Supplementary materials:

MATERIALS AND METHODS

Cell culture

A549, H441 and CCD-8Lu cells were maintained in continuous culture in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, USA) supplemented with 10% foetal bovine serum (FBS) (Lonza, Belgium), 1% L-glutamine (Lonza) and 1% non-essential amino acids (NEAA) (Lonza) in a humidified incubator at 37^0C, 5% CO_2, and 95% air. Human primary alveolar epithelial cells (pAEpiC) were purchased from ScienceCell Research Laboratories, USA and cultured on poly-L-lysine (Sigma-Aldrich, MO, USA) coated culture flasks in alveolar epithelial cell media (AEpiCM) supplemented with epithelial cell growth supplements (EpiCGS) (ScienceCell Research Laboratory, USA) following the manufacturer protocol. Human primary small airway epithelial cells (SAEC) were purchased from Lonza, USA and cultured in SAGM Bullet Kit (Lonza, USA) according to manufacturer's instruction. SAECs were harvested and passaged using Subculture Reagent Pack (Lonza, USA). Passage 3 to 4 SAECs were used for all experiments.

Determination of doubling time of A549 and H441 cells

The doubling time of A549 and H441 cells in serum-free and 10% FBS supplemented DMEM was calculated via a cell counting method with the following formula:

PD = t x Log2/(LogC2-LogC1)

[PD = Population doubling, t = 24 hours, Log = 10 based Log, C1 = 1^{st} cell count, C2 = 2^{nd} cell count].

For doubling time calculation in 10% FBS supplemented DMEM, 10^5 A549 or H441 cells were seeded in T25 tissue culture flasks in 10% FBS supplemented DMEM (complete media) and cultured for 5 days with media changed on alternate days. Cells were harvested by

trypsinisation and counted by haemocytometer every 24 hours from Day 1 to Day 5. For calculation of doubling time in serum-free DMEM, 10^5 A549 or H441 cells were seeded in T25 culture flasks in complete media and grown for 24 hours, washed with PBS twice before the addition of serum-free DMEM into each flask, and allowed to grow for further 4 days. Cells were harvested and quantified as above every 24 hours from Day 2 to day 5. Due to poor recovery after seeding into serum-free DMEM, cells were initially recovered in complete media. Population doubling (PD) time was calculated using the above formula from Day 1 to Day 5 in 10% FBS supplemented DMEM and from Day 2 to Day 5 for serum-free DMEM. Mean PD of each time points was the determined population doubling time for each cell-type in each culture condition. (n=3) (fig. E2).

***In vitro* epithelial cell wound repair assay**

The *in vitro* wound repair assay was performed with some modification as described elsewhere [26]. Briefly, A549 and H441 cells were cultured in 24-well plates (Nunclon) in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% NEAA (complete growth media) to confluence as monolayers under standard culture conditions. Linear wounds were made on cell monolayers with a 200 µl plastic pipette tip followed by wash once with pre-heated PBS (Phosphate Buffered Saline) and once with pre-heated serum-free DMEM (SF-DMEM) to remove cell debris. Then samples were replenished with either SF-DMEM, 10% FBS supplemented DMEM, serum-free conditioned media (SF-CM) for 24 hours. Wound images were recorded with a digital camera (Canon, Japan) attached to an inverted light microscope (Nikon Eclipse, TS100, Japan) at 0 and after 24 hours of wounding. Circumferential wound gaps were measured by Image J software (NIH, USA) and percentage of wound repair after 24 hours was calculated.

For preparation of SF-CM, A549 and H441 cells were grown separately to confluent monolayers on 24-well plates. To prepare SF-CM from unwounded A549 and H441 cells, cells were first washed repeatedly with PBS before a final addition of 1ml media per well per unwounded monolayer and cultured for 24 hours in the incubator at 37^0C in presence of 5% CO_2 and 95% of air. To prepare SF-CM from wounded A549 and H441 cells, monolayers were wounded with a 200µl plastic pipette tip followed by two PBS washes and subsequent replenishment with SF-DMEM and cultured for 24 hours as described above. SF-CM were collected and stored at -80^0C. Prior to use, all SF-CM were sterile filtered using 0.2 µm Cellulose Acetate syringe filter.

