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Title: Cigarette smoke affects IL-17A, IL-17F and IL-17 receptor expression in the lung tissue: ex vivo and in vitro studies

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Corresponding Author: Dr. Mirella Profita, PhD

Corresponding Author's Institution: IBIM

First Author: Angela M Montalbano, Dr

Order of Authors: Angela M Montalbano, Dr; Loredana Riccobono, Dr; Liboria Siena, Dr; Giuseppina Chiappara, Dr; Caterina Di Sano, Dr; Giulia Anzalone, Dr; Rosalia Gagliardo, Dr; Fabio L Ricciardolo, MD; Valentina Corsello, Dr; Loredana Pipitone, Dr; Patrizio Vitulo; Mirella Profita, PhD

Abstract: Cigarette smoke is a risk factor for Chronic Obstructive Pulmonary Disease (COPD). Th-17 cytokines are involved in the pathogenesis of COPD. To evaluate the role of cigarette smoke on the expression of IL-17A, IL-17F and IL-17R in airways of COPD patients. Epithelial and subepithelial immunoreactivity for IL-17A, IL-17F and IL-17R was assessed in surgical specimens from COPD patients (n=15) and from healthy subjects (HC) (n=10) by immunohistochemistry. In vitro, human epithelial cell line 16HBE and A549 as well as PBMC from normal donors were stimulated with cigarette smoke extract (CSE) (0, 2.5, 5, 10%) to evaluate the IL-17A, IL-17F and IL-17R expression by flow-cytometry. Furthermore, rhIL-17A and CSE stimulation was evaluated on proliferation and apoptosis in 16HBE and in A549. In central and distal airways immunoreactivity for IL-17A, IL-17F and IL-17R significantly increased in the epithelium and IL-17A in the subepithelium from COPD than in HC. In distal airway, immunoreactivity for IL-17F increased in the subepithelium of COPD than in HC. IL-17A immunoreactivity positively correlate with IL-17R and total pack years in the epithelium from central and distal airways of COPD patients. In vitro, CSE stimulation significantly increased IL-17F and IL-17R in 16HBE (2.5%) and A549 (5%) while IL-17A and IL-17F in PBMC (10%). IL-17A and CSE stimulation, rather than CSE or rhIL-17A alone, significantly increased proliferation in 16HBE and apoptosis in A549. Cigarette smoke increases Th17 Immunity in lung tissue of COPD patients, promoting the mechanism of proliferation and apoptosis in airway epithelial cells.

Suggested Reviewers: Dong Qiu
Department of Clinical Laboratory, Henan Medical CollegeNo. 8 Xinzhenglonghu Zhe
Dong-Qiu451191@163.com
He studied the activity of IL-17

Y Chang Y
changyingcn@hotmail.com
He studied the proinflammatory activity of IL-17

T Hallstrand
tealh@uw.edu

He studied the proinflammatory activity of IL-17

L Zhang
zhangcatli1979@sina.com
He studied the proinflammatory activity of IL-17

F Bucchieri
fabiobuk@hotmail.com
He is a specialist of airway inflammation

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Editor in Chief of Cytokine

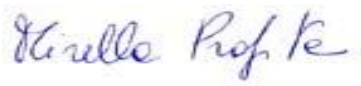
RE: Submission of the manuscript "Cigarette smoke affects IL-17A, IL-17F and IL-17 receptor expression in the lung tissue: ex vivo and in vitro studies".

Dear Editor,

Enclosed please find the paper entitled "Cigarette smoke affects IL-17A, IL-17F and IL-17 receptor expression in the lung tissue: ex vivo and in vitro studies" together with figures and tables which we would like to submit to the journal "Cytokine". The paper is not under consideration by any other journal at the same time, and it has not been accepted for publication elsewhere. I declare the integrity of the data and the accuracy of the data analysis. Each author listed on the manuscript has seen and approved the submission of this version of the manuscript and takes full responsibility for the manuscript.

The special consideration regarding the paper is that this study describes, using an ex vivo/in vitro experimental model, the potential role of cigarette smoke habit in the activation of epithelial cells affecting IL-17A immunology in Chronic Obstructive Pulmonary Disease (COPD).

