COPD lungs show an attached stratified mucus layer that separate bacteria from the epithelial cells resembling the protective colonic mucus

SUPPLEMENTARY TABLES AND FIGURES

Tables S1 – S8, page 1 and separate Excel files

Figure Legends S1 – S9, pages 2 – 5

Figures S1 – S9, pages 6 - 13

Supplementary Tables are given as separate Excel files.

Table S1. Patients from whom BALF samples were collected.

Table S2. Mass spectrometry based proteomics of BALF obtained from human never smoker (n=5) asymptomatic smokers (n=12) and COPD (n=42).

Table S3. Mass spectrometry based absolute quantification of MUC5AC, MUC5B, AGR2, CLCA1, DMBT1, FCGBP, MSLN, MUC1, MUC2, PSCA, TFF3, TGM2 and ZG16B in BALF obtained from human never smoker (n=5) asymptomatic smokers (n=12) and COPD (n=42).

Table S4. Mass spectrometry based proteomics of whole BALF and BALF supernatants (SN) after centrifugation obtained from saline- and PPE-exposed mice.

Table S5. Mass spectrometry based absolute quantification of Muc5ac and Muc5b in whole BALF, BALF supernatants after centrifugation (SN), mucus plugs, and isolated epithelial cells obtained from saline- and PPE-exposed mice.

Table S6. Mass spectrometry based proteomics of isolated airway mucus plugs as stained by Alcian blue and obtained from PPE-exposed mice.

Table S7. Mass spectrometry based proteomics of isolated airway epithelial cells obtained from saline- and PPE-exposed mice.

Table S8. Isotopically labelled peptides used for absolute quantification of human and mouse proteins.
Figure S1. **Heat map of the BALF proteome.** The localization of some mucus proteins discussed is marked. Never smokers (n=5), asymptomatic smokers (n=12) and chronic obstructive pulmonary disease (COPD) patients (n=42).

Figure S2. **Heat map of proteins absolutely quantified with isotopically labelled peptides by mass spectrometry in BALF.** Never smokers (n=5), asymptomatic smokers (n=12) and chronic obstructive pulmonary disease (COPD) patients (n=42).

Figure S3. **The enzymatic activity of PPE was inactivated by PMSF.** (A) Graph showing fluorescence measured every 5 min for 1 h by cleavage of the BODIPY® casein substrate as a measure of serine peptidase activity (EnzChek Protease Assay Kit; Invitrogen). Enzymatic activity of PPE was evaluated before and after incubation with 1 mM PMSF overnight at 4°C. PMSF fully inhibited PPE peptidase activity, as the fluorescence signal was equivalent to that measured for a 0.9% saline solution, used as a blank. Results shown are representative of two experiments. Triplicate wells were used for each sample. Mean ± SEM. Two-way repeated measures ANOVA and Tukey’s post-test. **** P<0.0001 vs. active PPE. (B and C) Low and high magnification images of lungs from mice exposed to inactivated PPE. Scale bar in C, 100 μm.

Figure S4. **The distribution of airway mucus obstruction induced by PPE in mice is related to the diameter of the airways.** (A) Image showing a transverse section of an airway and the representation of its minimum Feret’s diameter (XFmin), defined as the shortest distance between any two parallel tangents on the airway.
Scale bar, 50 μm. (B) The XFmin was assessed for each airway section classified depending on its level of obstruction in mice exposed to PPE. Individual data, medians and interquartile ranges. P < 0.05*, P < 0.01** and P < 0.0001**** vs. the 10-30 % obstruction range, Kruskal-Wallis with Dunn´s post hoc test. (C) The same data as in B, but the y-axis was cut at 50 μm to illustrate airways smaller than 50 μm in diameter. (D) The correlation between XFmin and obstruction levels was studied both including all airway sections \([r_{(1)}]\) or only airway sections with a percentage of obstruction higher than 10 % \([r_{(2)}]\). Two-tailed Spearman’s correlation. Each dot represents an airway section. n=591 airway sections from 9 animals. (E) The same data as in D but the y-axis was cut at 50 μm to illustrate airways smaller than 50 μm in diameter.