Direct and indirect-contact co-culture wound repair assays

For direct-contact co-culture wound repair experiment, DiO-labeled (Vybrant™ Multicolor Cell Labeling Kit, Invitrogen, USA) 2 x 10^5 H441 cells or 10^5 CCD-8Lu cells were grown on inverted 24-well plate-format 3 µm pore transwell PET (polyethylene terephthalate) membranes (BD Bioscience, NJ, USA) in 100µL complete growth media for 4 hours in a humidified incubator at 37^0C allowing them to adhere with the PET transwell membrane. After 4 hours, the transwells were reverted and placed back into the companion well plate (BD Bioscience, NJ, USA) and then DiI-labeled (Vybrant™ Multicolor Cell Labeling Kit, Invitrogen, USA) 10^5 A549 or 10^5 SAEC cells were seeded onto the upper surface of the transwell PET membrane and sufficient complete growth media (10% FBS supplemented DMEM for A549-H441 direct contact and complete SAGM media for SAEC-H441 direct contact) added (300µl media on upper chamber and 700µl in the lower chamber of the transwell). For indirect-contact co-culture, 10^5 A549 cells were grown on the upper surface of 0.4 µm porous PET membrane of 24-well plate format transwells (BD Bioscience, NJ, USA) and 3 x 10^5 H441 cells or 2 x 10^5 CCD-8Lu cells on the bottom surface of the companion

well plate in complete growth media. Cells were grown in normal culture condition for 24 hours to achieve confluent layers. Linear wounds were made on A549 cell or SAEC monolayers using 200 µl plastic pipette tip followed by washing (once with PBS and once with serum-free media) to remove cell debris. H441 or CCD-8Lu cells were also washed to remove all serum. Wounded A549 cells or SAECs along with their accompanying H441 cells or CCD-8Lu cells were then cultured in SF-DMEM for A549-H441 or A549-CCD 8Lu co-culture or small airway basal media (SABM, Lonza, USA) for SAEC-H441 co-culture for a further 24 hours. Prior to imaging the direct-contact co-culture H441 cells or CCD-8Lu cells were removed carefully from the undersurface of the PET membrane with a cotton swab and washed with warm PBS. The cells of the upper surface of PET membrane were then fixed with 4% paraformaldehyde (4% PFA) for 30 minutes. The PET membranes were cut from transwell housing and mounted on glass slides and fixed with cover slips for microscopy. Images of direct-contact co-culture were acquired with a laser scanning confocal microscope (Olympus Fluoview, Japan) at both 0 and 24 hours of wounding. Images of indirect-contact co-culture wounds were taken by an inverted light microscope as described above. Wound gap measurement and wound repair analysis were performed as previously. For negative and positive control A549 or SAEC monolayers were wounded and cultured in SF-DMEM or SF-SABM and complete 10% FBS supplemented DMEM or complete SAGM, respectively. Control cells were grown on the respective PET membranes specific to the direct or indirect contact co-culture model used.

For assessment of H441 cell migration in A549-H441 or SAEC-H441 cell direct-contact co-culture DiO-labelled H441 cells and DiI-labelled A549 cells or SAECs were counted at the respective wound sites at 0 and after 24 hours of wounding. Confocal Z-scanning was performed to confirm the migration.

Assessment of apoptosis in alveolar A549 cells or SAECs

Ligand-induced apoptosis in wounded A549 cell monolayers was assessed by treating the cells with recombinant human soluble TRAIL or FasL (Peprotech, NJ, USA) for 24 hours in SF-DMEM. The apoptosis was blocked through pre-incubation of A549 cells with TRAIL-R1 (HS101, 10 μg/ml) and/or TRAIL-R2 (HS201, 10 μg/ml); and Fas (SM1/23, 10 μg/ml) receptor blocking antagonistic monoclonal antibodies (mAbs) (Enzo Life Science, Switzerland). For induction of apoptosis in SAEC with soluble TRAIL, SAEC monolayers were formed over 24 hours in complete SAGM growth media. After 24 hours cells were treated with different concentration of soluble TRAIL in SF-SABM for 24 hours (fig. E8). Apoptotic induction was evaluated with the TUNEL method. To assess H441 or CCD-8Lu cell direct contact induced apoptosis in A549 cells or SAEC, DiI-labelled A549 cells or SAECs were cultured in direct-contact with un-labelled H441 or CCD-8Lu cells for 24 hours in complete growth media as described above; linear wounds were made on A549 cell and SAEC confluent monolayers as described above and cultured for an additional 24 hours in serum free basal media (SF-DMEM for A549-H441 or A549-CCD-8Lu and SF-SABM for SAEC-H441 co-culture). To block apoptosis, samples were treated with of Fas (SM1/23, 10μg/ml) or TRAIL-R1/R2 (HS101, HS201; 10 μg/ml) receptor blocking antagonistic mAbs throughout the entire co-culture wound repair period. Apoptosis was assessed after 24 hours by the TUNEL method using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science, USA) according to the manufacturer's protocol. For positive control, apoptosis was induced in A549 or SAECs by 200 μM H_2O_2 treatment in SF-DMEM/SABM respectively for 24 hours. For negative control cells were cultured in serum free basal media (SF-DMEM/SABM). All control cells were cultured on identical cultures surfaces that were used for respective experimentation.

