Macrophage-derived IL-6 trans-signaling as a novel target in the pathogenesis of bronchopulmonary dysplasia

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Runnning title: Targeting IL-6 trans-signaling in BPD

Summary: M1-like macrophage activation is linked to IL-6/STAT3 axis in clinical and experimental BPD. Inhibition of macrophage-related IL-6 trans-signaling promotes ATII survival and lung growth in experimental BPD as a new therapy for preterm infants.

Online Data Supplement

Material and Methods

Animal studies

All animal studies were approved by the local government authorities (LANUV, NRW, Germany; 87-51.04.2010.A372; 84-02.04.2015.A120; 84-02.04.2016.A119). Adult and neonatal male and female C57BL/6N and C57BL/6J (WT) and B6.129S2*Il6*tm1Kopf/J (*Il6*$^{-/-}$) mice were housed in humidity- and temperature-controlled rooms exposed to a 12 h dark/light cycle and were allowed food and water ad libitum.

Neonatal hyperoxia-induced lung injury model

Newborn mice were pooled and randomized to dams at the day of birth (born within 12 h of each other). Half of the litters were exposed to 85 % or 40 % O_2 (hyperoxia, HYX) for 3, 7, and 28 days, whereas the other pups were at room air [21% O_2; normoxia, NOX] as described previously (1). Every 24 h the nursing dams were rotated between hyperoxia and normoxia litters to avoid O_2-related toxic effects on the dams. Exposure to hyperoxia was performed in a 90 × 42 × 38 cm plexiglas chamber. Oxygen concentrations were monitored with a Miniox II monitor (Catalyst Research, Owing Mills, MD).

Treatment with sgp130Fc and IL-6 mAb

To determine if inhibition of IL-6 signaling protects from neonatal hyperoxia-induced lung injury and promotes alveolarization, newborn wildtype (C57Bl/6N) mice were treated with intraperitoneal injection of either sgp130Fc (~10µg/g bw), IL-6 mAb (~10µg/g bw) or vehicle (PBS) at postnatal day 3 (P3), P7, P14, and P21. At P28, mice were sacrificed. The dose was based on previous studies (2).

Mouse model of hyperoxic lung injury for RNA Seq

All animal procedures were approved by the Institutional Animal Care and Use Committee (IUCAC) at Stanford University. As part of a separate study, mice contain loxP sites flanking critical exons of the Ikk2 (IKKβ) gene, described previously (3), were crossed to transgenic $Pdgfb^{WT}$ and $Pdgfb^{CreERT2}$ mice (4), which express a tamoxifen inducible form of Cre-recombinase under the control of the *Pdgfb* promoter, to generate $Ikk\beta^{WT}$ and $Ikk^{\Delta Endo}$ mice. For these studies, within 24 h of birth, male and female $Ikk\beta^{WT}$ pups were exposed to 80 % O_2 (hyperoxia) or 21 % O_2 (normoxia) for two weeks (P14), after receiving 300 µg of 4-OHT suspended in corn oil via daily intragastric injection from P1-P3.

RNA-Seq

Lungs were obtained from $Ikk\beta^{WT}$ pups maintained in normoxia or hyperoxia for 14 days. The pulmonary circulation was perfused with saline, and total RNA extracted using RNeasy Mini kit (Qiagen, Germantown, MD). RNA-sequencing was performed by Quick Biology (Pasadena, CA). Briefly, RNA integrity was confirmed by Agilent Bioanalyzer 2100, and libraries were prepared according to KAPA Stranded mRNA-Seq poly(A) selected kit (KAPA Biosystems, Wilmington, MA). Final library quality and quantity was analyzed, and 150 bp paired end reads sequenced on Illumina HighSeq 4000 (Illumina Inc., San Diego, CA). Reads were mapped to the latest UCSC transcript set using Bowtie2 version 2.1.0 (5), and the gene expression was estimated using RSEM v1.2.15 (6). TMM (trimmed mean of M-values) was used to normalize the gene expression. Differentially expressed genes were identified using edgeR (7). Genes showing altered expression with $p< 0.05$ and more than 1.5-fold change were considered differentially expressed.

Pulmonary function test

To assess airway resistance at P28, we used direct plethysmography for mice (FinePointe™RC; Buxco, Wellington, NC, USA) as previously described (8-10). First, mice were deeply

anesthetized by intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight), followed by tracheotomy and ventilation. Respiratory system compliance (Cdyn) was measured at baseline.

Tissue preparation

Mice were sacrificed as previously described (11). Lungs were excised; the right lung was immediately snap-frozen for subsequent molecular assessment; the left lung was pressure-fixed in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) as described below.

Tissue assays

Protein extraction and immunoblots: After isolation of lungs, lungs were snap frozen and stored at -80 °C for protein extraction; lungs were homogenized with Chaps buffer along with 1x Halt™ protease and phosphatase inhibitor cocktail (Cat. No. 78442, Rockford, USA) using a plastic pistil. Afterwards, sonication (Bandelin, SONOPULS HD 2070, Germany) was performed for separation of tissue cells. Samples were placed on ice for 1 h, followed by centrifugation and collection of the supernatant. The protein concentration was measured using BCA Protein assay (Pierce, Thermo Fisher Scientific, #23225, Rockford, USA). For SDS-PAGE, 20-40 µg of protein was incubated with sample loading buffer at 70 °C for 10 min, and loaded on a 10 % acrylamide gel. The gel was then transferred onto a 0.45 µm nitrocellulose membrane (Amersham™ Protran® 0.45 NC nitrocellulose, GE Helathcare, #10600002, MA, USA) using semi-dry electro-blotter (Avantor, #700-1220, Vienna, Austria); blocking of the membrane was performed with 5 % milk and 2 % bovine serum albumin (BSA) in TBS-Tween (0.1 % Tween®20, Sigma Aldrich, #P1379, Germany). The blot was then incubated with the following primary antibodies at 4 °C overnight: rat anti-interleukin 6 (IL-6; R&D System, #MAB406, Minneapolis, USA, 1:2000), rabbit anti-phosphorylated signal transducer and activator of transcription 3 (pSTAT3; Cell Signaling, #9145, Danvers, USA, 1:1000), rabbit anti-signal transducer and activator of transcription 3 (STAT3; Cell Signaling, #9139, Danvers,

USA, 1:1000), rabbit anti-suppressor of cytokine signaling 3 (SOCS3; Abcam, Cat.No.ab16030, UK, 1:1000), rabbit anti-pro-surfactant protein C (SFPTC; Merck, Cat.No.AB3786, Germany, 1:2000), rabbit anti-Aquaporin 5 (AQP5; Sigma-Aldrich,Cat. No. A4979, USA, 1:2000), rabbit anti-Krüppel-like factor 4 (Klf4; Cell Signaling, #4038, Danvers, USA, 1:1000), mouse anti-β-actin (Cell Signaling, #3700, Danvers, USA, 1:10,000). Next, horseradish-peroxidase (HRP)-linked secondary antibody (anti-mouse IgG, HRP-linked antibody #7076 or anti-rabbit IgG, HRP-linked antibody #7074, Cell Signaling, Danvers, MA, USA) was applied at RT for 1 h. The membrane was then incubated with ECL™ (GE Healthcare, #RPN2232, UK) for 1 min. Protein bands were visualized by UV imager (Bio-Rad Universal Hood II Gel Doc, Cambridge, MA, USA). Densitometry analysis was performed to quantify protein amounts using Image Lab software 5.2.1 (BioRad, Germany).

