**Supplementary data**

**Altered cholesterol immunometabolism activates the macrophage NLRP3-inflammasome in lung fibrosis**

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**Running title: Macrophage inflammasome activation in lung fibrosis**

**Supplementary methodology**

**Murine sample collection**

The animals were anesthetized via intraperitoneal injection of 80mg/kg ketamine and 5mg/kg xylazine in 0.9% NaCl. Following laparotomy, blood sample was collected from the vena cava using a needle coated with Na-Heparine (17units/mL) and moved to a Na-Heparine-coated tube. Blood samples were centrifuged at 1000xg for 10min at 4°C and the resulting plasma was transferred to a new tube, frozen in liquid nitrogen and stored at -80°C until further use.

Cell-free bronchoalveolar lavage fluid (BALF) and BALF-cells were obtained via bronchoalveolar lavage (BAL), after animal sacrifice by exsanguination. Following a tracheotomy, a 20G cannula was inserted in the trachea. Afterwards lung was perfused with 0.9% NaCl through the right ventricle. BAL was acquired by instilling twice 1.5mL of 0.9% NaCl and BALFs were collected in 15mL tubes placed on ice. The samples were centrifuged at 300xg, for 10min at 4°C. The harvested cell-free BALFs were aliquoted in vials, frozen in liquid nitrogen and stored at -80°C until further use. The BALF cell pellet was treated accordingly to the analysis of interest (see below).

**Technical controls**

Technical controls were included to verify the validity of a method (e.g. ELISA, WB) and obtained as follow. THP-1 monocytes from the ATCC® TIB202™ (Virginia, USA) were cultured in RPMI-1640 complete medium supplemented with L-glutamine, 25mM HEPES (Corning, Virginia, USA), 10% fetal bovine serum (FBS) (Sigma Aldrich, Germany), and 1% penicillin/streptomycin (10000 units penicillin + 10mg streptomycin/mL, Sigma Aldrich, Germany) and differentiated into macrophage-like cells (MLC) with 5ng/mL phorbol 12-myristate 13-acetate (PMA, Stemcell technology, Switzerland) in dimethyl sulfoxide (final concentration 0.1%, DMSO, Sigma Aldrich, Germany) for 48h, followed by 24h rest in fresh medium (1) . MLC incubated at 37°C, 5%CO2 in the presence of 25µg/ml OxLDL for 24h served as technical control (oxLDL+) for the reverse cholesterol transport (RCT) pathway. MLCs treated with 5μg/mL lipopolysaccharide (LPS, E. coli O26: B6 ≥10,000 EU/mg, Sigma Aldrich, Germany) for 3h followed by 10μM Nigericin (NIG, #66419, Cell Signaling Technology) for 45min served as positive control for NLRP3 inflammasome. Technical controls were not intended as a comparison group for the analysis of the outcome, but for confirmation of the specificity of the response and corresponding band analyzed.

**Western Blot (WB) analysis**

Samples of frozen human lung tissue (N=6 PPF/IPF and N=6 control) from patient cohort 1, were lysed in 400µL cold radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Germany) containing protease inhibitor cocktail (cOmplete, Sigma Aldrich, Germany) and phosphatase inhibitor (PhosStop, Roche, Switzerland), using tissue lyser (TissueLyser III, QIAGEN, Germany) twice for 1min at 20MHz. For mouse samples, cell pellets were obtained by BAL from 10, 30, and 60 weeks old SP-C-/- mice (N=4/group) as described above, resuspended in RIPA buffer containing cOmplete and lysed using a thermomixer at 4°C, 1400rpm for 30min. All lysates were centrifuged at 4°C, 13000xg for 20min to pellet down cell debris. Protein concentration was measured using a bicinchonic acid kit (BCA), following manufacturer’s instructions (Pierce BCA Protein Assay Kit, Thermo Scientific, Germany). Samples were normalized to the same protein concentration, boiled at 95°C for 10min in 1x Laemmli buffer (Tris-HCl 63mM, glycerol 10%, SDS 2%, bromophenol blue 0.01%, 2-mercaptoethanol 5%; pH 6.8) and loaded into 10, 12, or 16% (wt/vol) sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, USA). Non-specific binding on PVDF membrane was blocked using 5% non-fat milk powder (VWR, Belgium), or 5% bovine serum albumin in Tris-buffered saline (TBS) with 0.1% Tween 20 (Acros Organics, Geel, Belgium) for 1h at RT. The membranes of human and mouse samples were incubated overnight at 4°C with primary antibodies as stated in supplementary table 1. Following washing and 1h incubation with HRP conjugated anti-rabbit (#P0399, Agilent DAKO, Santa Clara, CA, USA), anti-mouse or anti-goat (#ab6728, #ab6885, Abcam, Cambridge, England, UK) secondary antibodies, specific bands were detected through chemiluminescence (ECL Chemocam imager, Intas science imaging, Germany) using ECL western blotting detection reagents (Amersham Pharmacia Biotech, UK). Densitometry analysis was conducted using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA).

