SUPPLEMENTAL METHODS and DATA

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

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SUPPLEMENTARY METHODS

SFTPC Expression Plasmids

For in vitro proSP-C trafficking studies, HEK293 cells (ATCC; CRL-3249) were grown to 85-90% confluency and transiently transfected with a family of *SFTPC* expression vectors encoding for either EGFP-fused wild-type (EGFP/SP-C^{WT}) or mutant (EGFP/SP-$C^{C121Y}$and EGFP/SP-C^{C121G}) isoforms using Lipofectamine 2000 (Thermo Fisher Scientific) as previously described (1-3). For co-transfection studies, DsRed/ABCA3 expression vector was incorporate into the transfection protocol as previously described (4).

Efficiency of Neomycin Cassette Excision

The efficiency of removal of the PGK-neo from *Sftpc* alleles of *$Sftpc^{C121G/C121G}$ / $R26^{Cre}$* mice was measured by q-PCR on DNA isolated from AT2 cells obtained 7 days after tamoxifen administration. Primers were obtained (Applied Biosystems) based on previously described protocol to amplify a 62 base-pair DNA sequence within the Neo-Cassette:

Neo Fwd: 5'-CGACAAGACCGGCTTCCAT-3'

Neo Rev: 5'-CGACCACCAAGCGAAACAT-3'

Relative quantification of intact *$sftpc^{C121Gneo}$* alleles was performed using the protocol previously described (5) with SYBR Green Master Mix (Applied Biosystems). Rpp30 served as a housekeeping control:

Rpp30 Fwd: 5'-TCCAGTGTGCAAGAAAGCTAAATG-3'

Rpp30 Rev: 5'-GGCAGTGCGTGGAGACTCA-3

Standard curves and melting point curves were constructed to validate assay specificity and efficiency. Copy number of the PGK-neo cassette relative to Rpp30 was calculated using the delta-Ct method.

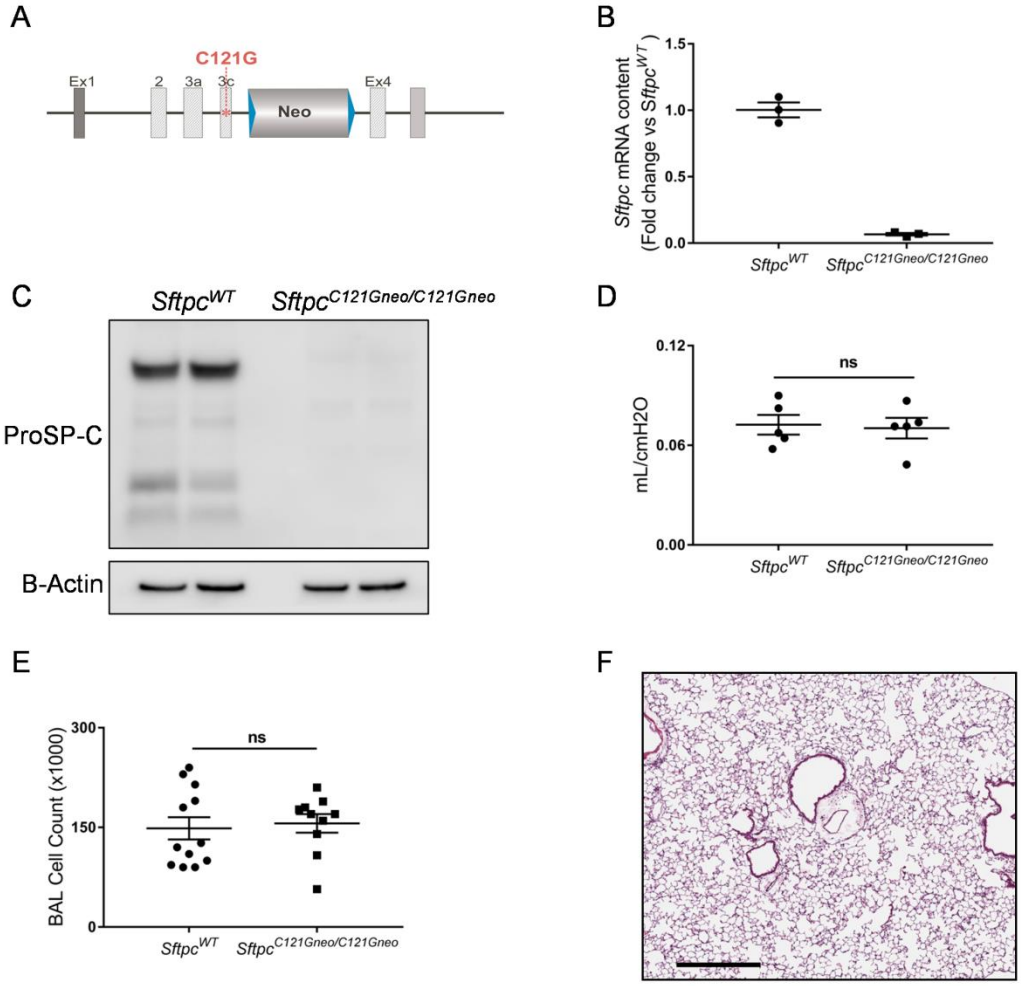
Measurement of Pulmonary Function and Pulse Oxymetery