RNA extraction and qRT-PCR: Lung tissue, mouse lung epithelial cells (MLE12), mouse ascites macrophages (J774A.1), primary murine macrophages (peritoneal), and human macrophages were used for RNA extraction. Samples were treated with TRI Reagent® (Sigma-Aldrich, cat. No. T9424, Germany) for cell lysis. The mRNA was precipitated by using isopropanol, washed with ethanol, and finally dissolved in RNAse free water. The mRNA concentration was measured by using Nano Quant (Tecan Infinite® 200 PRO, Switzerland), and 1 µg of mRNA was used for cDNA synthesis. To this end, mRNA was first treated with RQ1 DNase 10x Reaction Buffer (Promega, #M198A, Madison, USA) and RQ1 RNase-Free DNase (Promega, #M610A, Madison, USA) to remove genomic DNA. DNase activity was stopped by adding DNase Stop Solution (Promega, #M199A, Madison, USA) and heating samples at 65 °C for 5 min. Random and oligo primers were added to the samples and heated at 70 °C for 15 min. Finally, M-MLV Reverse Transcriptase 5x reaction buffer (Promega, #M531A, USA), M-MLV Reverse Transcriptase (Promega, #M1701, Madison, USA), recombinant Ribonuclease Inhibitor (Promega, #N2511, Madison, USA), and recombinant dNTP Mix (Thermo Fisher Sientific, #R1092, Lithuania) were mixed with the sample and

incubated at 37 °C for 1 h for reverse transcription. Quantitative RT-PCR (qRT-PCR) was performed in 96 well plates (FrameStar, 96 well plate #4ti-0770/C, UK) with DNA-DYE (GoTaq® qPCR Master Mix, #A600A, Madison, USA or Platinum™ Quantitative PCR SuperMix-UDG w/ROX, #11743500, Netherlands) using a 7500 Real-Time PCR System (Applied Bioscience) as previously described (12). The Primers were designed with Primer Express Software and are listed in supplemental table 1. Gene expression was calculated based on the ΔΔCt-method, and expressed as fold induction of mRNA expression. The housekeeping gene β-actin was used to normalize genes of interest.

Gel electroporation of PCR products

1.5 % agarose (Biozym, # 850471, Austria) gel was prepared with 8 µl DNA staining solution (Midori Green Advance, #MG04, Nippon Genetics, Japan). Final product of the qRT-PCR after 40 cycle of expansion for different genes (*Sftpc*, *Aqp5* and β-actin) from the hAEC, A549 and hSMA were loaded on the gel. Electrophoresis was performed at 120 V for 1 h. Gel was then transfer to UV imager (Biometra, # 032-001,302,303, Germany) and Image was taken to determine the DNA products.

Quantitative lung histomorphometric analysis

Lungs were inflated by pressure fixation at a constant pressure of 20 cm H_2O using fixative agent [4 % (mass/volume) of PFA] for 15 min. For PFA fixation, lungs were left in fixative for 30 min at RT and then stored in fixative agent at 4°C overnight. Next day, lungs were embedded in paraffin using automatic tissue embedder. For Isotropic Uniform Random (IUR) orientation, paraffin-embedded lungs were cut at random angles in 2 perpendicular planes using a random number chart to select the angles. The paraffin blocks were re-mounted on blocks for sectioning. A random number from 1 to 100 defined how many 3 µm slices from the edge of the lung tissue were discarded to determine the start of the first series. Subsequently, series of thirty 3 µm sections were generated every 200 µm, and the lung sections were mounted on poly-l-lysine-coated glass slides. Randomly, four tissue sections were selected and

deparaffinized using NeoClear rank (NeoClear, #1098435000, Merck, Darmstadt, Germany), and rehydrated in a gradual series of ethanol (100 %, 96 %, and 70 %, and finally PBS or destilled water). Next, tissue sections were stained with hematoxylin for 6 min, washed with distilled water and rinsed in regular tap water for 6 min. Afterwards, tissue was exposed to eosin for 5 min, followed by tissue dehydration in gradual series of ethanol (70 %, 80 %, 96 %, and 100 %, and Neo Clear), and finally tissue mounting using Neo-Mount® (Merck, # 109016, Germany). Images of lung sections were taken using a slide scanner (Leica SCN400 Slide Scanner, Houston, USA); up to ten fields of view per lung section (20x) were used for further analyses. We studied four randomly selected slides per animal and a total of five to six animals per group. Images were not taken in account when airways and vessels covered more than 15 % of the total area; atelectatic or not well inflated lungs were avoided.

Radial alveolar count: The radial alveolar count (RAC) method was described by Emery and Mithal, and used to count the number of alveoli across a terminal bronchus (13). Briefly, a line was drawn from terminal bronchiole to the nearest interlobular septum, and the number of distal air spaces the line crossed was counted.

Alveolar surface area: ImageJ-win64 (NIH, USA) was used to measure the alveolar surface area (ASA). Up to ten fields of view per lung section were selected for ASA; in each field of view up to ten random alveoli were selected and the alveolar diameter was measured. The alveolar diameter was used to calculate the surface area of an alveolus [4 *(pi) * r^2].

Alveolar septal thickness: As described previously (11), up to ten random alveoli per field of view were selected. The distance from the inner to the outer surface of the alveolar septum was measured to determine the septal thickness.

Mean Liner Intercept: Cell D 3.4 Olympus soft image solutions (Olympus, Hamburg, Germany) was used to determine the mean linear intercept (MLI). As described previously (1, 11), a grid of lines was used and each intercept of the lines and alveolar walls was counted and the total number of intercepts per field was divided through the total length of lines.

Elastic fibre quantification: Lung sections were stained for elastic fibers (purple) using Resorcin Fuchsin from Weigert (Weigert's Iron Resorcin and Fuchsin Solution; Carl Roth, X877.3) and counterstained yellow with Tartrazine [0.5 % in 0.25 % acetic acid (Dianova, cat. no. TZQ999, USA)]. The color thresholds were set to differentiate between elastic fibers and lung tissue. Elastic fiber density as an index of parenchymal elastin content was analyzed in up to ten fields of view per tissue section and four random tissue sections per animal (five to seven animals per group). Elastic fiber density of alveoli was measured using Cell D 3.4 Olympus Soft Imaging Solutions (Olympus; CellSens, Germany). First, the positive-stained area was defined manually as elastic fibers; next, an automatic quantification of the total elastic fiber-positive area was performed and related to the total area of tissue on the slide. The amount of elastic fibers relative to the total tissue served as a surrogate parameter for elastic fiber density. Areas where lung tissue was not well inflated or airway and/or blood vessel covered more than 15 % of the tissue area were excluded from the analysis. All images for calculation were taken at 40x magnification using a bright field microscope (Olympus, Hamburg, Germany).