Supplementary table 1.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Primary antibodies** | **Specificity** | **Species** | **Supplier** | **Catalogue #** | **Concentration**  **used** |
| CD36 | human | Rabbit | Abcam | ab252922 | 0.5µg/mL |
| LXRα+β | human | Goat | Abcam | ab24362 | 1µg/mL |
| IL-1β | human | Rabbit | Abcam | ab216995 | 0.5µg/mL |
| pro Caspase-1 | human/mouse | Rabbit | Abcam | ab179515 | 2.0µg/mL |
| LDLR | human | Rabbit | Invitrogen | PA5-22976 | 1µg/mL |
| ApoE | human | Rabbit | Invitrogen | PA5-27088 | 1.3µg/mL |
| NLRP3 | human/mouse | Mouse | AdipoGen Life Science | AG-20B-0014 | 1µg/mL |
| ASC/TMS1 | human | Rabbit | Cell Signaling Technology | 13833 | 0.812µg/mL |
| ASC/TMS1 | mouse | Rabbit | Cell Signaling Technology | 67824 | 0.1µg/mL |
| IL-18 | mouse | Rabbit | Cell Signaling Technology | 57058 | 0.1µg/mL |
| CES1 | mouse | Rabbit | Abcam | ab68190 | 0.7µg/mL |
| Perilipin-1 | mouse | Rabbit | Abcam | ab3526 | 2µg/mL |
| LXRα | mouse | Mouse | Abcam | ab41902 | 1µg/mL |
| CD36 | mouse | Goat | R&D systems | AF2519 | 0.1µg/mL |
| β-actin | human/mouse | Mouse | Sigma-Aldrich | A2228 | 0.2µg/mL |
| β-actin | human/mouse | Goat | Abcam | ab8229 | 0.25µg/mL |
|  |  |  |  |  |  |

Supplementary table 2.

|  |  |  |  |
| --- | --- | --- | --- |
| **Secondary antibodies** | **Species** | **Supplier** | **Catalog #** |
| HRP conjugated anti-rabbit | Swine | Agilent DAKO | P0399 |
| HRP conjugated anti-mouse | Rabbit | Abcam | ab6728 |
| HRP conjugated anti-goat | Donkey | Abcam | ab6885 |

**Lipid extraction and phosphorus assay**

Phospholipid (PL) concentration was used to normalize the cholesterol concentration in BALF obtained from 10, 30, and 60 weeks old SP-C -/- mice (N=8/group). Therefore, a lipid extraction and a colorimetric phosphorus assay were performed. For each sample, the lipid extraction was conducted in duplicate by adding an “acidic mixture” composed of methanol:chloroform:HCl (2:3:0.005, v/v/v) (2), followed by centrifugation at 785xg for 10min. The resulting hydrophobic phase was dried under nitrogen stream and the lipids were then subjected to the phosphorus assay as following (3). Standard curve with 0.2, 0.5, 1, 2, 5, and 10µg phosphorus (0.439mg/ml KH2PO4 asP source) was prepared. In every standard and sample tube, 70% perchloric acid was added and allowed to incubate at 180°C for 2h. Subsequently, they were incubated with 4.6mM ammonium molybdate and ascorbic acid at 60°C for 1h and then plated in duplicate. The absorbance was measured at 820nm using the microplate reader (Fluostar Omega, BMG Biotech, Germany) and the concentration was calculated in reference to the standard curve.

