-6TS components (e.g. sIL-6R) in the serum of emphysema patients, have considerable translational potential for biomarker discovery and early disease detection, as well as patient stratification for potential responders who may gain benefit from selective therapies against the IL-6TS/mTORC1 axis.

While our data reveal that mTORC1 hyper-activation is associated with elevated alveolar cell apoptosis, mTORC1 is also a negative regulator of autophagy, a process which has been implicated in emphysematous lung destruction. However, we have previously reported that IL-6-driven emphysematous changes in the lung (which are associated with elevated mTORC1 activation) are independent of autophagy, and for that matter inflammation (7). Furthermore, considering that mTORC1 is activated by a vast range of stimuli (e.g. cytokines, growth factors, stress, oxygen, nutrients) and regulates a plethora of cellular
responses in the lung in both a cell-type and stimulus-dependent manner (35, 40), our current findings suggest that mTORC1 activation in the alveolar epithelium specifically by IL-6 (unlike other stimuli) does not affect autophagy and/or inflammation.

Hyper-activation of mTORC1 can promote IL-6TS by upregulating sIL-6R levels \textit{in vivo} (41), which not only supports mTORC1 being an integral component of the IL-6TS network (at least in the lung), but also suggests a feed-forward regulatory loop involving IL-6TS and mTORC1. A role for mTORC1 in promoting emphysema is also supported by the demonstration that peripheral blood mononuclear cells from COPD patients and CS extract-stimulated human monocytes display increased mTOR activity, which is associated with corticosteroid resistance in COPD patients (42). However, in contrast to our current findings, expression of the CS-induced stress response protein, RTP801, can increase septal cell death leading to pulmonary injury and emphysema via inhibition of mTORC1 and NF-κB activation (43). Since the upstream activators of mTORC1 in the above study were not documented, they may differ from IL-6 which could cause differences in the kinetics of mTORC1 activation and subsequently the net signal output and cellular response within the lung. In this regard, mTORC1 can exhibit pro- and anti-apoptotic activities (35), and although the ability of hyper-activated mTORC1 to promote apoptosis has been linked to a negative feedback loop which suppresses the AKT survival pathway (35, 44), AKT activation in the lungs of \textit{gp130}^{F/F} mice is unaltered (data not shown). Collectively, these observations not only highlight the importance of identifying the molecular drivers and cellular context of mTORC1 activity during disease pathogenesis prior to the potential application of therapeutics, but also warrant investigations into the existence of alternate signalling pathways (e.g. ERK MAPK) and target genes (e.g. p53, Bcl-2, Nox4, Bad) which can promote the pro-apoptotic actions of mTORC1 (45) during emphysema.
Current strategies aimed at targeting IL-6 in the clinic have largely resulted from earlier experimentally-induced mouse models for arthritis and inflammatory bowel diseases, whereby antibody–mediated neutralization of IL-6R or IL-6 suppressed disease development (46). In this respect, the humanized neutralizing IL-6R monoclonal antibody, tocilizumab, has shown considerable promise in clinical trials in the management of rheumatoid arthritis (47). However, tocilizumab inhibits the mIL-6R mediated pathway, which most likely accounts for the increased incidence of infection associated with its use due to suppressing the “immune-protective” IL-6 classical signalling pathway. Indeed, studies using IL-6 knock-out mice have suggested that IL-6 (classical) signalling protects against mucosal ulcerations from bacterial pathogens, such as *Citrobacter rodentium* infection (48). Such studies highlight how, under certain situations, blocking IL-6 (classical) signalling may have detrimental consequences, and also imply that other modes of IL-6 signalling promote chronic inflammatory responses associated with disease pathogenesis.

