Multi-omics links IL-6 trans-signalling with neutrophil extracellular trap formation and *Haemophilus* infection in COPD

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Online supplementary Methods and Material

Patient cohorts

Southampton cohort: All COPD patients characterized as previously described [1], were smokers with chronic productive cough and were classified according to GOLD (Global Initiative for Chronic Obstructive Lung Disease) criteria [2] into the following stages: stage 0 (n = 18), stage I (n = 7), and stages II to 4 (n = 13). All patients gave informed written consent and the study was approved by the Southampton University and General Hospitals ethics committee. The patients underwent bronchoscopy and bronchial brushings and provided samples of epithelial cells of greater than 95% purity. Transcriptomic data from brushings were obtained by using a microarray platform (Affymetrix, Santa Clara, CA). Nonlinear normalization and probe set reduction were used to obtain gene expression data [3].

BEAT-COPD cohort: Patients from BEAT-COPD were recruited from the Glenfield Hospital, Leicester, United Kingdom [4]. All BEAT-COPD patients fulfilled the inclusion criteria of age greater than 40 years, GOLD stage I–IV, and ≥ one exacerbation in the

preceding 12 months defined as the requirement of emergency health care. All patients gave informed written consent and the study was approved by the local ethics committee. COPD sputum samples were collected at stable state (defined as 8-weeks free from an exacerbation; Stable visit 1), exacerbation, and at recovery (6-weeks post-exacerbation visit; Stable visit 2).

For 16S rRNA gene sequencing, bacterial genomic DNA was extracted from sputum samples using the Qiagen DNA Mini kit (Qiagen) and the V3-V5 hypervariable regions of the 16S rRNA gene were amplified and pyrosequenced using the 454 Genome sequencer FLX platform (454 Life Sciences; Roche Diagnostics) to obtain microbiome communities. Sequencing reads were processed using QIIME (Quantitative Insights Into Microbial Ecology; Version 1.7) and stringent criteria were used to remove low-quality reads (e.g. reads <150 bp and >500 bp and reads with quality score <50), adaptors, chimeras and potential human sequences. Taxonomic classification, within sample (α- diversity) and between sample (β- diversity) microbiome measures were performed at normalized sequence read depth of 1666 and 97% sequence identity. A sample with a known microbial profile from previous analyses and molecular grade water were used as positive and negative reagent controls, respectively. To ensure that reagent-based contamination risk was minimized, a qPCR analysis of total 16S rRNA abundance was performed. Sequence data are deposited at the National Center for Biotechnology Information Sequence Read Archive (SRP065072).

Manchester cohort 1: 23 patients with COPD older than 40 years with postbronchodilator FEV1/forced vital capacity ratio of less than 0.7 and 10 or more pack-year smoking history were recruited [5]. All patients gave informed written consent and the study was approved by the Greater Manchester Ethics Committees. 9 patients were blood eosinophil low (<150 cells/μL) and 14 were blood eosinophil high (>250 cells/μL). Sputum and bronchial brushings were collected at stable state (defined as being 6 weeks free from an exacerbation). Inflammatory proteins in sputum were measured using a multiplex RBM panel (Myriad Rules

Based Medicine, Austin, Tex). Bronchial epithelial brushings were collected from the right and left lower lobes and lysed in RLT buffer (Qiagen, Manchester, UK). Total RNA was extracted from bronchial epithelial brushings using ZR RNA MicroPrep kit (Zymo Research, Orange, CA, USA) and RNA-seq libraries were prepared using TruSeq Stranded mRNA Prep kit (Illumina, San Diego, CA, USA) per manufacturers' protocols. To confirm RNA quality, RIN scores were evaluated using the 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA), with an acceptable cut-off criteria of ≥7.0. Paired-end sequencing (75 base pairs per end) with sequencing depth at 80 million reads was performed on the HiSeq2000 platform (Illumina, San Diego, CA, USA) to generate FASTQ files. These were aligned to human genome (version HG19) using HiSAT2 (John Hopkin's University, Baltimore, MA, USA) and SAMtools (Genome Research Limited, Cambridge, UK). Normalized read counts were generated per transcript using DESeq2.

The expression of the IL-6TS 8-gene epithelial signature in bronchial brushings (*TNFAIP6*, *PDE4B*, *IL1R2*, *S100A9*, *S100A8*, *S100A12*, *CHI3L1*, and *SPP1*) and the IL-6TS 5-protein sputum signature in sputum (IL-6, sIL-6R, MIP-1β, IL-1β, and IL-8) were combined into single score for each signature according to a previously described method [6]. First the biomarker (gene or protein) expression data was log2-transformed and a mean expression value for each biomarker was calculated. The expression levels were centred by subtraction of the mean value for each biomarker and scaled using the following equation: x'=x-min(x)/max(x)-min(x); where x' is the scaled (normalised) value and x is the centred value. The final scores are the mean of the scaled biomarker values for each patient, herein referred to as "8 gene-mean" and "5 protein-mean". Pearson's sample correlation coefficient was used to corelate the IL-6TS signature scores.