**Cholesterol assay**

Cholesterol content in lung tissue samples of control (N=10) and PF (N=11) patients, in BALFs and BALF-cells of age-matched 10, 30 and 60 weeks old SP-C -/- and WT mice (N=8/group) was quantified using a commercially available fluorimetric assay (Total Cholesterol Assay Kit, STA-390, Cell Biolabs Inc., California, USA), following manufacturer’s instructions. Human lung tissue extracts were obtained with chloroform:isopropanol:NP-40 (7:11:0.1, v:v:v) using tissue lyser twice for 1min at 20MHz. Mouse cell extracts were obtained with the same extraction buffer by ultrasonication (Bandelin Sonopuls GM70, Berlin, Germany) with 3 bursts of 70% of maximum cycling. Extracts were centrifuged for 10min at 13000xg at 4°C. The organic fraction was collected in 10mL pyrex tubes, dried at 50°C under nitrogen flow and lastly reconstituted in Assay diluent. Mouse BALF were directly analysed, without extraction. All samples were analysed in duplicate for total cholesterol by addition of reaction reagent solution containing cholesterol esterase, cholesterol oxidase, fluorescence probe, and HRP. Fluorescence was measured in a fluorimetric microplate reader (Fluostar Omega, BMG Biotech, Germany) with excitation range 530-570nm and emission range 590-600nm. For the analysis of cholesterol ester fraction in BALF from mice and human lung samples, the free cholesterol was first determined by omitting the addition of cholesterol esterase from the cholesterol reaction reagent solution. Then the cholesterol ester fraction was calculated as:

Total cholesterol and cholesterol esters in human lung tissue were normalized per tissue weight. For mouse BALF cells, data were normalized by cell lysate total protein concentration measured by BCA, as reported above. For mouse BALF, data were normalized by BALF total phospholipid content.

**Enzyme-linked immunosorbent assay (ELISA)**

Levels of IL-18 and IL-1β were assessed in lung lysates of control, PPF/IPF patients (N=12/group) and in non-diluted BALFs of 10, 30 and 60 weeks old SP-C-/- mouse (N=6/group) using commercially available ELISA kits (Human Total IL-18, DY318-05; Mouse IL-18, DY7625-05; Human IL-1β/IL1F2, DY201; Mouse IL-1β/IL-1F2, DY401-05, R&D System, Minnesota, USA), following manufacturer's instructions.

**ASC speck immunofluorescence**

ASC specks oligomerization was detected by immunofluorescence as a readout of inflammasome activation, as previously described (4) in human tissue and mouse BALF cells.

Formalin fixed-paraffin embedded human tissue was first deparaffinized in Xylol and re-hydrated in a row of descending alcohol percentage. Antigen retrieval was achieved under pressure cooking the slides in citric buffer (pH 6) during 8min. Slides were then washed briefly with PBS and tissue samples were permeabilized with Triton 0.1% in PBS for 10 min. The blocking was performed with 1%BSA in PBS for 1h at RT. The primary antibodies (anti-ASC, 13833, Cell Signaling, Leiden, The Nederlands and anti-CD68, 14-0688-82, Invitrogen, USA) was incubated in 1%BSA in PBS overnight at 4°C at a final concentration of 25µg/mL. After washing, the secondary antibody (Goat-anti-Rabbit-PE, 79408, Cell Signaling, Leiden, The Nederlands or goat anti-mouse-APC, 405308, Biolegend, CA, USA) was incubated for 1h at RT at a final concentration of 15µg/mL. The nucleus was labeled with DAPI (9542, Sigma Aldrich, Taufkirchen, Germany) at a final concentration of 1µg/mL and visualized as explained below. The quantification of ASC speck in CD68+ cells was performed counting the number of ASC speck in 50 CD68+ cells per sample, in an N=5 healthy and N=9 IPF patient samples.

BALF cells were collected from 10, 30, and 60 week old SP-C -/- mice following the above-mentioned method. Total number of cells present in BALFs was counted in the Cytosmart (Axion BioSystems, Inc., Atlanta, Georgia, USA). Cell pellet was resuspended in a calculated volume of PBS in order to have 100000 cells/100µL. Cells were placed on slides using cytospin (Cellspin III, Tharmac, Hessen, Germany) at 1500rpm for 10min, followed by fixation in PBS-buffered formalin 4% for 5min. Slides were stored at 4°C until further use. After rinsing the slides with PBS, they were subjected to blocking in permeabilization/blocking (p/b) buffer, consisting of 10% goat serum, 1% FBS, and 0.5% Triton-X100 in PBS, for 30min at 37°C. Next, the slides underwent an overnight incubation at 4°C with anti-ASC/TMS1 (0.4µg/mL, #67824, Cell Signalling) primary antibody in p/b buffer. This was followed by a 1h incubation at room temperature, with a conjugated secondary antibody in p/b buffer protected from light to avoid bleaching of fluorophores conjugated to the secondary antibody. Nuclei staining was achieved by incubation in Hoechst 34580 (1µg/mL, Chemodex, Switzerland) diluted in PBS for a duration of 10min. Sample incubation only with the secondary antibody was used as a control for the exclusion of non-specific binding due to secondary antibody. The slides were covered with a mounting solution and observed under the Axio 196 Imager.Z2 microscope (Zeiss, Germany). The acquired data were then processed utilizing ZEN software (v3.4, blue edition, Zeiss, Germany).

