**ONLINE SUPPLEMENT**

# CCSP counterbalances airway epithelial-driven neutrophilic chemotaxis

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Supplementary Methods

*Chemotaxis assay and analysis*

The chemotaxis assays were performed using Dunn chambers [24]. Peripheral neutrophils were placed on a coverslip and incubated for 20 min at 37°C. The coverslip was placed inverted onto the Dunn chamber. This slide comprises a bridge separating inner and outer concentric rings. The outer circle was filled with the studied solution. Subsequently, neutrophil chemotaxis along the chemoattractant gradient was observed on the bridge. Healthy neutrophil chemotaxis phenomena were studied using a negative control (PBS), a positive control (fMLP), and cell supernatants with or without previous stimulation, or with drugs alone. One part of the bridge region was filmed and snapshots were acquired at 1-min intervals during 30 min using a Zeiss inverted microscope equipped with a 20x objective. Chemotaxis was quantified using the XFMI value, which is the ratio of the distance travelled during the acquisition and the final position of the neutrophils on the x-axis in the direction of the chemoattractant gradient.

ImageJ software was used to track cells and analyse chemotaxis. All analyses were performed by a single analyst, blinded to the subject group.

*Cell culture*

Human primary bronchial epithelial cells (HBECs) were cultured under Air-Liquid Interface (ALI) conditions, adapted from Gras et al. and Gamez et al. [17, 22]. Bronchial epithelial cells from 4 control subjects, 10 smokers, and 13 COPD patients were mechanically dissociated and suspended in Bronchial Epithelial Growth Medium (BEGM). After seeding in multiwell plates coated with a solution of fibronectin and collagen, cells were expanded in a flask (0.75 cm2) and then plated (250,000 cells per well) on uncoated nucleopore membranes in a 1:1 mixture of BEGM and Dulbecco Modified Eagle Medium (DMEM) applied at the basal side only to establish the ALI. Cells were maintained in culture for 28 days to obtain a differentiated cell

population with a pseudostratified mucociliary epithelium. Cell cultures were maintained at 37°C under 5% CO2. Cells were treated for 24 hours with or without cigarette smoke extract (CSE) and with or without CCSP supplementation (at the physiological concentration of 3µg/ml according to Gamez et al., Chest, 2015), applied at the apical surface. The supernatants were collected and stored until used for neutrophil experiments and CCSP/IL8 ELISA test (Biovendor/Diaclone).

*Reagents and antibodies*

Recombinant human (rh)CCSP and anti-CCSP antibody were purchased from Biovendor (Brno, Czech Republic). rhIL8 and fMLP were purchased from Sigma-Aldrich. Anti-IL8 antibody was purchased from Santa Cruz. Anti-CXCR1 antibody was purchased from R&D systems and CXCR2 inhibitor from Tocris.

*Co-immunoprecipitation*

To detect whether a potential interaction exists between CCSP and IL8, co-immunoprecipitation and immunoblotting were performed using anti-Protein A Sepharose 4 Fast Flow magnetic beads (GE Healthcare). Briefly, mouse anti-human IL8 antibodies were used to immunoprecipitate rhIL8 and rhCCSP. If rhIL8/rhCCSP bound covalently, a complex will be formed and will attach to anti-IL8 antibodies. To confirm our results, rabbit anti-human CCSP antibodies were also used to immunoprecipitate the rh proteins. The immune complexes were incubated with anti-Protein A Sepharose magnetic beads and captured on a magnetic beaded support to immobilize them. Any proteins not precipitated on the beads were washed away. Finally, the complexes were recovered, denatured and analysed by SDS-PAGE gel electrophoresis and Western blotting. The amount of rhIL8/rhCCSP that co-immunoprecipitated with anti-IL8 antibodies was determined from immunoblots developed with anti-CCSP antibodies (1:500). Inversely, the amount of rhIL8/rhCCSP that co-

immunoprecipitated with anti-CCSP antibodies was determined from immunoblots developed with anti-IL8 antibodies (1:500). Rh proteins without antibody were used as a negative control. A solution of rhCCSP/rhIL8 was used as a positive control for CCSP and IL8 expressions. All immunoblots were developed and quantified by using the Odyssey Infrared Imaging System (LICOR Biosystems) and infrared-labelled secondary antibodies.

