

Supplement

Methods

Generation of heat-inactivated *NTHi*

NTHi bacteria (3198-R strain) were grown overnight at 37°C in MaxQ 4450 Benchtop Orbital Shaker (Thermo Scientific) in Mueller-Hinton broth medium. Fresh broth medium was inoculated with the overnight culture and grown to OD₄₉₀ of 0.11. Subsequently, bacteria were washed twice with 10ml PBS and adjusted to OD₄₉₀ of 0.11 in PBS (equivalent to approximately 10⁹ colony forming units (CFU)/ml). Next *NTHi* were heat-inactivated by incubation at 80°C for 45min.

Enzyme-linked immunosorbent assay (ELISA)

SAEC secretions from apical and basolateral compartment of ALI cultures were collected and S100A8/A9 and BD2 protein was measured according to the manufacturers' instructions (S100A8/A9, S-1011, BMA BIOMEDICALS; BD2, EK-072-37, Phoenix Pharmaceuticals).

RNA isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR, qPCR)

Total RNA was extracted using RNeasy Plus Mini kit (Qiagen) according to the manufacturer's protocol and cDNA was generated by reverse transcription using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Quantitative (q)RT-PCR was performed using fluorogenic SYBR Green and the Light Cycler 480 detection system (Roche). PCR was performed using the primers: Interleukin 6 (*IL6*), assay ID 144013; *AXIN2*, assay ID 102998, secretory leukocyte peptidase inhibitor (*SLPI*), assay ID 108143; S100 calcium binding protein A7 (*S100A7*), assay ID 115051, S100 calcium binding protein A8 (*S100A8*), assay ID 105537; S100 calcium binding protein A9 (*S100A9*), assay ID 113434; Elafin (*PI3*), assay ID 103876; human beta defensin 1 (*DEFB1*), assay ID 114433, human beta defensin 2 (*DEFB4A*), assay ID 148831, Macrophage Receptor With Collagenous Structure (MARCO), (CD163), assay ID 114683, Interleukin 10 (IL10), assay ID 137154, High Affinity Immunoglobulin Gamma Fc Receptor I (FCGR1A), assay ID 103507, Low Affinity Immunoglobulin Gamma Fc Region Receptor II-A (FCGR2A), assay ID

113119, Toll Like Receptor 4 (TLR4) assay ID 149447 and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), assay ID 141139 (Roche) and Antigen Peptide Transporter 1 (TAP1), TAP_1, Antigen Peptide Transporter 2 (TAP2), TAP2_1, Toll Like Receptor 2 (TLR2), TLR2_1 from Sigma Aldrich. *GAPDH* was used as a reference gene.

Relative gene expression is presented as ΔC_p value ($\Delta C_p = C_p \text{ GAPDH} - C_p \text{ gene of interest}$). Relative change in transcript level upon treatment is expressed as $\Delta\Delta C_p$ value ($\Delta\Delta C_p = \Delta C_p \text{ of treated sample} - \Delta C_p \text{ of control}$).

Immunohistochemistry

Transwell cultures were fixed for 10min with ice-cold acetone-methanol (1:1) and the whole membrane was cut out for further processing. Following standard procedures, inserts were dehydrated and embedded in paraffin. Next 3 μ m thick sections were prepared and inserts were rehydrated with descending series of ethanol. Staining with haematoxylin and eosin (H&E) and alcian blue/PAS staining were prepared according to standard protocols. Images were acquired with the use of AxioCam MR3 (Zeiss).

For fluorescence staining the sections were incubated for 1h with primary antibody (E-cadherin, BD 610181, 1:200), followed by secondary antibody (Invitrogen A-11029, 1:1000) for 2h and DAPI for 10 min to visualize cell nuclei. Next, the sections were mounted with Fluoromount-G™ (Invitrogen, 00495802). Images were acquired with the use of LSM 710 (Zeiss).

