Supplementary Material

Microbial and Host Immune Factors as Drivers of Chronic Obstructive Pulmonary Disease (COPD)

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Supplementary Methods

Supplementary methods to Study Design and Sample Collection

Controls (n=10) were participants with a bronchoscopy indicated for work-up of chronic cough, hemoptysis, or suspicion of foreign body aspiration, without evidence for an active inflammatory or infective process. In more details, the indications for bronchoscopies were the following: suspected tumor (20), endoscopic lung volume reduction (13), chronic cough (3), hemoptysis (2), unclear infiltrates (2), suspected foreign body (1). For tracheobronchial sampling, broncho-alveolar lavage and brushings were systematically performed on the contralateral side (i.e. away from a suspected tumor foreign body, or radiological finding). The final diagnoses retained for bronchoscopies other than lung volume reduction were: lung cancer (9), metastatic lung disease (2), non-active chronic inflammation (5), and no abnormal findings (12). In the more severe COPD 3/4 groups ELVR was the main indication in 13/16 patients with no potential interference by another lung disorder. Though we cannot exclude an effect of lung cancer on the microbiome and/or gene expression profiles, we have taken every possible precaution to minimize this bias by systematically sampling the uninvolved side in the lung. Also, evidence for an active infection or inflammation were exclusion criteria for participants in the study. Exclusion criteria were: pregnancy, severe comorbidities, active respiratory tract infection or antibiotic therapy in the previous month, systemic immunosuppression and chemotherapy, refractory severe respiratory insufficiency, and non-reversible anti-coagulation.

Supplementary methods to minimize contamination during bronchoscopies

Particular state-of-the-art care was taken to minimize contamination during bronchoscopies: First, all brushings were performed with one dedicated protected microbiology brush per

anatomical site (Conmed Microbiology Bruch, CON-130, Lasermed AG, Roggwil, Siwtzerland) and the bronchoscopist took specific caution to extrude an retract the brushes at a distance of several centimeters from the bronchoscope tip. Second, to reduce contamination of the working channel to a minimum, no suctioning was performed prior to wedging the bronchoscope in the segmental bronchus to perform broncho-alveolar lavage. However, negative control samples were also obtained by placing a tube containing 1.4 ml PBS with open lid in the bronchoscopy suite to investigate potential contamination.

Supplementary Methods to Sample Procedures and 16S *rRNA* Sequencing of the Microbiota

All samples were immediately placed on ice upon collection during bronchoscopy. Until further processing, samples were stored at -80°C. Samples were then thawed at room temperature and vortexed for subsequent DNA extraction. DNA was extracted directly out of the swab using 200 µl of PBS from the sample tube (QIAamp DNA Minikit, Qiagen, Hilden, Germany). PCR of the V3-V5 region of the *16S rRNA* gene was performed using primer pair 341F/907R. Primer sequences were as follows: 341F 5`-*CGTATCGCCTCCCTCGCGCCA*TCAGXXXXXXXXXX**ACTCCTACGGGAGGCAGCAG**-3`, and 907R 5`-*CTATGCGCCTTGCCAGCCCGC*TCAGXXXXXXXXXX**CCGTCAATTCMTTTGAGTTT**-3` where the adaptor sequences are italicized, the template-specific sequences are in bold, and the XXXXXXXXXX sequences describe the sample-specific multiplex-identifier barcode. For every patient a negative amplification control was included using PCR-grade double-distilled H_2O as template instead of DNA. PCR products were cleaned-up (Wizard SV Gel and PCR Clean-Up System, Promega, Madison, WI) and eluted in 40 µl of double-distilled H_2O. Concentration of the PCR products was measured by the Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). We additionally treated a subset of BAL samples (n=10)