*Surgical lung samples and induced sputum collection*

10 COPD patients scheduled for lung removal because of small peripheral lung lesions consented to participate in this study (AOI 2000, CPP 000901, DGS 2001/0075). All adequate samples were collected 3 to 5 days before surgery according to international guidelines and processed for analyses (Djukanovic et al., Eur Respir J Suppl , 2002). Before sputum induction, all subjects underwent spirometry before and 10 min after inhalation of 200 µg salbutamol by metered-dose inhaler. Hypertonic saline was nebulised with an ultrasonic nebuliser (DP 100 Syst'am; Paris, France). This nebuliser generates particles with a mean mass aerodynamic diameter of 4.5 µm and has an output of 2.4 $mL \cdot min^{-1}$. Subjects inhaled hypertonic saline solution for 5-min periods up to 30 min, and were asked to rinse their mouth out with water before induction to avoid salivary contamination. The concentration of saline was increased, if possible, at intervals of 10 min (two nebulisations of each concentration) from 3% to 4% to 5%. Spirometry was repeated at 5-min intervals throughout the procedure and immediately after sputum induction was completed. At the end of the test, a nebulisation with bronchodilators was given.

The concentration of saline was not increased if the $FEV_1$ fell by 10% or more from the post-bronchodilator value. Sputum induction was discontinued if the $FEV_1$ declined >20% or if troublesome symptoms occurred (i.e., dyspnoea, wheezing, severe cough). Selected sputum plugs from saliva were then analysed. The volume of the induced sputum plugs was determined and overlaid with an equal volume of 0.1% dithiothreitol (Sputalysin 10%; Behring Diagnostics

Inc., Somerville, NJ, USA). The sample was then vortexed and placed in a shaking water bath at 37°C for 30 min. The homogenised sample was centrifuged (GR4.22; Jouan, St Herblain, France) at 2,000 rpm (400 × *g*) for 10 min. The supernatant was aspirated and frozen at −80°C for later analysis. ELISA tests were used in order to assess CCSP (Biovendor) and IL-8 (Diaclone) after adequate dilution of the samples (1:1000 and 1:2).

*Lung histology*

At the time of surgery, a lung slice at distance from the neoplastic lesion was provided by the surgeon. It was first inflated with a needle mounted on a syringe and then fixed by immersion in formalyn 4% then embedded in paraffin. Samples of fixed tissue were processed into paraffin blocks, cut into sections that were 4 to 5 micrometers thick, and placed on glass slides. Blocks were fully cut until exhausted in order to reach at least 6 airways per patient. Hematoxylin eosin staining was used to perform small airway morphometry analysis. Briefly, adequately orientated small airways were delineated using an image analyzer (AnalySIS 7.2 for Windows, Olympus Soft Imaging Solutions, Muenster, Germany) linked to a CCD camera (Sony DXC950P, Sony Group, Tokyo, Japan) connected to a light microscope (Olympus BHS, Olympus Optical, Tokyo, Japan).

*Immunohistochemistry*

Sections of pre-identified small airways were stained separately to identify polymorphonuclear neutrophils (Neutrophil Elastase NE, Dako-cytomation, dilution 1:50). Control sections were treated with mouse IgG1 isotype at the same dilution. Manual cells counts were performed within each bronchioli wall at 100 micrometers maximal depth and expressed as a number/$mm^2$ of submucosal area.

*Statistical analysis*

Clinical data were expressed as means ± SEM. The effect of supernatants obtained from COPD patients, smokers and control subjects were compared using Kruskal-Wallis tests for quantitative data and Fisher tests for qualitative data. Chemotactic data were expressed as means ± SEM. Paired comparisons were made using Mann-Whitney tests. Data were considered statistically significant at a p-value $<0.05$. All graphical data and statistical analyses were generated with GraphPAD Prism software (Version 6.0).

Supplementary Tables and Figures

Table S1. Subject baseline characteristics: neutrophil donors.

| | Healthy controls | COPD |
|---|---|---|
| N | 5 | 5 |
| Age, years (mean ± SEM) | 42 ± 5.8 | 68 ±1.2 |
| Gender [n (% male)] | 1 (20) | 4 (80) |
| Smoking history, pack years (mean ± SEM) | 0 ± 0.0 | 60 ± 13 |
| Smoking status [n (%weaned >1 year)] | NA | 5 (100) |
| $FEV_1$, % predicted (mean ± SEM) | 106 ± 4.0 | 29.0 ± 5.3 |
| $FEV_1$/FVC (mean ± SEM) | 83.8 ± 3.6 | 44.0 ± 6.1 |
| **Treatments:** | | |
| ICS (n) | 0 | 0 |
| LABA (n) | 0 | 5 |
| LAMA (n) | 0 | 5 |