Flow Cytometry

For live-dead staining cells were incubated with Pacific Orange (1:3000) for 10min at RT. For surface staining, cells were incubated with antibodies in 0.2% BSA-PBS for 10 min at 4°C. Following antibodies were used: CD14 (BD 564054, concentration 1:50), CD16 (BD 565421, 1:50), CD40 (BD 565258, 1:50), CD80 (BD 565210, 1:50), CD163 (BD 562643, 1:50), CD206 (BD 550889, concentration 1:50), CD64 (Biolegend 305042, 1:50), CD32 (BD 564838, 1:50), MARCO (Invitrogen 25-5447-42, 2.5:50), CD163 (BD 562643, 1:50), CD206 (BD 550889, 1:50), CD11C (Biolegend 337220, 0.5:50), CD40 (BD 565258, 1:50), CD80 (BD 565210, 1:50), CD38

(BD 562325, 2:50) In order to avoid Fc-receptor binding, stainings were performed in the presence of 2µg/mL αhFcγ-receptor. Subsequently, cells were analyzed using flow cytometer LSRII (BD Bioscience).

MSD assay

For the assessment of the protein levels in apical and basolateral supernatants V-PLEX ELISA as used according to the manufacturer's protocol (Cytokine Panel 1 (K15050D), Proinflammatory Panel 1 (K15049D).

WST-1 assay

MDMs were seeded in 96-well plates and treated with apical or basolateral SAEC SN for 24h. Metabolic activity was monitored using the WST-1 assay kit according to the manufacturer's protocol (Roche, 11644807001). Media was aspirated and 100µl of 10% WST-1 solution was added to each well and cells were incubated for 2h. Absorbance (wavelength 450nm) was measured using microplate reader (EnVision, PerkinElmer).

Phospho-kinase array

MDMs were treated with 5µg/ml of S100A8/A9 or vehicle for 30min. Equal amounts of cell lysates (300µg) were used to simultaneously detect the relative levels of phosphorylation of kinases according to the manufacturer's protocol (dot blot array ARY003C; R&D Systems). Phosphorylation was visualized using an ImageQuant LAS 4000 (GE Healthcare). A pixel density module in ImageJ was used to quantify phosphorylation levels of the proteins.

Seahorse analysis

One hundred MDMs per well were seeded into a Seahorse XF96 plate and left for 30min at room temperature. Then cells were stimulated with 5 µg/ml S100A8/A9 (triplicates) for 24 h. For analysis of glycolysis parameters, Seahorse assays were performed according to the

manufacturer's instructions of the Seahorse Bioscience XF cell glycolysis stress test kit (#103020–100). During the assay cells were treated with 5mM Glucose, 1μM Oligomycin and 50mM 2-Deoxyglucose. Density of the cells at the end of experiment was assessed with the use of CyQUANT® Direct Cell Proliferation Assay (Thermo Fisher C35011) and the glycolysis parameters were normalized to the cell density.

Transepithelial electric resistance (TEER) measurement

TEER measurement was performed on ALI cultures at the end of experiment. 150μl of PBS was added to the insert and TEER was measure with the Voltohmmeter.

Binding assay

To assess the binding of S100A8/A9 recombinant protein to MDMs, S100A8/A9 and control protein (soluble Thy1) was labelled with RPE (RPE Conjugation Kit, LNK024RPE, Bio-Rad). MDMs were incubated on ice with labelled proteins for 30 min. Afterwards cells were washed with PBS and centrifuged 3 times and the fresh media was added on top. Cells were scanned with IncuCyte and the RPE signal was measured. Values were normalized to the cell density (brightfield image).

Promoter analysis

In order to perform the analysis of S100A8 and S100A9 promoter for TCF/LEF (β-catenin) tool provided by alggen.lsi.upc.es was used.

GEO analyses

To reveal the most deregulated pathways between nonCOPD and nonCOPD small airway epithelial cells the GSE19407 dataset was also used.