with Propidium Monoazide (PMA, Biotium, Fremont, CA) in order to remove dead bacterial cells before DNA extraction and PCR. Briefly, dead bacterial cells release DNA, which intercalates with the photoreactive PMA. Upon light exposure, the DNA is permanently modified by PMA and is no longer accessible to the PCR polymerase (1). The protocol was as follows: 0.5 µl of PMA was added before DNA extraction to 200 µl of BAL. Samples were covered by tin foil and incubated at room temperature for five minutes on a rocker (300 rpm). Thereafter, samples were exposed for 15 minutes to a halogen light (distance: 20 cm), while keeping them on ice with a tin foil underneath to reflect the light. Dead DNA was then pelleted by centrifuging at 5000xG for ten minutes before subsequent DNA extraction. A paired analysis of the PMA-treated samples and the untreated samples from the same patients was performed. Microbiota sequencing was performed on the 454 GS FLX Titanium platform (Roche, Basel, Switzerland). Sequence reads were analyzed using the PyroTagger pipeline, which comprised quality filtering, removing of chimeras, the definition of 97% Operational Taxonomic Units (OTUs), and taxonomic assignment (2). The read length was set to 230 nucleotides, because a loss of quality scores was observed for subsequent nucleotides (3). Quality controls were as follows: Samples with a PCR product lower than 1.0 ng/µl, corresponding to less than 1 pg/µl bacterial DNA (4), which was recommended as the threshold when working with low-density samples (5), were excluded, as well as samples with less than 120 sequence reads, and samples with >10% relative abundance in common with the dominating OTU from one negative control sample that could get amplified and sequenced. Overall, after *16S rRNA* sequencing we excluded n=2 samples, which had <120 sequence reads and n=13 samples, which had >10% relative abundance in common with the dominating OTU (*Flavobacterium sp*.) of the negative control sample.

Species richness, Shannon Diversity Index (SDI), and beta-diversity were calculated in R version 3.3.0 (http://www.R-project.org) using the *vegan* package as described before (4, 6). In brief, the richness was calculated using the *specnumber*, and the SDI via the *diversity* function. Beta-diversity was calculated based on the Jaccard distances using non-metric multidimensional scaling (NMDS) as ordination method (function *vegdist* and *metaMDS* in R). Arrows indicating the clustering of samples according to the sampling site were received and were fitted using the envfit function of R. Note that even though the Jaccard index is by definition binary-based, vegan calculates either the binary-, or the abundance-based distances under the same name. The *adonis* function was used to calculate permutational MANOVA tests.

Supplementary Methods for Characterization of BAL Cell Gene Expression

Characterization of the BAL cell gene expression was described in detail by Bernasconi et al. (7). Briefly, cell pellets obtained after centrifugation of fresh BAL were snap frozen after cell lysis in RLT buffer (Qiagen, Hilden, Germany) and stored at minus 80°C until further processing. BAL cell lysates were transferred into a QIAshredder column (Qiagen) for homogenization, and total RNA was extracted using RNeasy Minikit (Qiagen) according to the manufacturer's instructions. RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) and reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Characterization of BAL cell gene expression profiles was based on multiplex real-time PCR analysis of 12 genes associated with innate immune cell function. Guanine nucleotide-binding protein, beta polypeptide 2-like 1 (GNB2L1) gene was employed as a reference gene. Custom oligonucleotide primers and probes were designed using NCBI primer blast software. Amplification was carried out using iQ Multiplex Powermix and a CFX96 Real-Time detection

system. Absolute quantification was performed using the CFX Manager Software 2.1 (all from Bio-Rad) based upon values obtained with a set of purified amplicons used as standards. Macrophages represent the predominant proportion of cells recovered by BAL, and thus, characterization of BAL cells gene expression is mainly a characterization of macrophage gene expression (8-10). Quantitative real-time PCR was performed of the following genes: Tumor necrosis factor alpha (TNF alpha), cyclooxygenase 2 (COX2), chemokine (C-X-C motif) ligand 11 (CXCL11), as genes involved in the inflammation process. As for genes involved in immunomodulation, indoleamine 2,3-dioxygenase 1 (IDO), interleukin 1 receptor antagonist (IL1RN), and interleukin 10 (IL10) were studied. And as for markers of remodeling and scavenging receptors, mannose receptor C type 1 (MRC1 or CD206), dendritic cell-specific intercellular adhesion molecules-3 grabbing non-integrin (DCSIGN or CD209), platelet-derived growth factor D (PDGFD), tissue inhibitor of metalloproteinase 1 (TIMP1), and matrix metallopeptidase 12 (MMP12) were analyzed.

Supplementary Methods for Univariate negative binomial Regression Model for the Correlation Analysis of Macrophage Gene Expression with the Abundance of Bacterial Families

In addition, in order to investigate whether the macrophage gene expression was associated with the abundance of bacterial families, regression analyses were performed. In detail, to investigate whether the data was normally distributed, the D`Agostino & Pearson normality test was performed in GraphPad Prism version 7.01 (GraphPad Software, La Jolla, CA).