Immunostaining

Tissue sections were deparaffinized and rehydrated as mentioned previously, then treated with MaxBlock™ reagent A (MaxVision Biosciences, #MB-L,Washington, USA) for 5 min at RT, and washed with 96 % ethanol and PBS. Antigen retrieval was performed by boiling with 10 mM citrate buffer (pH 6; Dako, #S2369, Germany) at 90 - 120 °C for 25 min. Afterwards, tissue sections were incubated with blocking solution (Sea Block, Thermo Scientific™, #37527, Netherlands) at RT for 1 h. The incubation with the primary antibody mouse anti-αSMA-CY3 (Sigma-Aldrich, #C6198, USA, 1:200) was performed in the dark at 4 °C overnight. Sections were then washed with PBS and treated with DAPI dye (Sigma-Aldrich, #D9542, Germany, 1:5000) to counterstain the nucleus. Lung tissue sections were incubated with Post-Detection Conditioner Reagent B (MaxVision Biosciences, #MB-L, Washington, USA) for 5 min at RT to enhance the fluorescent signal, and then mounted in fluoromount

aqueous (Sigma Aldrich, #F4680, USA). Images of lung sections were taken using a fluorescence microscope (Olympus, Hamburg, Germany) at 40x and 100x magnification. These images were used to count αSMA^+ cells in four fields of view in two random sections per animal (four animals per group).

TUNEL and SFTPC staining

Tissue section and Precision-Cut Lung Slices (PCLS) were treated with MaxBlock™ reagent A (MaxVision Biosciences, #MB-L,Washington, USA) for 5 min at RT and washed with 60 % ethanol and PBS. Antigen retrieval was performed by treating the sections with proteinase K solution (Thermo Scientific™, #EO0491, Lithuania) for 15 min at 37°C. The tissue sections were then incubated with blocking solution (Sea Block, Thermo Scientific™, #37527, Netherlands). Subsequently, the lung sections were exposed to terminal uridine deoxynucleotidyl transferase dUTP terminal nick end labeling (TUNEL) solution according to the manufacturer's instructions (*In Situ* Cell Death Detection Kit, Roche, #11684795910, Germany) for 1 h at 37 °C. Next, the sections were washed with PBS-T and then incubated with rabbit anti-pro-SFTPC antibody (Merck, #AB3786, Germany, 1:200) in dark at 4 °C overnight. The following day, the sections were washed with PBS-T and treated with secondary antibody conjugated with CY3 (Dianova, #111-165-003, USA) for 1 h at RT; cell nuclei were stained with DAPI (Sigma-Aldrich, #D9542, Germany). Finally, the tissue sections were mounted (Fluoromount™ Aqueous Mounting Medium, Sigma-Aldrich, #F4680, USA) and images were taken directly at 40x and 100x magnification using a fluorescence microscope (Olympus, Hamburg, Germany). These images were used to calculate the total $TUNEL^+$, $SFTPC^+$ and $TUNEL^+/SFTPC^+$ cells in four fields of view in one or two random sections per animal (four animals per group for *in vivo* studies and three to four animals per group for PCLS).

Precision Cut Lung Slices (PCLS):

Murine lungs were isolated from 14-days old mice after intratracheal instillation of agarose (2 % agarose in HBSS). Lungs were sliced with a thickness of 200 µm and cultured with serum-rich medium [Dulbecco's Modified Eagle Medium, Gibco, #41966-029, Netherlands) supplemented with 10 % FBS and 1 % Penicillin/Streptomycin (P/S)] for 24 h. The lung sections were then treated with 100 ng of IL-6 (Sigma-Aldrich, #I9646, Germany) + 20 ng IL-6Ra (IL-6Ra Protein, R&D Systems, #1830-SR-025, USA) (IL-6/sIL-6R), or vehicle (0.1 % BSA) and exposed to either normoxia (21 % O_2, NOX) or hyperoxia (85 % O_2, HYX) for 48 h, followed by fixation of the PCLS with 4 % PFA and paraffin-embedding. Subsequently, sections of 3 µm thickness were produced and immunofluorescence staining for SFTPC (Merck, #AB3786, Germany) was combined with TUNEL detection (*in situ* Cell Death Detection Kit, Roche, #11684795910, Germany). The total number of $TUNEL^+$ cells as well as the number of $TUNEL^+$ and $SFTPC^+$ cells (double-positive) were counted from 4 different areas per lung section; lungs of 3-5 different mice were used.

Cell culture experiments

Mouse Lung Epithelial cells

Mouse Lung Epithelial cells (MLE12; ATCC, CRL-2110; Manassas, VA) were cultured according to the ATCC recommendations in Dulbecco's medium, Ham's F12 (DMEM/F12) (Gibco, #11039-021, Netherlands) supplemented with fetal bovine serum (FBS; Merck, #S0615, Germany), hydrocortisone (Sigma, #H6909; St Louis, MO), insulin-transferrin-selenit-X-supplement (Gibco™, #51500-056, Netherlands), β-estradiol (Sigma, #E2758, Germany) and Penicillin/Streptomycin solution (P/S; Sigma-Aldrich, #P4458-100ML, Germany). Cells (passage 2-10) were subcultured every 3-4 days at a 1:8 ratio. MLE12 cells were grown in 6-well plates and 96-well plates to 80 % confluency in a medium with 2 % FBS (FBS-rich medium) and starved overnight for 12 h in a medium with 0.2 % FBS (FBS-reduced medium). Subsequently, MLE12 cells were treated 1) for 24 h at either normoxia or hyperoxia

with 100 ng IL-6 (Sigma-Aldrich, #I9646, Germany) + 20 ng IL-6Ra (IL-6Ra Protein, R&D Systems, #1830-SR-025, USA) (IL-6/sIL-6R), 2) for 24 h with conditioned media (secretome, supernatant) from macrophages, which were exposed to normoxia (21 % O_2) or hyperoxia (85 % O_2) or 3) the respective vehicles. BSA (0.1%) served as vehicle for IL-6/sIL-6R. At the end of the experiment, cell viability was assessed using MTT-assay or mRNA was extracted.