The specific IL-6TS antagonist sgp130Fc used in our current study can exhibit significant therapeutic activity in IL-6TS-driven animal models of inflammation, autoimmunity and cancer, with very low immunogenicity and ~7 day *in vivo* half-life, as well as no interference with IL-6 classical signalling events (18, 21, 49). Notably, blocking IL-6TS with sgp130Fc can inhibit sIL-6R production by macrophages contributing to attenuation of idiopathic pulmonary fibrosis (50), which is consistent with our findings that sgp130Fc suppressed augmented levels of sIL-6R in the lungs of *gp130<sup>+/−</sup>* and CS-induced emphysema models. Moreover, the efficacy of sgp130Fc, which will undergo future phase II clinical trials in Europe for Crohn’s Disease, at suppressing emphysematous changes in the lungs of both models provides translational implications directly to preventatives for human emphysema. From a therapeutic perspective the selective blockade of IL-6TS, and lung-directed mTORC1 suppression, offer promising new strategies to ameliorate emphysema (and COPD), and our
current findings will fast-track further pre-clinical testing of sgp130Fc and the development of humanized anti-IL-6R mAbs which target IL-6TS for clinical use in emphysema.
REFERENCES


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FIGURE LEGENDS

Figure 1  Augmented expression of IL-6TS components promotes emphysema in gp130^{F/F} mice. Q-PCR analyses of (A) Adam17 and Adam10 gene expression were performed on cDNA derived from total RNA prepared from lung tissue of 6 month old (mo) gp130^{+/+} (+/+) , gp130^{+/+}:sgp130Fc^{tg/tg} (+/+:tg) , gp130^{F/F} (F/F) and gp130^{F/F}:sgp130Fc^{tg/tg} (F/F:tg) mice. Expression data are shown following normalization for 18S expression, and are presented as the mean ± SEM from triplicate analyses from n = 5 mice per genotype. (B) IL-6, sIL-6R and sgp130 protein concentrations in lung lysates as determined by ELISA. n = 5 mice per genotype. (C) Representative methylene blue-stained cross-sections of lungs from 6mo +/+, +/+:tg, F/F and F/F:tg mice. Arrows indicate airspace enlargement. Scale bars = 100μm. (D) Static compliance as determined by flexiVent lung function analysis, and (E) lung volume per body total weight (grams), of the above 6mo mice. Data are expressed as mean ± SEM. n = 10 mice per genotype. *P< 0.05, **P< 0.01, ***P< 0.001 and ****P< 0.0001.

Figure 2  IL-6TS-mediated apoptosis of ATII cells leads to the development of emphysema. Stereological quantification of the number of (A) TUNEL- and (B) SPC-positive ATII cells, and corresponding representative photomicrographs showing positive cells in cross-sections of lungs in 6 month old (mo) gp130^{+/+} (+/+) , gp130^{+/+}:sgp130Fc^{tg/tg} (+/+:tg) , gp130^{F/F} (F/F) and gp130^{F/F}:sgp130Fc^{tg/tg} (F/F:tg) mice. Scale bars = 25μm. (C) Representative confocal immunofluorescence photomicrographs of cells positive for either caspase-3 alone (green), SPC alone (red), or both caspase-3 and SPC (yellow, white circles) in cross-sections of the lung in 6mo F/F mice. The graph depicts stereological quantification of dual labelled caspase-3- and SPC-positive cells in the lungs of 6mo mice of the indicated genotypes. Scale bars = 10μm. Data are expressed as the mean ± SEM. n = 5 mice per genotype. *P< 0.05 and **P< 0.01.
Figure 3 Therapeutic blockade of IL-6TS prevents emphysema development in gp130\textsuperscript{F/F} mice. (A) Representative methylene blue-stained cross-sections (arrows indicate airspace enlargement), (B) static compliance and (C) lung volume, of lungs from 6 month old (mo) \textit{gp130\textsuperscript{+/+}} (+/+, n = 8) and \textit{gp130\textsuperscript{F/F}} (F/F, n = 8) mice, and 3mo F/F mice treated with sgp130Fc inhibitor for 12 weeks (F/F+sgp130Fc, n = 5). Scale bars = 100\textmu m. (D) Stereological quantification of the number of TUNEL-positive cells, and corresponding representative photomicrographs showing positive cells in cross-sections of lungs, from \textit{+/+} (n = 8), F/F (n = 8) and F/F+sgp130Fc (n = 5) mice. Data are expressed as the mean ± SEM. *\textit{P}< 0.05, **\textit{P}< 0.01 and ***\textit{P}< 0.001.