Definition of abbreviations: $FEV_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroid; LABA, long acting β agonist; LAMA, long acting muscarinic agonist

Table S2. Subject baseline characteristics: ALI cultures.

| | Healthy controls | Smokers | COPD | p- value |
|---|---|---|---|---|
| n | 4 | 10 | 13 | NA |
| Age, years (mean ± SEM) | 48 ± 9.9 | 59 ± 4.7 | 61 ± 2.0 | 0.405 |
| Gender [n(%male)] | 2 (50) | 7 (70) | 8 (62) | 0.774 |
| Smoking history, pack years (mean ± SEM) | 0.75 ± 0.7 | 34 ± 5.6 | 46 ± 6.6 | 0.005 |
| Smoking status [n (%weaned >1 year)] | 4 (100) | 5 (50) | 7 (54) | 0.196 |
| $FEV_1$, % predicted (mean ± SEM) | 87.8 ± 3.7 | 109 ± 5.3 | 50.8 ± 7.2 | < 0.0001 |
| $FEV_1$/FVC (mean ± SEM) | 82.3 ± 4.3 | 82.0 ± 3.0 | 53.4 ± 5.8 | 0.0015 |
| **Treatments** | | | | |
| ICS (n) | 0 | 0 | 0 | NA |
| LABA (n) | 0 | 0 | 13 | NA |
| LAMA (n) | 0 | 0 | 13 | NA |

Definition of abbreviations: $FEV_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroid; LABA, long acting β agonist; LAMA, long acting muscarinic agonist

Figure S1

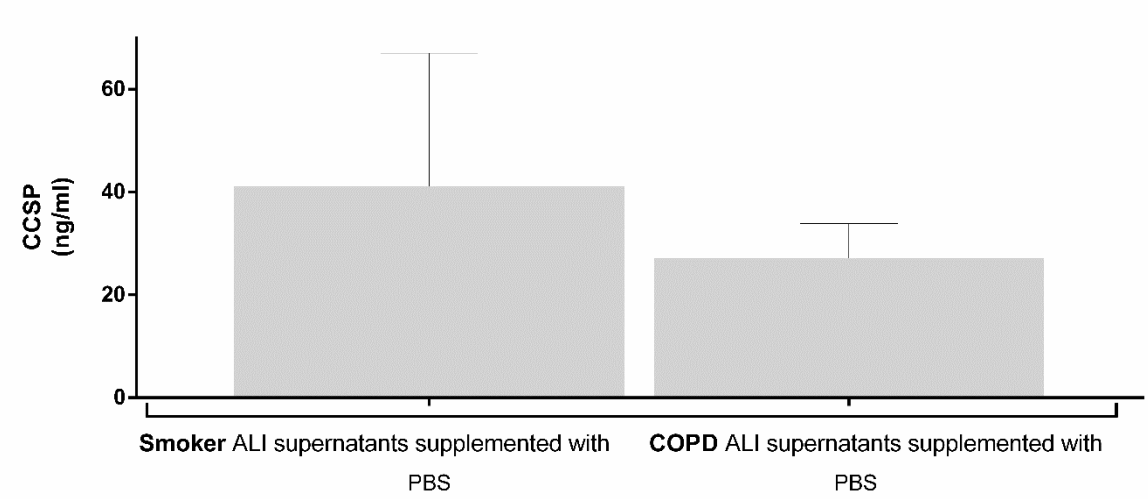

CCSP (ng/ml)
60
40
20
0
Smoker ALI supernatants supplemented with PBS
COPD ALI supernatants supplemented with PBS