Primary human alveolar epithelial cells (hAEC)

Primary human alveolar epithelial cells (PELOBiotech, #PB-H-6053, Germany) were cultured according to the recommendations in medium provided by PELOBiotech (CellBiologics; PELOBiotech; #PB-H6621). 10.000 hAECs per well were seeded in a 96 well plate and allowed to grow for 24 h. Subsequently, hAECs were treated 1) for 24 h under normoxia (21 % O_2) or hyperoxia (85 % O_2) with 100 ng IL-6 (Sigma-Aldrich, # I1395, Germany) + 20 ng IL-6Ra (IL-6Ra Protein, R&D Systems, #227-SR, USA) (IL-6/sIL-6R), 2) for 24 h with conditioned media (secretome, supernatant) from human macrophages of three different donors (M0-like and M1-like), which were exposed to normoxia (21 % O_2) or hyperoxia (85 % O_2) for 48 h, or 3) the respective vehicles. BSA (0.1%) served as vehicle for IL-6/sIL-6R. At the end of the experiment, cell proliferation was assessed using MTT-assay (ATCC; #30-1010K).

A549 cells

Human lung carcinoma cell line (A549) (ATCC, #CCL-185™, USA) cells were cultured with DMEM/F12 (Dulbecco's Modified Eagle Medium/F12, Gibco, #11320-033, Netherlands) supplemented with 10 % FBS (serum-rich medium) and 1 % Penicillin/Streptomycin (P/S) according to the recommendation of ATCC. Cells were grown at on glass slides and in T75 flask at 37 °C and 5 % CO_2. When cells were 80 % confluent, cells were used for immunostaining and mRNA isolation.

Primary human bronchial smooth muscle cells (hSMC)

Human bronchial smooth muscle cells (hSMC) (PromoCell, #C-12561, Heidelberg, Germany) and grown according to the recommendations (PromoCell; Smooth Muscle Cell Growth Medium 2: Lot#427M002; Supplement-Mix: Lot#425M235, Heidelberg, Germany) at 37 °C and 5 % CO_2. When cells were 80 % confluent, cells were used for mRNA isolation.

Macrophages

Mouse ascites macrophages (J774.A1, ATCC® TIB-67™) cells were cultured with DMEM (Dulbecco's Modified Eagle Medium, Gibco, #41966-029, Netherlands) supplemented with 10 % FBS (serum-rich medium) and 1% Penicillin/Streptomycin (P/S) according to the recommendation of ATCC. Cells were grown on plates (Falcon®, #353003, USA) at 37 °C, 5 % CO_2. When cells were 80 % confluent, media was changed to DMEM with 1 % FBS (serum-reduced medium) and exposed to either normoxia or hyperoxia for 48 h. At the end of the experiment, the supernatant (conditioned media; secretome of the macrophages) was collected for ELISA or for treatment of MLE12 cells, and macrophages were harvested for further analyses.

Primary murine macrophages

Isolation of peritoneal macrophages was performed as described previously (14). Briefly, WT C56BL/6J mice received an intraperitoneal injection of 1 ml Brewer's thioglycolate medium (BD-BBL™, #221195, USA) and were sacrificed four days after. A peritoneal lavage of 5 times 5 ml of serum-free medium (Dulbecco's Modified Eagle Medium, Gibco, #41966-029, Netherlands) was performed. Macrophages from the peritoneal cavity were centrifuged at 1500 rpm or 300 g for 10 min at 4°C. The cell pellets were resuspended in 1 ml of serum-rich medium. 500.000 macrophages per well were seeded in a 6-well plate and cultured with serum-reduced medium (1% FBS) under normoxia (21 % O_2) or hyperoxia (85 % O_2) for 48 h. At the end of the experiments, the conditioned medium was collected to treat hAEC; mRNA was isolated from macrophages and gene expression was assessed.

Human monocyte-derived macrophages and differentiation

Human blood was collected from the hospital blood bank (for ethical consent see below).

M0 Macrophages

Human macrophages were generated from peripheral blood mononuclear cells (PBMCs) as previously described (15, 16). Briefly, PBMCs were isolated from buffy coats obtained from the blood bank of the Universities of Giessen and Marburg Lung Center (AZ 58/15) using Ficoll density gradient centrifugation. Platelets and red blood cells (RBC) were removed by two washing steps with RBC lysis buffer (BD Biosciences) and phosphate-buffered saline (PBS), respectively. Finally, monocytes were differentiated to macrophages during 10 days in RPMI containing 2.5% human serum, 4 mML-glutamine, and penicillin/streptomycin in six-well tissue culture plates. Next, we exposed M0 macrophages to normoxia (21 % O_2) or hyperoxia (85 % O_2) for 48 h. At the end of the experiments, the conditioned medium was collected to treat hAEC; mRNA was isolated from macrophages and gene expression was assessed.

M1 Macrophages

Isolation of monocyte-derived macrophages was performed as described previously (17). Buffy coat from the blood was collected from the hospital blood bank of the University Hospital Cologne (approval No 06.062) and treated with the magnetic beads-containing antibody against CD14 cell marker (Miltenyi Biotec #130-110-520, Germany). $CD14^+$ cells were collected and kept in serum-rich medium [Roswell Park Memorial Institute (RPMI) Medium, Gibco, #RPMI1640) supplemented with 10 % FBS and 1 % Penicillin/Streptomycin (P/S)]. For differentiation in M1-like monocyte-derived macrophages, $CD14^+$ cells were treated with Granulocyte-macrophage colony-stimulating factor (GM-CSF; 100 ng/ml) for 7 days in serum-rich medium; the media was changed on alternate days. After 7 days, monocyte-

derived macrophages were cultured with GM-CSF in serum-reduced (1 % FBS) medium and exposed to normoxia (21 % O_2) or hyperoxia (85 % O_2) for 48 h. At the end of the experiments, the conditioned medium was collected to treat hAEC; mRNA was isolated from macrophages and gene expression was assessed.

Immunocytochemistry of hAEC, A549, and hSMC

Cells (hAEC, A549 and hSMA) were cultured overnight on glass slides that were coated with 0.2 % gelatin (30 min at 37°C). Cells were then fixed with pre-cooled methanol for 5 min , subsequently washed with PBS, and treated with blocking solution for 1 h. Primary antibody for rabbit anti-pro-surfactant protein C (SFPTC; Merck, #AB3786, Germany, 1:200) and rabbit anti-Aquaporin 5 (AQP5; Sigma-aldrich, #A4979-200UL, Germany, 1:200) were applied for overnight. Next day, cells were washed with PBS and treated with secondary antibody conjugated with CY3 (Dianova, #111-165-003, USA) and Alexa (Jacksonimmuno, #111-485-003, USA) for 1 h at RT. Cell nuclei were stained with DAPI (Sigma-Aldrich, #D9542, Germany). Finally, cells were mounted (Fluoromount™ Aqueous Mounting Medium, Sigma-Aldrich, #F4680, USA) and images were taken directly at 100x magnification using a fluorescence microscope (Olympus, Hamburg, Germany).