Certain of your consideration, we look forward to hearing from you and remain
Sincerely yours
Mirella Profita, PhD



Consiglio Nazionale delle Ricerche - Istituto di Biomedicina ed Immunologia Molecolare "Alberto Monroy"
Via Ugo La Malfa, 153 - 90146 Palermo C.F. 80054330586 – P. IVA 02118311006
TEL: (+39) 0916809194 – FAX: (+39) 0916809122 – e-mail: segreteria@ibim.cnr.it – Sito web: www.ibim.cnr.it

Highlights of the manuscript

- IL-17A, IL-17F and the related receptor IL-17R are increased in COPD.
- Cigarette smoke habit affect the proinflammatory activity of IL-17A.
- IL-17A rather than IL-17F is able to activate airway epithelial cells in COPD patients.
- IL-17A might be able to affect epithelial cell proliferation and apoptosis involved in the tissue damage and repair during the pathogenesis of COPD.

Cigarette smoke affects IL-17A, IL-17F and IL-17 receptor expression in the lung tissue: ex vivo and *in vitro studies*

Angela Marina Montalbano[1], Loredana Riccobono[1], Liboria Siena[1], Giuseppina Chiappara[1], Caterina Di Sano[1], Giulia Anzalone[1], Rosalia Gagliardo[1], Fabio L. M Ricciardolo[2], Valentina Sorbello[2], Loredana Pipitone[3], Patrizio Vitulo[3], Mirella Profita[1].

[1]Unit: "Ex vivo/In vitro models to study the Immunopathology and Pharmacology of airway diseases", Institute of Biomedicine and Molecular Immunology (IBIM), Italian National Research Council (CNR), Palermo, Italy.

[2]Department of Clinical and Biological Sciences, University of Torino, Torino, Italy.

[3]Istituto Mediterraneo per i Trapianti e Terapie ad Alta Specializzazione (ISMETT), Palermo, Italy

Corresponding Author:
Mirella Profita, PhD
Institute of Biomedicine and Molecular Immunology "A. Monroy"
Italian National Research Council, Palermo, Italy.
E-mail: *profita@ibim.cnr.it*

Abstract

Cigarette smoke is a risk factor for Chronic Obstructive Pulmonary Disease (COPD). Th-17 cytokines are involved in the pathogenesis of COPD. To evaluate the role of cigarette smoke on the expression of IL-17A, IL-17F and IL-17R in airways of COPD patients. Epithelial and subepithelial immunoreactivity for IL-17A, IL-17F and IL-17R was assessed in surgical specimens from COPD patients (n=15) and from healthy subjects (HC) (n=10) by immunohistochemistry. In vitro, human epithelial cell line 16HBE and A549 as well as PBMC from normal donors were stimulated with cigarette smoke extract (CSE) (0, 2.5, 5, 10%) to evaluate the IL-17A, IL-17F and IL-17R expression by flow-cytometry. Furthermore, rhIL-17A and CSE stimulation was evaluated on proliferation and apoptosis in 16HBE and in A549. In central and distal airways immunoreactivity for IL-17A, IL-17F and IL-17R significantly increased in the epithelium and IL-17A in the subepithelium from COPD than in HC. In distal airway, immunoreactivity for IL-17F increased in the subepithelium of COPD than in HC. IL-17A immunoreactivity positively correlate with IL-17R and total pack years in the epithelium from central and distal airways of COPD patients. In vitro, CSE stimulation significantly increased IL-17F and IL-17R in 16HBE (2.5%) and A549 (5%) while IL-17A and IL-17F in PBMC (10%). IL-17A and CSE stimulation, rather than CSE or rhIL-17A alone, significantly increased proliferation in 16HBE and apoptosis in A549. Cigarette smoke increases Th17 Immunity in lung tissue of COPD patients, promoting the mechanism of proliferation and apoptosis in airway epithelial cells.

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is a complex disease characterized by chronic innate and adaptive inflammatory immune responses. Cigarette smoke is the major risk factor for the development of COPD, cigarette smokers constituting more than 90% of all COPD patients in developed countries [1]. The pathological hallmarks of COPD are destruction of the lung parenchyma, which characterizes pulmonary emphysema, inflammation of the peripheral airways, respiratory bronchiolitis and inflammation of the central airways [1, 2, 3]. However, there are evidences of increased inflammation in both central and peripheral airways, involving a range of cell types, including neutrophils, macrophages, lymphocytes and epithelial cells. To what extent central airways may mirror events, occurring in distal lung is uncertain.