**Single cell sequencing data analysis from Adams et al 2020**

Publically available scRNA-Seq data published by Adams et al 2020 (5) was downloaded from GEO (accession number GSE136831). We re-analyzed the dataset from Adams et al 2020 (5) by following the original cell annotation reported by the authors and as plotted in Suppl Figure 1b.The data was analyzed using R package Seurat (6). The raw counts and metadata were converted into a Seurat object and the count matrix was normalized using the NormalizeData() function, which then was followed by FindVariableFeatures() function (nfeatures=3000) and ScaleData() functions to find and scale highly variable features. Dimensionality reduction was performed using RunPCA() and RunUMAP() functions with dims=1:30. We have used the cell type annotations already available in the metadata. Differential gene expression analysis between control and IPF conditions (for each cell type) were performed using a pseudo-bulk approach, which aggregates counts of all cells of each cell type (7) before testing for significant differences in gene expression across conditions. After generating pseudo-bulk counts, DESeq2 (8) and the Wald test were used to identify differentially expressed genes. P values were adjusted for multiple testing using the Benjamini-Hochberg method (and can be found in [www.zenodo.org/records/17665327](http://www.zenodo.org/records/17665327)). The code used for data analysis is available through github.com at <https://github.com/Lopez-Giambelluca/LungChol>.

**Characterization of lung leukocytes by flow cytometry**

Lungs of 10, 30, and 60 weeks old SP-C-/- sacrificed mice (N=3/group) were perfused through the heart, dissected and minced with scissors in a tissue culture dish containing 1mL digestion medium (Dispase (Corning, Cat# 354235) with 0.4mg/mL DNase I (Sigma-Aldrich DN251G)) per lung. The minced lung suspensions were incubated in a rocking water bath at 37 °C for 45 min. Digested lung suspensions were dissociated by passing 3-times through a 20G syringe followed by a 100μm-filter. Cells were pelleted by centrifugation and red blood cells cleared by exposure to erythrocyte lysis solution (150mM NH4Cl, 10mM KHCO3, 1mM EDTA) for 2 min. The lung suspensions were then washed, centrifuged and resuspended in 450µL FACS buffer (0.1% BSA, 0.1M EDTA in 1x PBS) and stored on ice until further analyses. Viable cells were determined by hemocytometer and trypan blue dye exclusion staining.

Cell surface antigen staining was performed on single cell suspensions to identify AM and inflammatory monocyte/macrophages (iM) (Figure 3h). Cells were stained in 100µL volume. First, cells were blocked with anti-CD16/anti-CD32 (clone: 2.4G2, BD) to block non-specific antibody binding. After 5 min, cells were stained with mAbs against surface antigens CD45 (clone: 30-F11, BD), CD11c (clone: N418, eBioscience), CD11b (clone: M1/70, eBioscience), F4/80 (clone: BM8, eBioscience), Siglec F (clone: E50-2440, BD), Ly6C (clone: HK1.4, BioLegend), and MHC Class II (I-A/I-E) (clone: M6/114.15.2, ebioscience) for 30 min at 4 °C in the dark. For ASC-1 staining we used a previously described protocol by Beilharz et al 2016 (4) where, shortly, cells were fixed in 4% formaldehyde (methanol-free, ROTI-HistoFix 4% Formaldehyde, P087.5, Carl Roth, Karlsruhe, Germany) at 37°C for 30 min. Next, cells were centrifuged and washed twice in 1x PBS and resuspended in permeabilization solution (1% FBS and 0.5% Triton-X100 in 1x PBS). Cells were incubated for 30 min at 37°C in permeabilization solution, centrifuged and resuspended in 100µL permeabilization solution supplemented with anti-ASC-1 (clone: AL177; primary antibody (10µg/mL, AdipoGen LifeSciences, Fuellinsdorf, Switzerland)) and left incubating 1h at RT. Cells were washed and centrifuged prior 1h incubation at RT with the secondary antibody (2.5µg/mL, anti-Rb-Cy5 (A10523, Thermo Fisher Scientific, Waltham, MA, USA)). Then samples were washed with 1x PBS, centrifuged and resuspended in 100 µL FACS buffer before analyzing total numbers and frequencies of innate leukocyte populations on the FACS Canto II (BD, Heidelberg, Germany). The gating strategy is depicted in Supp Figure 3a-b.