Invasive measurement of static lung compliance was performed with mice anesthetized with intraperitoneal pentobarbital. The mouse tracheas were cannulated with a 20-gauge metal stub adapter and then placed on a small-animal ventilator (flexiVent; SCIREQ, Inc. Toronto Canada) at 150 breaths per min and a tidal volume of 10 mL/kg of body weight. Static lung compliance was determined with the manufacturer's software using a 2 second breath pause maneuver. Arterial hemoglobin oxygen saturations were measured in anesthetized, spontaneously ventilating mice using a rodent pulse oximeter (PhysioSuite with MouseSTAT Pulse Oximeter Kent Scientific, Torrington, CT) per the manufacturer's instructions.

REFERENCES FOR SUPPLEMENTAL METHODS

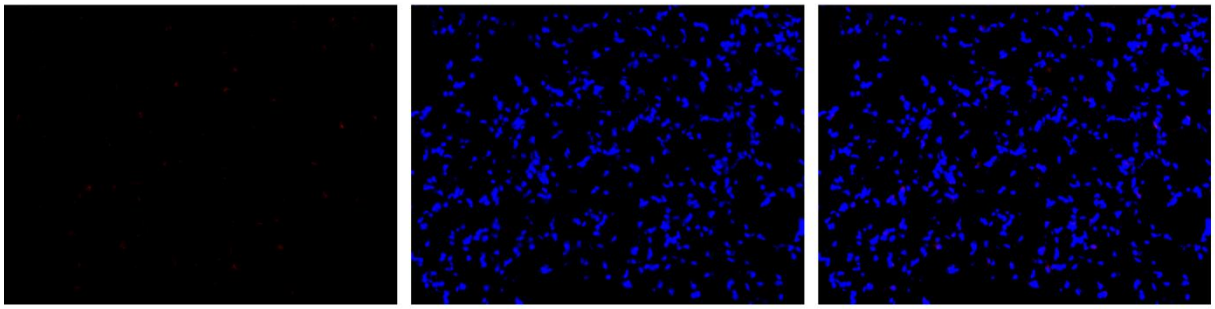
1. Wang WJ, Mulugeta S, Russo SJ, Beers MF. Deletion of exon 4 from human surfactant protein C results in aggresome formation and generation of a dominant negative. *J Cell Sci.* 2003;116(4):683-692.
2. Maguire JA, Mulugeta S, Beers MF. Multiple ways to die: Delineation of the unfolded protein response and apoptosis induced by Surfactant Protein C BRICHOS mutants. *Int J Biochem Cell Biol.* 2012;44(1):101-112.
3. Maguire JA, Mulugeta S, Beers MF. Endoplasmic Reticulum Stress Induced by Surfactant Protein C BRICHOS Mutants Promotes Proinflammatory Signaling by Epithelial Cells. *Am J Respir Cell Mol Biol.* 2011;44(3):404-414.
4. Beers MF, et al. A novel conserved targeting motif found in ABCA transporters mediates trafficking to early post-Golgi compartments. *J Lipid Res.* 2011;52(8):1471-1482.
5. Mancini C, Messana E, Turco E, Brussino A, Brusco A. Gene-targeted embryonic stem cells: real-time PCR assay for estimation of the number of neomycin selection cassettes. *Biological Procedures Online.* 2011;13(1):10.

SUPPLEMENTAL FIGURES



Supplemental Figure 1. The hypomorphic *Sftpc*$^{C121Gneo/C121Gneo}$ founder line does not produce mutant proSP-C protein due to a retained intronic PGK-neo cassette and lacks a spontaneous lung phenotype.
(A) Schematic diagram of the knocked-in *Sftpc*^{C121Gneo} allele showing cysteine-to-glycine substitution at codon 121 (C121G) at the 3' end of exon 3c and the PGK-Neomycin cassette in Intron 4 flanked by locus of X-over P1 (lox-P) sites (blue arrows). **(B)** qRT-PCR analysis for *Sftpc* expression in the purified AT2 cells from homozygous *Sftpc*$^{C121Gneo/C121Gneo}$ and *Sftpc*^{WT} mice. Data normalized to 18S RNA are expressed as fold change in *Sftpc* mRNA from control mice. **(C)** Western blot analysis of the AT2 cell lysate from *Sftpc*^{WT} and *Sftpc*$^{C121Gneo/C121Gneo}$ mice showing an absence of proSP-C in the founder line. **(D)** *Sftpc*$^{C121Gneo/C121Gneo}$ and *Sftpc*^{WT} mice were subjected to pulmonary function testing at 52 weeks with calculated static compliance demonstrating no difference between genotypes by students t-test. **(E)** Dot-plot of BAL cell counts in *Sftpc*$^{C121Gneo/C121Gneo}$ and *Sftpc*^{WT} mice at combined time points of 16, 32, and 52 weeks show no difference in total cell counts between genotypes by students t-test. **(F)** Representative 10x magnification photomicrograph of H&E stained section from a 52 week *Sftpc*$^{C121Gneo/C121Gneo}$ mouse. Bar = 500 μM.