Figure 4 Blockade of IL-6TS with sgp130Fc suppresses apoptosis induced by CS exposure to wild-type mice. (A) Q-PCR expression analyses of the indicated genes were performed on lung cDNA, and (B) protein expression analyses of the indicated proteins were performed on lung lysates, from 1 month old (mo) \textit{gp130\textsuperscript{+/+}} (+/+, n = 8) mice with (+) or without (-) CS exposure for 4 days. Data are expressed as the mean ± SEM. n = 5 mice per genotype. *\textit{P}< 0.05. (C) Stereological quantification of the number of TUNEL-positive cells, and corresponding representative photomicrographs showing positive cells in cross-sections of lungs, from the following mouse groups; \textit{+/+} (- CS), \textit{+/+} (+ CS), \textit{+/+} concurrently treated with sgp130Fc (+/+, Fc + CS)) and \textit{gp130\textsuperscript{+/+}:sgp130Fc\textsuperscript{tg/tg}} (+/+:tg) mice with or without CS exposure for 4 days. (D) Static compliance, (E) lung volume, (F) mean linear intercept, and (G) stereological quantification of the number of TUNEL-positive cells and corresponding representative photomicrographs showing positive cells in cross-sections of the lungs from 1mo \textit{gp130\textsuperscript{+/+}} mice (+/+) treated with or without sgp130Fc inhibitor or CS for 12 weeks. Data are expressed as the mean ± SEM. n = 5 mice per genotype. *\textit{P}< 0.05, **\textit{P}< 0.01, ***\textit{P}< 0.001 and ****\textit{P}< 0.0001.
Figure 5 IL-6-induced mTORC1 pathway hyper-activation promotes emphysematous changes in the lungs of mice. (A) Stereological quantification of the number of P-rpS6-stained cells, and corresponding representative photomicrographs showing positive cells in cross-sections of lungs, from 6 month old (mo) gp130+/+, gp130<sup>F/F</sup> (F/F) and F/F:Il6<sup>-/-</sup> mice. n = 5 mice per genotype. *P < 0.05. (B) Immunoblotting with indicated antibodies on whole lung lysates from 6mo F/F and F/F:Il6<sup>-/-</sup> mice. Each lane represents tissue from an individual mouse. Densitometric quantification of P-rpS6 and total S6 protein levels per genotype was performed and normalized against Actin protein levels present in each sample. Data are presented as the mean fold induction ± SEM from n = 7 mice per genotype. Stereological quantification of the number of P-rpS6-positive cells in the lungs of (C) 6 month old (mo) +/+, gp130<sup>+/+;sgp130Fc<sup>tg/tg</sup></sup> (+/+:tg), F/F and gp130<sup>F/F;sgp130Fc<sup>tg/tg</sup></sup> (F/F:tg) mice and (D) 3mo F/F mice treated with sgp130Fc inhibitor for 12 weeks (F/F + Fc). n = 5 mice per group. *P < 0.05, **P < 0.01. (E) Representative methylene blue-stained cross-sections of lungs from F/F mice (aged 1 month) that were treated with or without rapamycin for 12 weeks. Arrows indicate airspace enlargement. Scale bars = 100μm. (F-I) Static compliance (F), lung volumes (G), and the stereological quantification of the number of TUNEL-positive cells (H) and P-rpS6-positive cells (I) in the lungs of F/F mice treated with or without rapamycin for 12 weeks. n = 4 mice per group. *P < 0.05 and **P < 0.01. (J) Stereological quantification of the number of P-rpS6-positive cells in the lungs of gp130<sup>+/+</sup> mice (+/+) aged 1 month treated with or without sgp130Fc inhibitor or CS for 12 weeks. n = 4 mice per group. **P < 0.01 and ***P < 0.001.