**Urea analysis**

BALFs collection procedure through instillation of saline solution causes an unknown dilution of the BALFs components. The ratio of plasma to BALFs urea concentration can be used as an estimation of the dilution factor (9). This approach was used for the pre-analysis normalization of the metabolomic and lipidomic data. To this aim, plasma and BALFs urea were analysed using a colorimetric assay (ChromaDazzle Urea Assay Kit, AssayGenie, Dublin, Ireland), according to manufacturer’s instructions.

**Untargeted metabolomics/lipidomics analysis**

BALFs and BALF cells of 30 weeks old SP-C -/- (N=12) and sex-matched WT (N=12) mice. Animals were kept under the same conditions and the same diet for at least 4 months before sampling. Blood was collected to estimate the BALFs dilution factor via urea assay (described above). Protein concentration of cell lysates was determined by BCA to normalize the cell data.

***Chemicals***

Acetonitrile, methanol, isopropanol and water (HPLC grade) were purchased from Fisher Scientific (Loughborough, U.K.). Formic acid and acetic acid (≥98.0% purity) was purchased from VWR International (Lutterworth, U.K.), and ammonium formate and ammonium acetate (≥98.0% purity) was purchased from Sigma-Aldrich (Poole, U.K).

***Sample preparation***

***Pulmonary alveolar cells***: Biphasic liquid-liquid extractions were performed to extract metabolites from the murine alveolar cells. To each cell pellet/sample, 480µL ice-cold methanol / 0.26µg protein and 170µL ice-cold water / 0.26µg protein were added. Samples were freeze/thawed by placing in a -80°C freezer for 20min and were subsequently vortexed for 30s; this procedure was repeated three times to release metabolites from the cells. To the cell extract 480µL chloroform / 0.26µg protein and 240µL water / 0.26µg protein was added to each sample, vortexed and centrifuged at 1800xg for 10min and then allowed to stand at room temperature for 5min to allow phase separation. Standard aliquots of the polar methanol/water/top (600µL) and non-polar chloroform bottom (400µL) phases were removed and dried using either a vacuum concentrator (polar) or nitrogen blow drier (non-polar) to normalise samples amounts according to measured cellular protein concentration. Aliquots of the remaining extracts were pooled together to create a pooled QC sample for each assay. Two extraction blank samples were prepared by performing the sample extraction as described but with no biological sample present. To reconstitute the process blanks, biological samples and pooled QCs the samples were either reconstituted in 100µL of methanol / acetonitrile / water (37.5:37.5:25) for HILIC UPLC-MS or 100 µL of isopropanol / water (75:25) for the lipidomics UHPLC-MS assay. Samples were vortexed for 15mins, centrifuged at 18,000xg for 20mins at 4°C and 90µL of the supernatant was transferred to a low recovery vial. Samples were randomised for sample extraction to ensure no correlation with sample class.

***Cell-free bronchoalveolar lavage fluid (BAL):*** Normalisation of the BAL volume extracted was performed according to the plasma urea / BAL urea ratio determined for each sample. The normalized volume of each BAL sample was dried and then reconstituted in 100µL of methanol / acetonitrile / water (37.5:37.5:25) for HILIC UHPLC-MS assay or 100µL of isopropanol / water (75:25) for the lipidomics UPLC-MS assay. Samples were vortexed for 15s, centrifuged at 18,000xg for 20min, and 90µL of the supernatant was transferred to a low recovery vial for analysis. A pooled QC sample was created by taking a normalized volume (determined by the plasma urea / BAL urea ratio for each sample) of each BAL sample, the samples were mixed together to create a pooled QC sample, and a standard volume (90µL) aliquoted to create 19 QC samples. The QC samples were dried and then reconstituted in appropriate solvent for each assay as described for the BAL samples. Two extraction blank samples were prepared by performing the sample extraction as described but with no biological material added. Samples were randomised for sample extraction to ensure no correlation with sample class.