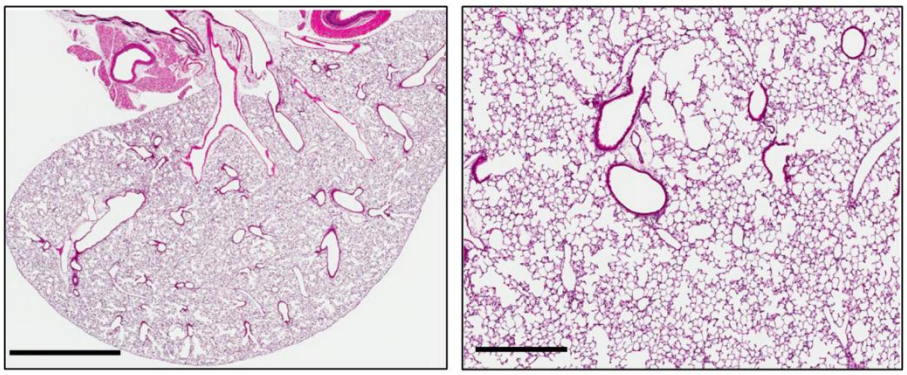
A



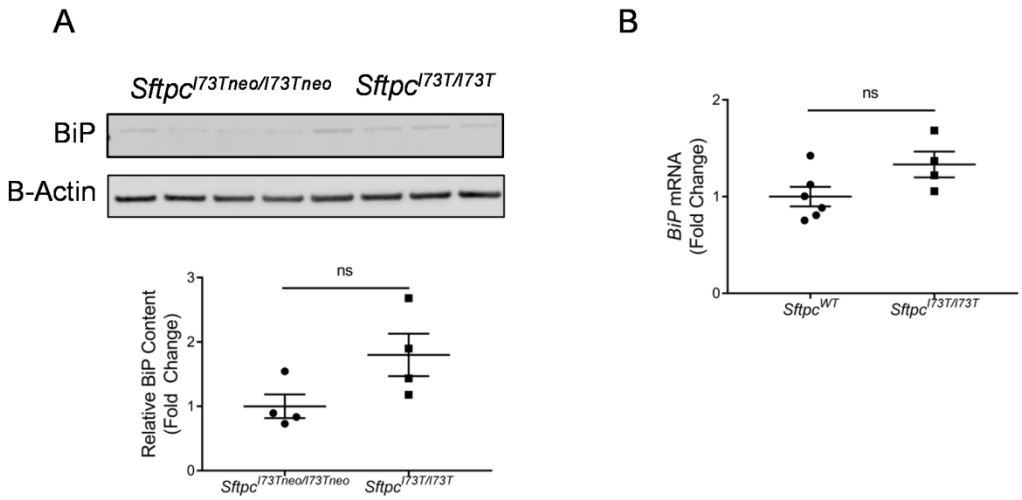
B

	BALF protein (ug/mL)	BALF Cell count (x1000)	Percents of BALF cells: Macrophage / Monocyte	Neutrophil	Lymphocyte	Eosinophil
#1	321	162	96.6	0.6	1.7	1.1
#2	335	99	97.3	1.3	0.7	1.3
#3	246	102	92.9	3.6	2.6	2.6
Mean	**300.7**	**121**	**95.6**	**1.8**	**1.6**	**1.7**

C

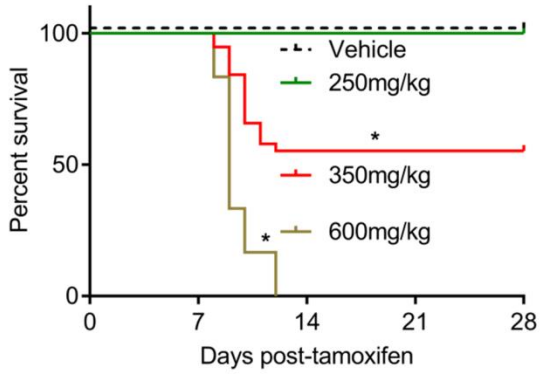


Supplemental Figure 2. *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice have no spontaneous phenotype.
(A) 20x immunoflorescent microscopy of vehicle (oil) intraperitoneal (IP) injected *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} for proSP-C and Dapi showing absence of proSP-C protein in AT2 cells. Representative of 5 x 20x fields per mouse in 3 individual mice. **(B)** Cell counts and differentials from 3 *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice following IP vehicle showing normal BALF cell numbers and differentials. **(C)** Representative low powered (left) and high powered (right) photomicrograph of H&E stained section from *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} showing normal alveolar architecture following IP vehicle (Left 5x magnification bar= 2 mM; right 10x magnification bar= 500 μM).