RT-PCR analysis

RT-PCR was performed to determine the mRNA expression of TRAIL, TRAIL-R1 and TRAIL-R2 in A549 and H441 cells. Total RNA was extracted using QIA RNeasy Mini Spin Column (Qiagen, Germany) according to the manufacturer protocol. Quality and quantity of yield RNA were determined by measurement of absorption at 260 and 280 nm using NanoDrop ND-1000 spectrophotometer (NanoDrop, USA). The ratio of optical density at 260 and 280nm was >1.8 in all cases. One-step RT-PCR was performed using SuperScript® III One-Step RT-PCR System with Platinum® *Taq* High Fidelity kit (Invitrogen, CA, USA) and amplified in DNA Engine® Thermal Cycler (MJ Research, MA, USA) in a single tube according to the manufacturer protocol. In short, 20 ng RNA of each sample was mixed with 6.25 μL 2X Reaction Mix, 3 μL RNAse-free water, 1 μL of each primer (10 μM) and 0.25 μL of Platinum® *Taq* polymerase enzyme. The thermal cycling protocol comprised an initial reverse transcription at 50^0C for 30 minutes, pre-denaturation at 94^0C for 2 minutes followed by 30 cycles of denaturation at 94^0C for 15 seconds, annealing at 53-55^0C (depending on primers) for 30 seconds and extension at 68^0C for 1 minute followed by a final extension at 64^0C for 5 minutes. The PCR fragments were separated by electrophoresis on 2% agarose gel (Fisher Scientific, NJ, USA) and visualized by staining with ethidium bromide (Sigma, MO, USA) under UV light. Primers for β-actin were used as a loading control. All primers were obtained from Invitrogen, USA (Table E1).

Fluorescent immunocytochemistry

Expression of *pro*SP-C in A549 cells was determined by immunostaining with anti-*pro*SP-C primary antibody (polyclonal, ab40879, dilution 1:250; Abcam, Cambridge, UK) and visualised by secondary antibody anti-rabbit IgG-NL493 (dilution 1:200, NorthernLights, R

& D System, MN, USA) according to manufacturer protocol. TRAIL, TRAIL-R1 and TRAIL-R2 expression were detected in unwounded A549 and H441 cells using anti-TRAIL primary antibody (polyclonal, ab2435, dilution 1:100; Abcam) and TRAIL-R1 (monoclonal, HS101, 10μg/ml) and TRAIL-R2 (monoclonal, HS201, 10μg/ml) (both Enzo Life Science, Switzerland) according to the manufacturer protocol. Anti-rabbit IgG-NL493 secondary antibody (1:200) against TRAIL antibody (ab2435) and anti-mouse IgG-NL557 (1:200) against TRAIL-R1 and –R2 antibodies were used (NorthernLights, R & D System). TRAIL evaluation was also conducted on unwounded SAEC. All primary antibody treatment incubation was for 24 hours at 4^0C and secondary treatment was for 2 hours at room temperature. DAPI was used for nuclear counterstaining (Invitrogen, USA). Images were acquired by a fluorescent microscope (Olympus Fluoview, Japan or Nikon Eclipse Ti-ST, Japan).