Figure 6 Augmented levels and correlations of IL-6TS components in human emphysema tissue and serum. (A) Q-PCR gene expression analyses of IL6 and ADAM17 were performed on human lung cDNA from emphysema patients (Emph, n = 20) or
emphysema-free individuals (Ctl, n = 10). Expression data are shown following normalization for 18S expression, and are presented as the mean ± SEM from triplicate analyses. (B) IL-6 and sIL-6R protein concentrations in lung lysates as determined by ELISA. Data are expressed as the mean ± SEM. (C) Immunoblotting with antibodies against ADAM17 and Actin on whole lung lysates from Emph and Ctl groups. Each lane represents tissue from an individual. (D) Linear correlation between sIL-6R and ADAM17 (normalized to Actin following densitometry) protein levels from individuals with emphysema (n = 9). (E) Representative cross-section of the lung from an emphysema patient displaying cleaved caspase-3-positive cells. Arrows indicate positive cells. Scale bar = 100μm. (F) The number of cleaved caspase-3-positive cells per 20 high power fields in the lungs of individuals with emphysema (n = 9) were measured, and then linear correlation was performed between sIL-6R levels and the number of cleaved caspase-3-positive cells. (G) Immunoblotting with the indicated antibodies on lung lysates from Emph and Ctl groups, and each lane represents tissue from an individual. Densitometric quantification of P-rpS6 protein levels per group was performed and normalized against Actin protein levels present in each sample. Data are presented as the mean fold induction ± SEM. (H) Linear correlation between IL-6 and P-rpS6 protein levels compared to sIL-6R protein levels in lung tissue lysates from individuals with emphysema (n = 20). (I) Protein levels of IL-6 and sIL-6R in the serum of Emph and Ctl groups, as determined by ELISA. *P< 0.05 and **P< 0.05 versus Ctl group.
Therapeutic targeting of the IL-6 trans-signalling/mTORC1 axis in pulmonary emphysema

Saleela M. Ruwanpura, Louise McLeod, Lovisa F. Dousha, Huei J. Seow, Sultan Alhayyani, Michelle D. Tate, Virginie Deswaerte, Gavin D. Brooks, Steven Bozinovski, Martin McDonald, Christoph Garbers, Paul T. King, Philip G. Bardin, Ross Vlahos, Stefan Rose-John, Gary P. Anderson and Brendan J. Jenkins

"Online Data Supplement"
METHODS

Human lung biopsy and serum samples

Lung tissue from resection surgery for treatment of a solitary peripheral carcinoma was collected from patients with evidence of emphysema, as defined by pulmonary function tests (gas exchange, FEV1) (Table E2). Tissue from the subpleural parenchyma avoiding tumor-bearing areas was either snap frozen for molecular analyses or formalin fixed by immersion and paraffin embedded for histological analyses.

For serum collection, emphysema patients (n = 17) with moderate to severe stable disease were defined by a TLCO (% of predicted post-oxygen transfer into the blood which determines emphysema severity) of 36.2 ± 10.4 and FEV1 (% of predicted post-bronchodilator as a measure of airflow obstruction which determines COPD severity) of 38.2 ± 12.7. These patients also had radiological assessments such as high resolution computerized tomography (CT) of the chest to verify emphysema. Control serum samples (n = 6) were donated by healthy individuals with no emphysema. Lung tissue and serum were collected from individuals upon written informed consent, and studies were approved by the Monash Health Human Research Ethics Committee.