Figure S2

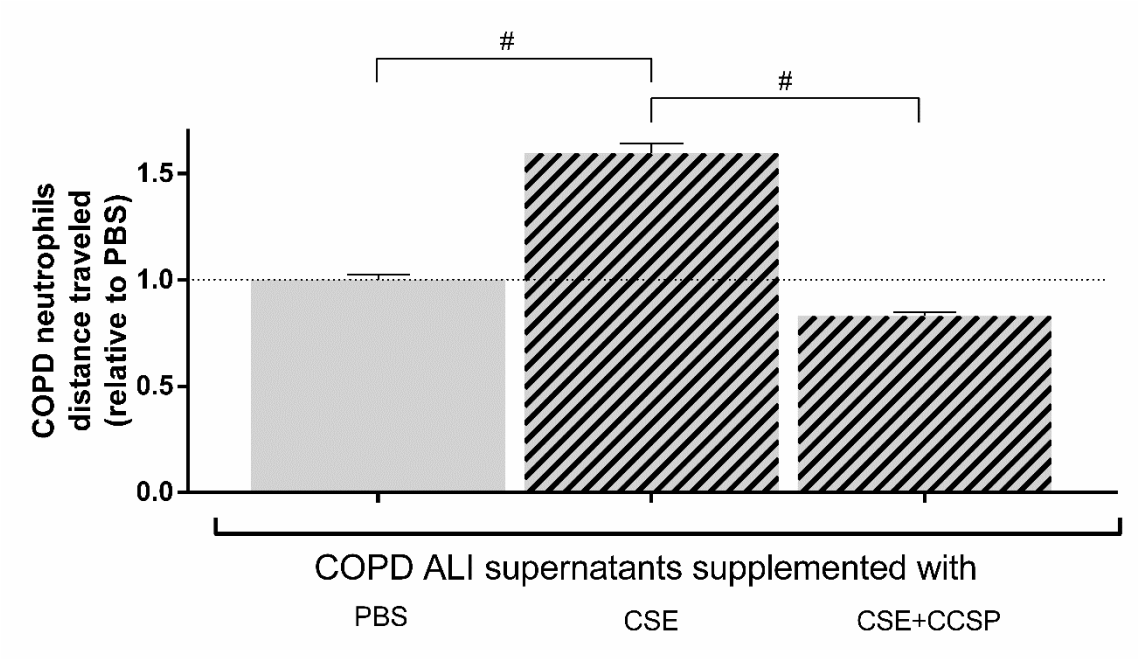
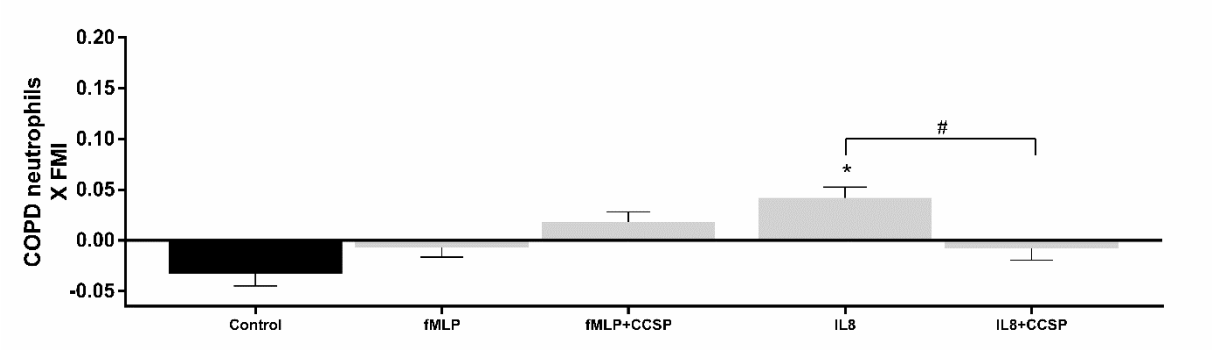
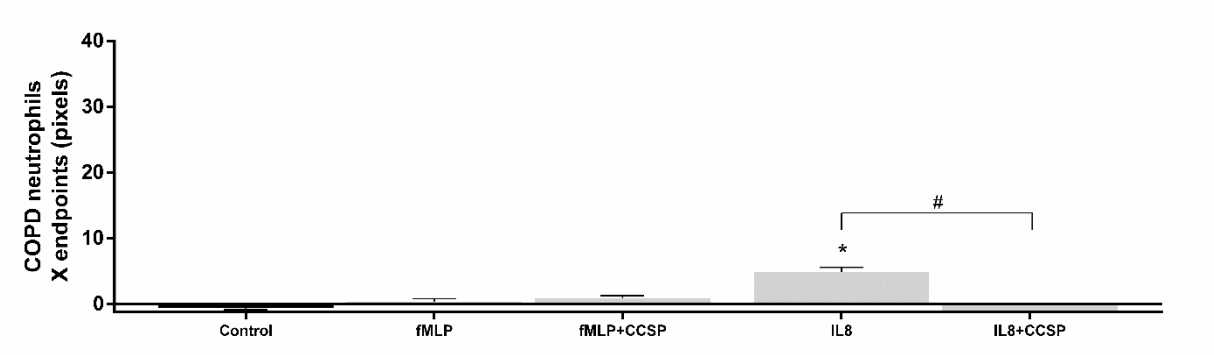

