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Alveolar type 2 (AT2) cell endoplasmic reticulum (ER) stress is a prominent feature in adult and pediatric interstitial lung disease (ILD and ChILD), but in vivo models linking AT2 cell ER stress to ILD have been elusive. Based on a clinical ChILD case we identified a critical cysteine residue in the Surfactant Protein C gene (*SFTPC*) BRICHOS domain whose mutation induced ER stress in vitro. To model this in vivo, we generated a knock-in model expressing a cysteine-to-glycine substitution at codon 121 (C121G) in the *Sftpc* gene. $Sftpc^{C121G}$ expression during fetal development resulted in a toxic gain of function resulting in fatal post-natal respiratory failure from disrupted lung morphogenesis. Induced $Sftpc^{C121G}$ expression in adult mice resulted in an ER retained pro-protein causing AT2 cell ER stress$Sftpc^{C121G}$ AT2 cells were a source of cytokines expressed in concert with development of a polycellular alveolitis. These cytokines were subsequently found in a high-dimensional proteomic screen of bronchoalveolar lavage fluid from ChILD patients with the same class of *SFTPC* mutations. Following alveolitis resolution, $Sftpc^{C121G}$ mice developed spontaneous pulmonary fibrosis and restrictive lung impairment. This model provides proof of concept linking AT2 cell ER stress to fibrotic lung disease coupled with translationally relevant biomarkers.

A *SFTPC* BRICHOS Mutant Links Epithelial ER Stress and Spontaneous Lung Fibrosis

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Running Title: ***Epithelial ER stress drives lung fibrosis***

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ABSTRACT

Alveolar type 2 (AT2) cell endoplasmic reticulum (ER) stress is a prominent feature in adult and pediatric interstitial lung disease (ILD and ChILD), but *in vivo* models linking AT2 cell ER stress to ILD have been elusive. Based on a clinical ChILD case we identified a critical cysteine residue in the Surfactant Protein C gene (*SFTPC*) BRICHOS domain whose mutation induced ER stress *in vitro*. To model this *in vivo*, we generated a knock-in model expressing a cysteine-to-glycine substitution at codon 121 (C121G) in the *Sftpc* gene. $Sftpc^{C121G}$ expression during fetal development resulted in a toxic gain of function resulting in fatal post-natal respiratory failure from disrupted lung morphogenesis. Induced $Sftpc^{C121G}$ expression in adult mice resulted in an ER retained pro-protein causing AT2 cell ER stress. $Sftpc^{C121G}$ AT2 cells were a source of cytokines expressed in concert with development of a polycellular alveolitis. These cytokines were subsequently found in a high-dimensional proteomic screen of bronchoalveolar lavage fluid from ChILD patients with the same class of *SFTPC* mutations. Following alveolitis resolution, $Sftpc^{C121G}$ mice developed spontaneous pulmonary fibrosis and restrictive lung impairment. This model provides proof of concept linking AT2 cell ER stress to fibrotic lung disease coupled with translationally relevant biomarkers.

INTRODUCTION

Adult Interstitial lung disease (ILD) and pediatric childhood ILD (ChILD) are defined pathologically by interstitial fibrosis, inflammation, or the combination of fibrosis and inflammation (1,2). With thickening of the alveolar interstitum causing impairment in effective gas exchange, the clinical course is often progression to chronic respiratory failure (3,4). Over the past decade, the alveolar type 2 (AT2) cell has come to the forefront as a key driver of lung fibrosis and ILD (5,6). The focus on the AT2 cell is in part due to the identification of disease causing mutations in the AT2 cell restricted Surfactant Protein C gene (*SFTPC*) in both pediatric patients with ChILD and in a subset of adult patients with familial ILD (7-9). The recent demonstration by our group of the occurrence of spontaneous lung fibrosis with *in vivo* expression of the $Sftpc^{I73T}$ trafficking mutation in a preclinical mouse mode has provided further proof of concept for the AT2 cell as a proximal driver of ILD (10). While the $SFTPC^{I73T}$ mutation, which causes a block in AT2 cell macroautophagy, is the most common clinical *SFTPC* mutation identified, a majority of the 60 described mutations in the *SFTPC* gene associated with human disease occur in the distal C-terminal (residues 94–197) "BRICHOS" domain of the Surfactant Protein C (SP-C) pro-protein (proSP-C). Their expression results in pro-protein aggregation and induction of endoplasmic reticulum (ER) stress (11-16). These cases further highlight the proximal position of the AT2 cell in the pathogenesis of pulmonary fibrosis and suggest AT2 cell ER stress may provide a broader mechanistic link between *SFTPC* BRICHOS mutation associated pediatric ChILD and adult ILD.

Markers of ER stress are also a prominent feature in the lung epithelium from patients with sporadic idiopathic pulmonary fibrosis (IPF), the most common adult ILD (17); however, the role of epithelial ER stress in disease pathogenesis is unclear. *In vivo* proof of concept studies linking *SFTPC* BRICHOS mutations, ER stress, and pulmonary fibrosis have been plagued by over and under expression of the mutant pro-protein isoforms, and have failed to establish a mechanistic connection. While the constitutive transgenic expression of a highly aggregation prone *SFTPC* mutation ($SFTPC^{\Delta Exon4}$) resulted in disrupted lung morphogenesis with prenatal lethality (18), inducible expression of a less severe mutation ($SFTPC^{L188Q}$) at lower levels produced an increased susceptibility to bleomycin fibrosis, but not

spontaneous fibrotic remodeling (19). This finding suggested that epithelial ER stress may not be a driver of fibrosis per se, but a marker of a dysfunctional and vulnerable epithelium, which requires a "second hit" to cause disease.

Given the low level of *SFTPC* expression induced in the *SFTPC*^{L188Q} mice, we sought to challenge this notion with the hypothesis that a more severe *SFTPC* BRICHOS mutation expressed at a more biologically relevant level would produce an ER stress based toxic gain of function resulting in a dysfunctional epithelial cell capable of driving a spontaneous lung phenotype. Based upon a pediatric case of ChILD with a mutation in the cysteine at codon 121 (C121), and previous ultrastructural analysis demonstrating that the disulfide bond formed between C121 and cysteine 189 of the BRICHOS domain mediates the primary folding of the proSP-C primary translation product (20), we generated a murine model (*Sftpc*^{C121G}). Consistent with a misfolded isoform, the *Sftpc*^{C121G} pro-protein is proximally retained in the ER and induces substantial epithelial ER stress both *in vitro* and *in vivo*. The resultant lung phenotype of AT2 cell driven spontaneous inflammation and subsequent fibrotic remodeling provides proof of concept linking *SFTPC* BRICHOS mutations and pulmonary fibrosis. The translational relevance of this model was further corroborated by a high-dimensional proteomic analysis of bronchoalveolar lavage fluid (BALF) from pediatric patients with *SFTPC* BRICHOS mutation-associated ChILD showing overlapping cytokine expression. The *Sftpc*^{C121G} model, with relevant biomarker elaboration, thus establishes that AT2 cell ER stress is a sufficient driver for spontaneous lung inflammation and aberrant fibrotic lung remodeling.

RESULTS

In vitro expression of a clinical SFTPC cysteine 121 mutation induces ER stress by a proximally retained SP-C pro-protein

A male child of an uncomplicated pregnancy was born at 38 weeks gestation by spontaneous vaginal delivery to a 27 year-old mother. He subsequently developed respiratory failure in the first day of life requiring endotracheal intubation and mechanical ventilation. He received exogenous surfactant and was extubated and transitioned to nasal canula supplemental oxygen. At 2 months of age, a second episode of respiratory failure occurred requiring tracheostomy for chronic mechanical ventilation. Chest imaging at 3 months demonstrated diffuse ground glass opacities **(Figure 1A)**. Subsequently, a clinical pathologic analysis of an open lung biopsy **(Figure 1B)** showed ChILD histology with enlargement of airspaces, interstitial widening, inflammatory infiltrate, and prominent AT2 cell hyperplasia. Bronchoalveolar lavage demonstrated an increased cellularity (755 cells/mL) with elevated neutrophils (20%). The patient was ultimately weaned from the ventilator and decannulated 2 years later. Genetic testing demonstrated a heterozygous *SFTPC* mutation in the BRICHOS domain: c.362 G>A (p.C121Y), without Surfactant Protein B (*SFTPB*) or ATP Binding Cassette A3 (*ABCA3*) mutations.

In vitro modeling was performed to define the functional consequences of C121 mutations on proSP-C biosynthesis. Plasmids containing EGFP tagged wild-type *SFTPC* (EGFP/SP-C^{WT}) and two *SFTPC* mutants, EGFP/SP-C^{C121Y} and EGFP/SP-C^{C121G}, were transfected into HEK293 cell lines and interrogated for cellular expression patterns and post-translational processing. The *SFTPC*^{C121G} mutation, which has also been described clinically (21), was selected in addition to the clinical *SFTPC*^{C121Y} mutation to generalize the importance of disulfide bonding in the pro-protein via the C121 thiol (20). In contrast to the wild-type isoforms, western blot analysis of cell lysates showed a primary proSP-C translation product containing either cysteine mutant failed to undergo normal processing to a cleaved intermediate pro-protein **(Figure 1C)**. Subsequent co-transfection of HEK293 cells with EGFP/SP-C^{WT} or EGFP/SP-C^{C121G} and the DsRed tagged *ABCA3* (DsRed/ABCA3) to mark lysosomal related organelles (LROs) demonstrated co-localization between EGFP/SP-C^{WT} and DsRed/ABCA3, but reticular patterning without co-localization of

the EGFP/SP-C^{C121G} isoform in LROs **(Figure 1D)**. Expression of the proximally retained mutant pro-proteins induced an ER stress response *in vitro* as transfected HEK293 cell lysates demonstrated increases in BiP (GRP78) protein in cells expressing either *SFTPC* mutation compared to wild-type *SFTPC* **(Figure 1E)**. This was consistent with previously published *in vitro* studies of *SFTPC* BRICHOS mutants, which have demonstrated BiP to be a reliable marker of the ER stress response (13,15). These data collectively define a critical role of the C121 residue in the biosynthetic routing and processing of the proSP-C, and reveal an ER stress toxic gain of function with *SFTPC^{C121}* mutations.

Constitutive expression of the Sftpc^{C121G} mutation in vivo phenocopies the human pediatric mutation

Given the importance of C121 in the processing of proSP-C *in vitro*, we utilized an ES cell recombineering strategy to selectively knock-in a homologous cysteine-to-glycine substitution at codon 121 (C121G) to the endogenous mouse *Sftpc* locus. Given that the functional consequences of each cysteine mutant isoform were equivalent *in vitro,* the C121G missense substitution was chosen for the *in vivo* knock-in model to limit confounding variables related to residue side chain size, hydrophobicity, or potential post-translational modification of a tyrosine residue. The resultant founder line (*Sftpc$^{C121Gneo/C121Gneo}$*) retained a PGK-Neomycin (PGK-neo) cassette in Intron 4 flanked by locus of X-over P1 (lox-P) sites **(Supplemental Figure 1A)**. Retention of the PGK-neo cassette rendered the *Sftpc^{C121Gneo}* gene hypomorphic, a strategy employed for knock-in models of toxic gain of function mutations (10,22,23), with mRNA levels in homozygous *Sftpc^{C121Gneo}* animals limited to ~ 6% of that of littermates expressing two *Sftpc^{WT}* alleles **(Supplemental Figure 1B)**. Despite AT2 cells lacking expression of proSP-C (**Supplemental Figure 1C)** and consistent with the phenotype of the *Sftpc* knockout mouse (24), the founder line was devoid of a spontaneous inflammatory or fibrotic phenotype when aged up to 52 weeks **(Supplemental Figure 1D, E, F)**.