Adaptive immune processes are implicated in the pathogenesis of COPD. It has been hypothesized that susceptibility to COPD may arise by a shift from the non-specific innate response present in every smoker toward an adaptive immune response with features typical of autoimmune processes [4, 5]. Th17-cells are effector T-cell subsets characterized by the production of IL-17A, IL-17F and IL-22, implicated in the pathogenesis of several inflammatory and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and psoriasis [6, 7]. IL-17A and IL-17F targeted the cells on IL-17 receptor (IL-17R). The IL-17R is expressed in blood cells as well as in structural cells such as airway epithelial cells [8, 9, 10, 11]. Th17 immunity and the related receptor are involved in both the innate and the adaptive aspects of airway immunity, which represent the crucial crosstalk between the immune system and structural cells [12]. It has been reported that IL-17A levels are increased in submucosal biopsy specimens from the large airways of patients with COPD compared with control subjects, but there is no difference for IL-17F [13] or in contrast that both IL-17A and IL-17F positive cells were higher in bronchial biopsies from COPD patients than in healthy control [14]. Furthermore, it was observed that the levels of IL-17A are increased in the submucosa of lung tissue from COPD than in S and HC while there were not differences in the

subepithelium [15]. However all these findings not clarify the role of IL-17 immunity in central or distal airway of COPD suggesting the necessity to compare the levels IL-17A, IL-17F and the related receptor (IL-17R) in the two lung districts of the same subject.

The aim of the present study is to evaluate the role of Cigarette Smoke on IL-17A, IL-17F and IL-17R using ex vivo/in vitro studies. Ex vivo, we investigated the immunoreactivity of IL-17A, IL-17F and IL-17R in the epithelium and in the subepithelium of the central and distal airways from surgical specimens of smoking COPD patients. In vitro, we evaluated the effect of cigarette smoke extract (CSE) on the expression of IL-17A, IL-17F and IL-17R in human bronchial epithelial cell line (16HBE), in alveolar cell line (A549) and in human peripheral blood mononuclear cells (PBMC) from normal donors. Finally, we studied whether CSE via the increased expression of IL-17R strengthen the IL-17A mediated activities on the proliferation and on the apoptosis of epithelial cell line from central and distal airways.

1. Materials and Methods

2.1. Patient Population

Patients underwent surgery for lung cancer and were recruited at ISMETT-Palermo, Italy. The study was approved by the ISMETT Ethic Committee (#217806-30/06/2008) and was in agreement with Helsinki Declaration. Written informed consent was obtained from each patient. The study was carried out using samples of three groups of subjects: 10 healthy asymptomatic non-smoking subjects with normal lung function (HC); 15 patients with chronic obstructive pulmonary disease (COPD). The diagnosis of COPD and the assessment of its severity were defined and classified according to the criteria reported by the Global Initiative for Obstructive Lung Disease (GOLD) guidelines for COPD management (GOLD stage ≥I) [16]. COPD subjects with exacerbations within 1-month prior to the study were excluded. Patients with COPD had a smoking history of 10 ≥ pack years or more.

COPD patients were treated with bronchodilators and were classified on the basis of preoperative lung function: FEV1 less than 80% of reference, FEV1/FVC less than 70%, and bronchodilatation effect less than 12%. The patients were not under corticosteroid therapy (neither inhaled nor systemic) and not under antibiotics and did not have exacerbations during the month preceding the study. Subjects had negative skin tests for common allergen extracts and had no past history of asthma or allergic *rhinitis.*

2.2. Immunohistochemistry of paraffine sections

Tissue specimens from tumor-free central bronchi and peripheral lung tissue were selected, fixed with 10% neutral buffer formalin and embedded in paraffin wax. Sequential sections (3µm thick) were placed on poly-L-lysine coated slides, deparaffinized in xylene, rehydrated in a descending ethanol series and stained with haematoxylin and eosin (HE). Immunoreactivity for Rabbit Polyclonal IL-17A Antibody (H-132): sc7927 (Santa Cruz Biotechnology, Santa Cruz, CA),

Human IL-17F Polyclonal Goat IgG (R&D System) and Monoclonal Anti-Human IL-17R Antibody (R&D System) was evaluated in Central (internal perimeter >6 mm) and Distal (internal perimeter, < or =6 mm) airways [17]. LSAB2 Dako kit (Code Nu K0674) (Dako, Glostrup, Denmark) and Fuchsin Substrate-Chromogen System Dako [18] were used for the staining for IL-17A and IL-17R Antibodies, meanwhile LSAB Dako kit Universal (Code Nu K0689) (Dako) and Fuchsin Substrate-Chromogen System Dako were used for staining of IL-17F antibody. Rabbit, mouse and goat negative control immunoglobulins (Dako) were used for negative controls. Sample immunoreactivity was evaluated blindly by two independent investigators, using image analysis (Leica microscope, Wetzlar, Germany) 400X magnification.