RNA isolation and gene expression analysis

Total RNA extraction and the preparation of cDNA for quantitative RT-PCR (Q-PCR) expression analyses of individual genes were performed as previously described (1, 2). Sequence information for primers against mouse genes 18s, Il-6, Il-6ra, Cxcl2, Ccl2 and Tnfa, as well as the human gene 18S, have been previously published (1). Primer sequences for the mouse gene Adam17 are; Adam17 forward 5’-GGCAACTCCAGGGTGGACGAAGGA-3’, Adam17 reverse 5’-ATCTTCAGCATCTCCTGTTGCGGG-3’. Sequences for human primers are as follows; IL-6R forward 5’-AAAGCTGGGCAGGTTGGTG-3’, IL-6R reverse 5’-
AGCTTGAGGAGATTGAGGAG-3’;  ADAM17 forward 5’-
GAAGTGCCAGGAGGCGATTA-3’,  ADAM17 reverse 5’-
GGGCACCTCCTGCTATTACC-3’.  Adam10/ADAM10 forward 5’-
CCTGAGCTCTGAGGAAAA-3’ and  Adam10/ADAM10 reverse 5’-
TGAGCAATCACAGGCTTCTCG-3’ primer sequences were used for both mouse and human genes.

**Protein extraction, ELISA and immunoblotting**

Total protein lysates were prepared from snap-frozen lung tissues (1, 2) and BALF, and were subjected to ELISA. The mouse IL-6 and human IL-6 ELISA sets were purchased from BD Sciences, (Franklin Lakes, NJ), while sIL-6R and sgp130 ELISA sets were purchased from R & D systems (Minneapolis, MN). The SAA ELISA set was purchased from Life technologies (Grand Island, NY). All ELISA sets were used as per the manufacturer’s instructions. Immunoblotting of P-rpS6 (Ser240/244, Cell Signaling Technology (CST), Denver, MA), rpS6 (CST), P-mTOR (Ser2448, CST), mTOR (CST) and ADAM17 (Millipore, Temecula, CA) were performed on lung lysates, and protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) and quantified using the Image J program (nih.gov). Antibody against actin was purchased from Sigma-Aldrich (St. Louis, MO).

**Immunohistochemistry and immunofluorescence**

Apoptosis was determined by the terminal deoxynucleotidyl transferase (tdT)-mediated dUDP nick-end labeling (TUNEL) technique using an ApopTag Peroxidase In Situ Apoptosis Detection kit (Millipore, Billerica, MA). Immunohistochemistry with antibodies against SPC (Santa Cruz Biotechnology, Dallas, Texas), P-rpS6 (Ser240/244, CST) and cleaved caspase-3
(CST) were performed as previously described (1). Stereological techniques were applied to determine the number positively-stained alveolar cells.

Dual immunofluorescence staining was performed on paraffin-embedded lung tissues using primary antibodies conjugated with fluorescent dyes or Alexa Fluor® secondary antibodies. Briefly, lung sections were antigen retrieved by microwave in 1mM Ethylenediaminetetraacetic acid (EDTA, pH8.0) buffer, following which they were blocked with universal CAS block (Invitrogen, Carlsbad, CA) for 1 hour. Antigens were detected with cleaved caspase-3 antibody conjugated with Alexa Fluor® 488 (CST), podoplanin antibody conjugated with Alexa Fluor® 594 (BioLegend, San Diego, CA), CD31 antibody conjugated with Alexa Fluor® 647 (BioLegend), P-rpS6 antibody conjugated with Alexa Fluor® 647 (CST), and SPC (Santa Cruz Biotechnology) and CD45 (BD BioSciences, Franklin Lakes, NJ) primary antibodies with Alexa 488/546-conjugated donkey anti-goat and Alexa 488-conjugated goat anti-rabbit secondary antibodies, respectively (Invitrogen). Slides were examined under a Nikon confocal microscope and analyzed for red and green fluorescence. Stereological techniques were applied to determine the number of dual stained cells.

**Cigarette smoke (CS) exposure**

Mice aged 4-6 weeks were subjected to whole body CS exposure for 4 days (acute) (1). This smoke exposure protocol caused acute inflammation with no acute lung injury. For chronic CS exposure, mice were subjected to whole body CS exposure over a 12 week period as per the protocol described for acute CS exposure.