To evaluate the effect of increasing *Sftpc^{C121G}* expression in the developing lung, we constitutively removed the Lox-P flanked PGK-neo cassette from *Sftpc^{C121Gneo}* alleles by crossing the *Sftpc$^{C121Gneo/C121Gneo}$* founder line to mice heterozygous for the CMV-Cre recombinase (a "deleter" line) (25). The resultant

progeny were either *Sftpc*$^{WT/C121Gneo}$ in mice not receiving the CMV-Cre allele, or heterozygous for the activated *Sftpc*^{C121G} allele (*Sftpc*$^{WT/C121G}$), in mice receiving the CMV-Cre allele (**Figure 2A**). Consistent with a dominant negative effect, heterozygous expression of *Sftpc*^{C121G} caused abnormal processing of the wild-type SP-C pro-protein with reduced processing intermediates on western blotting for proSP-C (**Figure 2B**) and an 84.7% ± 3.2% (X ± SD, n=4) reduction in mature SP-C (mSP-C) protein in P2.5 lung homogenates (**Figure 2C**). This finding recapitulates data from *SFTPC* BRICHOS mutation patients whereby heterozygous expression of the *SFTPC* mutation dramatically blocks pro-protein processing, reducing the amount of mSP-C in patients' BALF (26-28). Previous *in vitro* studies have shown mechanistically that heteromeric association between mutant and wild-type proSP-C proteins is responsible for the disruption of the intracellular trafficking of the wild-type proSP-C isoform (16,29,30).

The constitutive *Sftpc*^{C121G} expression model phenocopied many of the features of the pediatric disease (27,31). Similar to some cases of ChILD, including the one presented here (**Figure 1**), a toxic gain of function was observed, with *Sftpc*$^{WT/C121G}$ mice developing a lethal post-natal respiratory failure with a median survival of 2.5 days (**Figure 2D**). *Sftpc*^{C121G} expression manifested as respiratory distress and cyanosis between P1.5 and P3.5 (**Figure 2E**), with lung histology of distorted architecture; enlargement of airspaces, interstitial widening, inflammatory infiltrate, and proteinacious fluid in the airspaces (**Figure 2F**) with features resembling the histology in the clinical *SFTPC*^{C121Y} case (**Figure 1B**).

Expression of mutant Sftpc^{C121G} in adult mice results in ER retained SP-C pro-protein and ER stress

To evaluate the cellular effect of *Sftpc*^{C121G} expression in the adult mouse, the *Sftpc*^{C121Gneo} founder line was crossed to the Rosa26-ERT2-Cre line (*R26*^{Cre}) to generate a line (*Sftpc*^{C121G} / *R26*^{Cre}) capable of tamoxifen mediated Cre recombinase removal of the inhibitory PGK-neo cassette from *Sftpc*^{C121Gneo} alleles **(Figure 3A)**. In the absence of tamoxifen, adult homozygous *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} animals injected with vehicle (intraperitoneal (IP) oil) retained a phenotype identical to the *Sftpc*$^{C121Gneo/C121Gneo}$ founder line without substantial leakiness of the *R26*^{Cre} locus as shown by the absence of detectable proSP-C on immunohistochemistry **(Supplemental Figure 2)**. Given the absence of a lung phenotype in *Sftpc*$^{C121G/C121G}$

/ *R26*^{Cre} mice with IP oil and the extreme hypomorphic *Sftpc* expression in the founder line, we selected tamoxifen injected *Sftpc*^{WT}/ *R26*^{Cre} mice as controls given previously described cre lung toxicity (32). At 7 days after administration of tamoxifen (500mg/kg) *Sftpc*^{C121G} mRNA increased to 26.2% ± 4.9% (X ± SD, n=7) versus *Sftpc*^{WT} controls (**Figure 3B**); a four-fold increase from the hypomorphic *Sftpc*$^{C121Gneo/C121Gneo}$ founder line. Using qRT-PCR to estimate PGK-neo cassette numbers, the efficiency of LoxP mediated recombination in purified AT2 cells isolated one week post-tamoxifen was 92.4 ± 3.1% (X ± SD, n=4) referenced against *Sftpc*$^{C121Gneo/C121Gneo}$ mice. Despite a substantial increase in the *Sftpc*^{C121G} mRNA, western blot analysis of the phospholipid enriched large aggregate surfactant fraction of BALF from tamoxifen treated *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice showed no evidence of extracellular mSP-C protein (**Figure 3C**). The absence of the mSP-C protein was the result of a failure to process translated proSP-C as AT2 cell lysates from these mice showed a single 21 KDa pro-protein band, corresponding to the unprocessed primary translational product. In contrast, 23 and 21 KDa pro-protein bands (23 KDa corresponding to the palmitoylated post-ER pro-protein) with processing intermediates from 8-15 KDa were observed in *Sftpc*^{WT} / *R26*^{Cre} controls (**Figure 3D**) (33,34). Furthermore, lung sections of tamoxifen treated *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice demonstrated a reticular pattern of the mutant proSP-C with significant co-localization with the ER maker KDEL, signifying substantial ER retention (**Figure 3E**). Thus, interrogation of the adult *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice revealed ER retention of the mutant proSP-C *in vivo* without evidence for post-translational palmitoylation from post-golgi or proteolytic processing consistent with the *in vitro* data (**Figure 1**) and what has been reported in *SFTPC* BRICHOS patients (27,35).

ER retention of mutant proSP-C C121G induced activation of the unfolded protein response (UPR) in the pulmonary epithelium as reflected by a substantial increase in *BiP* mRNA in AT2 cells isolated from mouse lungs as early as 7 days post-tamoxifen (**Figure 4A**). In a corresponding fashion, BiP protein was also detected in AT2 cells by both immunohistochemical staining of cells localized to corners of alveoli (**Figure 4B**), and by western blotting of AT2 cell lysates (**Figure 4C**). Consistent with our prior *in vitro* modeling (36), the *Sftpc*^{C121G} AT2 cell phenotype was distinct from the *Sftpc*^{I73T} model where expression

of that mutant isoform caused a block in macroautophagy and failed to generate substantial AT2 cell BiP expression (**Supplemental Figure 3**)(10).

The UPR utilizes three signaling pathways: IRE1/XBP1, PERK/ATF4, and ATF6. Each pathway was interrogated in the AT2 cells from *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} and control mice after tamoxifen. The IRE1 pathway was activated as demonstrated by an increase in the ratio of active spliced *Xbp1(S)* to un-spliced *Xbp1(U)* mRNA (**Figure 4D**), and downstream activation of C-Jun N-terminal kinase (JNK) signaling as shown by increased phosphorylated JNK (**Figure 4E**). The PERK/ATF4 pathway was found to be activated by an increase in both ATF4 and its downstream target CCAAT-enhancer-binding protein homologous protein (CHOP) (**Figure 4C**). Interrogation of the ATF6 pathway revealed a marked decrease in the ATF6 P90 precursor band in *Sftpc*^{C121G} AT2 cells (**Figure 4C**).

While initiation of UPR signaling is viewed as an adaptive cellular response, sustained over activation can cause deleterious responses such as apoptosis, a feature of the epithelium in both sporadic and *SFTPC* mutation-related ILD (26,37,38). Previously, we have shown that *in vitro* expression of *SFTPC* BRICHOS mutations induce apoptosis via multiple UPR pathways including ATF4/CHOP and IRE1/JNK (14). Double label fluorescence immunohistochemistry of lung sections for proSP-C and cleaved Caspase 3 at 7 days post-tamoxifen, revealed a significant increase in the apoptotic index with 9.4% ±1.7% (X ± SD, n=4) AT2 cells undergoing apoptosis in the *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} lungs compared to 1.6% ± 0.5% (X ± SD, n=4) in controls (p = 0.001) (**Figure 4F**). Thus, *Sftpc*^{C121G} expression induced the UPR and produced an ER stress, pro-apoptotic AT2 cell phenotype.

Expression of the Sftpc^{C121G} *mutation causes early morbidity and mortality from lung injury and AT2 cell driven polycellular alveolitis*

Sftpc$^{C121G/C121G}$ / *R26*^{Cre} mice demonstrated tamoxifen dose dependent morbidity and mortality. At a dose of 350mg/kg tamoxifen the mice developed weight loss starting at 7 days post-tamoxifen with a nadir of 84.8% ± 9.6% (X ± SD, n=13) from baseline at 13 days (**Figure 5A**) in association with 40% early mortality (**Figure 5B**). Lower doses were non-fatal and a higher dose (600mg/kg) was 100% fatal

(**Supplemental Figure 4**). The weight loss and death were commensurate with hypoxemia (**Figure 5C**) and increased BALF total protein (**Figure 5D**). Histologically, the *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice developed acute, diffuse lung injury by day 14 (**Figure 5E**).

The parenchymal lung injury was accompanied by a polycellular alveolitis, with a substantial increase in total BALF cell counts beginning 7 days after tamoxifen and peaking at 2 weeks (**Figure 5F**). BALF cytospins demonstrated a multiphasic inflammatory cell alveolitis with an early and sustained increase in macrophage/monocyte lineages beginning 7 days after tamoxifen, a rise in neutrophils starting 7 days after tamoxifen and peaking by 2 weeks, and an increase in eosinophils at 2 weeks (**Figure 5G and Supplemental Table 1**). This pattern of granulocyte alveolitis mirrored that found in the *Sftpc*^{I73T} model (10). Total lymphocytes also increased by day 14 and were sustained through 28 days; however, at numbers one log-fold less than the macrophage/monocyte and granulocyte cell populations. The inflammatory phenotype was dependent on *Sftpc*^{C121G} allele status with mice heterozygous for the *Sftpc*^{C121G} allele (*Sftpc*$^{C121G/WT}$/ *R26*^{Cre}) developing a less severe alveolitis 2 weeks after tamoxifen that was sustained to 4 weeks, but in the absence of weight loss or early mortality (**Supplemental Figure 5**).

To define the effector cell populations that precede the overt alveolitis, flow cytometry analysis of whole lung tissue was performed at 3 days post-tamoxifen using a gating strategy shown in **Supplemental Figure 6**. By this algorithm, an early decrease in the relative percent of SigF^{+}CD11b^{-} resident alveolar macrophages was found (**Figure 5H**) commensurate with an influx of inflammatory CD11b^{+}Ly6C^{hi} monocytes (**Figure 5I**), a population recently implicated in fibrotic lung remodeling (39,40).

In order to define the potential mediators of effector cell recruitment, BALF samples at early and late stage alveolitis were analyzed by multiplex assay and ELISA (**Supplemental Table 2**). CCL2 (MCP1), CCL17 (TARC), and CCL7 (MCP-3) were found to be increased in the BALF of *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice compared to *Sftpc*^{WT} / *R26*^{Cre} controls at the early time point (**Figure 6A**). Importantly, increases in AT2 cell mRNA expression of *Ccl2*, *Ccl17*, and *Ccl7* occurred by 7 days suggesting a role for this cellular compartment as a source of these cytokines (**Figure 6B**). Notably, although elevations in cytokine levels for CCL2, CCL17 and CCL7 were not statistically significant at 3 days post-tamoxifen, there was a 2.6 ±

1.2 (X ± SD, n=4, p=0.01), 3.1 ± 1.0 (X ± SD, n=4, p=0.01), and a 3.0 ± 0.5 (X ± SD, n=4, p=0.005) fold increase in AT2 cell mRNA expression, respectively. These data coupled with the early influx of inflammatory monocytes suggest that local elaboration of these cytokines may contribute to the recruitment.