Subsequently, as relative abundances of bacterial families were not normally distributed, we used a univariate negative binomial regression model, as described before (11). Regression models were performed using the *glm.nb* function from the *MASS* package in R. Outcome

variables were the different macrophage genes, and as explanatory variable the relative abundance of bacterial families in BAL samples with >0.5% mean relative abundance per BAL sample was used. The macrophage gene expression counts were naturally logarithmic transformed before regression analysis. Graphical representations were either performed in R or in GraphPad Prism.

Supplementary Results

Supplementary Results Propidium Monoazide (PMA) Pre-Teatment for the Removal of Dead Cells

A subset of BAL samples (n=10) was treated with PMA before DNA extraction and *16SrRNA* PCR for microbiota analysis, in order to remove DNA from dead bacterial cells. We found a significantly decreased bacterial density, as measured by the *16S rRNA* PCR concentration, in PMA-treated samples as compared to untreated samples (Supplementary Figure 1A; Mann-Whitney test; *P*=0.02). In contrast, we did not find a difference between the treated and the untreated samples in alpha-diversity (within-sample diversity). Analyzing the bacterial composition we only found a slight increase of Acidaminococcaceae in PMA-treated samples (data not shown; paired t-test; *P*=0.02). There was, however, no general difference in beta-diversity (between-sample diversity) between treated and untreated samples (Supplementary Figure 1B). All in all, these results indicated only a minor impact of dead bacterial cells on the final sequencing output in the underlying data.

Supplementary Results for the Standard Culture of BAL Samples

Regarding the clinical bacteriology by standard culture of BAL samples, we found four positive cultures in COPD GOLD2 patients (3x *Haemophilus influenzae* and one co-colonization of *Moraxella catarrhalis* and *Streptococcus pneumoniae*), one positive culture in a COPD GOLD3 patient (*M. catarrhalis*), and one positive culture in a COPD GOLD4 patient (*S. pneumoniae*) (Supplementary Table 1). No pathogens were isolated from non-COPD controls and COPD GOLD1 patients.

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Supplementary Tables

Table S1: Clinical Bacteriology in Comparison to Microbiota Analysis.

Indicated is the number of isolates, the identified species, and the relative abundance of the corresponding bacterial family in the same sample identified by sequencing the microbiota via the *16S rRNA* gene.

Nr.	Culture	Microbiota Family (relative abundance [%])
1	*H. influenzae*	Pasteurellacaeae (0.84)
2	*M. catarrhalis / S. pneumoniae*	Moraxellaceae (16.67) / Streptococcaceae (58.83)
3	*H. influenzae*	Pasteurellaceae (40.22)
4	*H. influenzae*	Pasteurellaceae (5.43)
5	*M. catarrhalis*	Moraxellaceae (98.45)
6	*S. pneumoniae*	Streptococcaceae (6.00)

Table S2. Univariate Negative Binomial Regression Analyses of Host Gene Expression and the Most Abundant Bacterial Families. Indicated are the inflammatory (TNF alpha, COX2, CXCL11), the immunoregulatory (IDO, IL1RN, IL10), and the remodeling and scavenging receptor (MRC1, DCSIGN, TIMP1, MMP12, PDGFD) markers of bronchoalveolar lavage (BAL) cells as outcome variables. Explanatory variables were the most abundant bacterial families of BAL samples (>0.5% mean relative abundance per sample). Shown are the estimated values (Est.), the standard error (Std.E), and the *P*-value (*P*). Significant associations are indicated in bold.