***Sample analysis***

Each biological sample, QC sample and blank sample were analysed applying four complementary UHPLC-MS assays; a HILIC assay in positive and negative ion modes to study water-soluble metabolites and a lipidomics assay in positive and negative ion modes to study lipids.

***HILIC assay:*** The samples (maintained at 4°C) were analysed applying a Vanquish binary pump H system coupled with a heated electrospray Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific, MA, USA). Sample extracts were analysed using an Accucore-150-Amide-HILIC column (100 x 2.1 mm, 2.6μm, Thermo Fisher Scientific, MA, USA). For positive ion mode mobile phase A was 95% acetonitrile/water (10mM ammonium formate, 0.1% formic acid) and mobile phase B was 50% acetonitrile/water (10 mM ammonium formate, 0.1% formic acid). For negative ion mode mobile phase A was 95% acetonitrile/water (10mM ammonium acetate, 0.1% acetic acid) and mobile phase B was 50% acetonitrile/water (10mM ammonium acetate, 0.1% acetic acid). The gradient elution applied was: t=0.0, 1% B; t=2.1, 1% B; t=4.6, 15% B; t=7.6, 50% B; t=10.1, 95% B; t=11.0, 95%B; t=11.5, 1%B, t=15.0, 1% B. All changes were linear (curve = 5) and the flow rate was 0.50 mL.min-1. Column temperature was 35°C and injection volume was 2μL. Data were acquired in positive and negative ionisation modes separately in the m/z range of 76 –1050 with a mass resolution of 120,000 (FWHM at *m/z* 200). Ion source parameters applied were: sheath gas = 40 arbitrary units, aux gas = 8 arbitrary units, sweep gas = 1 arbitrary units, spray voltage = 3.2kV (positive ion mode) and 2.7kV (negative ion mode), vaporizer temperature = 320°C and ion transfer tube temperature = 250 °C. All samples were collected as MS1 data in the profile mode applying scan time = 100ms, microscans = 1, RF lens = 50% and normalised AGC target=100%. For peak annotation purposes, MS/MS data were collected in the “Data dependent mode” setting for five QC samples analysed as injections 7-11 in each batch over different m/z ranges (76−210 *m/z*; 200−310 *m/z*; 300−410 *m/z*; 400-510 *m/z* and 510-1010 *m/z*) using stepped normalized collision energies of 20/50/130% (negative ion mode) and 20/40/100% (positive ion mode). MS/MS data were applied with the number of dependent scans = 3, a mass resolution of 15,000 (FWHM at *m/z* 200) and an isolation width = 3 *m/z*. Orbitrap Exploris 240 Tune application software controlled the instrument.

BAL samples were analysed first followed by cell sample extracts. Samples were randomised for data collection to ensure no correlation with sample class. All samples were analysed in one continuous batch for each assay. Each batch was started with 12 pooled QC samples with an extraction blank sample analysed between QC5 and QC6. A pooled QC sample was analysed after six biological samples. Two pooled QC samples and then an extraction blank sample were analysed at the end of the batch.

**Lipidomics assay:** The samples (maintained at 4°C) were analysed applying a Vanquish binary pump H system coupled with a heated electrospray Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific, MA, USA). Sample extracts were analysed using a Hypersil GOLD C18 column (100 x 2.1mm, 1.9μm; Thermo Fisher Scientific, MA, USA). For positive and negative ion modes mobile phase A was 60% acetonitrile/40% water (10 mM ammonium formate, 0.1% formic acid) and mobile phase B was 85.5% propan-2-ol /9.5% acetonitrile/5% water (10 mM ammonium formate, 0.1% formic acid). The gradient elution applied was: t=0.0, 20% B; t=1.5, 20% B; t=3.0, 25% B; t=9.2, 100% B; t=10.5, 100% B; t=12.5, 20% B; t=15.0, 20% B. All changes were linear (curve = 5) and the flow rate was 0.40mL.min-1. Column temperature was 55°C and injection volume was 2μL. Data were acquired in positive and negative ionisation modes separately in the m/z range of 150 –2000 with a mass resolution of 120,000 (FWHM at *m/z* 200). Ion source parameters applied were sheath gas = 40 arbitrary units, aux gas = 8 arbitrary units, sweep gas = 1 arbitrary units, spray voltage = 3.2kV (positive ion mode) and 2.7kV (negative ion mode), vaporizer temperature = 320°C and ion transfer tube temperature = 250°C. All samples were collected as MS1 data in the profile mode applying: scan time = 100ms, microscans = 1, RF lens = 60% and normalised AGC target=100%. For peak annotation purposes, MS/MS data were collected in the “Data dependent mode” setting on five QC samples analysed as injections 7-11 in each batch over different m/z ranges (150-500 *m/z*; 500-710 *m/z*; 700-860 *m/z*; 850-1010 *m/z* and 1000-2000 *m/z*) using stepped normalized collision energies of 20/50/130% (negative ion mode) and 20/40/100% (positive ion mode). MS/MS data were applied with the number of dependent scans = 3, a mass resolution of 15,000 (FWHM at *m/z* 200) and an isolation width = 3 *m/z*. Orbitrap Exploris 240 Tune application software controlled the instrument.