**Figure S5. Intranasal PPE induced lung inflammation and structural damage.**

(A) Transmission electron micrographs showing secretory cells, which in PPE-exposed mice contained electron lucent vesicles and released their content to the airway lumen. Representative of 3 animals/group. Scale bars, 2 μm. (B) Fomaldehyde fixed paraffin sections stained with H&E revealed alveolar breakdown could be observed in mice challenged with PPE compared with controls instilled with saline. (A and B) Representative of 4-5 animals/group. Scale bars, 100 μm. (C) Comparison of cytokine and chemokine levels in BALF from vehicle and PPE-exposed mice, n = 9-17 animals/group, data presented as median ± interquartile range, IL-1β \(P = 0.001**\), IL-4 \(P < 0.0001****\), IL-5 \(P < 0.0001****\), IL-6 \(P = 0.02*\), KC, TNFα, TARC and MDC \(P < 0.0001****\), EGF \(P = 0.01**\), Mann-Whitney U test. KC: Keratinocyte-derived chemokine; TARC: Thymus and activation-regulated chemokine; MDC: Macrophage-derived chemokine.
Figure S6. Characterization of isolated mouse airway epithelial cells. (A) Initial percentage of epithelial cells (EpCAM+), leukocytes (CD45+) and neutrophils (Ly-6G+) in a pooled cellular suspension recovered from three mice induced by intranasal administration of LPS. (B and C) Percentage of CD45+ cells and Ly-6G+ cells recovered by magnetic beads and by this depleted from the initial cellular suspension. (D) Purity of the recovered epithelial cellular suspension after the two negative selections removal of CD45+ and Ly-6G+ cells by magnetic beads.

Figure S7. Immunostainings confirm increased production of mucus-related proteins. (A – D) Representative low magnification images of lung sections from vehicle (Saline)- and PPE- exposed mice stained with Muc5b (red; A), Muc5ac (red; B), Clca1 (red; C) and Fcgbp (red; D). Dot plots graphs quantifying the fluorescence intensity of proteins stain expressed as percentage of the mean intensity in saline-exposed mice (taken as 100%). n = 9 animals/group, data presented as median ± interquartile range, (A) P = 0.0002***, (B) P < 0.0001****, (C) P < 0.0001****, (D) P = 0.001**, Mann-Whitney U test. Scale bars, 500 µm. (E) Confocal microscope images showing the colocalization of Muc5ac and Muc5b in the airways after administration of saline (left) or PPE (right). Representative of 4 mice/group. Scale bars, 50 µm. Nuclei are stained blue.

Figure S8. Mucus-related proteins overexpression induced by PPE in mice depends on the proteolytic activity of PPE. (A–F) Representative low magnification images of lung sections from mice exposed to PPE inactivated by
PMSF. Sections were stained for Muc5b (red; A), Muc5ac (red; B), Clca1 (red; C) and Fcgbp (red; D). Scale bars, 500 µm. Confocal microscope high magnification images showing the overproduction of Clca1 (E) and Fcgbp (F) by airway epithelial cells and their accumulation in mucus plugs after PPE intranasal instillation. Scale bars, 50 µm. (A-F) Representative of 4-9 animals/group. Nuclei are stained blue.

Figure S9. Immunostaining by Muc5b of lungs of control mice and by Muc5ac on PPE-treated mice followed by treatment with saline or hypertonic saline and the instillation of Pseudomonas bacterial. A) Immunostaining of Muc5b mucin (green) in non-treated animals. Staining of goblet cells is shown, but no mucus layer. Nuclei stained blue. Representative of 3 mice, scale 30 µm. B) PPE-exposed lungs were instilled with P. aeruginosa, BAL was collected after 10 min by instilling 0.8 ml PBS and aspirating it back twice, then the lungs were isolated, fixed in Carnoy and immunostained for Muc5ac (red), P. aeruginosa (white), and nuclei (blue). Bacteria are trapped by the mucus and was not removed by washing with PBS. Representative of 3 mice, scale 30 µm C) PPE-exposed lungs were instilled with P. aeruginosa, BAL was collected after 10 min by instilling for two times 0.8 ml hypertonic saline (7%) and aspirating it back after 20 min. Then, the lungs were isolated, fixed in Carnoy and immunostained for Muc5ac (red), P. aeruginosa (white), and nuclei (blue). The bacteria are trapped in the stagnated adherent lung mucus that persisted after treatment with 7% hypertonic saline. Representative of 4 mice, scale 30 µm.
Figure S2