In addition to the monocyte/macrophage lineages, *Sftpc*^{C121G} AT2 cells also contributed to granulocyte recruitment. *Sftpc*^{C121G} AT2 cells had increased mRNA expression of *Ccl11 and Il5* compared to control AT2 cells, and BALF showed an increase in the corresponding eosinophil chemokines CCL11 (Eotaxin) and IL5 at 1 week post-tamoxifen (**Supplemental Figure 7**). This occurred in the absence of detectable increases in canonical Th2 cytokines IL4 and IL13. Additionally, a 10 fold increase in *Sftpc*^{C121G} AT2 cell expression of the murine homolog to *IL8* (neutrophil chemoattractant KC (*Cxcl1*/*GroA*)) occurred and was also reflected by elevated BALF KC/CXCL1 content at 1 week post-tamoxifen (**Supplemental Figure 7**). There was also a significant increase in BALF IL6, which along with IL8 has been touted as a biomarker for acute exacerbations of ILD (AE-ILD) (41,42) (**Supplemental Table 2**). In contrast to IL8, there was no increase in *Il6* mRNA expression in *Sftpc*^{C121G} AT2 cells, suggesting a non-AT2 cell source of IL6 such as the distal lung mesenchyme (43). In total, *Sftpc*^{C121G} AT2 cells were an early source of cytokines that mediate the recruitment of multiple immune effector cell populations and are an important contributor to the early lung inflammatory phenotype.

High-dimensional proteomic screen of a pediatric SFTPC BRICHOS mutation cohort translationally overlaps with the Sftpc^{C121G} cytokine profile

The translational relevance of the *Sftpc*^{C121G} model was assessed in a human pediatric cohort of patients with ChILD. Five patients with ChILD carrying *SFTPC* BRICHOS mutations and nine control subjects underwent BALF analysis. Subjects with *SFTPC* BRICHOS mutations had an increase in the total BALF cell count and an increase in the percentage of BALF eosinophils compared to controls (**Supplemental Table 3**). BALF supernatants were analyzed using a SOMAscan aptamer proteomics platform for 1129 proteins, which identified seven proteins increased in the *SFTPC* BRICHOS subjects' BALF that met conservative significance criteria (p-value < 0.001; difference greater than 1 on $\log_2$ scale).

Five of these proteins were associated with the recruitment of immune cells (**Figure 7A**). Three of these were also increased in the *Sftpc*^{C121G} model (**Figure 6A**) and have been well described in adult ILD cohorts (44-49): CCL2 (MCP-1), CCL17 (TARC) and CCL7 (MCP-3) (**Figure 7B-D**). All three of these cytokines are associated with macrophage/monocyte recruitment, the initial effector population identified in the *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} lungs.

Expression of Sftpc^{C121G} results in the development of spontaneous fibrotic lung remodeling

The lung injury and morbidity observed two weeks post-tamoxifen in the *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice was followed by partial recovery of weight loss and decrease in BALF cell counts. However, four weeks post-tamoxifen, lung sections from *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice demonstrated spatially heterogeneous areas of dense Trichrome positive parenchymal remodeling (**Figure 8A and B**) in association with the accumulation of SMA positive cells adjacent to hyperplastic AT2 cells (**Figure 8C**). There was also a demonstrable quantitative increase in lung collagen deposition, measured as both soluble collagen content of the lungs by Sircol assay (**Figure 8D**) as well as fibrillar collagen content assessed by picrosirius red staining (**Figure 8E**). Additionally, total lung *Col1a1* and *Col3a1* gene expression were increased (**Figure 8F**), demonstrating active fibrotic remodeling four weeks post-tamoxifen. The observed structural and biochemical changes correlated with restrictive lung impairment on lung mechanics, with flow volume curves exhibiting a 30% decline in static lung compliance compared to control (**Figure 8G**). Quantification of AT2 cells in serial lung sections identified by either proSP-B staining or proSP-C staining at four weeks post-tamoxifen demonstrated a 23.3% ±4.0% (X ± SD, n=3) and 27.5±3.8% (X ± SD, n=3) decline in AT2 cell number from one week post-tamoxifen, respectively, with heterogeneous regions of higher and lower AT2 cell density (**Supplemental Figure 8**). Given the concordance in cell number quantified by each AT2 cell specific maker, it appeared that at four weeks after tamoxifen the majority of AT2 cells continue to express *Sftpc*^{C121G}.

An increase in the potent anti-inflammatory and profibrotic mediator TGF-β1 preceded the appearance of the fibrotic remodeling and was commensurate with the resolution of inflammation at two

weeks post-tamoxifen (**Figure 8H**). *Sftpc*^{C121G} AT2 cells were a contributing source of TGF-β1, with increases in both gene expression for *Tgfb1* (**Figure 8G**) and in AT2 cell TGF-β1 staining (**Supplemental Figure 9**). Concurrently, MMP-7, a component of the pro-fibrotic milieu, which was elevated in *SFTPC* BRICHOS patients' BALF (**Supplemental Figure 10A)** and has been described in adults with IPF (50), was also increased in the *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice BALF during this transitional period to fibrosis (**Supplemental Figure 10B)**. Thus, following the resolution of the alveolitis, the *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice express fibrotic mediators and biomarkers found in ChILD and ILD, and develop spontaneous fibrotic lung remodeling with a substantial restrictive impairment in respiratory mechanics.

DISCUSSION

Endoplasmic reticulum stress is a recognized pathway in the pathogenesis of fibrosis in multiple organs, including heart, kidney, liver, and lung (51). AT2 cell ER stress is also an appreciated feature of the epithelium in sporadic ILD and IPF (17,52). There are multiple postulated etiologies for this, including exogenous factors such as air pollution, cigarette smoke exposure (52), and chronic viral infections (53,54). Additionally, it is hypothesized that epithelial ER stress can arise from dysfunctional epithelial proteostasis in an aging or senescent epithelium (55-57). However, *in vivo* modeling to derive proof of concept linking AT2 cell ER stress and fibrotic lung remodeling has been elusive. Here we present a new model that provides evidence that AT2 expression of a disease-associated *SFTPC* BRICHOS mutation is sufficient to activate the UPR and produce spontaneous lung inflammation and fibrotic remodeling. Thus, AT2 cell ER stress and our $Sftpc^{C121G}$ model fit within the growing paradigm that disturbance in epithelial cell quality control drives fibrotic lung disease (7,58). In support of this, we recently reported on the non-BRICHOS $Sftpc^{I73T}$ mouse model where expression of this trafficking mutation causes defective AT2 cell macroautophagy in the absence of ER stress with resultant spontaneous inflammation and fibrotic lung remodeling (10). These two models taken together strongly support the notion that distinct endophenotypes of AT2 cell dysfunction may each contribute to ChILD and ILD through both overlapping and distinct pathways.

Previous *in vivo SFTPC* BRICHOS mutation models have been limited by technical difficulties with the control of relevant gene dosages. Constitutive $SFTPC^{\Delta Exon4}$ mutation expression in mice resulted in dose dependent disrupted lung morphogenesis with prenatal lethality (18). While that model recapitulated the cell biology of the *SFTPC* mutation and established a toxic gain of function for *SFTPC* BRICHOS mutations, the transgenic expression of this highly aggregation prone mutation using a human *SFTPC* promoter fragment is the likely cause of the severe developmental phenotype, limiting its translational relevance. Whereas the $SFTPC^{\Delta Exon4}$ model demonstrated a severe developmental phenotype, the inducible transgenic $SFTPC^{L188Q}$ model lacked a spontaneous phenotype, but had increased

susceptibility to bleomycin induced fibrotic remodeling (19). This model was limited by both low (10% of wild-type) *SFTPC*^{L188Q} mRNA expression and the observation *in vitro* that this mutated pro-protein was less aggregation prone than other BRICHOS mutations, suggesting a less severe cellular response to this isoform (59).

Based on the importance of the disulfide cysteine bonds in the BRICHOS domain for primary folding and processing (20) and our *in vitro* modeling of C121 mutant proSP-C isoforms (**Figure 1**), we selected an *Sftpc* mutation that we anticipated would cause significant ER stress. We developed a hypomorphic founder line which expressed low *Sftpc*^{C121G} mRNA, no detectable mutant proSP-C and lacked a spontaneous phenotype. This was in contrast to the hypomorphic *Sftpc*^{I73T} line, where an age dependent lung phenotype developed in parallel with the steady accumulation of mutant proSP-C isoforms due to their mistrafficking and evasion of proteasomal quality control (10). In contrast, SP-C BRICHOS mutations are substrates for proteasomal degradation, limiting their expression until transcription/translation exceeds proteasomal capacity (15), which likely accounts for the difference in protein expression in the two hypomorphic models. Since the founder *Sftpc*^{C121G} line failed to generate a phenotype, we developed complimentary models with sufficient gene expression to recapitulate varying aspects of the human lung phenotype. Constitutive heterozygous *Sftpc*^{C121G} expression allowed both proof of concept of the dominant negative effect of the mutant pro-protein on wild-type proSP-C processing (**Figure 2B and 2C**), and a pediatric/neonatal lung phenotype that mimicked clinical cases of ChILD with *SFTPC* BRICHOS mutations (**Figure 2D-F**). However, in the adult inducible model we discovered that heterozygous *Sftpc*^{C121G} expression was insufficient to develop a robust phenotype. This was similar to what was described in the *Sftpc*^{I73T} model and likely reflects that either the developing epithelium is more sensitive to the mutated isoforms or that low *Sftpc* ^{C121G} transcription in the heterozygous adult model was inadequate to produce sufficient mutant pro-protein capable of generating a toxic gain of function.

To overcome the limitation of low *Sftpc* mRNA in the adult inducible model, we selected homozygous *Sftpc*^{C121G} expression with inducible Cre-driven expression under a strong *Rosa26* promoter. While using this Cre-ERT2 system limited the ability to perform AT2 cell lineage tracing experiments (as

could performed with an *Sftpc*^{ERT2Cre}), it did produce a gene-dose level closer to predicted levels for heterozygous *SFTPC* BRICHOS mutation patients, and was capable of inducing a stronge ER stress cellular phenotype. Thus, by modulating gene-dose in both the constitutive and inducible *in vivo* models, we were able to produce lung phenotypes that recapitulated the spectrum of pediatric and adult *SFTPC* BRICHOS associated ILD, providing proof of concept linking AT2 cell ER stress and spontaneous fibrotic lung remodeling.

The translational relevance of this model was supported by the discovery of multiple biomarkers associated with human ILD in the *Sftpc*^{C121G} BALF. These included MMP7, which is a well described marker of fibrotic tissue remodeling in IPF (60) and was identified both in our pediatric proteomic analysis (**Supplemental Figure 10**) and in the *Sftpc*^{I73T} model. Additionally, we identified IL6 and IL8 in the *Sftpc*^{C121G} BALF during the inflammatory phase of the model (**Supplemental Table 2**), consistent with analyses of patient samples during AE-ILD that have identified increased plasma levels of IL8 and IL6 as biomarkers associated with worse prognosis (41,42).

The role of inflammation and immune effector cell populations in ILD is an area of ongoing research, but the importance of this is highlighted clinically by AE-ILDs. Key cellular features of acute exacerbations include epithelial injury and apoptosis, and the recruitment of a mixed granulocytic cell population (61,62). In this regard, the AT2 cell apoptosis (**Figure 4F**), alveolitis (**Figure 5F and 5G**), and subsequent fibrotic remodeling (**Figure 8**) in our model, recapitulates the human disease. We found that the *Sftpc*^{C121G} AT2 cell was responsible for early cytokine production associated with immune cell recruitment (**Supplemental Figure 7**). This corroborated previous *in vitro* studies where expression of *SFTPC* BRICHOS mutations caused the secretion of cytokines associated with immune cell recruitment (13,14). The relationship between ER stress and inflammation has been an active area of study in multiple organ systems, including in the pathogenesis of diabetes in the pancreatic B cell (63), hepatic steatosis liver disease (64,65), and vascular atherosclerosis (66). However, that the *Sftpc*^{I73T} model had both a similar immune cell ontogeny and overlapping AT2 cell produced cytokines suggests that AT2 cell ER stress per se may not underlay the effector cell recruitment (10), but rather that distinct AT2 cell dysfunction

endophenotypes converge to produce a common cytokine profile. The regulation of both this immune cell recruitment and then the transition to resolution of alveolitis represent two important areas of future research requiring additional understanding of epithelial-immune cell crosstalk.

