

# **Impairment of neutrophil reactivity to elastin peptides in COPD**

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## **ONLINE DATA SUPPLEMENT**

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### **1. Supplemental methods**

#### **Study population**

Controls (n=20) were recruited from subjects attending the department of respiratory diseases and allergy for suspicion of allergy. They were invited to participate in the study when they did not present any respiratory symptoms, and had normal spirometric values and normal blood tests. They were matched with COPD patients for smoking, age and gender. COPD patients (n=16) were enrolled in the study on the basis of the following criteria: over 45 years old, current or ex-smoker for  $\geq 20$  pack-years, forced expiratory volume in 1-second ( $FEV_1$ )/forced vital capacity (FVC)  $< 70\%$  after bronchodilatation. COPD patients were included during an exacerbation phase defined by the presence of at least two of the following criteria: increased dyspnoea, increased sputum production and sputum purulence. Blood samples were drawn from all exacerbated COPD patients before any specific therapy (i.e. antibiotics possibly in combination with corticosteroids, and nebulization of bronchodilators for one week). After inclusion, exacerbated COPD patients were followed clinically. Four to

six weeks after treatment ending, further blood samples were drawn from eleven of the former exacerbated COPD patients that returned to a stable phase. Patients with a diagnosis of asthma, extensive pulmonary tuberculosis or neoplasia were not eligible for the study.

### **Neutrophil isolation and culture**

Neutrophils were shown to be > 98% by FACS analysis and cell viability was over 95% as determined by trypan blue exclusion (data not shown). Isolated neutrophils ( $1 \times 10^6$  cells/mL) were cultured at 37°C in 5% CO<sub>2</sub> in 24-well culture plates and in RPMI 1640 medium containing 5 mM L-glutamine (Fisher Bioblock Scientific, France).

### **Neutrophil chemotaxis assay**

Briefly, 27 µl of RANTES (0.1 µg/mL) or VGVAPG (1 and 10 µg/mL) were added to the lower wells of a 48-well Boyden chamber (Neuro Probe, Maryland, USA) and covered with a 5 µm polycarbonate filter (VWR International, France). 50 µl of neutrophil suspension ( $5 \times 10^5$  cells/mL) were then added to the upper wells of the chamber and incubated for 45 min at 37°C in 5% CO<sub>2</sub>. Migrating cells, fixed and stained with eosin and bromophenol blue at the lower part of the filter, were counted by microscopy in five randomly selected high-power (40 x) fields per well. The experiments were performed in triplicate and results are reported as the mean number of cells per field.

### **Real-time RT-PCR analysis**

Total RNA was extracted from  $5 \times 10^6$  neutrophils using *MasterPur*<sup>TM</sup> RNA Purification Kit (Tebu-bio, France) in accordance with the manufacturer's technical data sheets. Then, 1 µg of total RNA was reverse transcribed into cDNA with reverse transcriptase using *SuperScript*<sup>TM</sup> First-Strand Synthesis System (Invitrogen Life Technologies, France), as previously described.<sup>1</sup> After reverse transcription, the cDNA product was amplified by real-time PCR. Using this approach, the S-Gal, IL-6, TNF-α, and IL-8 mRNA levels were

determined with the 7000 sequence detection system ABI Prism sequence detector (Applied Biosystems, California, USA), using the double-strand-specific SYBR Green (Applied Biosystems) dye system. Data analysis was performed with SDS software (Applied Biosystems). The amount of the target gene mRNA was calculated relative to the  $\beta$ -actin gene mRNA and an internal control ( $2^{-\Delta\Delta C_t}$ , where  $\Delta$  is change and  $C_t$  is the cycle threshold).

### **Phagocytosis assay**

The red fluorescent latex beads (L-3030 carboxylate-modified, 2.0- $\mu$ m mean diameter; Sigma-Aldrich, France) were first pre-coated with 10% human AB serum (SAB) before adding 1 mL of SAB-coated beads to neutrophils at a final cell/bead ratio of 1:25. The cells were then incubated with the fluorescent latex beads for 30 or 90 min at 37°C in 5% CO<sub>2</sub> atmosphere. Following incubation, cells were washed three times with 0.5% Tween 20 in PBS and resuspended in PBS containing 1% PFA. Fluorescence emission was assessed by flow cytometric analysis using a FACSCalibur<sup>TM</sup> Instrument (BD Biosciences). To account for extracellular binding, phagocytosis was inhibited by adding 5  $\mu$ M Cytochalasin D (Sigma-Aldrich) 30 min before contact with latex beads to block F-actin-dependent phagocytosis. The mean fluorescence intensity of these samples was subtracted from samples exposed to beads in the absence of Cytochalasin D.