Manchester cohort 2: Biobanked bronchoalveolar lavage fluid (BALF) from COPD patients (n=29), healthy smokers (n=8) and healthy non-smoking subjects (n=27), who had previously been recruited for a mixture of research studies at the Medicines Evaluation Unit, Manchester, UK [7], were assessed for sIL-6R and NETosis markers. COPD patients had post-bronchodilator forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) ratio < 0.7 and a percent predicted FEV_1 of 40-70% (Gold II and III). Frequent exacerbating patients (n=13) required to have had ≥2 exacerbations in the previous 12 months, while infrequent exacerbating patients (n=16) required no exacerbations. Smokers and non-smokers had FEV_1/FVC ratios >0.7 and FEV_1 percent predicted of >80%. COPD patients and smokers had a smoking history of >10 pack years, while non-smokers had a smoking history of <1 pack year. COPD patients and smokers were all aged over 40 years, while non-smokers were aged over 28 years. All subjects had no history of asthma or other respiratory disorders (except COPD), were non-atopic as determined by skin prick test to house dust mite, grass pollen and cat dander and had not received antibiotics or suffered a respiratory illness in the preceding 6 weeks. Clinical features are summarised in Supplementary Table S1. For BALF collection, the bronchoscope was wedged in the bronchus and a maximum of 4 × 60 mL aliquots of pre-warmed sterile 0.9% NaCl solution were instilled into the right and/or left upper lobes. The aspirated BAL sample was stored on ice, and then filtered using a 100 μm filter (Becton Dickenson, UK). The filtrate was centrifuged (at 400 g for 10 min at 4°C) and the BALF sample was removed, and stored in aliquots at -80^{o}C for further analysis. Sample collection was approved by the local research ethics committee (NRES Committee North West – Greater Manchester South; REC Ref: 06/Q1403/156). All subjects provided written informed consent.

Supplementary Table S1:

Clinical characteristics of Manchester cohort 2.

	Healthy non-smokers (n=27)	Healthy smokers (n=8)	COPD (n=29)	Infrequent Exacerbators (n=16)	Frequent exacerbators (n=13)
Age (y)	48 ± 9	54 ± 8	64 ± 6*$^{/+}$	62 ± 6	66 ± 4$^{\$}$
Gender (m/f)	16/11	4/4	23/6	14/2	9/4
Current smokers (n)	0	8	12	7	5
Pack-years history	0	29 ± 16	44 ± 24	50 ± 29	36 ± 11
Post bronchodilator FEV_1 (% predicted)	105 ± 12	105 ± 11	59 ± 9.5*$^{/+}$	62 ± 8.3	54 ± 9.4$^{\$}$
Post bronchodilator FEV_1/FVC (%)	79 ± 4.6	78 ± 3.8	46 ± 8.1*$^{/+}$	49 ± 7.2	43 ± 8.3
ICS users (n)	0	0	23	11	12
BDP equivalent	0	0	1042 ± 848*$^{/+}$	772 ± 852	1415 ± 714$^{\$}$
Total SGRQ	NA	NA	39 ± 25	38 ± 25	36 ± 26
mMRC	NA	NA	1.2 ± 1.0	1.3 ± 0.9	1.1 ± 1.2
CAT	NA	NA	16 ± 11	16 ± 11	16 ± 11
Exacerbation rate in previous 12 months	0	0	0 (0-3)*$^{/+}$	0 (0 – 0)	2 (2 – 3)$^{\$}$

Data presented as %, mean ± standard deviation, or median (range). Comparisons between Healthy non-smokers, healthy smokers and COPD were by ANOVA and, if significant, with Bonferoni post-hoc test: Healthy non-smokers vs COPD: *$p<0.01$; healthy smokers vs COPD: $^{+}p<0.01$. Comparisons between Infrequent and frequent exacerbating COPD patients was by T-test or Fisher's exact test: $^{\$}p<0.05$. Abbreviations: BDP: beclomethasone dipropionate; CAT: COPD Assessment Test; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; ICS: inhaled corticosteroids; mMRC: modified Medical Research Council; SGRQ: St George's Respiratory Questionnaire.

PAD4 inhibitor

The peptidyl arginine deiminase 4 (PAD4) inhibitor is a novel covalent inhibitor which has been developed internally at AstraZeneca. The compound demonstrated protection against NETosis in the human neutrophil assay (IC50 = 0.9 µM, n = 21), as well as significant inhibition in the PAD4 AMI-MS enzyme assay (IC50 = 0.6 µM, n = 3). AMI-MS data towards other PADs shows that the inhibitor is >20-fold selective over PAD3 and >100-fold selective over PAD1 and PAD2.

Bacteria

Haemophilus influenzae (CCUG; 23946; Biotype IV [6107]; Pittman type b) was grown in brain heart infusion media (VWR) supplemented with 10 µg/mL beta-nicotinamide adenine dinucleotide and 10 µg/mL Hemin (Sigma-Aldrich) until the optical density reached 0.9 (~2 x 10^9 CFU/mL) at 600 nm. Bacterial numbers were confirmed by plate count and seeded at

different dilutions in culture medium together with primary human neutrophils or fresh human lung tissue.