#
#
COPD neutrophils distance traveled (relative to PBS)
1.5
1.0
0.5
0.0
COPD ALI supernatants supplemented with
PBS
CSE
CSE+CCSP

Figure S3

A




B




C

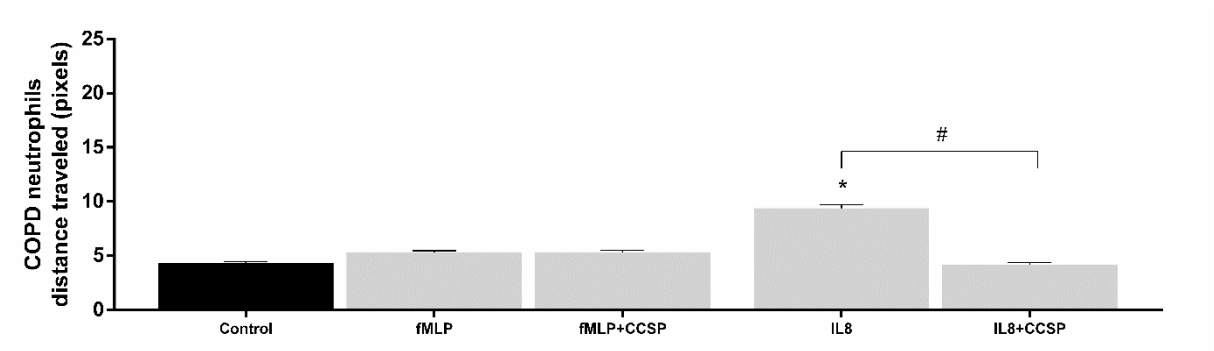


Table S1: **Subject baseline characteristics: neutrophil donors.** The characteristics of the subjects who provided blood for neutrophil isolation for the chemotactic studies.

Table S2: **Subject baseline characteristics: ALI cultures.** The characteristics of the subjects who provided bronchial epithelial cells. As expected, control subjects had no airflow obstruction. COPD patients and smokers differed as expected by their level of airflow obstruction, whereas cumulative smoking history and smoking status were almost similar. COPD patients had had no exacerbations since at least 2 months and all subjects were infection- and medication-free for at least 8 weeks prior to the time of blood sampling.

Figure S1: **Baseline CCSP secretion.**

CCSP secretion by epithelial cells from smokers (n=7) and COPD patients (n=8). CCSP secretion tended to be reduced in COPD cells compared to those from smokers.

Figure S2: **Treating the airway epithelium with exogenous CCSP prevented CSE-induced COPD neutrophil chemotaxis.**

The distance travelled by COPD neutrophils on a gradient of COPD ALI supernatants (n=8) stimulated by CSE with or without CCSP supplementation. Since the latter are disoriented, considering the distance travelled by the neutrophils was a better measure for demonstrating that CCSP had the same effect on COPD neutrophils as on healthy neutrophils. Indeed, CCSP inhibited CODP neutrophil recruitment when exposed to CSE ($p < 0.0001$).

Approximately 100 tracked neutrophils by condition. Data were expressed as means ± SEM. Paired comparisons were made using Mann-Whitney tests indicated by hash signs (#).

Figure S3: **Stimulated-COPD neutrophil migration was slowed down by CCSP.**

A: The XFMI of COPD neutrophils on fMLP- or IL8- gradients with or without CCSP. CCSP inhibited the chemotaxis of neutrophils stimulated by IL8 ($p < 0.0001$).

B: The X endpoints of COPD neutrophils on fMLP- or IL8- gradients with or without CCSP. CCSP inhibited the neutrophil movement in the direction of the IL8-induced gradient ($p < 0.0001$).

C: The distance travelled by COPD neutrophils on fMLP- or IL8- gradients with or without CCSP. CCSP inhibited the neutrophil movement induced by IL8 ($p < 0.0001$).

Approximately 100 tracked neutrophils by condition. All conditions were reproduced three times. Data were expressed as means ± SEM. Paired comparisons were made using Mann-Whitney tests. Significant differences compared to controls were indicated by stars. Hash signs (#) indicated significant differences between compared groups.