Preceding the overt alveolitis, we identified the recruitment of monocyte/macrophage effector cells (**Figure 5I and Figure 6**). $CD11b^{+}Ly6C^{hi}$ monocytes have been experimentally shown to participate in the development of fibrotic lung disease in exogenous models of injury/repair such as bleomycin and were found to be an early immune population recruited in the $Sftpc^{I73T}$ model (10,39,67). Our models of spontaneous injury suggest that $CD11b^{+}Ly6C^{hi}$ monocytes are recruited to replenish the alveolar macrophage niche, which is consistent with recent evidence showing monocyte-derived alveolar macrophages as a key fibrogenic subset (39). We found the monocyte/macrophage cytokine CCL2/MCP-1 in the $Sftpc^{C121G}$ BALF, which has previously been identified in fibrotic lung disease and preclinical models (10,48,49,68-70). However, we also uncovered elevated levels of CCL17/TARC and CCL7/MCP-3, cytokines with overlapping functional redundancies with CCL2/MCP-1. All three of these cytokines were elaborated in the pediatric proteomic analysis (**Figure 7**), proving a translational context to this discovered redundancy, which may account for the phase-two clinical trial failure of pharmacologic MCP-1 neutralization as a therapy in IPF (71).

There are multiple possible mechanisms by which fibrosis develops in this model. During the inflammatory phase of the model there is AT2 cell apoptosis, which has been associated with the development of fibrosis in AT2 cell ablation models (72). However the 20-30% decline in AT2 cell number we observed in this model at four weeks post-tamoxifen was a degree of apoptosis found to be insufficient to induce spontaneous fibrosis in multiple AT2 cell ablation models (73,74). Alternatively our data shows that the $Sftpc^{C121G}$ AT2 cell is a source of the potent fibrotic mediator TGF-β1 (**Figure 8G**), albeit the true contribution of TGF-β1 to the development of fibrosis in this model would require TGF-β1 depletion. In the lung there has been limited mechanistic work on the link between epithelial ER stress and TGF-β1 (57) (75). However, UPR pathways such as ATF4/CHOP have been implicated in TGF-β1 production in

epithelial injury models of liver and kidney fibrosis (76,77). That AT2 cell *Tgfb1* expression in the *Sftpc*^{C121G} model increased two weeks post-tamoxifen demonstrates a temporal sequence of events, with early AT2 cell pro-inflammatory cytokine production followed by a transition to the release of anti-inflammatory and pro-fibrotic TGF-β1. The regulation of this phenotypic transition may provide additional insights into the accelerated fibrosis that occurs during recovery from an AE-ILD.

In conclusion, the *Sftpc*^{C121G} model provides a proof of principle for the role of AT2 cell ER stress in pediatric ChILD and adult ILD. We have demonstrated that *Sftpc*^{C121G} AT2 cells are a source of multiple relevant chemokines/cytokines associated with the recruitment of significant effector cell populations supporting a proximal position for the AT2 cell in the pathway to fibrosis. While the precise UPR pathways responsible for the expression of these cytokines and the fibrotic mediator TGF-β1 would require genetic or pharmacologic inhibition, this model represents a new tool for therapeutic intervention studies such as these. The translational relevance of the model is further supported by the identification of these cytokines and other biomarkers in the high-dimensional BALF proteomic analysis of patients with ChILD from *SFTPC* BRICHOS mutations. The *Sftpc*^{C121G} model thus connects AT2 cell dysfunction manifesting as ER stress with lung inflammation and aberrant lung remodeling, supporting its role as a novel and relevant preclinical model.

MATERIALS and METHODS

Mouse Models

The *Sftpc*^{C121Gneo} founder line was commercially produced (Genoway, Inc. Lyon, France) using targeting vector based embryonic stem cell electroporation transfection strategies to knock-in coding sequences for the cysteine-to-glycine substitution at codon 121 into the endogenous mouse *Sftpc* locus (depicted in **Supplemental Figure 1A**). For constitutive removal of the lox-P flanked PGK-neo cassette from *Sftpc*^{C121Gneo} allele in utero homozygous *Sftpc*$^{C121Gneo/\ C121Gneo}$ female mice were bred to the heterozygous X-linked CMV-Cre recombinase line (Strain B6.C-Tg(CMV-cre)1Cgn/J; Stock# 006054; The Jackson Laboratory, Bar Harbor, ME) (25). For tamoxifen inducible removal of PGK-neo from *Sftpc*^{C121Gneo} alleles, the *Sftpc*^{C121Gneo} founder line was crossed to the Rosa26-ERT2-Cre (Strain B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J; Stock# 008463; The Jackson Laboratory, Bar Harbor, ME). The progeny was backcrossed to homozygosity of the *Sftpc*^{C121Gneo} allele and the *R26*^{Cre} allele. All mouse strains and genotypes generated for these studies were congenic with C57/B6/J. Both male and female animals (aged 8-12 weeks) were utilized in tamoxifen induction protocols. Mice were housed under pathogen free conditions in a barrier facility, and all experimental and breeding protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Perelman School of Medicine at the University of Pennsylvania.

Materials and Reagents

Tamoxifen (non-pharmaceutical grade) was purchased from Sigma Aldrich (St Louis, MO). Cytological slides were stained with Giemsa (#GS500; Sigma Aldrich). All other reagents were electrophoretic or immunological grade and purchased from either Sigma Aldrich or Thermo-Fisher Scientific (Waltham, MA).

Lung Histology

Whole lungs were fixed by tracheal instillation of 1.5% glutaraldehyde/1.5% paraformaldehyde mixture in 0.15 M HEPES buffer at a constant pressure of 25 cm H2O and removed en bloc. Paraffin embedded 6μM lung sections were stained with Hematoxylin & Eosin (H&E) or Masson's Trichrome stains by the Pathology Core Laboratory of Children's Hospital of Philadelphia.

Immunohistochemistry and Immunofluorescence

Immunohistochemical staining of paraffin-embedded lung sections was performed with primary antibodies at indicated dilutions as described in **Supplemental Table 4**. A commercial horseradish peroxidase based method (Vectastain Elite Rabbit IgG ABC Kit #PK6101; Vectastain DAB Substrate Kit #SK410; Vector Laboratories, Burlingame, CA) was used to visualize immunoreactivity. Following immunoreactivity staining, sections were counterstained with Mayer's hematoxylin.

Immunofluorescence staining of lung sections was performed with combinations of primary antibodies and secondary Alexafluor conjugated anti-IgG (**Supplemental Table 4**). Images were visualized on an Olympus I-70 inverted fluorescent microscope (Olympus, Melville, NY), captured with a Hamamatsu 12-bit coupled-charged camera (Hamamatsu, Japan), and processed using Metamorph, Version 7.8.4.0 (Universal Imaging, West Chester, PA).

Quantitative determination of proSP-C and ProSP-C positive cells was performed by fluorescent immunostaining of serial lung sections with anti-proSP-C and anti-proSP-B. Five consecutive 20x images were obtained from 2 different lobes (10 peripheral lung fields per section), with manual counting followed by quantification of positive cells per field. Apoptotic AT2 cell index was performed using double label fluorescent immunostaining of lung sections with anti-proSP-C and anti-cleaved caspase 3. Five consecutive 20x images were obtained from 2 different lobes (10 peripheral lung fields per section). Apoptotic (percentage cleaved caspase 3 / proSP-C double positive cells per total proSP-C positive cells) indices were determined by manual counting.

Picrosirius Red Staining

Staining for collagen was performed using the Picosirius Red Stain Kit (Polysciences, Inc., Warrington PA) according to the manufacturer's instructions. Following lung sections staining, five consecutive 20x images were obtained from 2 different lobes (10 peripheral lung fields per section without large airways) for analysis. Digital morphometric measurements were performed using Image J. The mean area of positive picrosirius staining of each lung field was expressed as a percentage of total section area (10,78).

Quantification of Lung Collagen Content

Total right lung acid soluble collagen content was determined using the Sircol® assay (Biocolor, Ltd; Carrickfergus, UK) according to the manufacturer's instructions and as previously described (69).

Bronchoalveolar Lavage Fluid Collection and Processing

BALF was collected from mice using five sequential 1 ml sterile saline lung lavages. 300μL aliquots of each cell-free, first 1mL of BALF return was removed and stored at -80°C for cytokine analysis. Cell pellets recovered from centrifugation of complete return were resuspended in 1 ml of sterile saline and total cell counts determined by a Z1 Coulter Counter (Beckman-Coulter, Indianapolis, IN). Differential cell counts were obtained by manually quantifying cytospins of BALF cell pellets stained with modified Giemsa (Sigma Aldrich, #GS500). Large-aggregate surfactant fractions were prepared from cell free BALF by centrifugation at 20,000 X g for 60 min at 4^{o}C as described previously (79). Total protein content of BALF, surfactant, cell lysate, and whole lung homogenate were assayed by the Bradford method (79).

Cytokine Analyses

Aliquots of first return, cell free BALF were analyzed for levels of a panel of cytokines (CCL2, CCL11, IL-4, IL-13, IL-5, IL-6, GM-CSF, IL-1β, KC(GroA); Panel MCYTOMAG-70K-9) using the Luminex (Luminex Corporation, Austin, TX) multiplex platform analyzed by the Human Immunology Core at the Perelman School of Medicine. Active TGF-β1 and CCL17 (TARC) were measured separately also using Luminex kits (Panel TGFBMAG-64K and MECY2MAG-73K). For select cohorts, CCL7 was measured using a MCP-3 ELISA kit (Cat #BM6006INST, Invitrogen, Waltham, MA).

Mouse Alveolar Type 2 Cell Isolation

Mouse AT2 cells were isolated as previously reported (69). Briefly, a single cell suspension was obtained by instilling Dispase (BD Biosciences) into perfused lungs, followed by mechanical dissociation with a McIlwain tissue chopper (Metrohm USA), Riverview, FL), and treatment with 20 μg/mL DNAase I (Sigma Aldrich). Differential adherence on plastic culture dishes negatively selected mesenchymal cells. CD45+ cells were depleted by negative selection using Dynabeads untouched mouse T cell kit (#11413D) and Dynabeads mouse DC enrichment kit (#11429D) (Thermo Fisher Scientific). Recovered cells were collected and flash frozen at -80^{o}C. Purity was determined by immunostaining preparations adhered overnight to 10% matrigel coated cover slips using DAPI in combination with primary antisera for proSP-C. Manual counts in five 20x fields per sample were done and purity defined as number of pro-SP-C+ cells (AT2 cells) divided by total nuclei showing >95% purity (n=4).

SDS-PAGE and Immunoblotting

SDS-PAGE using Novex Bis-Tris gels (#NP0301 ThermoFisher Scientific) and immunoblotting of PVDF membranes with primary antibody (**Supplemental Table 4**) was performed. This was followed by species specific horseradish peroxidase conjugated secondary antibody and band detection by enhanced

chemiluminescence (ECL2 #80196 ThermoFisher or WesternSure #926-95000, LI-COR Biotechnology, Lincoln, NE) using a LiCor Odyssey Fc Imaging Station and quantized using the manufacturers' software.

Antibodies

A polyclonal proSP-C antiserum ("NPRO-SP-C") raised against rat proSP-C and polyclonal anti-proSP-B ("PT3") raised against bovine proSP-B were each produced in rabbits in-house and previously validated as published (80,81). The other antibodies used for these studies were obtained from commercial sources (**Supplemental Table 4**).