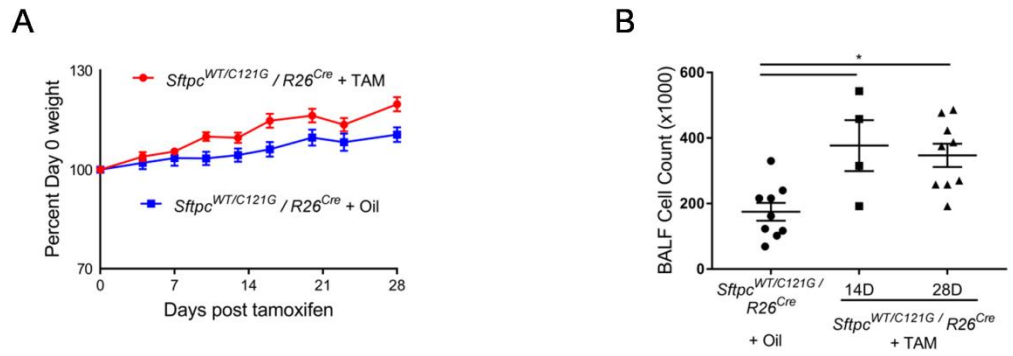


Supplemental Figure 3. Expression of the *Sftpc^{I73T}* mutation *in vivo* does not generate substantial ER stress

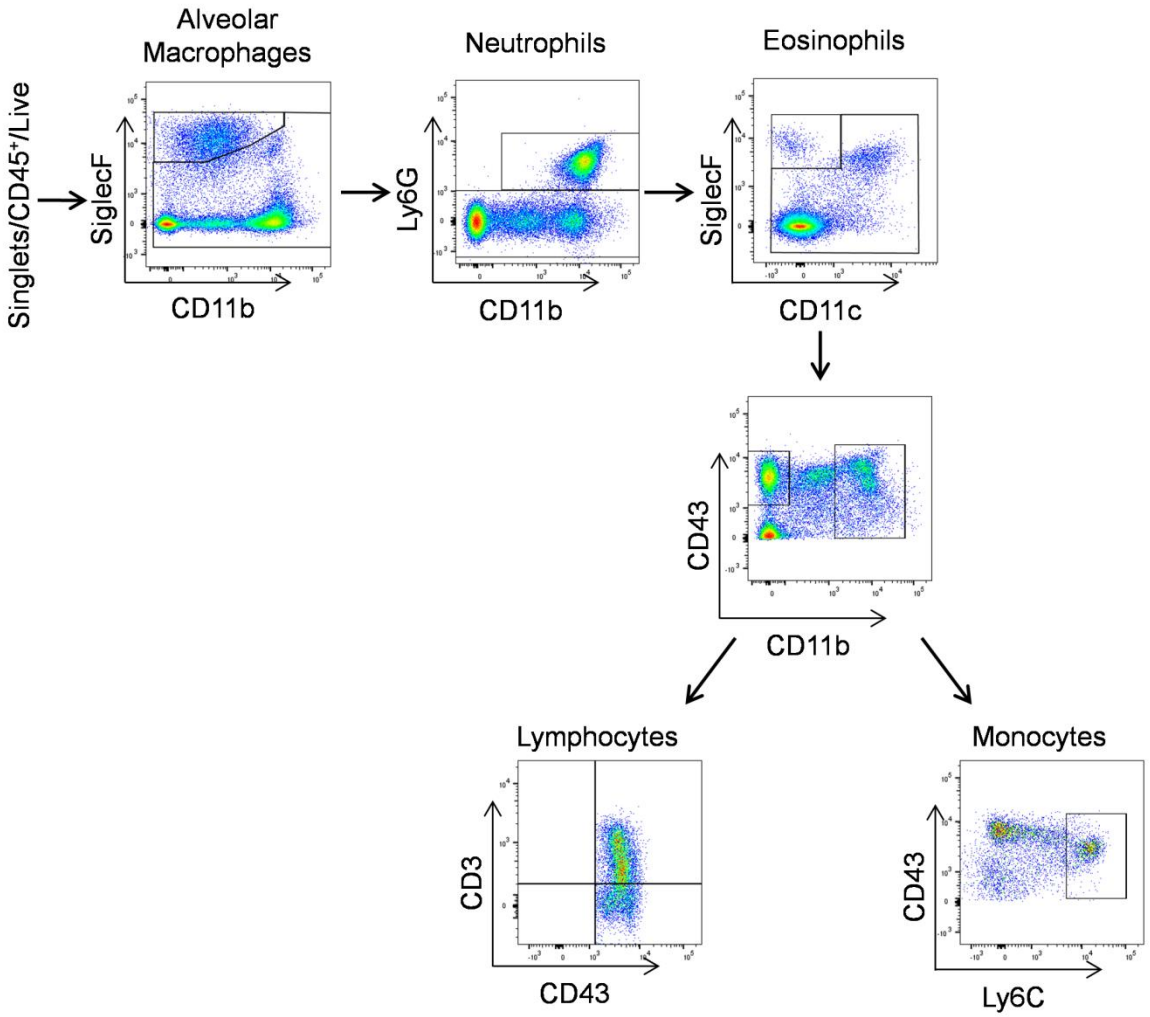
(A) Western blot analysis (upper) of AT2 cell lysate isolated 2 weeks post-tamoxifen from *Sftpc$^{I73Tneo/I73Tneo}$* control mice and homozygous *Sftpc^{I73T}* expressing mice demonstrate no significant difference in relative BiP content (lower) by densitomrry. Non-significant vs control using unpaired two tailed t-test. **(B)** Dot-plots with mean and SEM of *Bip* mRNA expression measured by qRT-PCR in AT2 cells from *Sftpc^{I73T}* expressing *Sftpc$^{I73T/I73T}$/ R26^{Flpo}* mice and *Sftpc^{WT} / R26^{Flpo}* controls 14 days following tamoxifen. Non-significant vs control using unpaired two tailed t-test.