Isolation of human neutrophils

Blood from healthy donors obtained from AstraZeneca blood donation program, were collected in vacutainer tubes with heparin (BD Bioscience) and mixed 1:1 with 2% dextran (Sigma). After sedimentation of erythrocytes, peripheral blood mononuclear cells (PBMCs) and granulocytes were separated by density gradient centrifugation in LymphoPrep (StemCell Technologies). Remaining erythrocytes were lysed in distilled water and the neutrophils were collected and resuspended in RPMI medium supplemented with 5 % fetal bovine serum (FBS, Gibco). Neutrophil purity was >95% as assessed by Sysmex XT 1800i (Sysmex Kobe Japan).

To further verify the purity and identity of the isolated neutrophils the cells were stained with antibodies against Myeloperoxidase (clone 2C7, ab25989; Abcam) followed by Goat anti-Mouse IgG (H+L, Alexa Fluor 633; Thermo Fisher Scientific) secondary antibodies. Fluorescence microscopy was performed using Carl Zeiss LSM 880 confocal microscope and images analysed by ZEN v.2.3 software.

NETosis (Full protocol)

Human blood neutrophils were plated (10^6 cells/mL) in Poly-D-lysin coated plates (Sigma, Greiner CELLCOAT®) and were left to sediment for 60 minutes at 37°C with 5% CO_2. Where described, cells were incubated with 5, 10 or 25 µM final PAD4i, or DMSO, 1 hour before addition of bacteria. Cells were then treated with *H. influenzae* ($1 \times 10^7 - 1 \times 10^8$ CFU/mL) for 3 hours or with 4 µM Ionomycin and 2 mM $CaCl_2$ for 1 hour. The levels of sIL-6R were measured in the cell-free supernatant with Human IL-6R alpha Quantikine ELISA Kit (R&D). The extracellular DNA associated with NETosis was measured by adding

the SYTOX Green Nucleic Acid Stain (Thermo Fisher Scientific), which is an cell impermeable DNA dye, to the live neutrophil culture at the time of *H. influenzae* or Ionomycin/CaCl2 challenge, and the green fluorescence signal was measured after the corresponding incubation times using PheraStar fluorescence plate reader (excitation 504/emission 523). To asses NETosis by the expression of citrullinated histone H3 (H3cit) the cells were fixed in 4% paraformaldehyde for 30 minutes, permeabilized with 0.25% Triton X-100 for 10 min and blocked in 1% BSA/2 % goat serum overnight at 4°C. Next, the cells were incubated for 90 minutes at room temperature with anti-citrullinated histone H3 (H3cit) antibody (ab5103; Abcam). The cells were then washed in PBS and stained with Goat anti-Rabbit IgG (H+L) secondary antibody (Alexa Fluor 568; Thermo Fisher Scientific) for 1 h. Nuclei and DNA (including extracellular DNA) were visualized by SYTOX Green Nucleic Acid Stain (Thermo Fisher Scientific). H3cit expression was analysed by ImageXpress reader micro XL and MetaXpress 5.1.0.41 software (Molecular Devices). The multi wavelength cell scoring was used for quantification of the number of H3cit-positive cells, in comparison to total number of cells. The cell IC50 of PAD4 inhibitor (PAD4i) was assessed using H3cit as target engagement marker in primary neutrophils stimulated with Ionomycin/$CaCl_2$. The above protocol was used with the following modification; the compound was diluted in a 10-point 1:2 dilution series, pre-incubated for 90 minutes with the neutrophils, which were then treated with 8 μM Ionomycin + 2mM $CaCl_2$ for 30 minutes. IC_{50} values were calculated using a four-parameter logistic fit. No cellular toxicity from PAD4i was observed using concentrations up to 25 μM.

Human lung tissue

Lung samples were from lung cancer patients undergoing resection of tumours and surrounding lung tissue at the Sahlgrenska University Hospital, Gothenburg, Sweden. Study and consent procedures were reviewed and approved by the Swedish Research Ethical

Committee in Gothenburg, Sweden (FEK 675-12/2012 and 1026-15, March 2016) in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained preoperatively. The explanted lung tissue, from areas distal to the tumour, was cut into 3 mm pieces, washed with PBS and plated in 96 well plates with X-VIVO^{TM}10, serum-free cell culture medium (Lonza) supplemented with and L-Glutamine (3 pieces/well). Fresh human lung tissue was treated with different concentrations of *H. influenzae* (1-200 x 10^8 CFU/mL) resuspended in culture medium and incubated for 24h at 37°C with 5% CO2. The same amount of sterile culture medium was used as control (0.0 CFU/mL). The cell culture supernatants were centrifuged at 2500 rpm for 5 min and sIL-6R levels were measured in the fluid fraction by Human IL-6R alpha Quantikine ELISA Kit (R&D). Three separate wells were analysed for each condition.

Supplementary references

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