RNA Isolation and Quantitative Real Time Polymerase Chain Reaction

cDNA was prepared from RNA template purified from AT2 cell or whole lung RNA using the RetroScript Kit. Quantitative single-plex polymerase chain reactions (qRT-PCR) were performed using Taq polymerase and TaqMan RT-PCR kits (Applied Biosystems/ ThermoFisher Scientific) with primer sets for mouse genes listed in **Supplemental Table 5** on an ABI Prism 7900 system and a QuantStudio 7 Flex Real-Time PCR System.

Multichannel Flow Cytometry

Tissue immune cell characterization was performed as previously described (10). Briefly, En bloc lungs were digested in Dulbecco's Modified Medium (DMEM) + 5% FBS + 2 mg/ml Collagenase D (Cat #11088866001, Roche, Indianapolis, IN), passed through 70-μm nylon mesh to obtain single-cell suspensions, and then mixed with Gibco RBC Lysis Buffer (Cat #A10492-01, Thermo Fisher Scientific). Cell pellets were resuspended in PBS+0.1% sodium azide and blocked with anti-mouse CD16/32 antibody (Fc block, eBiosciences, San Diego, CA) followed by incubation with antibody mixtures (or isotype controls) and conjugated viability dye (see **Supplemental Table 6**). Single cell suspensions were then analyzed on a LSR Fortessa (BD Biociences, San Jose, CA). They were then gated on viability and singlets

and analyzed with FlowJo software (FlowJo, LLC, Ashland, Oregon) based on a modification of the strategy of Misharin, et al (82) as published (10) (**Supplemental Figure 6**).

Pediatric SFTPC Mutation Cohort For Comparative BALF Proteomics

Clinically indicated flexible bronchoscopy was performed and BALF was collected, processed and stored in standard fashion. Disease control subjects were defined as patients with pulmonary symptoms necessitating flexible bronchoscopy including unexplained wheeze, cough, congestion, and recurrent pneumonia, but who had normal-appearing bronchoscopy. BALF cytology and microbiologic testing were performed as part of routine clinical care (83). Briefly, the area of lavage was picked by the pulmonologist performing the procedure, and in general was performed in areas where mucus had collected, or in area where infiltrate was seen on chest imaging. Typically, three lavages were performed using sterile nonbacteriostatic saline at room temperature. Each lavage aliquot consisted of 1 milliliter per kilogram of body weight, with a maximum of 30 milliliters of normal saline per lavage. After BALF collection, any excess fluid not required for clinical laboratory testing was processed for research study. Collected BALF was processed in a standard manner. It was initially centrifuged for 10 minutes at 1200 revolutions per minute (rpms) at 4^{o}C. The pellet was saved, and the supernatant was spun again for 20 minutes at 4700 rpms at 4^{o}C. The second pellet was saved. This supernatant was split in half, with half stored 0.5 mL aliquots at -70^{o}C. The other half of the supernatant was treated with the protease inhibitors phenylmethelsulfonyl (PMSF), and Ethylenediaminetetra acetic acid (EDTA) to inhibit protease activity. The samples are frozen at -70^{o}C for further study.

SOMAmer®proteomic analysis (84,85): SOMAscan technology was used to simultaneously measure 1129 proteins from each sample at SomaLogic, Inc. (Boulder, CO, USA). BALF was diluted with buffer to a standard protein concentration of 20ug/mL and 100uL of diluted BALF was equilibrated with a SOMAmers® mix (Gold 2010). The SOMAmer® mixture allowed for the detection and quantitation of 1129 protein aptamers simultaneously in each sample recorded as relative florescent units (RFU). Raw

RFUs were adjusted based on the individual dilution factor for each sample to reflect the initial protein amounts.

Statistical Analysis

For *in vitro* and *in vivo* studies all data are presented with dot-plots and group mean ± SEM unless otherwise indicated. Statistical analyses were performed with GraphPad PRISM (SanDiego, CA). Student's t-test (1 or 2 tailed as appropriate) were used for 2 groups and multiple comparisons were done ANOVA with post hoc testing as indicated; survival analyses was performed using log-rank test. In all cases statistical significance was considered at p-values <0.05.

For the pediatric BALF analysis, adjusted RFUs were log (base 2) transformed and quantile normalization was used to obtain final values for analysis. RFUs for each protein aptamer were compared univariately between groups using difference in $\log_2$ means and a two-sample t-test assuming unequal variances. Conservative selection of aptamers included the intersection set with a difference greater than 1 on $\log_2$ scale and a p-value < 0.001. This approach provides balance between selecting aptamers with a large absolute change and significant p-value.

Study Approval

Mice were housed in pathogen free facilities according to protocols approved by the IACUC of the Perelman School of Medicine at the University of Pennsylvania. For the pediatric proteomics data, the study was approved by the Colorado Multiple Institutional Review Board (#99-113 and #10-0472). Informed consent was obtained from all subjects for sample collection and study enrollment. If the patient was a minor, informed consent was obtained from the subject's legal guardian. In subjects aged 12 to 17 years, informed assent was obtained.

AUTHOR CONTRIBUTIONS:

MFB and SM developed the concept. MFB, SM, JK, YT, MK, and AV designed the experiments. YT, JK and MK performed *in vivo* animal experiments; JK, AV, SJR, ACH, MCB, MFB, and SM performed *in vitro* experiments and endpoint analyses for *in vivo* studies; RRB and BDW designed the human cohort study and performed the analysis; MFB, JK, AV, RRB and BDW analyzed data, generated figures, and interpreted results; JK and MFB drafted manuscript; MFB, AV, MCB, JMS, and SM edited the manuscript. All authors reviewed and approved the final version prior to submission.

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FIGURES

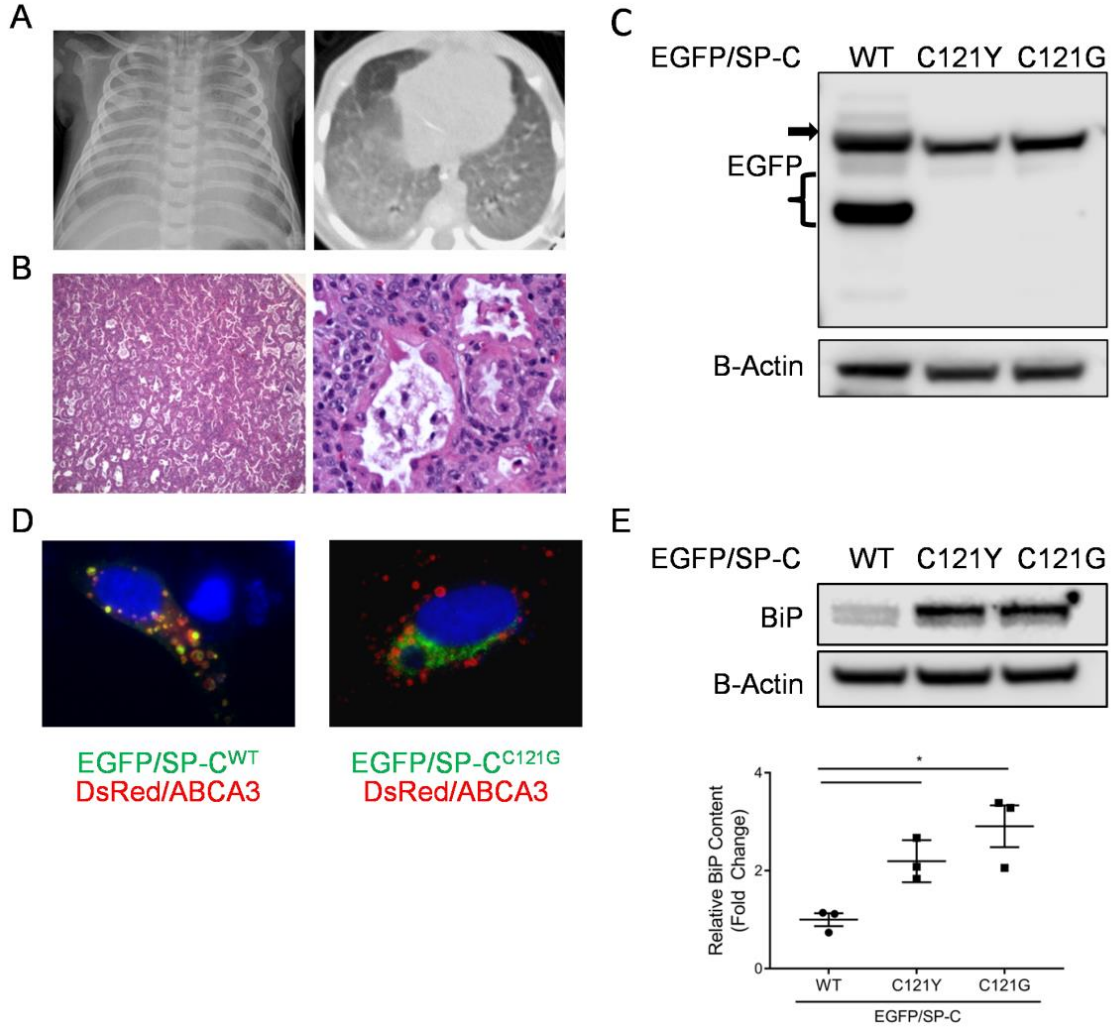

Figure 1. In vitro modeling of a clinical *SFTPC^{C121Y}* mutation identifies mistrafficked pro-protein and epithelial ER stress with mutagenesis of codon 121 in *SFTPC*.
(A) Chest imaging of a 3 month old patient with heterozygous *SFTPC^{C121Y}* mutation shows bilateral diffuse hazy opacity on chest radiograph (left) and diffuse ground glass opacities on chest CT (right). **(B)** H&E staining of patient lung biopsy demonstrates diffuse abnormality in lobular architecture with mild enlargement of airspaces and mild to moderate interstitial widening, airspaces filled by foamy macrophages, scattered neutrophils, and eosinophilic material suggestive of proteinosis (original magnification 4x left and 20x right). **(C)** HEK293 cells were transiently transfected with either EGFP/SP-C^{WT} or EGFP/SP-C^{C121} mutants as indicated and cell lysates harvested at 48 hours were subjected to western blotting for EGFP. An SP-C primary translation product (black arrow) was detected in all transfections, however, no processing intermediates (black brackets) were observed with either EGFP/SP-C^{C121} mutation. Representative of 3 independent experiments. **(D)** HEK293 cells transiently co-transfected with either EGFP/SP-C^{WT} or EGFP/SP-C^{C121G} and DsRed/ABCA3 and subjected to immunflorescence microscopy demonstrating co-localization of EGFP/SP-C^{WT} with DsRed/ABCA3, but a reticular expression pattern of EGFP/SP-C^{C121G} without DsRed/ABCA3 co-localization (original magnification 60x). **(E)** Cell lysates of HEK293 cells 48 hours after transfection with either EGFP/SP-C^{WT} or EGFP/SP-C^{C121} mutants as indicated were subjected to western analysis for BiP (upper). Representative western blot of n=3. Quantitative densitometry (lower) normalized to B-Actin showing increases in BiP with the cysteine mutants compared to EGFP/SP-C^{WT}. n=3 * $p < 0.05$ vs EGFP/SP-C^{WT} by One Way ANOVA with Dunnett's multiple comparison test.