The length of the basement membrane was evaluated using a Leica Application Suite V3.3 (LAS) software (Leica) for Image Analysis. Results were expressed as the number of positive epithelial cells/mm basement membrane as previously described [19]. Finally, immunostained cells were quantified in the subepithelium of central and distal airway, and the final results were expressed as the number of positive cell/mm^2 [15, 13].

2.3. Epithelial cell cultures

The SV40 large T antigen-transformed 16HBE cell line (16HBE), from normal bronchial epithelial cells [20] was used for this study. The 16HBE cell line retains the differentiated morphology and function of normal airway epithelial cells. The cells represent a clonal diploid (2n¼6) cell line isolated from human lungs previously used to study the functional properties of bronchial epithelial cells in inflammation and repair processes [21]. 16HBE cells were cultured as adherent monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated (56°C, 30 min) foetal bovine serum (FBS), 1% MEM (non-essential aminoacids, Euroclone), 2 mML-glutamine and gentamicin 250μg/ml at 37°C in a humidified 5% CO2 atmosphere [22]. Type II alveolar epithelial cell-derived A549, cell line were purchased from American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in complete

culture medium (RPMI 1640 containing 10% FCS and 200 IU/mL penicillin/100 µg/mL streptomycin).

2.4. Preparation of cigarette smoke extracts (CSE)

Commercial cigarettes (Marlboro; Philip Morris USA, Richmond, VA) were used in this study. CSE was prepared as described previously described [22]. Cigarette smoke extract was used to stimulate cultured 16-HBE and A549 cell line and Peripheral Blood Mononuclear Cells (PBMC) obtained from healthy control.

2.5. Isolation of human peripheral blood mononuclear cells (PBMC)

Blood samples were collected in EDTA vacutainer tubes (BD Biosciences) and used for plasma selection and PBMC isolation from Healthy donors. The cells were isolated using a density gradient centrifugation (Ficoll-paque^{TM} PLUS; Amersham Biosciences SE-751 84, Uppsala, Sweden) and, after two washes, the cells were suspended in RPMI 1640 cell culture medium (Invitrogen Life Technologies, Italy) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mML-glutamine, 20 mM HEPES,100 U/ml penicillin, 100 µg/ml streptomycin, 5 x 10^{-5} M 2-ME and 85 µg/ml gentamicin. Purity of PBMC was assessed by May–Grünwald–Giemsa staining and was 93 + 3% accordingly with the purity declared in the data sheet of the Ficoll-Paque^{TM} PLUS density gradient. The viability of the PBMC was tested using trypan blue exclusion and was 93$\pm$5% as previously described [23].

2.6. PBMC stimulation

PBMC (1x10^6 cells/ml) were cultured in the presence of CSE (2,5%, 5%, 10%) for 48 hours in 24-well cell culture plates in complete medium RPMI 1640 (Invitrogen Life Technologies, Italy) plus 10% heat-inactivated FBS. Cultured PBMC were assessed for intracellular cytokines IL-17A,

IL-17F, IL-17R expression, by immunocytochemistry, immunofluorescence and flowcytometric analysis. After the stimulation, the viability of the cells was tested using trypan blue exclusion.

2.7. 16HBE and A549 cells stimulation

16HBE and A549 were plated (200,000 cells/well) in standard six-well plates, in a suitable medium at 10% FBS and grown to confluence (70–80%). After, the mediums were replaced and the 16HBE and A549 cells were stimulated in the presence or absence of different concentration of CSE (2.5%, 5%, 10%) for 24h to evaluate IL-17A, IL-17F, IL-17R expression by immunocytochemistry, immunofluorescence, and flowcytometric analysis. Furthermore, 16HBE and A549 were stimulated in the presence or absence of CSE (2.5 %, 5% respectively) with or without hrIL-17A (20ng/ml) to study cell apoptosis after 24 hrs and to study cell proliferation after 192 hrs.