**Primary lung cell cultures, and flow cytometry**

Mouse primary alveolar type II (ATII) epithelial cells were prepared as previously described (3). Following the digestion of lungs, single cell suspensions were prepared and then incubated
with purified CD45 antibody (BD Biosciences) and epithelial cells negatively enriched with BioMag goat anti-rat immunoglobulin-coupled magnetic beads (Qiagen, Hilden, Germany). The negative selection for ATI epithelial cells was performed by incubation of podoplanin-coated petri dishes for 30 minutes at $37^\circ$C. Cell purity was routinely assessed by epithelial cell morphology and flow cytometry analysis with anti-EpCAM, anti-podoplanin and anti-SPC (ATII) antibodies. Cells were cultured on collagen-coated (MP Biomedicals, Santa Ana, CA) plates and stimulated with 100ng/ml Hyper-IL-6, an IL-6/soluble IL-6R fusion protein (4) over the indicated times. Monolayers were detached with either 3mM EDTA for flow cytometry analysis with Annexin-V (BD Biosciences) and 7-AAD to examine the level of apoptosis, or RIPA buffer for immunoblot analyses of mTORC1 activity. Cells were analysed on a FACS-CantoII flow cytometer (BD Biosciences) and data was performed using FACSDiva software (BD Biosciences).
REFERENCES


SUPPLEMENTARY FIGURE LEGENDS

Figure E1: Expression of IL-6 signalling components in emphysematous gp130<sup>F/F</sup> and emphysema-free gp130<sup>F/F</sup>:sgp130<sup>Fc<sup>tg/tg</sup> mice. (A) IL-6, sIL-6R and sgp130 protein concentrations were determined by ELISA in the BALF of 6 month old (mo) gp130<sup>+/+</sup> (+/+) and gp130<sup>F/F</sup> (F/F) mice. (B) Mean linear intercept of +/+, gp130<sup>+/+</sup>:sgp130FC<sup>tg/tg</sup> (+/+:tg), F/F and gp130<sup>F/F</sup>:sgp130FC<sup>tg/tg</sup> (F/F:tg) mice aged 6 months. (C) Serum amyloid A (SAA) levels in the serum and lungs of the indicated 6mo mice. Data are expressed as mean ± SEM. n = 5 mice per genotype. *P < 0.05, **P < 0.01 and ***P < 0.001. ND = not within assay detectable level (assumed 0 for statistical analyses).

Figure E2: IL-6TS-mediated apoptosis is not observed in alveolar type I (ATI) and endothelial cells from gp130<sup>F/F</sup> mice. Representative confocal immunofluorescence photomicrographs of cells stained for (A) cleaved caspase-3 (green) and podoplanin (red, ATI cell marker) either alone or together, or (B) cleaved caspase-3 (green) and CD31 (red, endothelial cell marker) either alone or together, in cross-sections of lungs from 6mo gp130<sup>F/F</sup> (F/F) mice. In both (A) and (B), sections are also stained with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI; blue) as indicated. White circles indicate examples of (A) podoplanin-positive cells (cell membrane) and (B) CD31-positive cells (cell junction in cell membrane) with cleaved caspase-3 (cytoplasm), while green circles (A and B) indicate examples of cleaved caspase-3 alone cells. Scale bars = 40μm. The graphs depict stereological quantification of dual labelled caspase-3/podoplanin- or caspase-3/CD31-positive cells in the lungs of 6mo mice of the indicated genotypes. Data are expressed as the mean ± SEM. n = 5 mice per genotype.
Figure E3: Therapeutic and genetic blockade of IL-6 trans-signalling in CS-exposed wild-type mice suppresses expression of inflammatory mediators. (A) Q-PCR expression analyses of the indicated genes were performed on lung cDNA, and (B) ELISAs for the indicated proteins were performed on lung lysates, from 1 month old gp130+/+;sgp130Fctg/tg (+/+;tg) mice with (+) or without (-) cigarette smoke (CS) exposure. (C) Q-PCR expression analyses of inflammatory mediators, Cxcl2, Tnfa and Ccl2 were performed on lung cDNA from gp130+/+ (+/) mice without CS (+/-CS), with CS (+/+ +CS), or concurrently treated with sgp130Fc and CS (+/+ Fc+CS), as well as +/+:tg mice with/without CS exposure. Data are expressed for n = 3 mice per genotype. *P < 0.05 and ** P < 0.01.