IL-6 ELISA: Conditioned media of macrophages was used for ELISA to determine the IL-6 concentration. ELISA was performed according to the manufacturer's guidance (IL-6 Mouse Uncoated ELISA Kit, Invitrogen, #88-7064-22, USA). In brief, ELISA plate was incubated with IL-6 capture antibody overnight at 4 °C, followed by adding 100 µl sample (conditioned media) per well and incubated for 2 h at RT. The plate was then washed with washing buffer, 100 µl of detection antibody was added to each well and incubated at RT for 1 h. Next, 100 µl of avidin-HRP conjugated antibody was added to the plate and incubated for 30 min at RT. Afterwards incubation with 100 µl of TMB solution (substrate for avidin-HRP) was performed for 15 min; the reaction was stopped by adding the stop solution (2N H_2SO_4). Finally, the optical density (OD) was measured at 450/570nm (Tecan Infinite® 200 PRO, Switzerland).

MTT assay: MTT assay was performed according to the guidelines of the manufacturer (ATCC, #30-1010K, USA). To this end, 10.000 MLE12 cells were incubated in a 96 well plate for 24 h, followed by 12 h starvation with serum-reduced medium. Cells were then washed with PBS, and serum-reduced MLE12 medium and conditioned media of macrophages were added to each well (1:1 ratio). For control, serum-reduced MLE12 medium a well as DMEM with 1% FBS (medium used for macrophages) was used (1:1 ratio). Cells were exposed to either normoxia (21 % O_2) or hyperoxia (85 % O_2) for 24 h. Afterwards, cells were incubated with 10 µl of tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 2 h, and treated with 100 µl of detergent provided by the manufacturer for 2 h. Absorbance was measured at 570 nm (Tecan Infinite® 200 PRO, Switzerland).

si-Klf4 transfection in macrophages: Cultured J774.a1 were transfected with either anti Klf4-siRNA (si-klf4; Dharmacon, Cat. No. SO-2589106G, USA) or scrambled siRNA (scr-siRNA; Dharmacon, Cat. No. SO-2588425G, USA) using Endo-Porter (GeneTools LLC, Philomath, Oregon, USA). Briefly, macrophages were grown to 70 % confluency in serum-rich medium, followed by transfection of 20 ng si-klf4 or scr-klf4 at 37 °C and 5 % CO_2 for 24 h using the following medium: 6 µl of 1 mM Endo-Porter (GeneTools LLC, Philomath, Oregon, USA) in 1 ml of antibiotic free complete media (DMEM with 10 % FBS). Afterwards, the media was changed and the transfected cells were maintained in serum-rich medium for 24 h. Finally, cells were starved with serum-reduced medium for 12 h, followed by exposure to either normoxia or hyperoxia for 48 h. At the end of the experiments, macrophages were harvested for further analysis.

Human lung tissue

BPD and control (non-diseased) postnatal human lung tissues were obtained through the NHLBI LungMAP Consortium Human Tissue Core Biorepository (BRINDL) through the work of the U.S.

Transplantation program, the International Institute for the Advancement of Medicine and the National Disease Research Interchange. The Biorepository is approved by the University of Rochester Research Subjects Review Board (RSRB00056775). Consent for research use has been provided for each sample. Clinical metadata and histopathology was assessed in each case to determine a combined diagnosis based on clinical history and pathology of the lung tissue (ClinPathDx, Table 1).

Immunostaining of human lungs

Paraffin embedded BPD and control (non-diseased) postnatal human lungs were sectioned at 5 µm, deparaffinized with xylene, rehydrated in decreasing graded ethanol concentrations, and boiled in a sodium citrate antigen retrieval solution (10 mM, pH 6.0) for 12 min. Slides were then washed in TBST (TBS and 0.1 % Tween). Endogenous peroxidase was quenched 3 % H_2O_2 for 15 min. Slides were then washed in TBST and blocked using 3 % bovine serum albumin/5 % Normal Goat Sera/0.1 % Triton (serum type was dependent on the secondary antibodies being used) for at least 1 h at RT. Slides were incubated with the following primary antibodies overnight at 4 °C: pSTAT3 (Cell Signaling, #9145, 1:200), CD45 (Invitrogen, #14-9457-82; 1:200), CDH1 (BD Biosciences, 610181; 1:200), CD68 (eBioscience, 14-0688-82; 1:200, San Diego, USA), and Surfactant protein C (SFTPC, LSBio,LS-B10952; 1:100, Seattle, USA). The following day slides were washed with TBST and incubated for 1 h at RT with appropriate Cy-3 and Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). Slides were then stained for DAPI (DE571; LifeTechnologies, Carlsbad, USA) and mounted using ProLong Diamond Antifade Mountant (LifeTechnologies, Carlsbad, USA).

In Situ Hybridization of Human Lungs

Fluorescent *in situ* hybridization was conducted using the Advanced Cell Diagnostics RNAscope Fluorescent Multiplex Assay following the manufacturer's instructions, with minor adjustments. Treatment time with Protease Plus was decreased to 22 min. Tissue was incubated

with *IL6* probe (Advanced Cell Diagnostics; 310371) for 2 h at 40 °C on protocol day 1, followed by washing in RNAscope wash buffer, and stored overnight at RT in 5X SSC. On day 2 the protocol was continued as described in the manual until the HRP blocker step was completed. Slides were then washed with RNAscope wash buffer and proceeded onto CDH1 immunofluorescent staining. Slides were blocked using 3 % bovine serum album/5 % Normal Goat Sera/0.1 % Triton for at least 1 h at RT, and incubated overnight at 4 °C with primary antibody CDH1 (BD Biosciences, 610181, 1:200). The following day slides were washed with TBST and incubated for 1 h at RT with Cy5-goat-anti-mouse-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). Slides were then stained for DAPI (DE571; LifeTechnologies) and mounted using ProLong Diamond Antifade Mountant (LifeTechnologies).

Human cohort study Stanford

Premature infants who were born at ≤ 32 weeks gestational age (GA) and admitted to the neonatal intensive care unit (NICU) at Lucile Packard Children's Hospital Stanford were prospectively enrolled into a longitudinal study designed to assess clinical biomarkers of BPD. We have previously reported on this cohort(10). Infants with complex congenital heart disease, inherited metabolic disorders and/or lethal congenital malformations were excluded from participation. Plasma specimens were obtained at weekly time points during their hospitalization, and clinical characteristics were abstracted from their medical records. Infants were followed to 36 weeks post-conceptual age (PCA) to determine if they acquired BPD based on NIH criteria, defined by the ongoing need for either supplemental O_2 and/or positive pressure respiratory support. The study was approved by the Stanford University Institutional Review Board (Protocol #20210). Supplemental table 2 shows maternal and infant characteristics at birth for infants enrolled in the study. Plasma specimens were dispensed into 50 µl aliquots that were immediately frozen at -80 °C for later batch processing. Plasma was assayed for cytokine profiling via the Human Immune Monitoring Center at Stanford

University using a validated custom-built Human 63-plex bead assay (eBiosciences/Affymetrix).