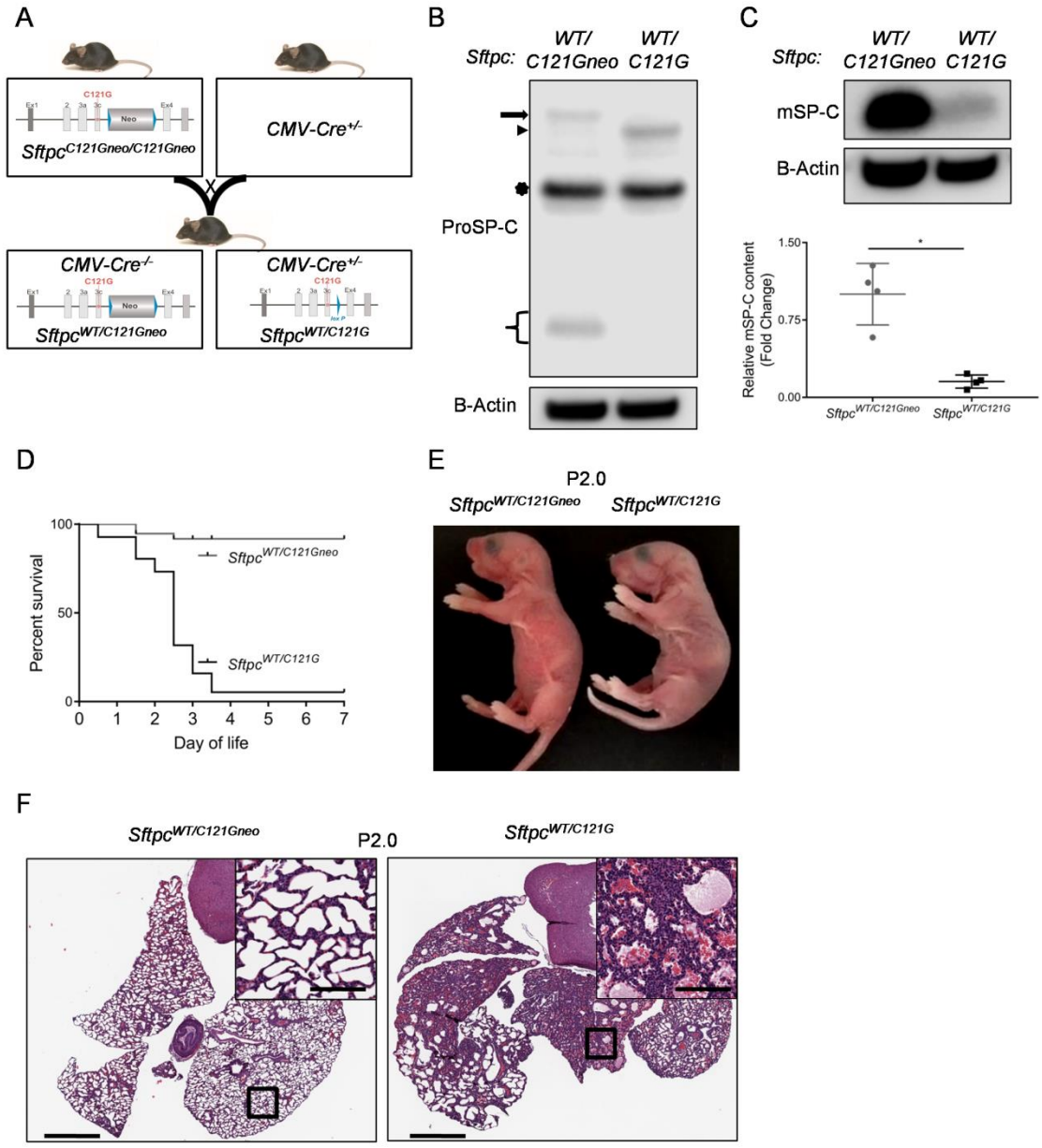

Figure 2. Constitutive expression of the *Sftpc*^{C121G} mutation *in vivo* causes a lethal toxic gain of function.
(A) Schematic for generation of mice constitutively expressing *Sftpc*^{C121G} alleles. Breeding of *Sftpc*$^{C121Gneo/C121Gneo}$ line to *CMV-Cre* mice yields litters with heterozygous constitutive expression of *Sftpc*^{C121G} mutation (*Sftpc*$^{WT/C121G}$). **(B)** Western blotting of lung homogenates from day P2.5 for proSP-C expression show aberrant banding pattern with heterozygous *Sftpc*^{C121G} expression with decreased processing intermediates. (arrow- *Sftpc*$^{WT/C121Gneo}$ proSP-C highest molecular weight translational product; arrowhead- *Sftpc*$^{WT/C121G}$ highest molecular weight translational product ; star is non-specific bands; brackets are processing intermediates). **(C)** (Upper) Representative western blot of lung homogenates from day P2.5 for mature SP-C (mSP-C); (Lower) Dot-plots of means and SEM of mSP-C densitomitry expressed as fold change to *Sftpc*$^{WT/C121Gneo}$. *p < 0.05 using unpaired two tailed t-test with normalization to B-Actin loading control. **(D)** Kaplan–Meir survival analysis showing high lethality with constitutive *Sftpc*^{C121G}expression (n=41 and 37 for *Sftpc*$^{WT/C121G}$ and *Sftpc*$^{WT/C121Gneo}$, respectively). p < 0.001 by Log-rank (Mantel-Cox) test. **(E)** Representative photographs of P.2.0 pups show the development of cyanosis in the *Sftpc*$^{WT/C121G}$ animal. **(F)** Representative lung histology (4x magnification) of P2.0 *Sftpc*$^{WT/C121G}$ pups with heterogeneous areas (10x magnification insert) of thickened septae with proteinaceous fluid in the alveolar space. Bar = 500 μM; Insert bar = 200μM.

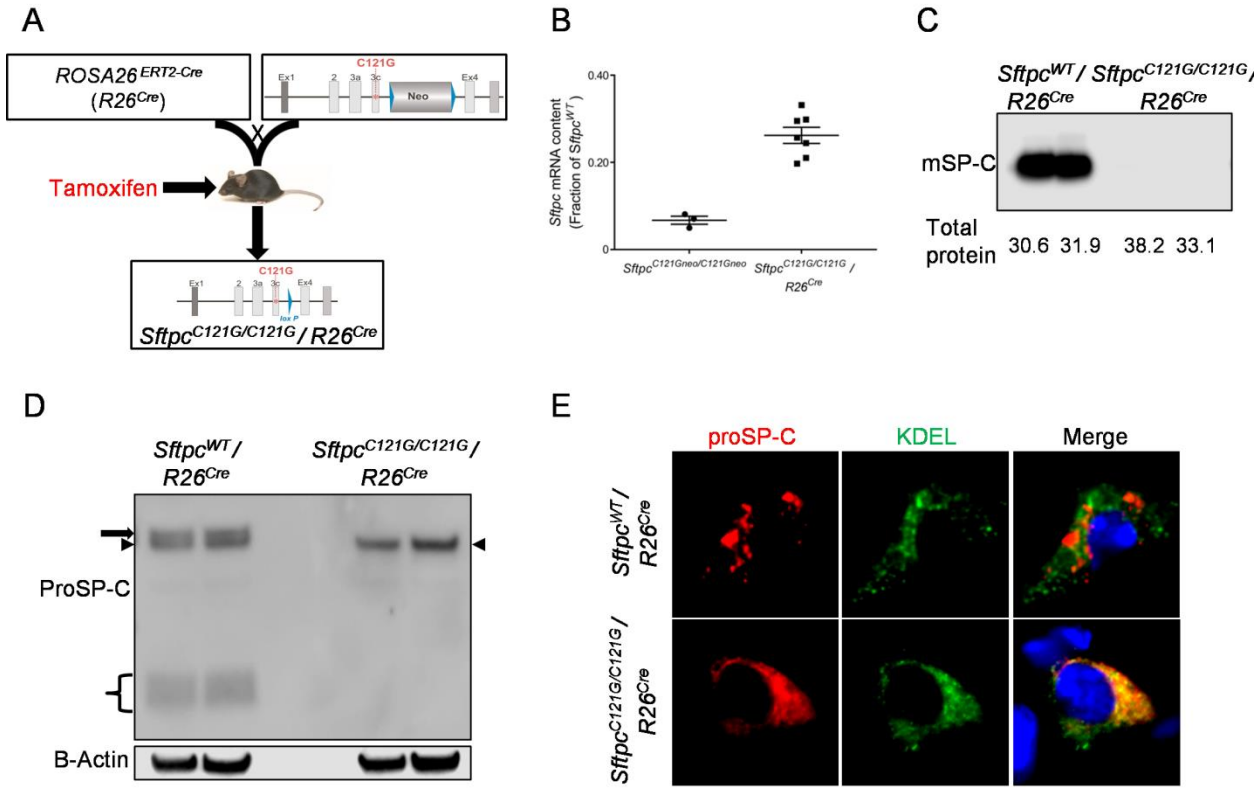

Figure 3. ***In vivo*** **inducible expression of the** ***Sftpc*** ^{C121G} **mutation in adult mice causes an ER retained SP-C pro-protein.**

(A) Strategy for generation of tamoxifen inducible mice in which tamoxifen treatment of *Sftpc* $^{C121G/C121G}$ / *R26* ^{Cre} line results in a removal of an inhibitory intronic PGK-neo cassette. (**B)** qRT-PCR analysis for *Sftpc* expression in the purified AT2 cells from homozygous *Sftpc* $^{C121Gneo/C121Gneo}$ and *Sftpc* $^{C121G/C121G}$ / *R26* ^{Cre} mice at 7 days after treatment with tamoxifen. Data normalized to 18S RNA are expressed as *Sftpc* mRNA as a fraction of *Sftpc* ^{WT}/ *R26* ^{Cre} mice. **(C)** Western blotting of BALF large aggregate fraction from *Sftpc* $^{C121G/C121G}$ / *R26* ^{Cre} and *Sftpc* ^{WT}/ *R26* ^{Cre} mice at Day 7 after tamoxifen showing the absence of mature SP-C (mSP-C) in the *Sftpc* $^{C121G/C121G}$ / *R26* ^{Cre} mice. **(D)** Western blotting of the AT2 cell lysates from *Sftpc* ^{WT} / *R26* ^{Cre} mice or *Sftpc* $^{C121G/C121G}$ / *R26* ^{Cre} 7 days after tamoxifen show *Sftpc* $^{C121G/C121G}$ / *R26* ^{Cre} AT2 cells with an endoplasmic reticulum (ER) retained pro-protein (arrowhead) without post-translational palmitoylation (arrow) or processing intermediates (bars) observed in *Sftpc* ^{WT}/ *R26* ^{Cre} AT2 cells. (**E**) Double label immunofluorescence staining of whole lung sections for proSP-C (red) and the ER marker KDEL, demonstrates a reticular proSP-C staining with significant co-localization with KDEL in *Sftpc* $^{C121G/C121G}$ / *R26* ^{Cre} mice, compared to the punctate pattern of proSP-C staining distinct from KDEL observed in the *Sftpc* ^{WT} / *R26* ^{Cre} mice (original 60x magnification).