Figure E4: Therapeutic and genetic blockade of IL-6TS in gp130F/F mice suppresses mTORC1 pathway activation in alveolar epithelial type II (ATII) cells. (A and B) Representative confocal immunofluorescence photomicrographs of cells positive for either P-rpS6 alone (red, A and B), SPC alone (green, A), CD45 alone (green, B) or both P-rpS6 and SPC (yellow, white circles, A) in cross-sections of lungs from 6mo gp130F/F (F/F) mice. Scale bars = 10μm. (C) Immunoblotting with indicated antibodies on whole lung lysates from 6mo F/F and F/F:Il6−/− mice. Each lane represents tissue from an individual mouse. Densitometric quantification of P-mTOR (Ser2448) protein levels per genotype was performed and normalized against Actin protein levels present in each sample. Data are presented as the mean expression ± SEM from n = 5 mice per genotype. *P < 0.05.

Figure E5: Activation of the IL-6TS/mTORC1 axis in cultured gp130F/F primary alveolar type II (ATII) epithelial cells stimulated with the IL-6TS agonist Hyper-IL-6. (A) Immunoblotting with indicated antibodies on lysates from cultured gp130F/F (F/F) ATII cells following stimulation with Hyper-IL-6 (10ng/ml) over the indicated time points. (B)
Representative histograms of flow cytometric analyses of Annexin-V and 7AAD staining showing percentages of Annexin-V-positive/7AAD-negative apoptotic ATII cells from wild-type (+/+) and F/F mice.

**Figure E6:** Blockade of mTORC1 using rapamycin prevents alveolar cell death in gp130<sup>F/F</sup> mice with hyper-activated IL-6TS/mTORC1 axis. (A and B) Representative photomicrographs of P-rpS6-positive cells in cross-sections of lungs from (A) 6mo gp130<sup>+/+</sup> (+/+), gp130<sup>+/+</sup>:sgp130Fc<sup>tg/tg</sup> (+/+:tg), F/F and F/F:tg mice, and (B) 3mo +/+ and F/F mice, and F/F mice treated with sgp130Fc inhibitor for 12 weeks (F/F + Fc). (C and D) Representative photomicrographs showing (C) TUNEL-positive cells and (D) P-rpS6-positive cells, in cross-sections of lungs from gp130<sup>F/F</sup> (F/F) mice aged 6 months, and 3 month old F/F mice treated with rapamycin for 12 weeks (F/F + Rapa). Scale bars = 100μm.