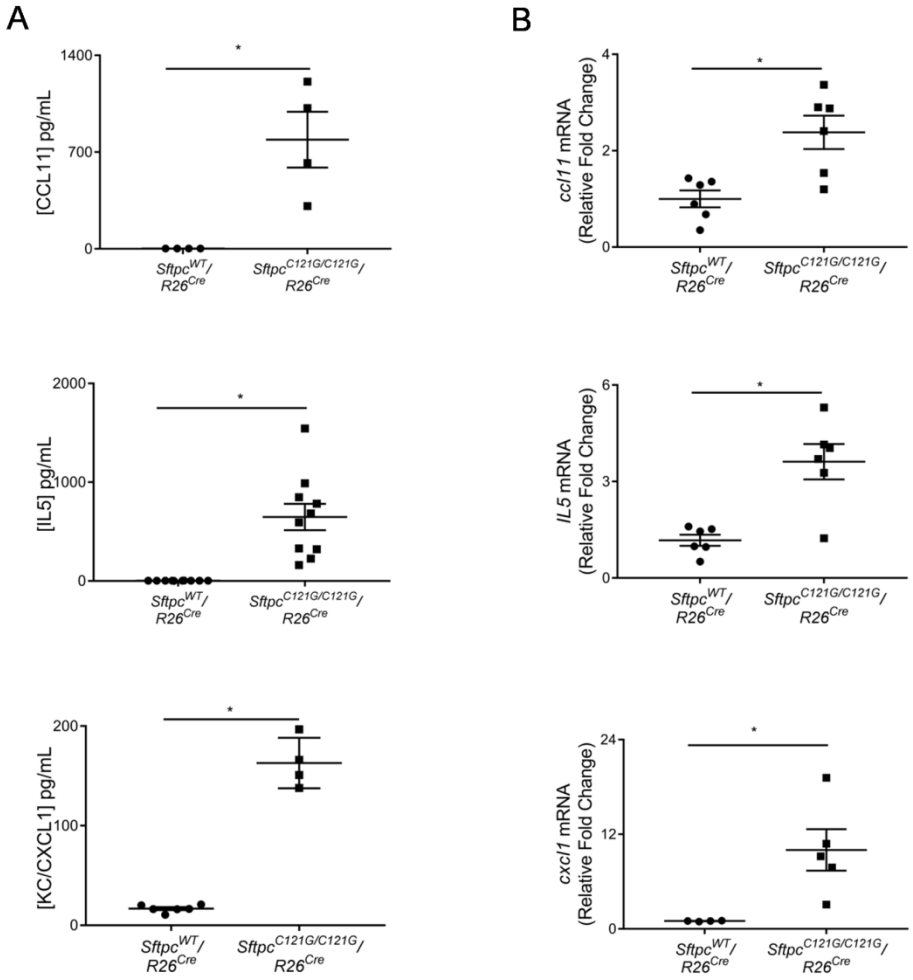
Supplemental Figure 4. Survival of *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice as a function of tamoxifen dose. Kaplan–Meier analysis for survival of *Sftpc*$^{C121G/C121G}$ / *R26*^{Cre} mice treated with vehicle (oil) or tamoxifen at the indicated doses. Endpoints were defined as death or body weight < 75% on 2 consecutive days. $p < 0.001$ vs vehicle control by Log-rank (Mantel-Cox) test.



Supplemental Figure 5. Tamoxifen treatment of heterozygous *Sftpc$^{WT/C121G}$ / R26^{Cre}* induced an inflammatory phenotype.
(A) Body weight measured in *Sftpc$^{WT/C121G}$ / R26^{Cre}* animals treated with tamoxifen (600mg/kg) or Oil (control). **(B)** BALF cell count determined in *Sftpc$^{WT/C121G}$ / R26^{Cre}* mice at 14 and 28 days after treatment with tamoxifen (600mg/kg) or Oil (controls). * p< 0.05 versus controls by One Way ANOVA followed by post-hoc Tukey test.