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Supplementary Table S1

Gene	Species m = mouse h = human	Primer	Sequence
αsma *(Acta2)*	m	for	ACATCAGGGAGTAATGGTTGGAAT
		rev	GGTGCCAGATCTTTTCCATGTC
		probe	CGATAGAACACGGCATCATCACCAACTG
Arg1	m	for	ACCCTGACCTATGTGTCATTTGG
		rev	TGGTACATCTGGGAACTTTCCTTT
		probe	ATGCTCACACTGACATCAACACTCCCCTG
Aqp5	m	for	TCACTGGGTCTTCTGGGTAGGA
		rev	CTGGCTCATATGTGCCTTTGAC
		probe	TACTTCTACTTGCTTTTCCCCTCCTCGCTG
**Aqp5*	h	for	CCACCTTGTCGGAATCTACTTCA
		rev	GGGCCCTACCCAGAAAACC
βactin	m	for	TGACAGGATGCAGAAGGAGATTACT
		rev	GCCACCGATCCACACAGAGT
		probe	ATCAAGATCATTGCTCCTCCTGAGCGC
**βactin*	h	for	GATGGCCACGGCTGCTT
		rev	ACCCTCATTGCCAATGGT
Ccl2	m	for	GGCTCAGCCAGATGCAGTTAAC
		rev	CTTGGTGACAAAAACTACAGCTTCTT
		probe	CCCCACTCACCTGCTGCTACTCATTCA
Ccl7	m	for	TGGGAAGCTGTTATCTTCAAGACA
		rev	TTCTGTTCAGGCACATTTCTTCA
		probe	CTTAGACATGAAAACCCCAACTCCAAAGCC
**Col1a1*	m	for	GCAGTGCTGTTGCGATCTTG
		rev	CAGAGGGACAGAGCACAGCTT
Cxcl5	m	for	TGGCATTTCTGTTGCTGTTCA
		rev	GCTCCGTTGCGGCTATGA
		probe	AGCATCTAGCTGAAGCTGCCCCTTCCT
**Fbn1*	m	for	GGTCAATGCAACGATCGAAA
		rev	AGTGTGACAAAGGCAGTAGAAGCTT
**Fbln5*	m	for	TACATCCTACTCAGGCCCATACC
		rev	GTTGCCTTCATCCATCTGATACC
Fizz1	m	for	CGTGGAGAATAAGGTCAAGGAACT
		rev	CACTAGTGCAAGAGAGAGTCTTCGTT
		probe	TTGCCAATCCAGCTAACTATCCCTCCACTG
**IL1b*	h	for	CTAAACAGATGAAGTGCTCC
		rev	GGTCATTCTCCTGGAAGG
**Il4*	m	for	GGAGATGGATGTGCCAAACG
		rev	GCACCTTGGAAGCCCTACAG
Il6	m	for	ACAAGTCGGAGGCTTAATTACACAT
		rev	AATCAGAATTGCCATTGCACAA
		probe	TCTTTTCTCATTTCCACGATTTCCCAGAGAA
**IL6*	h	for	GTACATCCTCGACGGCATCTC
		rev	GCTGCTTTCACACATGTTACTCTTG
Il17a	m	for	CCAGAAGGCCCTCAGACTACCT

		rev	GGGATATCTATCAGGGTCTTCATTG
		probe	AACCGTTCCACGTCACCCTGGACTC
Mmp12	m	for	GCAGCAGTTCTTTGGGCTAGA
		rev	GTACATCGGGCACTCCACATC
		probe	CTGGGCAACTGGACAACTCAACTCTGG
**MMP12*	h	for	GTCCCTGTATGGAGACCCAAAA
		rev	ACGGTAGTGACAGCATCAAAACTC
**Sftpa*	m	for	TCAAACATCAGATTCTGCAAACAA
		rev	TGACTGCCCATTGGTGGAA
**Sftpb*	m	for	CTGCTGGCTTTGCAGAACTCT
		rev	GAGGACAAGGCCACAGACTAGCT
**Sftpc*	m	for	CCTCGTTGTCGTGGTGATTGTA
		rev	GCTCATCTCAAGGACCATCTCAGT
**Sftpc*	h	for	GCACCTGCTGCTACATCATGA
		rev	CCATCTGGAAGTTGTGGACTTTT
**Sftpd*	m	for	CAGCAGATGGAGGCCTTAAAA
		rev	GGGAACAATGCAGCTTTCTGA
Socs3	m	for	CCACCCTCCAGCATCTTTGT
		rev	TCCAGGAACTCCCGAATGG
		probe	ACTGTCAACGGCCACCTGGACTCCT
Tlr4	m	for	GGTGAGAAATGAGCTGGTAAAGAATT
		rev	GCAATGGCTACACCAGGAATAAA
		probe	TGCCCCGCTTTCACCTCTGCC
**TLR4*	h	for	CATTTCAGCTCTGCCTTCACTACA
		rev	ATGGAAACCTTCATGGATGATGT
**TNFa*	h	for	CCCAGGGACCTCTCTCTAATCAG
		rev	TCAGCTTGAGGGTTTGCTACAA

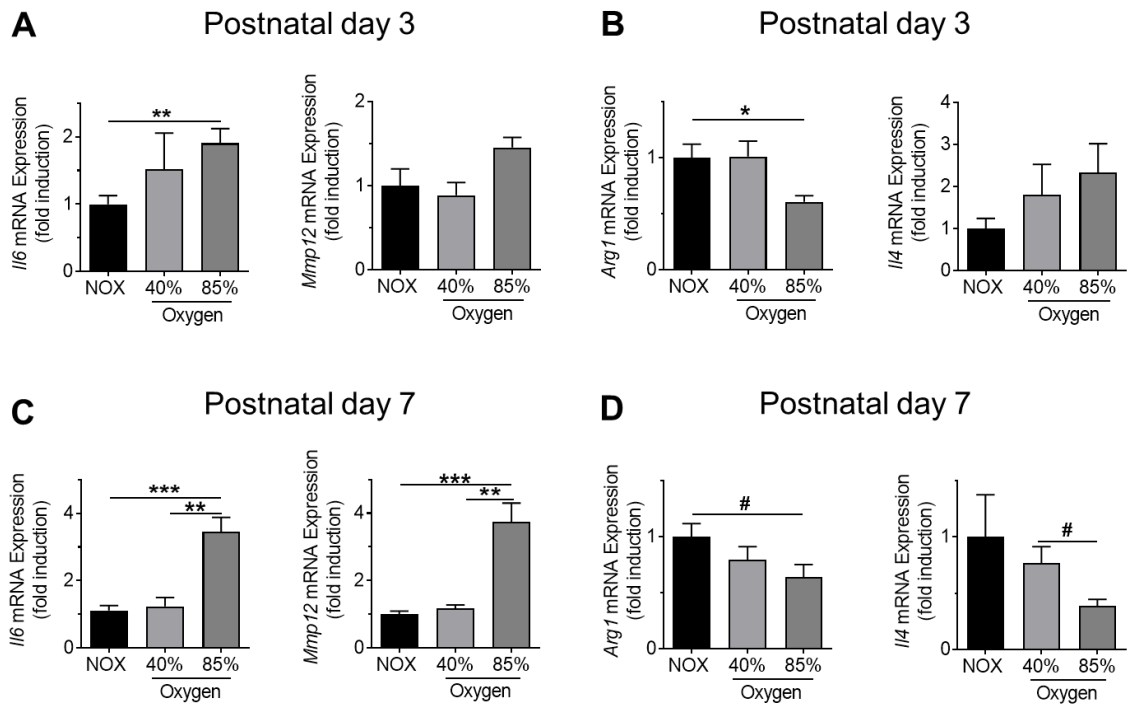
Supplementary table S1. List of primers used for real-time RT-PCR; *SYBR-Green primer.