**Figure E7:** Hyper-activation of the IL-6TS/mTORC1 pathway correlates with augmented cellular apoptosis in the lungs of CS-exposed wild-type mice. (A) Stereological quantification of the number of P-rpS6-positive cells, and corresponding representative photomicrographs showing positive cells in a cross-section of lungs from +/+ -CS, +/+ +CS, and +/+ mice concurrently treated with CS and sgp130Fc (+/+ Fc+CS), as well as gp130<sup>+/+</sup>:sgp130Fc<sup>tg/tg</sup> (+/+:tg) mice with or without CS exposure. (B) Stereological quantification of the number of P-rpS6-positive cells, and corresponding representative photomicrographs showing positive cells in a cross-section of lungs from +/+ mice concurrently treated with CS and either vehicle (Veh) or rapamycin (Rapa). (C) Stereological quantification of the number of TUNEL-positive cells, and corresponding representative photomicrographs showing positive cells in a cross-section of lungs from +/+ mice concurrently treated with CS and either Veh or Rapa. Data are expressed as mean ± SEM. n =
4 mice per genotype. Scale bars = 100μm. \(*P < 0.05\), \(**P < 0.01\) and \(***P < 0.001\). (D) Representative photomicrographs showing P-rpS6-positive cells in cross-sections of the lungs from \(gp130^{+/+}\) mice (+/+) aged 1 month treated with or without sgp130Fc inhibitor or CS for 12 weeks.
Table E1: Comparative stereological analyses of lungs from the indicated mice. Data are expressed as the mean \( \pm \) SEM. \( n = 5 \) mice per genotype per age group. \# \( P < 0.05 \) and \### \( P < 0.001 \) versus age-matched +/- mice. * \( P < 0.05 \), *** \( P < 0.001 \) and **** \( P < 0.0001 \) versus age-matched F/F mice. \( V_v \) = volume fraction; \( \text{par} = \text{parenchyma}; \text{air} = \text{air space}; \text{sep} = \text{septal tissue}; \text{Sv} = \text{surface density}; \text{S} = \text{surface area.} \)
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**Table E2: Clinical characteristics of the patients.** Data are expressed as the mean ± SEM. FEV1 denotes forced expiratory volume in one second.
Figure E1

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[B] IL-6

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[B] sgp130

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B

[B] Mean linear intercept

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C

[B] SAA

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<tr>
<td>F/F:tg</td>
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Figure E2

A

Cleaved Casp-3 + Podoplanin

Podoplanin

Cleaved Casp-3

Dapi

B

Cleaved Casp-3 + CD31

CD31

Cleaved Casp-3

Dapi

Cleaved Casp-3+/Podoplanin* cells/20 fields

Cleaved Casp-3+/CD31* cells/20 fields
Figure E3

A

B

C

Il6

Adam17

sIL-6R

sgp130

Cxcl2

Tnfa

Ccl2

Relative Expression

Relative Expression

Relative Expression

Relative Expression

Relative Expression

Relative Expression

Relative Expression

Relative Expression

-0.5
0
0.5
1.0
-0.5
0
0.5
1.0
-0.5
0
0.5
1.0
-0.5
0
0.5
1.0
-0.5
0
0.5
1.0
-0.5
0
0.5
1.0
Figure E4

A

SPC+ P-rpS6

F/F

SPC

P-rpS6

F/F

B

CD45+ P-rpS6

F/F

CD45

P-rpS6

F/F

C

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<th>F/F</th>
<th>F/F:Il6/-</th>
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<td><img src="image3.png" alt="Image" /></td>
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Ratio to Actin

P-mTOR (ser2448)

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<th>F/F</th>
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*
Figure E5

A

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<th>Hyper-IL-6 (hours)</th>
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<td>P-rpS6</td>
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<td>S6</td>
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<td>Actin</td>
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B

Count

Annexin-V-positive/7AAD-negative

+++

35.2%

Apoptotic

+++

45.3%

Apoptotic
Figure E6

A

P-rpS6

+/+

+/+:tg

F/F

F/F:tg

B

P-rpS6

+/+

F/F

F/F + Fc

C

TUNEL

F/F

F/F + Rapa

D

P-rpS6

F/F

F/F + Rapa
Figure E7

**A**

![Image of immunohistochemical staining for P-rpS6+ cells/20 fields](image)

+/- (-CS)  
+/- (+CS)  
+/- (Fc +CS)

+/-:tg (-CS)  
+/-:tg (+CS)

**B**

![Image of immunohistochemical staining for P-rpS6+ cells/20 fields](image)

+/- (Veh +CS)  
+/- (Rapa +CS)

**C**

![Image of immunohistochemical staining for TUNEL+ cells/20 fields](image)

+/- (Veh +CS)  
+/- (Rapa +CS)

**D**

![Image of immunohistochemical staining for P-rpS6](image)

+/- (-CS)  
+/- (+CS)  
+/- (Fc+CS)