Fmo/uL, Log10 scale

0.01 10 100

TFF3  TGFβ  MSLN  FCGBP  ZG16B  MUC1  PSCA  MUC5B  DMBT1  MUC5AC  AGR2

TFF3  TGFβ  MSLN  FCGBP  ZG16B  MUC1  PSCA  MUC5B  DMBT1  MUC5AC  AGR2

COPD 5_34  COPD 5_16  COPD 5_21  COPD 5_19  COPD 5_08  COPD 5_23  COPD 5_21  COPD 5_30  COPD 1_14  COPD 5_20  COPD 5_15  COPD 5_31  COPD 5_17  COPD 5_05  COPD 5_37  COPD 5_18  COPD 5_12  COPD 5_25  COPD 5_11  COPD 5_26  COPD 5_03  COPD 1_13  COPD 5_13  COPD 1_13  COPD 5_04  COPD 1_20  COPD 5_18  COPD 1_18  COPD 5_35  COPD 5_26  COPD 5_19  COPD 1_19  COPD 5_33  COPD 1_17  COPD 5_32  COPD 5_10  COPD 5_22  COPD 5_01  COPD 5_04  COPD 1_30  COPD 5_24  COPD 1_14  COPD 5_12  COPD 5_07  COPD 1_11  COPD 5_28  COPD 1_10  COPD 5_38  COPD 1_0  COPD 5_36  COPD 5_29  COPD 5_06  COPD 1_16  COPD 5_27  COPD 1_06  COPD 5_18  COPD 1_04  COPD 5_02  COPD 5_09  COPD 1_09  COPD 5_07  COPD 1_15  COPD 5_01  COPD 1_07  COPD 1_03  COPD 1_05  COPD 1_02  COPD 1_08  COPD 1_01

Asymptomatic smoker 5_09  COPD 1_04  Asymptomatic smoker 5_02  COPD 1_09  COPD 1_15  COPD 1_07  Never smoker 1_03  Never smoker 1_05  Never smoker 1_02  Asymptomatic smoker 1_08  Never smoker 1_01

Asymptomatic smoker 5_33  COPD 1_17  Asymptomatic smoker 5_32  Asymptomatic smoker 5_10  Asymptomatic smoker 5_22  Asymptomatic smoker 5_01  COPD 5_24  COPD 1_14  COPD 1_12  COPD 5_07  COPD 1_11  Asymptomatic smoker 5_28  COPD 1_10  Asymptomatic smoker 5_38  COPD 5_36  COPD 5_29  COPD 5_06  COPD 1_16  COPD 5_27  Asymptomatic smoker 1_06  Never smoker 1_04  Asymptomatic smoker 5_02  COPD 1_15  Asymptomatic smoker 1_09  COPD 1_15  Asymptomatic smoker 1_07  Never smoker 1_03  Never smoker 1_05  Never smoker 1_02  Asymptomatic smoker 1_08  Never smoker 1_01

Never smoker 1_04  Asymptomatic smoker 5_02  COPD 1_15  Asymptomatic smoker 1_09  COPD 1_15  Asymptomatic smoker 1_07  Never smoker 1_03  Never smoker 1_05  Never smoker 1_02  Asymptomatic smoker 1_08  Never smoker 1_01

Never smoker 1_04  Asymptomatic smoker 5_02  COPD 1_15  Asymptomatic smoker 1_09  COPD 1_15  Asymptomatic smoker 1_07  Never smoker 1_03  Never smoker 1_05  Never smoker 1_02  Asymptomatic smoker 1_08  Never smoker 1_01

Never smoker 1_04  Asymptomatic smoker 5_02  COPD 1_15  Asymptomatic smoker 1_09  COPD 1_15  Asymptomatic smoker 1_07  Never smoker 1_03  Never smoker 1_05  Never smoker 1_02  Asymptomatic smoker 1_08  Never smoker 1_01

Never smoker 1_04  Asymptomatic smoker 5_02  COPD 1_15  Asymptomatic smoker 1_09  COPD 1_15  Asymptomatic smoker 1_07  Never smoker 1_03  Never smoker 1_05  Never smoker 1_02  Asymptomatic smoker 1_08  Never smoker 1_01

Figure S2
Figure S3

A

Fluorescence at 30 min

0 15 30 45 60

Time (min)

Active PPE

Inactivated PPE

Blank

B

C

Histological images
Figure S4

A

B

C

D

E

r_1 = 0.2173
P_1 < 0.0001

r_2 = 0.3517
P_2 < 0.0001
Figure S5

A

Saline

PPE

B

Saline

PPE

C

pg/ml in BALF

pg/ml in BALF

IL-1β  IL-2  IL-4  IL-5  IL-6  KC  IL-10  IL-12  TNFα (p70)

Saline  PPE

IL-9  IL-13  IL-17  TARC  MDC

Saline  PPE

EGF
Figure S6

A Airway cellular suspension before depletion of immune cells

B Cellular fraction CD45⁺

C Cellular fraction Ly-6G⁺

D Recovered fraction Ly-6G⁻ CD45⁻
Figure S7
Figure S8
Figure S9

A

Muc5b  Nuclei

B

C

Muc5ac  Nuclei  Pseudomonas