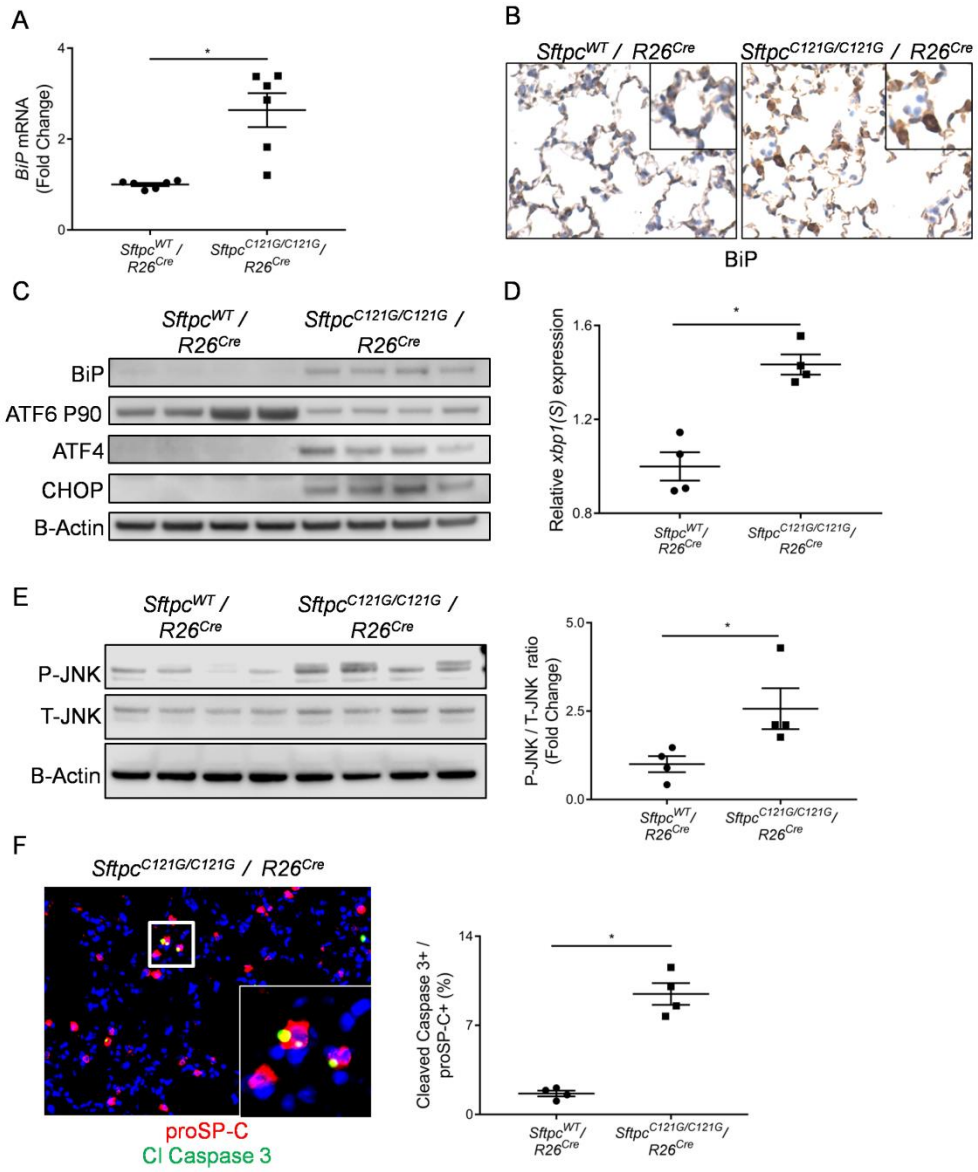

Figure 4. Expression of the SP-C C121G pro-protein causes activation of multiple epithelial ER stress pathways and induces AT2 cell apoptosis.
(A) qRT-PCR for *BiP* expression in purified AT2 cells from *Sftpc*^{WT}/ *R26*^{Cre} controls and *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice at 7 days after tamoxifen. Data normalized to 18S RNA are expressed as fold change in *BiP* normalized to control mice. *p < 0.05 vs control using unpaired two tailed t-test. **(B)** Representative immunohistochemical staining for BiP in lung sections (20x magnification) from control and *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice at 7 days after tamoxifen shows increased BiP staining in (60x magnification insert) AT2 cells. **(C)** Western blotting of AT2 cell lysates at 7 days after tamoxifen for BiP, ATF6 (P90), ATF4, CHOP, and B-Actin. **(D)** qRT-PCR for *XBP1* splicing ratio in AT2 cells at 7 days after tamoxifen shows an increase in the spliced fraction in *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} AT2 cells compared to controls. *p < 0.05 vs control using unpaired two tailed t-test. **(E)** Western blot (left) of AT2 cell lysates at 7 days after tamoxifen for phosphorolated JNK (P-JNK; upper band), total JNK (T-JNK; lower band), and B-Actin. Densitometry ratio (right) of P-JNK/T-JNK, reveals increase in P-JNK in *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice compared to control. *p < 0.05 vs control using unpaired two tailed t-test. **(F)** Representative 20X fluorescence micrograph from *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} lung 7 days after tamoxifen stained with proSP-C (red) and Cleaved Caspase 3 (green) (left). Dot-plots of double-positive (Cleaved Caspase 3+ / proSP-C+) cells expressed as a percentage of total proSP-C+ AT2 cells are shown with means and SEM (right). *p < 0.05 vs control using unpaired two tailed t-test.

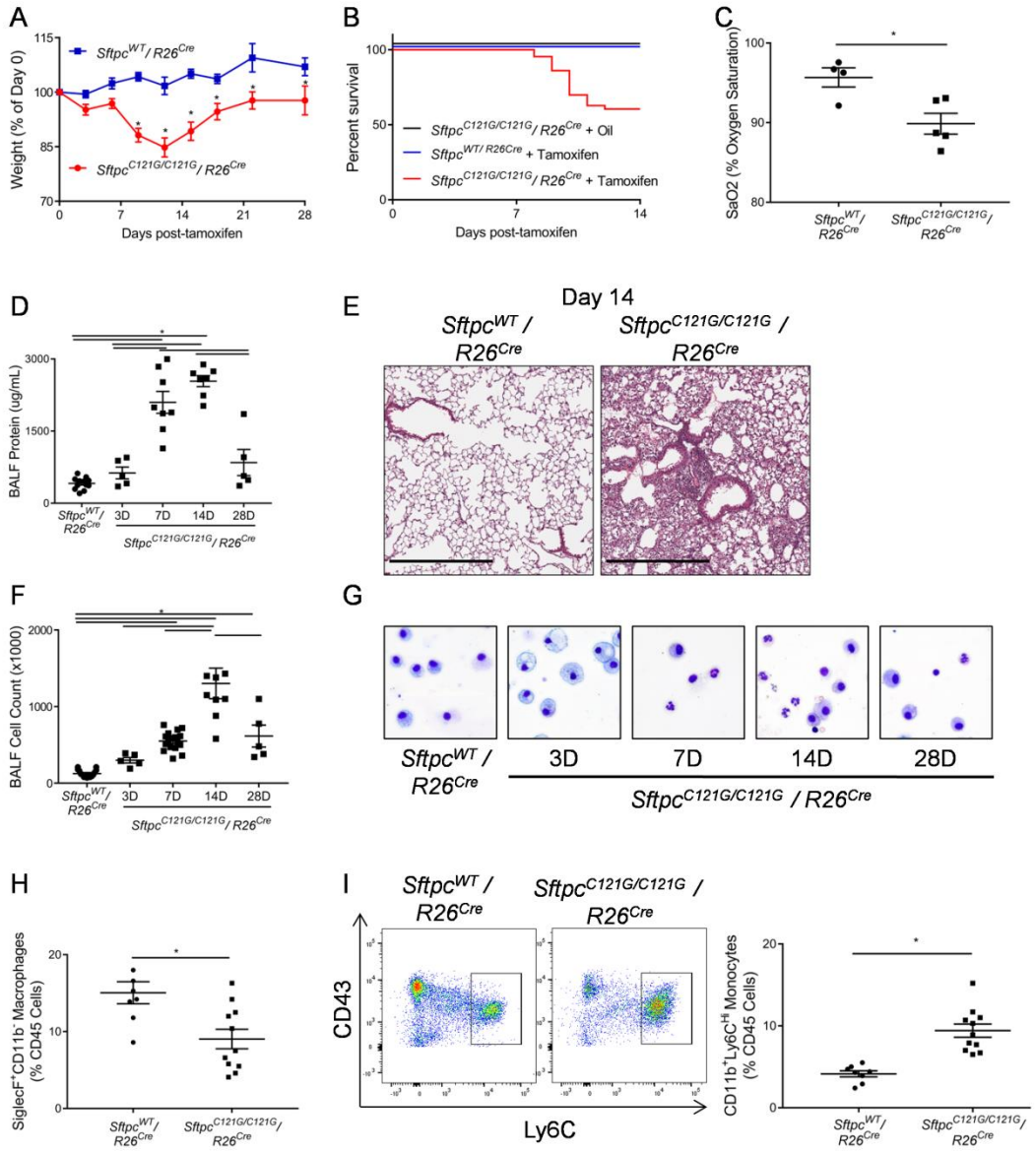

Figure 5. Expression of mutant *Sftpc*^{C121G} causes lung injury with a polycellular alveolitis.
(A) Weight loss curve in surviving *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice and *Sftpc*^{WT} / *R26*^{Cre} controls treated with tamoxifen. *p < 0.05 vs control group using unpaired two tailed t-test. **(B)** Kaplan–Meier survival curve of *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice treated with tamoxifen (n=43) or vehicle (oil) (n=16) and *Sftpc*^{WT} / *R26*^{Cre} mice treated with tamoxifen (n=32). Endpoints were defined as death or body weight < 75% on 2 consecutive days. p < 0.001 by Log-rank (Mantel-Cox) test. **(C)** Pulse oximetry of *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice and controls 7 days post-tamoxifen. *p < 0.05 vs control using unpaired two tailed t-test. **(D)** BALF protein content following tamoxifen. Controls represent pooled samples from all four time points. * p< 0.05 by One Way ANOVA with post-hoc Tukey test. **(E)** Representative 10x magnification H&E histology at 14 days after tamoxifen. Bar = 500 μM. **(F)** Dot-plots with mean and SEM of BALF cell count following tamoxifen. Controls represent pooled samples from all four time points. *p < 0.05 by One Way ANOVA with post-hoc Tukey. **(G)** Representative Giemsa stained BALF cytospins from control and *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice after tamoxifen (20x magnification). **(H)** Dot-plots with mean and SEM of percentage of total lung digest immune cells (CD45^{+}) that were alveolar macrophages (SiglecF^{+}CD11b^{-}) at 3 days post-tamoxifen. *p < 0.05 vs control using unpaired two tailed t-test. **(I)** Representative control and *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} flow cytometry gating for CD11b^{+}Ly6C^{Hi} monocytes at day 3 post-tamoxifen (left). Dot-plots with mean and SEM of percentage of total lung digest immune cells (CD45^{+}) that were CD11b^{+}Ly6C^{Hi} monocytes. *p < 0.05 vs control using unpaired two tailed t-test.

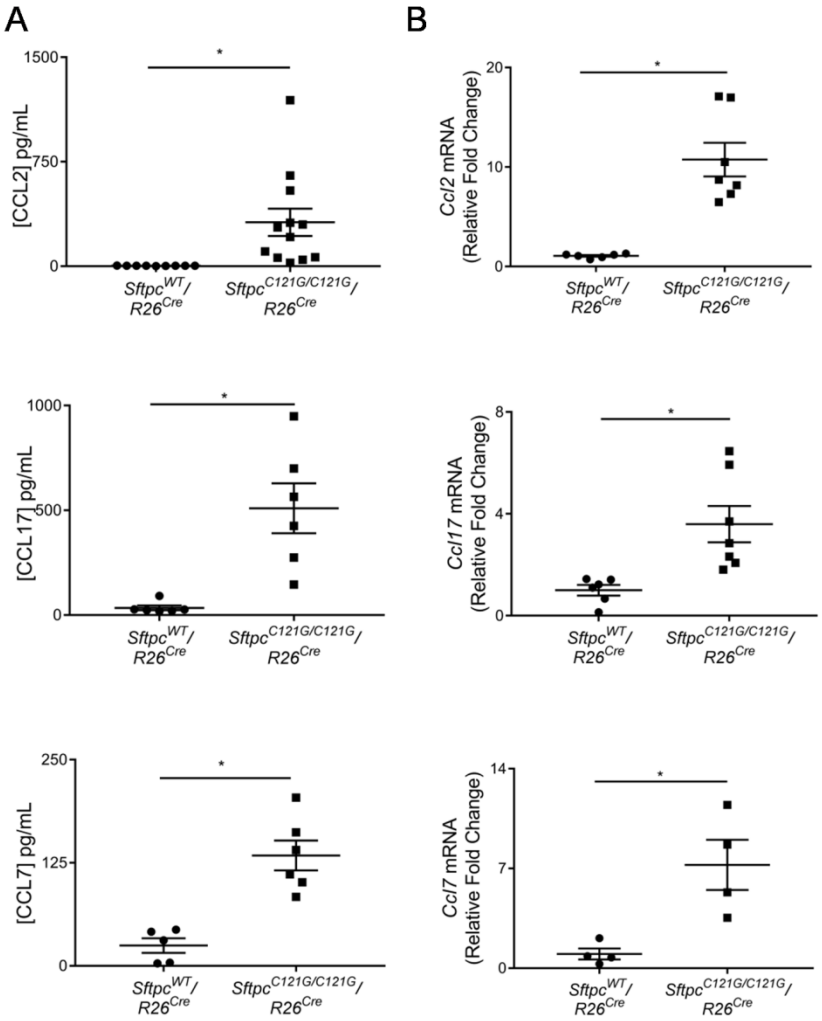

Figure 6. *Sftpc*^{C121G} AT2 cells are a source of cytokines associated with macrophage/monocyte recruitment. **(A)** Dot-plots with mean and SEM of BALF CCL2 (upper), CCL17 (middle), and CCL7 (lower) protein in *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice and *Sftpc*^{WT} / *R26*^{Cre} controls 7 days post-tamoxifen determined by Luminex assay (CCL2 and CCL17) and ELISA (CCL7). * $p < 0.05$ versus controls by One Way ANOVA (see **Supplemental Table 2**) followed by post-hoc Tukey test. **(B)** qRT-PCR determination of *Ccl2* (upper), *Ccl17* (middle), and *Ccl7* (lower) mRNA expression in AT2 cells 7 days following tamoxifen. Dot-plots with mean and SEM. *$p < 0.05$ vs control using unpaired two tailed t-test.