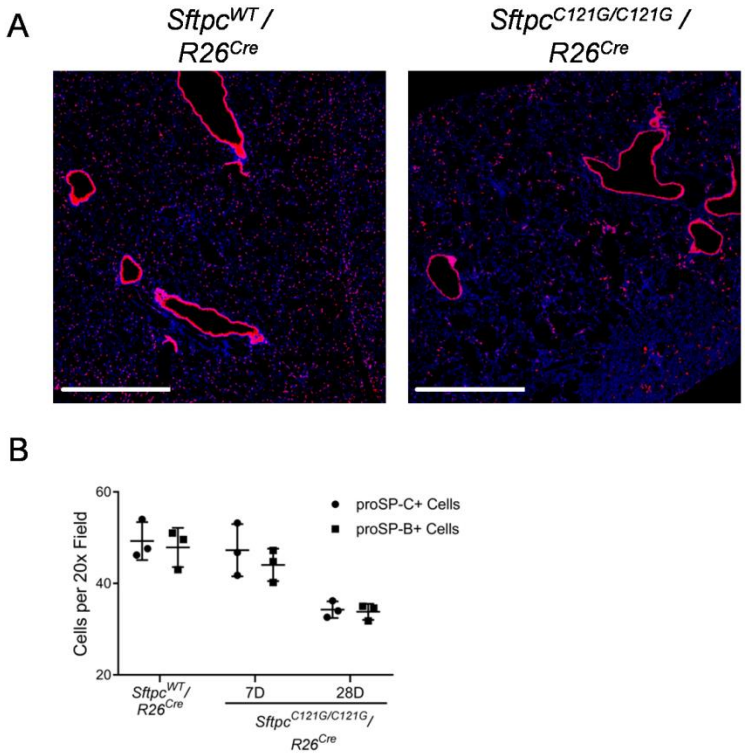
Supplemental Figure 6. Flow cytometric gating strategy for analysis of whole lung digest. Representative flow cytometric analysis for identification of immune cell populations in whole lung digest at 3 days post-tamoxifen. Immune cells were identified by singlet identification of $CD45^+$ viable cells followed by gating strategy as above with antibodies listed in **Supplemental Table 6.**



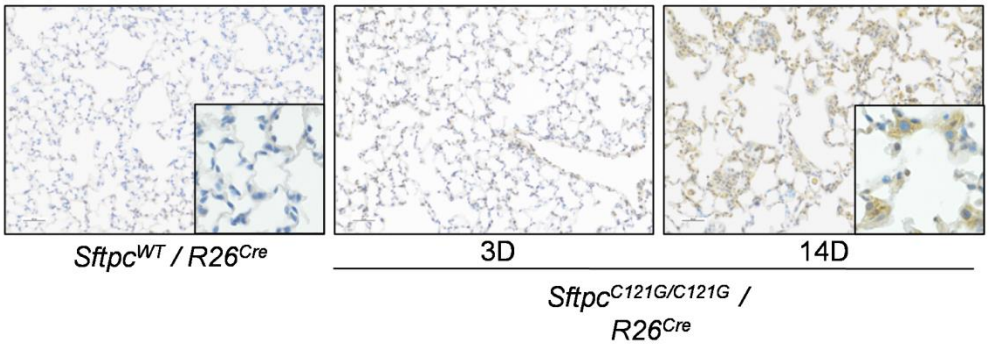
Supplemental Figure 7. *Sftpc^{C121G}* AT2 cells are the source of multiple granulocyte recruitment cytokines.

(A) Dot-plots with mean and SEM of BALF content of CCL11 (upper), IL5 (middle), and CXCL1 (KC/IL8) (lower) protein in *Sftpc$^{C121G/C121G}$ / R26^{Cre}* mice and *Sftpc^{WT} / R26^{Cre}* controls 7 days following tamoxifen determined by Luminex assay. * $p < 0.05$ versus controls by One Way ANOVA followed by post-hoc Tukey test (see **Supplemental Table 2** for complete time course). **(B)** Dot-plots with mean and SEM of *Ccl11* (upper), *Il5* (middle), and *Cxcl1* (lower) mRNA expression measured by qRT-PCR in samples of AT2 cells from *Sftpc$^{C121G/C121G}$ / R26^{Cre}* mice and controls 7 days following tamoxifen. *$p < 0.05$ vs control using unpaired two tailed t-test.