### **Neutrophil-associated bacteria and intracellular ROS measurements**

Neutrophils were cultured in 96-well culture plates (2.5 x 10<sup>4</sup>/well) and incubated with 0.1 mL of *P. aeruginosa* (3-5 10<sup>7</sup> CFU/mL) for 30 or 90 minutes at 37°C in 5% CO<sub>2</sub> atmosphere. For neutrophil-associated bacteria analysis, the plates were placed in ice following incubation in order to stop bacteria uptake. Cells were then centrifuged at 400 g for 5 min at 4°C to remove non-phagocytosed *P. aeruginosa*, and thereafter washed twice with PBS and lysed with 1% Triton X-100 (Sigma-Aldrich) to disrupt eukaryotic cells. The pellet was

resuspended in 0.5 ml of saline, and the suspension was cultured on a standard Trypic Soy Agar (Fisher Bioblock Scientific). After 24 h of incubation at 37°C, the number of colony-forming unit (CFU) was counted to determine the number of viable neutrophil-associated bacteria. For measurement of intracellular ROS, cells were exposed to 10 µM of the cell-permeant 2',7'-dichlorofluoresceindiacetate compound (Invitrogen Life Technologies). This molecule is oxidized by hydrogen peroxide, peroxynitrite (ONOO<sup>-</sup>), and hydroxyl radicals (OH<sup>•</sup>) to yield the fluorescent molecule 2',7'-dichlorofluorescein. After 15 min of incubation at 37°C, cells were washed twice in PBS and resuspended in 500 µl of PBS containing 1% PFA. Fluorescence emission, directly proportional to the presence of the abovementioned ROS, was assessed by flow cytometric analysis using a FACSCalibur<sup>TM</sup> Instrument (BD Biosciences).

#### **Determination of serum elastin-derived peptide concentration**

Briefly, the wells of a microtitre plate (Nunc, Fisher Bioblock Scientific) were coated with 150 µL (1.5 µg/mL) soluble human lung elastin peptides (Elastin Products Company) in 0.1 M sodium carbonate buffer (pH 9.0) for 18 h at 4°C. The plate was then washed four times with 300 µL PBS (pH 7.0) containing 0.05% (vol/vol) Tween 20 (PBST). A standard curve was generated using dilutions of human lung elastin peptides (0-200 ng/mL) in PBST containing 8% (wt/vol) bovine serum albumin (PBST-8% BSA). These dilutions were pre-incubated with rabbit anti-elastin polyclonal antibodies (1:500, Elastin Products Company) in PBST-8% BSA overnight at 4°C. 100 µL of these mixtures were then transferred to the pre-coated plate and incubated at room temperature for 1 h. After washing the plate four times with PBST, 150 µL HRP-conjugated goat anti-rabbit polyclonal IgG antibody (Santa Cruz Biotechnology, California, USA) were added to each well (1:1000 dilution in PBST-8% BSA). The plate was incubated for 1 h at room temperature and again washed four times with PBST. Thereafter, 100 µL of ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

in 0.05 M phosphate-citrate buffer (pH 5.0) were added to each well, and the plate was incubated for another hour at room temperature before measuring absorbance at 495 nm. Standards and variable dilutions of serum samples were analysed in triplicate. The serum elastin-derived peptide concentrations were calculated from the standard curve and expressed as micrograms per millilitre.

## **2. Supplemental references**

1. **Esnault S**, Benbernou N, Lavaud F, *et al.* Differential spontaneous expression of mRNA for IL-4, IL-10, IL-13, IL-2 and interferon-gamma (IFN-gamma) in peripheral blood mononuclear cells (PBMC) from atopic patients. *Clin Exp Immunol* 1996;**103**:111–18.