2.8. Flowcytometric analysis for IL-17A, IL-17F, and IL-17 Receptor

Monensin 1mM was added to 16HBE, A549 cells and PBMC (800.000 cells/well) stimulated with CSE for 12h before the collection. The cells were washed in PBS and collected in FACS tubes. After wards, cells were washed in staining buffer (PBS containing 1% FCS and 0.1% Na azide) and then incubated with PBS containing 4% paraformaldehyde for 20 min. Fixation was followed by two washes in permeabilization buffer (PBS containing 1% FCS, 0.3% saponin, and 0.1% Na azide) and fixed permeabilized cells were stained with the primary antibody, 1h, 4°C. The 16HBE, A549 cells and PBMC were analyzed for rabbit-polyclonal antibody anti IL-17 (H-132), Santa Cruz Biotechnology, Santa Cruz, Calif.), anti-human IL-17F Antibody (R&D System), and Monoclonal anti-Human IL-17Receptor Antibody (R&D Systems, Inc. Minneapolis), expression by FACS analysis. Non-immune IgG at the same titre as the primary antibody was used as a negative control. Cells were washed in cold PBS and incubated with FITC-conjugated polyclonal swine anti-Rabbit

Ig (DAKO Glostrup, Denmark), Polyclonal Rabbit anti mouse/FITC IgG (DAKO Glostrup, Denmark) and Polyclonal Rabbit Anti-goat IgG/FITC (DAKO Glostrup, Denmark) in the dark 1h at 4°C before flowcytometric analysis. After washing, the cells were analysed by FACS Calibur (Becton Dickinson, Mountain View, CA, USA) flowcytometer. Fluorescence-positive cells were quantified. Percentages of positive cells for IL-17A, IL-17F and IL-17R of positive cells were determined from forward (FS) and sideways (SS) scatter patterns, after gating on the cells, excluding debris. Non-specific binding and background fluorescence were quantified by analyzing negative control.

2.9. Immunocytochemistry

Cytospins were made from cultured PBMC stimulated with and without CSE 10% and fixed and permeabilized as previously described. After washing in PBS, slides were incubated with blocking solutions (0.5% BSA in PBS) 1h RT, and then slides were incubated with the primary antibodies IL17 (1:50 dilution), IL17F (1:10 dilution), and IL17R (1:10 dilution) overnight 4°C. The reaction was revealed by LSAB and LSAB2 KIT phosphatase method (DAKO Glostrup Denmark) in 16-HBE, A549 and PBMC according to the manufacturer's instructions. Non-immune IgG at the same titre as the primary antibody were used as a negative controls. Cytospins were examined under light microscopy with a final magnification of 400X.

2.10. Immunofluorescence Co-Localization of IL-17A/IL-17R and IL-17F/IL-17R on airway epithelium

Double immunofluorescence tests were performed to assess the co-localization of rabbit polyclonal antibody IL-17 (H-132), Santa Cruz Biotechnology or the goat polyclonal antibody IL17 F (R&D System), with mouse monoclonal IL-17 R Antibody (R&D System). Frozen sections of

Bronchial ring (6 μm) and Parenchimal sections (9 μm) were subjected to indirect immunofluorescence as follow described. Briefly, the samples were fixed in 4% paraformaldehyde for 15 min at room temperature, followed by 3 washes in PBS, 3 min each. The samples were then permeabilized with Saponin 0.05% in PBS-plus 3% BSA for 5 min at room temperature and after 3 washes in PBS, were fixed in cold Aceton at -20°C for 7 minutes. After washes in PBS, samples were blocked in 3% BSA in PBS for 1hour at room temperature. Primary antibody incubation was performed in 3% BSA in PBS overnight. The following primary antibodies were used: anti IL-17A (rabbit, 1:50), anti IL-17R (mouse, 1:50) and anti IL-17F (goat, 1:50) all overnight at 4°C. The day after, slides were wash in PBS and the secondary antibodies incubation was in 3% BSA in PBS at room temperature for 1 hour at room temperature, followed by three PBS washes. FITC-conjugated sheep anti-rabbit IgG (Sigma Aldrich, F7512), R-Phycoerythrin-conjugated goat anti-mouse IgG (Sigma P9287) and Alexa 488 conjugated rabbit anti-goat IgG secondary antibodies (Thermo Scientific Cat. N. SA5-10078) were used all 1: 200 1 hour in the dark at room temperature. The samples were then incubated with Hoechst (Sigma-Aldrich, Inc, Milan, Italy) 1:1000 in PBS for 10 min at room temperature, followed by PBS wash. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA), and images were analyzed by using a laser scanning microscope ZEISS at a final magnification of 400X. All immunofluorescence tests were performed with negative controls where no primary antibody was added.