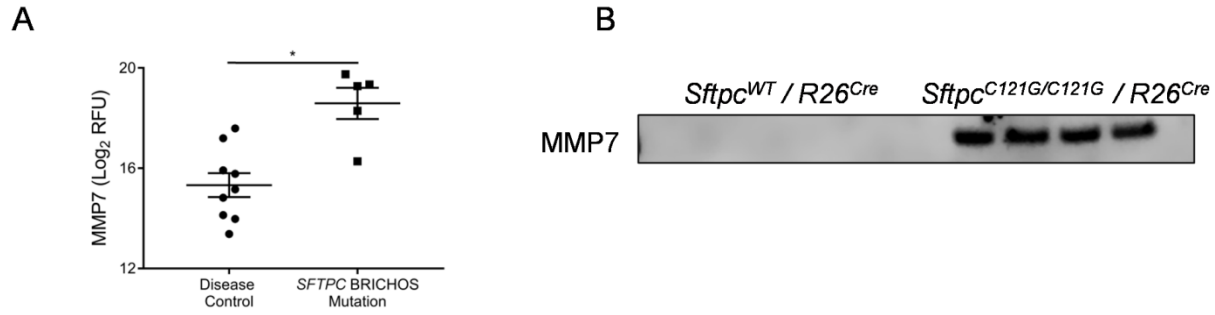
Supplemental Figure 8



Suppmental Figure 8. For weeks post-tamoxifen *Sftpc^{C121G}* expressing mice have decreased AT2 cell density with heterogenous distrubution.
(A) Representative 2X immunoflorescent microscopy stained for proSP-C (red) and Dapi (blue) showing decreased AT2 cell density in the *Sftpc$^{C121G/C121G}$/ R26^{Cre}* lung section compared to control. **(B)** ProSP-B and proSP-C positive cells were quantified in *Sftpc^{WT} / R26^{Cre}* and *Sftpc$^{C121G/C121G}$/ R26^{Cre} mice* at 7 and 28 days after tamoxifen by manually counting 20X immunoflorescent stained micrograph. Data is shown as individual dot-plots of mean and SD.



Supplemental Figure 9. The *Sftpc*^{C121G} AT2 cell is a source of TGF- β1.
Representative immunohistochemistry for TGF-β1 protein in paraffin imbedded lung sections from *Sftpc*^{WT} / *R26*^{Cre} and *Sftpc*$^{C121G/C121G}$/ *R26*^{Cre} at 3 and 14 days after tamoxifen shows increased TGF-β1 staining in (insert) *Sftpc*$^{C121G/C121G}$/ *R26*^{Cre} AT2 cells at 14 days.



Supplemental Figure 10. MMP7 is elevated in the BALF of *SFTPC* BRICHOS pediatric cohort and *Sftpc*^{C121G} mice during transition to fibrotic remodeling.
(A) Individual Dot-plots of mean and SEM log2 RFUs for MMP7 from disease control (n=9) and *SFTPC* BRICHOS mutation patients (n=5). * p< 0.05 *SFTPC* BRICHOS mutations cases and disease control by unpaired two tailed t-test. **(B)** Western blotting of BALF (15µl/lane) for MMP-7 protein in *Sftpc*^{WT}/ *R26*^{Cre} controls and *Sftpc*$^{C121G/C121G}$/ *R26*^{Cre} mice 14 days after tamoxifen.

Supplemental Table 1. Ontonagy of BALF effector cell populations following *Sftpc^{C121G}* expression.

Effector Cell (Total Recovered)	*Sftpc^{WT}/ R26^{Cre}* (n=15)	*Sftpc$^{C121G/C121G}$ / R26^{Cre}*			
		3 Days (n=5)	7 Days (n=13)	14 Days (n=9)	28 Days (n=5)
Macrophage/ Monocytes	120.8 ± 11.8	281.0 ± 31.7	300.9 ± 23.3*	522.5 ± 60.7*	480.0 ± 115.1*
Neutrophils	2.6 ± 0.7	12.5 ± 6.1	137.4 ± 25.1*	368.3 ± 83.2*	38.1 ± 16.2
Eosinophils	0.7 ± 0.2	0.8 ± 0.8	90.9 ± 10.4	366.5 ± 81.6*	45.4 ± 9.0
Lymphocytes	2.0 ± 0.2	5.7 ± 1.4	11.6 ± 1.3	44.0 ± 11.1*	52.5 ± 7.3*

BALF isolated from control (*Sftpc^{WT}/ R26^{Cre}*) and *Sftpc$^{C121G/C121G}$/ R26^{Cre}* mice 3, 7, 14, and 28 days tamoxifen treatment were analyzed for total cell numbers by Z1 Coulter Counter. Individual cell populations were determined by manual differential counts of Giemsa stained cytospins (>200 cells/slide). Data are expressed as cell number for each population (mean ± SEM) *p< 0.05 versus controls by One Way ANOVA followed by post-hoc Tukey test.

Supplemental Table 2. Time course for BALF cytokines induced by *Sftpc^{C121G}* expression.

	CCL2	CCL7	CCL17	GM-CSF	IL-1β	IL-4	IL-5	IL-6	IL-13	KC/ CXCL1
Sftpc^{WT}/ R26^{Cre}	2.8 ± .4	24.6± 8.9	34.1 ± 11.5	3.2 ± 0	6.1 ± 0.4	0.9 ± 0.2	2.7 ± 0.4	3.4 ± 0.4	9.5 ± 3.1	16.7 ± 1.5
Sftpc$^{C121G/C121G}$ / R26^{Cre}										
3 Days	4.0 ± 1.0	40.9± 10.8	211.8 ± 43.1	5.9 ± 1.3	5.3 ± 0.3	1.1 ±0.2	14.5 ± 4.8	171 ± 22.11	9.6 ± 3.0	51.1 ± 9.9
7 Days	315.4 ± 98.3 *	133.8± 18.1*	390.3 ± 103.6*	13.14 ± 2.7*	6.8 ± 0.4	2.3 ±0.3	828.6 ±165.2*	5824± 1349*	8.2 ± 1.6	162.9 ±12.6*
14 Days	182.6 ± 77.3*	---	518 ± 109.9*	14.7 ± 3.8*	6.2 ± 0.7	5.0± 2.28	508.4 ± 156.4	3421 ±1170	7.3 ± 1.4	204.7 ±61.9*
28 Days	3.97 ± 0.8	---	214.2 ± 114.3	3.2 ± 0	4.5 ± 0.5	0.9 ± 0.3	6.8 ± 1.3	22.24 ± 13.7	6.5 ± 0.9	34.1 ± 6.8