Supplementary Table S2

	Number (% of cohort)
Male / Female	37 (67 %) / 18 (33 %)
Race: White Asian Black Pacific Islander	37 (67 %) 12 (22 %) 3 (5.5 %) 3 (5.5 %)
Hispanic ethnicity	24 (43 %)
Cesarean delivery	37 (67 %)
Multiple gestation	21 (38 %)
Vaginal bleeding Placenta Previa Abruptio Placenta	8 (14.5 %) 2 6
Maternal pre-eclampsia	17 (31 %)
Maternal prolonged rupture of membranes (PROM)	14 (25 %)
Intrauterine growth restriction (IUGR)	4 (7 %)
Antenatal corticosteroid treatment	36 (65 %)
Maternal group B streptococcal infection	4 (7 %)
APGAR at 1 minute (mean, SD)	5.6 (2.6)
APGAR at 5 minute (mean, SD)	7.6 (1.8)
Surfactant treatment	24 (43 %)
Bronchopulmonary dysplasia (BPD)	15 (27 %)
MV/CPAP (days, median & [range])[§]	2 [0-217]
nCPAP (days, median & [range])[¶]	17 [0-55)]
High-frequency ventilation treatment (n)	8
Days on respiratory support (mean ± SD)	61 ± 46
Days in hospital (mean ± SD)	86 ± 47
Patent ductus arteriosus (n)	16
Indomethacin treatment (n)	15
Caffeine treatment (n)	26

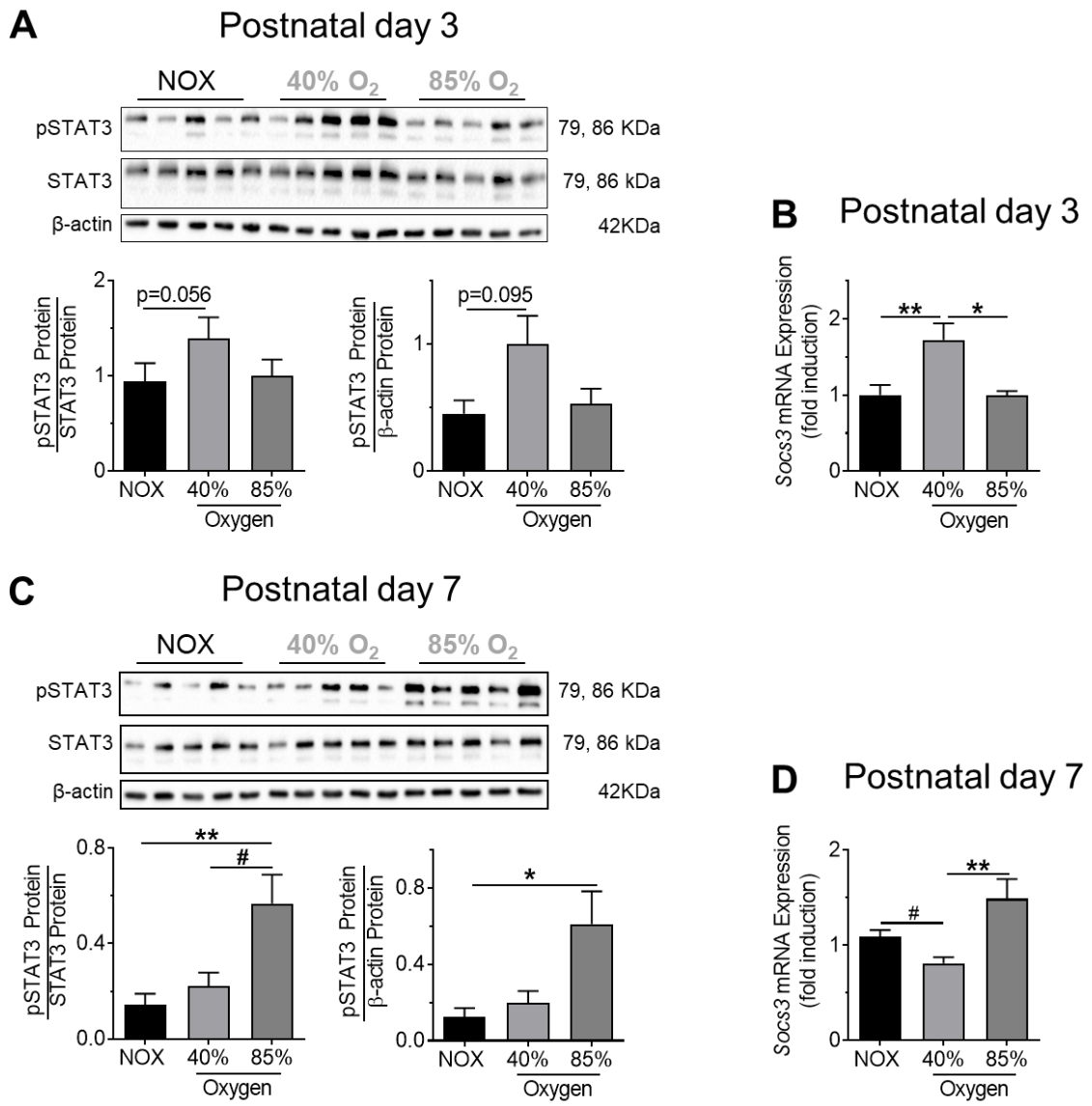
Supplementary table S2. Demographic and relevant clinical data for the infants enrolled, including maternal/pregnancy related data.