Immunohistochemistry on lung tissue sample

Formalin-fixed, paraffin embedded lung tissue samples were cut to 3 μm thick sections. Sections were deparaffinised and pre-treated for heat mediated antigen retrieval in citrate buffer (pH 6). Samples were stained on a Dako autostainer using Dako REAL detection kit (Dako, Denmark) according to the manufacturer protocol. Type II AEC and Clara cells were differentiated by dual immunostaining of samples with *pro*SP-C antibody (polyclonal, ab40879, dilution 1:1500; Abcam) and CC10 antibody (polyclonal, ab40873, dilution 1:200; Abcam). For dual immunostaining with TRAIL and CC10 antibodies samples were first treated with TRAIL primary antibody (polyclonal, ab2435, dilution 1:80; Abcam) and visualised by DAB followed by second antibody treatment by CC10 antibody and visualised by VIP (Very Intense Purple, Lab Vision, UK) in dual immunostaing and by DAB in mono-staining. For dual-immunohistochemistry staining an avidin biotin block was performed after

completion of the first antibody labelling to prevent cross reaction with the detection kit. The Negative controls included omission of the primary antibody. Sections were counterstained using haematoxylin Z (CellPath, UK).

Sections were examined for the expression of above- mentioned markers at the sites of interest in target IPF and control lung tissues and independently scored by two pathologists and their results compared. At least 300 epithelial cells were counted in successive high power fields on each whole section; and the number of cells positively expressing CC10, TRAIL and CC10/TRAIL was determined and then expressed as a percentage of the total number of cells counted per section. Where there was minor disagreement between the 2 observers, the number of CC10 / TRAIL / and/or CC10/TRAIL positive cells were averaged; if the disagreement was >15%, the cells were counted again.

TUNEL assay on A549 cells, SAEC and lung tissue samples

TUNEL (terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end-labelling) was performed using *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science, USA) according to the manufacturer's protocol. Briefly, cells were fixed with 4% paraformaldehyde (Fisher Scientific, UK) for 30 minutes followed by permeabilisation of samples with 0.1% Triton-X100 (Sigma, MO, USA) for 5 minutes. Cells were then treated with TUNEL reagent for 1 hour at 37^0C in the incubator. For positive control apoptosis was induced in A549 cells and SAECs with 200µM H_2O_2 for 24 hours. Samples were then examined under laser scanning confocal microscope (Olympus Fluoview, Japan). For combined TUNEL assay and immunohistochemistry with *pro*SP-C antibody on lung tissue, samples were treated with *pro*SP-C primary antibody as described above. For visualisation, samples were treated with anti-rabbit IgG-NL557 secondary antibody (R & D systems).

Afterwards, TUNEL was performed as described before. Samples were examined under fluorescence microscope and TUNEL positive nuclei were counted. Quantification of TUNEL positive AECs in each tissue sample was performed as described above.

Table E1

Genes	Forward primers	Reverse primers	Annealing temp (^{0}C)	Amplicon size (bp)
TNFSF10 (TRAIL)	CTGCAGTCTCTCTGTGTGG	TCTTTCTAACGAGCTGACG	55	196
TNFRSF10A (TRAIL-R1)	CAAAGAATCAGGCAATGG	GTGAGCATTGTCCTCAGC	55	196
TNFRSF10B (TRAIL-R2)	CACCAGGTGTGATTCAGG	CCCACTGTGCTTTGTACC	53	220
ACTB (β-Actin)	GCCACGGCTGCTTCCAGC	AGGGTGTAACGCAACTAAGTC	55	477

Table E1 Primer sequences

Official name is listed in gene column with alternative name in parentheses. Forward and reverse primers are listed in 5' to 3' orientation.

FIGURE LEGENDS

FIGURE E1. Cell morphology and *pro*SP-C expression in human alveolar A549 cells. a) Inverted light microscopic image of A549 cells at 100x magnification showing a homogeneous monolayer. b) Inverted light microscopic image of human Clara cell line H441 at 100x showing a homogeneous monolayer. c) Inverted light microscopic image of human primary SAEC at 100x magnification. d) Expression of *pro*SP-C in alveolar A549 cells, e) merged with DAPI; magnification 200x. Scale bars = 100 µm.

FIGURE E2: A549 and H441 cell growth and population doubling time. a) A549 and H441 cell growth in 10% FBS serum supplemented complete DMEM culture media. b) A549 and H441 cell growth in serum-free DMEM. c) Population doubling time (PD) of A549 and H441 cell lines in serum-free and 10% FBS supplemented DMEM (n=3). ***$p<0.001$. Results are presented as mean±SD.

FIGURE E3: H441 cell wound repair after 24 hours with SF-CM obtained from wounded and un-wounded A549 cell monolayers (n=3, 3 replicates per n). **$p<0.01$. Result is presented as mean±SD.