BAL samples were analysed first followed by cell sample extracts. Samples were randomised for data collection to ensure no correlation with sample class. All samples were analysed in one continuous batch for each assay. Each batch was started with 12 pooled QC samples with an extraction blank sample analysed between QC5 and QC6. A pooled QC sample was analysed after six biological samples. Two pooled QC samples and then an extraction blank sample were analysed at the end of the batch.

***Raw data processing***

Data files (in the .RAW file format) were converted to mzML file formats applying msConvert in Proteowizard (10). Data (mzML format) for each sample were processed applying the R package XCMS to construct a single data matrix for all samples (metabolite features as rows and samples as columns) for quality assessment and statistical analysis [2]. XCMS applied three steps. Step1: Peak detection (“findChromPeaks”) using the “centWave” algorithm was employed with parameter settings of m/z deviation = 25ppm, peakwidth = 5 ~ 20, snthresh = 10, prefilter = 3 ~ 100, mzCenterFun = "wMean") and mzdiff = 0.001. Step 2: Alignment (“adjustRtime”) was applied to perform retention time correction (alignment) between chromatograms of different samples using the Obiwarp method with parameters of binSize = 1, gapInit = 0.4) and gapExtend = 2.4. Step 3: Peak grouping (“groupChromPeaks”) was performed to group the chromatographic peaks within and between samples. The sample/replicate category group, such as “sample”, “QC” and “blank” was used as peak group information. Putative metabolite‬ annotation applying MS1 data was performed by applying the Python package‬ BEAMSpy (https://github.com/computational-

***Univariate analysis***

Univariate analysis was performed in the software MetaboAnalyst (12). For statistical analysis, data were normalized to total sample response and log10 transformed. Statistical analysis applied Student’s t-test. All p-values were corrected for multiple testing applying the Benjamini-Hochberg method. Fold changes were calculated using the mean of each class being studied.

**Cholesterol ester (CE) treatment of macrophage-like cells**

CE stock solution in chloroform was added to borosilicate glass tubes (Fisherbrand, North Rhine-Westphalia, Germany). Chloroform was evaporated under N₂ flow until forming dried film on the tube bottom. Then, lipids were resuspended in absolute ethanol at 40°C in the shaker at 700rpm for 30min in constant agitation. Once resuspended, the solution was immediately added to the serum free medium at final concentration of 30µg/mL.

THP-1 cells were differentiated into MLC, as described above. Then, cells were treated with either CE or vehicle (ethanol) for 48h. After treatment, supernatant was collected and centrifuged at 300xg for 5min at 21°C and stored at -20°C, for further IL-1β analysis by ELISA, as explained above.

**Statistical analysis**

GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Significant p-values are depicted as asterisk (\*p<0.05; \*\*p<0.01). Data are expressed as median. Statistical analysis was performed using a Mann-Whitney, when comparing 2 groups, and a Kruskal-Wallis nonparametric test, followed by Dunn`s multiple comparison (>2 groups). Significant p-values are depicted as asterisk (\*, p<0.05; \*\*, p<0.01).

**Supplementary results**

**Immunofluorescence of ASC specks in lungs of control and PPF/IPF patients**

Oligomerization of ASC into specks, as a readout for inflammasome activation, and its localization in macrophages as CD68+ cells, was investigated by immunofluorescence in lung slides of IPF and controls from patient cohort 2 (Supp Figure 1a). the absence of ASC speck in other cells and other regions of the lung was confirmed in Supp. Figure 2b. In addition, the quantification of ASC speck in CD68+ cells showed an increase in IPF patient samples when compared to healthy samples (supp figure 1c).