BALF isolated from control (*Sftpc^{WT}/ R26^{Cre}*) and *Sftpc$^{C121G/C121G}$ / R26^{Cre}* mice 3, 7, 14, and 28 days tamoxifen treatment were analyzed by multiplex assay (ELISA for CCL7 performed at 3 and 7 days only) for expression of cytokines shown above as described in Method. Mean ± SEM, n = 4-12 samples per group in pg/mL. * $p < 0.05$ versus controls by One Way ANOVA followed by post-hoc Tukey test.

Supplemental Table 3. Demographics and BALF cell counts in pediatric *SFTPC* BRICHOS cohort and disease controls.

	Disease Controls (n = 9)	*SFTPC* BRICHOS Mutation (n = 5)	p-value
Age at Diagnosis (years)	1.8 (0.5, 6.8)	2.0 (0.2, 10.8)	0.79
WBC, cells/ml x 10^3	178 (59, 358)	389 (141, 950)	0.05
% Neutrophils	2 (0, 18)	10 (7, 13)	0.25
% Lymphocytes	9 (0, 35)	3 (0, 20)	0.50
% Macrophage/Monocyte	82 (58, 97)	87 (53, 89)	0.99
% Eosinophils	0 (0, 0)	1 (0, 15)	0.02
% Epithelium	4 (1, 11)	0 (0, 7)	0.06

Median (and range) for age at ChILD diganosis, BALF cell count per mL, and percent of total WBCs by effector cell type for disease controls and *SFTPC* BRICHOS mutation patients. P-value reflects two-sample t-test assuming unequal variances between groups.

Supplemental Table 4. Antibodies.

Western Blot				
Antibody	**Clonality**	**Dilution**	**Catalog Number**	**Manufacturer**
EGFP	Monoclonal	1:2000	632381	Living Color
ProSP-C	Polyclonal	1:3000	22871	In house
β-Actin	Monoclonal	1:10000	A1978	Sigma Aldrich
BiP	Monoclonal	1:1000	3177	Cell Signaling
Mature SP-C	Polyclonal	1:2500	WRAB-76694	Seven Hills Bioreagents
SP-B	Polyclonal	1:500	PT3	In house
Phos-JNK	Monoclonal	1:1000	9255	Cell Signaling
Total-JNK	Monoclonal	1:1000	9258	Cell Signaling
ATF4	Monoclonal	1:1000	11815	Cell Signaling
CHOP	Monoclonal	1:500	5554	Cell Signaling
ATF6	Monoclonal	1:500	70B1413.1	Novus
MMP-7	Monoclonal	1:1000	3801	Cell Signaling
Histochemistry/Immunofluorescnce				
ProSP-C	Polyclonal	1:200	22871	In house
ProSP-C	Polyclonal	1:50	SC-7750	Santa Cruz
ProSP-B	Polyclonal	1:200	PT-3	In house
K-del	Monocloncal	1:200	ADI-SPA-827	Enzo
Cleaved Caspase 3	Polyclonal	1:10	CP-229A	Biocare Maedical
TGF-β1	Polycloncal	1:250	BS-0086R	Bioss Antibodies
Smooth Muscle Actin	Monoclonal	1:500	A2547	Sigma

Supplemental Table 5. qPCR Primers.

Taqman Primers	
18S	Mm03928990_g1
BiP	Mm00517691_m1
Ccl2 (MCP-1)	Mm00441242_m1
Ccl7 (MCP-3)	Mm00443113_m1
Ccl17 (TARC)	Mm01244826_g1
Cxcl1(GroA/KC)	Mm04207460_m1
Cxcl11(eotaxin)	Mm0041238_m1
Col1a1	Mm00801666_g1
Col3a1	Mm01254476_m1
Il5	Mm00439646_m1
Il6	Mm00446190_m1
Sftpc	Mm00488144_m1
tgfb1 (TGF–β1)	Mm01178820_m1
XBP1 Spliced	Mm03464496_m1
XBP1 Unspliced	Mm03464497_s1

Supplemental Table 6. Flow Cytometric Analysis Antibodies.

Antibody	Fluorochrome	Clone	Catalog Number	Manufacturer
CD16/32	-	93	14-0161-85	eBioscience
CD45	PerCP	30-F11	103130	Biolegend
SiglecF	PE-CF594	E50-2440	562757	BD Biosciences
CD11b	BV421	M1/70	101235	Biolegend
Ly6G	AF700	1A8	127622	Biolegend
CD11c	B711	HL3	563048	BD Biosciences
CD43	PE	S11	143205	Biolegend
CD3	BUV395	17A2	740268	Biolegend
Ly6C	BV510	HK1.4	128033	Biolegend
Viability Dye	eFluo780	NA	65-0865-14	eBioscience