FIGURE E4. H441 cell migration towards A549 cell layers in un-wounded A549-H441 direct contact co-culture system. a) Number of migrated H441 cells at A549 cell layer at 24 hours (equivalent duration of 0 hour wound) and 48 hours (equivalent duration of 24 hours wound) of A549-H441 direct contact co-culture (n=3). b, c) Laser scanning confocal microscopic images show migrated DiO-labelled H441 cells (Green) at DiI-labelled A549

cell layer (Red) at 24 (b) and 48 (c) hours of A549-H441 direct contact co-culture. Scale bar = 200 µm.

FIGURE E5. H441 cell migration in absence of A549 cells in transwell system. a) Laser scanning confocal microscopic image of DiI-labelled H441 cells after 24 hours on the undersurface of transwell 3 µm porous PET membrane. b) Laser scanning confocal microscopic image shows very few migrated DiI-labelled H441 cells on the opposite surface of transwell PET membrane after 24 hours of culture.. Scale bar = 200 µm.

FIGURE E6. A549-CCD 8Lu cell direct and indirect-contact co-culture wound repair. a) A549 cell wound repair after 24 hours in direct and indirect-contact co-culture with CCD 8Lu cells. Positive and negative control represent the A549 cell wound repair with 10% FBS supplemented and serum-free media respectively in monoculture (n=3). b) Laser scanning confocal microscopic images (100X) of A549-CCD 8Lu direct-contact co-culture at 0 and 24 hours. DiI labelled red cells are A549 cells and DiO labelled green cells are CCD-8Lu cells. Vertical side bars represent Z-slicing through the juxta-wound monolayers and horizontal bars represent Z-slicing through wound gaps. A negligible number of CCD-8Lu cells migrated to the A549 wound site after 24 hours of wounding. c) TUNEL assay on A549-CCD 8Lu direct-contact co-culture wound repair system. TUNEL positive cells were not observed in A549 cell population in the A549-CCD 8Lu direct contact co-culture wound repair system and A549 monoculture (negative control). In positive control, apoptosis was induced on A549 cell monoculture with 200 µM H_2O_2 . A549 cells were labelled with DiI and green signal indicate TUNEL positive apoptotic nuclei. *$p<0.05$, **$p<0.01$, ns=not significant. Results are presented as mean± SD. Scale bar = 150 µm (b), 200 µm (c).

FIGURE E7. Assessment of apoptosis in migrated H441 cells in A549-H441 direct contact co-culture wound repair. a) Apoptosis in migrated H441 cells at juxta-wound monolayers after 24 hours of direct contact co-culture (n=3). Negative control: apoptosis in A549 wounded monolayers cultured in SF-DMEM and positive control: apoptosis in A549 wounded monolayers cultured in 200 µM H_2O_2 for 24 hours. b) Confocal laser Z-scanning shows juxta-position of cluster of TUNEL-positive un-labelled A549 cells (Green) and TUNEL-negative migrated DiI-labelled H441 cells (Red). c) Laser scanning confocal microscopic image show TUNEL positive un-labelled A549 cells in negative control. d) Red cells are DiI-labelled migrated H441 cells and green nuclei are TUNEL positive nuclei in A549-H441 direct contact co-culture wound repair system after 24 hours. e) Positive control. Scale bar = 100 µm.

FIGURE E8. Induction of apoptosis in SAEC with soluble TRAIL. a) Dose response of apoptosis induction in SAEC with soluble TRAIL. 1.6 µg/ml of soluble TRAIL induced 16.8% apoptosis after 24 hours of treatment (n=3; **p<0.01, ***p<0.001 vs 0 ng/ml of TRAIL). b) Negative control: SAECs were treated with serum-free basal media. c) SAECs were treated with 1.6µg/ml of soluble TRAIL for 24 hours. d) Positive control: SAEC cells were treated with 200 µM H_2O_2 for 24 hours. Green signals are TUNEL positive nuclei. Scale bar = 150 µm.

FIGURE E9. Immunocytochemistry for TRAIL in SAEC.

FIGURE E10. SAEC apoptosis in SAEC-H441 direct contact co-culture wound repair model. Laser scanning confocal Z-slicing demonstrates that TUNEL positive nuclei were confined within the DiI-labelled cells and hence confirms the apoptotic cells are SAECs.