	Acidaminococcaceae			Flavobacteriaceae			Fusobacteriaceae			Moraxellaceae			Neisseriaceae		
	Est.	Std.E	*P*	Est.	Std.E	*P*	Est.	Std.E	*P*	Est.	Std.E	*P*	Est.	Std.E	*P*
COX2	0.0001	0.009	0.99	-0.05	0.05	0.35	0.02	0.03	0.50	0.0001	0.004	0.96	0.009	0.01	0.46
TNF alpha	-0.001	0.009	0.91	-0.02	0.04	0.68	0.003	0.03	0.92	0.002	0.003	0.52	0.002	0.02	0.91
CXCL11	-0.004	0.008	0.64	-0.001	0.03	0.98	0.0002	0.02	0.99	0.002	0.003	0.55	0.004	0.01	0.75
IDO	-0.002	0.009	0.82	-0.005	0.04	0.90	-0.0003	0.03	0.99	0.002	0.003	0.60	0.002	0.02	0.89
IL1RN	0.0003	0.01	0.98	0.0006	0.05	0.99	-0.0009	0.03	0.98	0.0009	0.004	0.82	-0.002	0.01	0.90
IL10	-0.002	0.009	0.80	-0.006	0.04	0.88	-0.002	0.03	0.94	0.002	0.003	0.60	0.001	0.02	0.93
MRC1	0.002	0.008	0.79	0.001	0.03	0.98	0.02	0.02	0.49	-0.001	0.01	0.74	-0.003	0.01	0.83
DCSIGN	-0.002	0.009	0.80	0.01	0.04	0.76	0.01	0.03	0.74	-0.001	0.004	0.76	-0.005	0.02	0.79
TIMP1	-0.0002	0.008	0.98	-0.009	0.04	0.81	0.007	0.03	0.79	0.0005	0.003	0.86	-0.0003	0.01	0.98
MMP12	0.0004	0.009	0.96	-0.03	0.05	0.58	-0.02	0.03	0.63	0.0004	0.004	0.91	-0.01	0.02	0.49
PDGFD	-0.001	0.01	0.89	0.07	0.03	**0.03**	-0.008	0.03	0.81	-0.01	0.01	0.17	-0.03	0.02	0.10

Table S2. Continuation.

	Pasteurellaceae			Porphyromonadaceae			Prevotellaceae			Streptococcaceae			Aerococcaceae		
	Est.	Std.E	*P*	Est.	Std.E	*P*	Est.	Std.E	*P*	Est.	Std.E	*P*	Est	Std.E	*P*
COX2	0.0008	0.008	0.92	-0.002	0.01	0.84	0.0006	0.004	0.87	-0.002	0.004	0.58	-0.08	0.09	0.37
TNF alpha	0.0002	0.007	0.97	-0.002	0.01	0.86	-0.0003	0.004	0.94	-0.002	0.003	0.64	-0.02	0.08	0.84
CXCL11	-0.002	0.006	0.77	0.004	0.008	0.59	-0.002	0.003	0.61	-0.0004	0.003	0.90	-0.01	0.07	0.85
IDO	-0.002	0.008	0.85	0.004	0.01	0.71	-0.001	0.004	0.74	-0.0005	0.003	0.88	-0.03	0.09	0.73
IL1RN	0.0005	0.009	0.96	-0.0001	0.01	0.99	-0.0006	0.004	0.89	0.0007	0.004	0.86	0.004	0.09	0.97
IL10	0.0004	0.0008	0.96	0.004	0.01	0.66	-0.002	0.004	0.64	<0.0001	0.003	0.99	-0.02	0.09	0.81
MRC1	0.0004	0.006	0.95	-0.01	0.01	0.35	0.002	0.003	0.58	-0.001	0.003	0.67	0.02	0.07	0.81
DCSIGN	<0.0001	0.008	1.00	-0.02	0.02	0.20	0.0002	0.004	0.96	0.001	0.004	0.69	0.02	0.09	0.82
TIMP1	0.001	0.007	0.94	-0.003	0.01	0.76	0.0007	0.004	0.84	-0.0009	0.003	0.77	0.01	0.08	0.90
MMP12	0.003	0.008	0.65	0.0004	0.01	0.97	0.0002	0.004	0.96	0.0006	0.003	0.87	-0.01	0.09	0.88
PDGFD	0.007	0.008	0.39	-0.002	0.01	0.85	-0.002	0.005	0.69	0.003	0.004	0.39	0.10	0.09	0.27

Table S2. Continuation.

	Eubacteriaceae			Staphylococaceae			Cellulomonadaceae			Others		
	Est.	Std.E	*P*	Est.	Std.E	*P*	Est.	Std.E	*P*	Est.	Std.E	*P*
COX2	-0.04	0.16	0.81	0.03	0.09	0.73	0.0002	0.005	0.96	0.004	0.009	0.64
TNF alpha	-0.02	0.14	0.91	0.01	0.09	0.88	0.11	0.48	0.83	0.0002	0.008	0.98
CXCL11	-0.04	0.12	0.73	0.03	0.07	0.71	0.10	0.42	0.81	0.001	0.007	0.86
IDO	-0.05	0.16	0.76	0.02	0.09	0.85	0.12	0.50	0.81	<0.0001	0.009	1.00
IL1RN	-0.04	0.17	0.82	0.005	0.10	0.97	-0.002	0.006	0.72	0.0002	0.01	0.98
IL10	-0.05	0.16	0.76	0.02	0.09	0.84	0.05	0.51	0.93	-0.003	0.009	0.74
MRC1	0.006	0.12	0.96	0.04	0.07	0.63	0.14	0.42	0.73	0.006	0.006	0.37
DCSIGN	-0.03	0.17	0.86	0.04	0.09	0.64	0.02	0.55	0.97	0.008	0.009	0.37
TIMP1	-0.002	0.14	0.99	0.006	0.08	0.94	0.09	0.45	0.84	0.0003	0.008	0.97
MMP12	-0.05	0.17	0.78	-0.02	0.10	0.86	-0.08	0.55	0.89	-0.007	0.009	0.49
PDGFD	0.12	0.18	0.51	-0.10	0.11	0.36	-2.80	3.00	0.35	-0.002	0.01	0.87