**Cholesterol content and lipidome alterations in aging SP-C-/- and WT mice**

Total cholesterol content was quantified in BALF cells of SP-C-/- mice (Suppl Figure 2a) showed a statistically significant and age dependent decrease. Total cholesterol in BALF (Supp Figure 2b) and BALF cells (Supp Figure 2c) showed no statistically significant changes with age in WT mice. Liquid chromatography/mass spectrometry-based untargeted metabolomic and lipidomic analyses were conducted in 12 BALF and 12 BALF cell samples of 30-week-old SP-C-/- and age- and sex-matched WT mice, to detect changes in water-soluble metabolites and lipids. Following data filtering and statistical analysis, a total of 561 (Suppl Figure 2e) and 120 (Suppl Figure 2f) metabolites in BALF cells and BALF, respectively, were present at statistically significant different levels in SP-C-/- compared to WT mice. Among the lipids at significant different level in BALF cells and BALF of SP-C -/-, the most represented annotated lipid classes were glycerophospholipids, although no differences were found in BALF levels of the main surfactant lipids, such as dipalmitoylphosphatidylcholine. Moreover, an overall increase of lipid species involved in the cytidine diphosphate-diacylglycerol pathway was assessed. Among the other lipids, major changes were found in triglycerides, cardiolipins, and ceramide and sphingolipid metabolism, with higher level in the BALF-cells of the SP-C-/- mice. Taken together, these findings indicate broad changes in the 30 weeks old SP-C-/- mouse BALF-cells lipidome.

**IL-18 levels**

IL-18 extracellular release in the BALFs of 10, 30, and 60 weeks old WT mice was detected via ELISA. Suppl Figure 2d shows statistically significant higher levels of IL-18 at the 60 weeks group, although the overall mean values were lower than what found for in BALF of SP-C-/- mice (Figure 2).

**Supplementary figures**

**Supp Figure** **1: Immunofluorescence of ASC specks in CD68+ cells in lungs of control and PPF/IPF patients and cell type annotation.** a-b) Representative micrographs of ASC oligomerization (white arrows) in CD68+ cells (red) in lung slides from control (N=5) and IPF (N=9) patients. Nuclei were stained with Hoechst (blue). All the micrographs were taken at the same magnification and reported with the same scale bar (scale bar = 20μm).   c) Quantification of ASC speck in CD68+ cells; statistical analysis according to Mann-Whitney test, \*=p<0.05. d) Dot plots displaying marker genes for lung cell type annotation of lung cell populations identified in the dataset from Adams et al 2020 (GSE136831). e) ASC WB blot densitometry and f) Pro-Caspase 1 (pro-Casp1) WB blot densitometry to β-actin as loading control in lung tissue from control (N=6) and PPF/IPF (N=6) patients.

**Supp Figure 2: Cholesterol content and lipidome alterations in SP-C****-/- compared to age-matched WT.** a) Total cholesterol content (µM) normalized by protein (µg) in BALF-cells of 10, 30, and 60 weeks old SP-C-/- mice (N≥6 mice/group). b) Total cholesterol normalized by phospholipid (PL) in BALFs of 10, 30, and 60 weeks old WT mice (N=6 mice/group). c) Total cholesterol content (µM) normalized by protein (µg) in BALF cells of 10, 30, and 60 weeks old WT mice (N=6 mice/group). d) IL-18 extracellular release assessed by ELISA in BALF samples of 10 (N=6), 30 (N=6) and 60 (N=5) weeks WT mice. Data are presented as median. The statistical test Kruskal-Wallis followed by Dunn`s multiple comparisons test. Significant p-values are depicted as asterisk (\*p<0.05; \*\*p<0.01). e-f) Pie diagrams showing the distribution of all metabolites at statistically significant different levels in e) BALF-cells and f) BALFs of 30 weeks old SP-C -/- mice compared to the WT. p values were adjusted for false discovery rate using the Benjamini-Hochberg correction, with an adjusted p-value <0.05 considered as statistically significant.

**Supp Figure 3: Flow cytometry analysis of lung leucocyte population.** a) gating strategy for the analysis of lung leukocytes in flow cytometry and b) gating strategy of ASC+ alveolar macrophages (AM) versus control (ctr) gate (only secondary antibody control).

**Supplementary references**

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