Supplementary Figure S1:



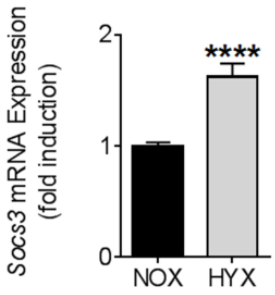
Supplementary Figure S1: *Effect of mild hyperoxia (40% O_2) and high hyperoxia (85% O_2) on gene expression of macrophage markers in mouse lungs at postnatal day 3 (P3) and P7.* Wildtype mice were exposed to 40 % O_2 or 85 % O_2 from birth until P3 or P7. A, C: Measurement of gene expression of M1-like markers in total lung homogenates at P3 (A) and P7 (C) using qRT-PCR; interleukin 6 (*Il6*) and metalloproteinase 12 (*Mmp12*); n=7 / group; β-actin served as housekeeping gene. B, D: Gene expression of M2-like markers in total lung homogenates at P3 (B) and P7 (D) using qRT-PCR: arginase 1 (*Arg1*) and interleukin 4 (*Il4*); n=7 / group. Mean±SEM; Kruskal-Wallis One-way ANOVA with Dunn's post-test: *p<0.05; **p<0.01; ***p<0.001; unpaired t-test: $^{\#}$p<0.05.

Supplementary Figure S2:



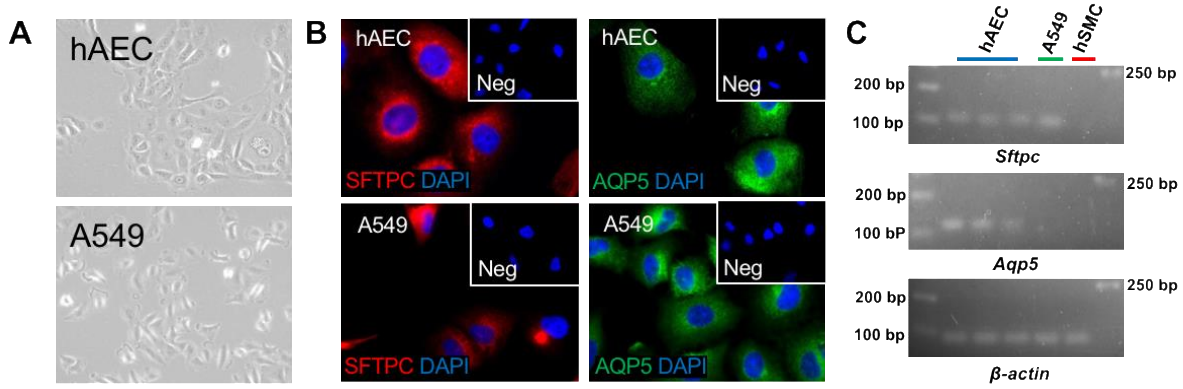
Supplementary Figure S2: *Effect of mild hyperoxia (40% O_2) and high hyperoxia (85% O_2) on activation of STAT3/SOCS3 signaling in mouse lungs at postnatal day 3 (P3) and P7.* Wildtype mice were exposed to 40% O_2 or 85% O_2 from birth until P3 or P7. A, C: Immunoblots showing phosphorylated STAT3 (pSTAT3), total STAT3 and β-actin protein abundance in total lung homogenates after exposure to Normoxia (NOX), 40% O_2 or 85% O_2; P3 (A), and P7 (C); pSTAT3 protein was related to STAT3 or β-Actin, which served as a loading control; densitometric data are displayed under the immunoblot; n = 5/group. B, D: Assessment of gene expression of suppressor of cytokine signaling 3 (*Socs3*) in total lung homogenates at P3 (B) and P7 (D); n=7-15/ group. Mean±SEM; Kruskal-Wallis One-way ANOVA with Dunn's post-test: *p<0.05; **p<0.01; Mann-Whitney test: $^{\#}$p<0.05.

Supplementary Figure S3:



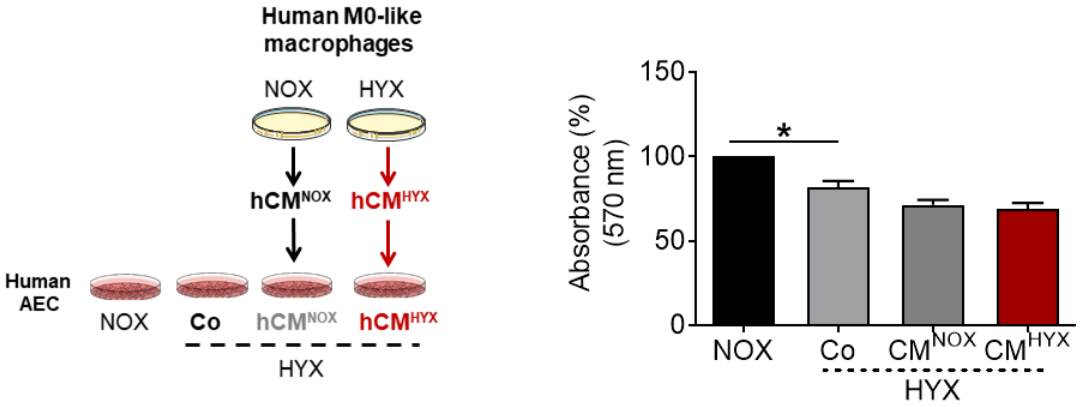
Supplementary Figure S3: Measurement of *Socs3* mRNA as IL-6/STAT3 target gene in lungs at P28 by qRT-PCR (n = 10-12/group); gene expression is shown as fold induction. Mean±SEM; non-parametric Mann-Whitney test: ****p<0.0001.

Supplementary Figure S4:



Supplementary Figure S4: (A): Morphological image of human alveolar epithelial cells (hAEC) and A549 cells. (B) Representative immunofluorescent staining for SFTPC (red) and AQP5 (green) in hAEC and A549, cultured for 24 h on glass slides, fixed with methanol, and subsequently stained. IgG antibody was used as negative control. (C) Gel electrophoresis of qRT-PCR products of *Sftpc*, *Apq5* and β-actin from hAEC, A549 and human bronchial smooth muscle cells (hSMC); the cells were cultured until they reached 80% of confluency and then harvested for mRNA isolation, While A549 served as positive control for alveolar epithelial cell markers, hSMC was used as a negative control. Scale = 100x.

Supplementary Figure S5:



Supplementary Figure S5: Human M0-like macrophages were exposed to normoxia (21 % O_2, NOX) or 85 % O_2, HYX) for 48 h and conditioned media (CM) was collected. Subsequently, human alveolar epithelial cells (hAEC) were exposed to HYX and treated with CM of NOX-exposed M0-like macrophages (CM^{NOX}), CM^{HYX} or vehicle (medium, Co) for 24 h; Control hAEC were exposed to NOX. At the end of the experiment, proliferation of hAEC was assessed using MTT assay; n = 4 /group. Mean±SEM; n = 4-5/group; Paired t test: *p<0.05.