FIGURE LEGENDS

Figure S1: PMA Treatment. A subset of BAL samples (n=10) was treated with Propidium Monoazide (PMA) in order to remove dead bacterial cells before DNA extraction and *16S rRNA* PCR. PMA-treated and untreated samples were pairwise compared. (A) Overall bacterial density, as measured by the concentration of the *16S rRNA* PCR product [ng/µl], was significantly decreased in PMA-treated samples (Mann-Whitney test; *P*=0.02). (B) Non-metric multidimensional scaling (NMDS) of PMA-treated (circles) and untreated BAL samples (triangles). NMDS based on the weighted Jaccard dissimilarity of the relative abundance of OTUs. Colors (n=10) represent the different sample pairs (n=10) of treated and untreated samples. Permutational MANOVA (*adonis*) revealed no difference between treated and untreated samples.

Figure S2: Alpha-Diversity values between groups and sampling sites. Means and 95% Confidence Intervals (CIs) indicated. **A)** Alpha diversity as measured by the sample richness. There were no significant differences between the groups or sampling sites. **B)** Alpha-diversity as measured by the Shannon Diversity Index (SDI). Controls had significantly increased SDI as compared to COPD GOLD3/GOLD4 in the pharynx (*P*=0.01), trachea (*P*=0.02), and BAL (*P*<0.0001), as calculated by 2way ANOVAs. COPD GOLD1/GOLD2 patients had significantly increased SDI as compared to COPD GOLD3/GOLD4 in the pharynx (*P*=0.01) and BAL (*P*=0.0004), as calculated by 2way ANOVAs.

Figure S3: Gene Expression in BAL cells. Inflammatory (A-C), immunomodulatory (D-F), and remodeling/scavenging receptor (G-K) markers are shown. Non-COPD controls had an increased expression of MRC1 and DCSIGN as compared to COPD GOLD3/GOLD4 patients (Mann-Whitney tests; *P*=0.003 and *P*=0.04). COPD GOLD1/GOLD2 patients had also an

increased expression of MRC1 and DCSIGN (Mann-Whitney tests; *P*=0.001 and *P*=0.0003). COPD GOLD3/GOLD4 patients had a decreased expression of TNF alpha as compared to COPD GOLD1/GOLD2 (Mann-Whitney test; *P*=0.04), but increased expression of CXCL11, IDO, and IL10 as compared to non-COPD controls (Mann-Whitney tests; *P*=0.01, *P*=0.03, and *P*=0.003, respectively), as well as increased expression of IL10 as compared to COPD GOLD1/GOLD2 patients (Mann-Whitney test; *P*=0.03).

Figure S4: Correlation of the Values of the Expression of BAL Cell Markers with Alpha-Diversity Measurements SDI (Shannon Diversity Index) in black and S (Richness) in grey. Illustrated are all cell markers which were investigated in this study. *P*-values were derived from Pearson correlation and are indicated if significant (*P*<0.05). The linear regressions (lines) with 95% confidence intervals (dots) are indicated for SDI (black) and S (grey).

Figure S5: Univariate Negative Binomial Regression Analyses of Host Gene Expression and the Bacterial Family Flavobacteriaceae. Indicated are the inflammatory (TNF alpha, COX2, CXCL11), the immunoregulatory (IDO, IL1RN, IL10), and the remodeling and scavenging receptor (MRC1, DCSIGN, TIMP1, MMP12, PDGFD) markers of bronchoalveolar lavage (BAL) cells as outcome variables. Shown are the estimated values (Est.), the standard error (Std.E), and the *P*-value (*P*). The significant association is indicated with an asterisk.