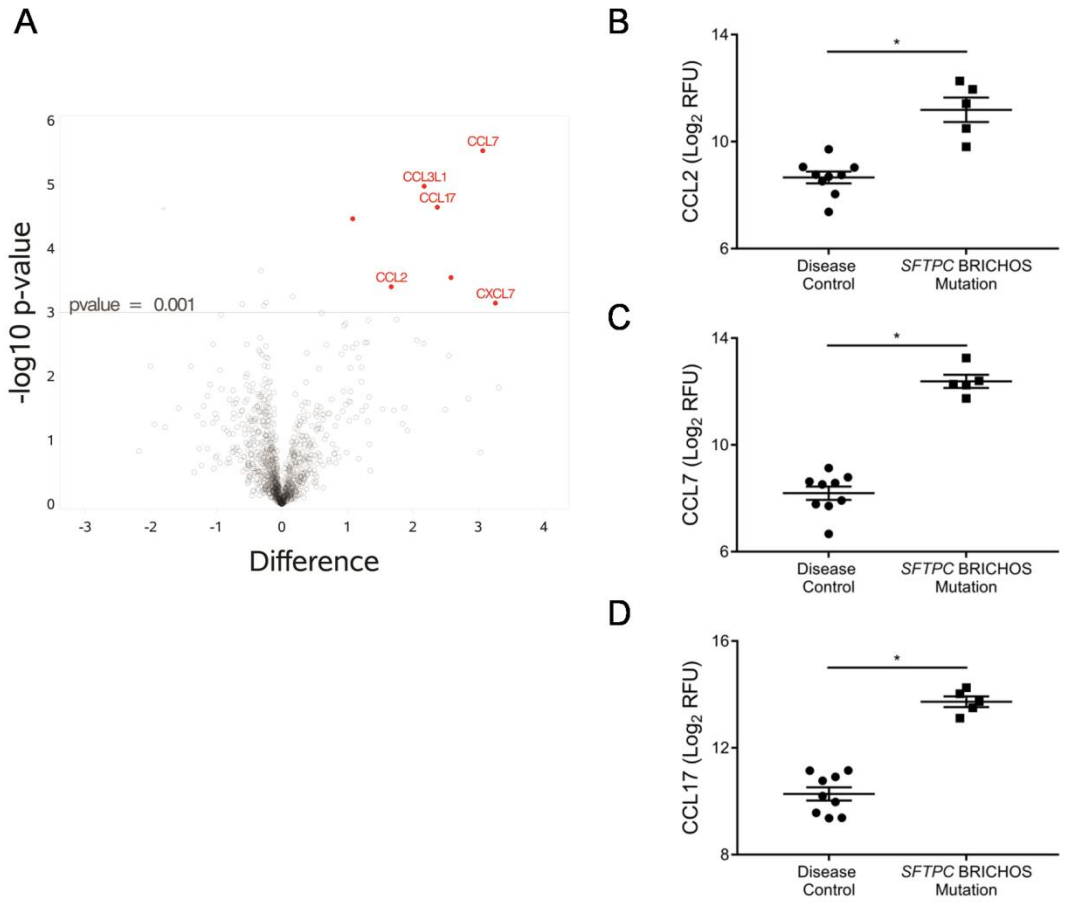

Figure 7. Pediatric *SFTPC* BRICHOS mutation patients elaborate multiple cytokines associated with macrophage/monocyte recruitment found in *Sftpc*^{C121G} mice.
(A) Volcano plot of SOMAscan proteomics platform analysis of BALF from *SFTPC* BRICHOS mutations cases (n=5) and Disease Control (n=9). Minus log (base 10) transformed p-value on y-axis, and Log_2 Difference on x-axis. Conservative selection of cytokines associated with immune cell recruitment with Relative Florescent Units (RFUs) difference greater than 1 on log2 scale and a p-value < 0.001 are shown in red. (**B-D)** Individual Dot-plots of mean ± SEM log2 RFUs for **(B)** CCL2, **(C)** CCL17, and **(D)** CCL7. *p < 0.05 for *SFTPC* BRICHOS mutations cases vs Disease Control using unpaired two tailed t-test.

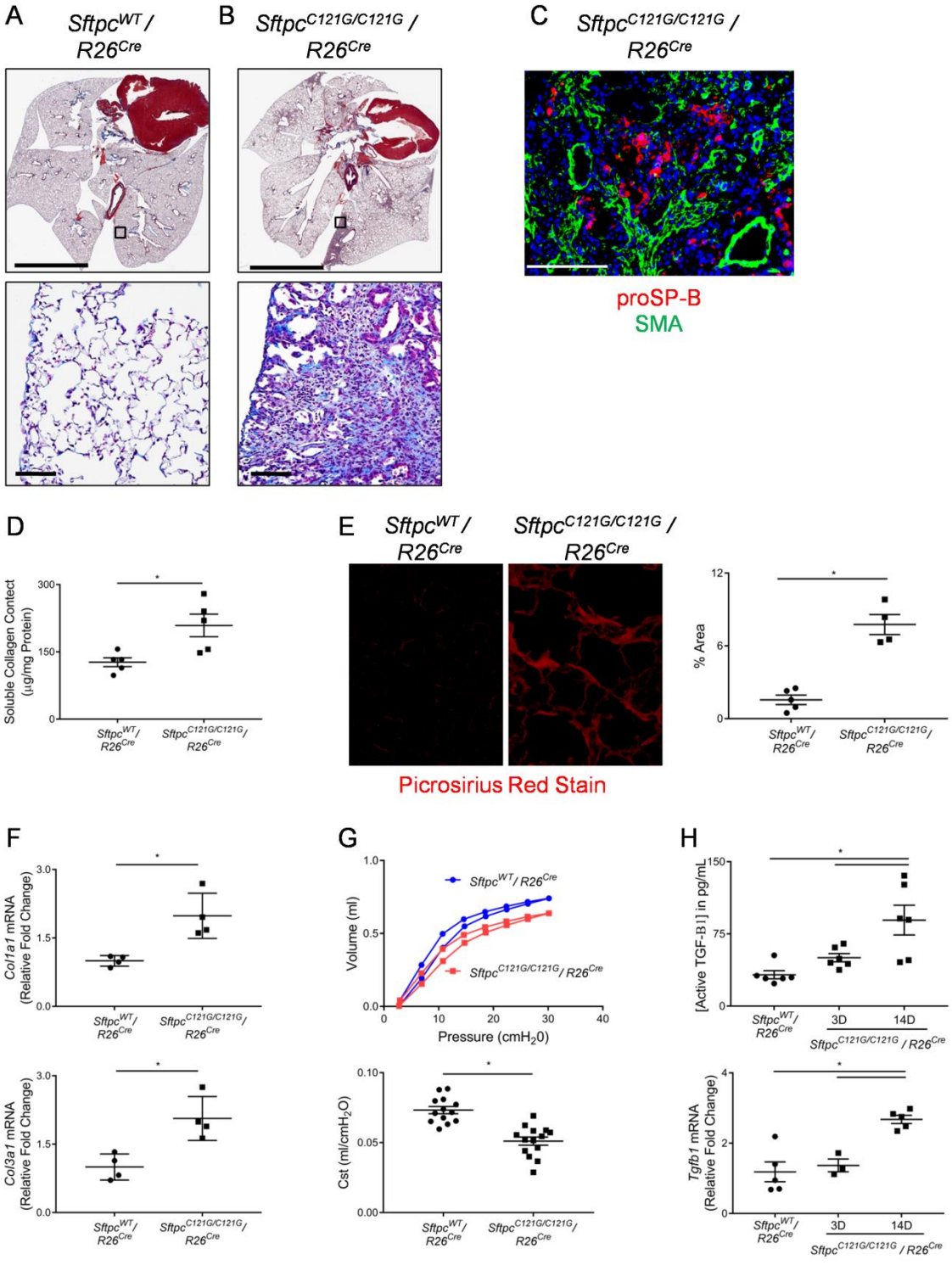

Figure 8. Mice expressing the *Sftpc^{C121G}* mutation develop spontaneous fibrotic lung remodeling.
Representative trichrome staining of *Sftpc^{WT}/ R26^{Cre}* **(A)** and *Sftpc$^{C121G/C121G}$ / R26^{Cre}* **(B)** lung section 28 days post-tamoxifen showing patchy areas of fibrotic remodeling in *Sftpc$^{C121G/C121G}$ / R26^{Cre}* mice. (Bar in 0.5X magnification upper panels = 5mm. Bar in 10X magnification lower panels = 100µM) **(C)** Double label immunohistochemistry of *Sftpc$^{C121G/C121G}$ / R26^{Cre}* lungs showing proSP-B+ AT2 cells (red) with adjacent smooth muscle actin (SMA) (green) myofibroblasts (20X magnification, bar = 100µM) **(D)** Soluble collagen in right lung homogenates measured by Sircol

assay. Shown are dot-plots with mean and SEM. *p < 0.05 vs control using unpaired two tailed t-test. **(E)** (Left) Representative picrosirius red stained 20x magnification fields. (Right) Quantification performed using Image J expressed as picrosirius staining as percentage of section area with dot-plots and mean and SEM shown. *p < 0.05 vs control using unpaired two tailed t-test. **(F)** Whole lung expression of *Col1a1* (top) and *Col3a1* (bottom) mRNA assayed by qRT-PCR and expressed as fold change from controls. Dot-plots and mean and SEM shown. *p < 0.05 vs control using unpaired two tailed t-test. **(G)** Pooled flow volume loops (top) (n=10) and calculated static compliance (Cst) (bottom) from pulmonary function testing at 28 days post-tamoxifen. *p < 0.05 vs control using unpaired two tailed t-test. **(H)** (Top) Active TGF-β1 levels in BALF at indicated times post-tamoxifen were measured using Luminex; shown are dot-plots with mean and SEM. *p < 0.05 vs control using unpaired two tailed t-test. (Bottom) qRT-PCR for *Tgfb1* mRNA expression in AT2 cells isolated at 3 and 14 days post-tamoxifen. Data expressed as fold change from control group are presented as dot-plots with mean and SEM shown. * p< 0.05 versus controls by One Way ANOVA with post-hoc Tukey test.