2.11. Immonofluorescence of 16HBE, A549 and PBMC

16HBE and A549 cells were seed in a 6 wells plates within we have insert a sterile cover slide. Cells growth until a 70-80% of confluence in presence or absence of CSE (2.5 and 5% respectively) for 24h and the cover slide was recovered. Cultured PBMC were stimulated with CSE (10%) for 24h and at the end of stimulation cytospins were performed to obtained the slides. The cells were fixed in Paraformaldehyde 4% 15 min. RT, wash in PBS and treated with

Permeabilization Buffer (0.05% Saponin in PBS- plus 3% BSA). Then, the cells were incubated with blocking Solution (BSA 0,5%in PBS) 1h RT. Immunofluorescent staining was performed with the rabbit polyclonal antibody IL-17A (H-132), Santa Cruz Biotechnology, the goat-polyclonal antibody IL17F (R&D System), and IL-17Receptor Antibody (R&D System) all at 1:25 dilution, overnight at 4°C. The primary antibodies were diluted in PBS plus 3% BSA. Non immune IgG was used as a negative control. Secondary antibodies, anti-Rabbit IgG (whole molecule)-FITC F7512 Sigma Aldrich, Anti Mouse IgG (whole Molecule) R-Phycoeritrin conjugate P9287 Sigma and Alexa Fluor 488 Donkey anti-goat IgG (A11055) was performed in the dark for 1 hour at room temperature. After wash in PBS, the slides were counterstained with DAPI and after wash in PBS and mounting with Mounting Medium Vectashield. Slides were coverslipped in Vectashield (Vector Laboratories, Burlingame, CA). Images were analyzed by using a laser scanning microscope ZEISS at a final magnification of 400X.

2.12. Cell apoptosis by flow cytometry

Cell apoptosis was evaluated in cell growth until 70% of confluence and stimulated with and without CSE (2.5% in 16HBE and 5% in A549) and recombinant human (rh) IL-17 A (20ng/ml), alone or in combination, for 24hrs. The cells were stained with a solution containing a mixture of Annexin V-FITC in binding buffer 1X. After incubation (15 min in total darkness, RT) was add Propidium Iodide just before analysis. The number of viable, apoptotic and necrotic cells were determined using the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Results were presented as a percentage of counted cells.

2.13. Cell proliferation assay

Cell proliferation was measured using carboxyfluorescein succinimidyl ester (CFSE) labeling assay. CFSE is used to fluorescently label live cells and is equally partitioned to daughter cells during division [24, 25]. Briefly, the cells were incubated with CFSE (Molecular Probes, Inc. Eugene, OR) (at a final concentration of 5 µM) at 37°C for 10 min. Labeling was blocked by the addition of an equal volume of heat inactivated FCS. Tubes were placed in ice for 5 min and then washed. The cells were plated at 8×10^5 cells/well in six-well plates and incubated at 37°C with 5% CO_2. After 5-7 hours cells were stimulated with and without CSE (2.5% in 16HBE and 5% in A549) and recombinant human (rh) IL-17 A (20ng/ml), alone or in combination, and incubated for 192 hours. Cell proliferation was assessed by flow-cytometry.

2.14. Statistical analysis

Statistical comparisons in order to test differences between the three groups (HC, COPD) were made by use of the Kruskall–Wallis test followed by Fisher's PLSD correction for multiple comparisons. Data were expressed as median and inter quartile range. ANOVA test was used for the analysis of the data obtained from *in vitro* experimental conditions expressed as mean ± S.D. Correlations were calculated according to Spearman test. All statistical analyses were performed using StatView® 5 software (SAS institute Inc.). A p value of less than 0.05 was considered to indicate statistical significance in these analyses.

3. Results

3.1. Demographic characteristics of the subjects

The demographic characteristics and the functional evaluations of the studied groups are shown in Table 1. All recruited patient groups were similar with regard to age.

3.2. IL-17A, IL17F and IL-17R expression in central airways and distal airways

We detected statistical higher levels of immunoreactivity for IL-17A, IL17F and IL17R expressed as positive cells/mm in the epithelium from the central airways of COPD patients compared with HC subjects ($p<0.0001$, $p=0.0001$ and $p=0.0005$, respectively) (Figure 1, A). The immunoreactivity for IL-17A and IL-17F expressed as positive cells/mm2 showed statistical higher levels in the subepithelium from the central airways of COPD subjects than in HC subjects ($p<0.0009$ and $p=0.0005$, respectively). No significant differences were observed for the IL-17R immunoreactivity in the cells of the subepithelial region from central airways of COPD patients than in HC subjects (Figure 1B).

Immunoreactivity for IL-17A ($p=0.0011$), IL-17F ($p<0.0005$) and IL-17R ($p=0.007$) were statistical significant higher in the epithelium from distal airways of COPD than in HC subjects (Figure 2, A). Additionally, immunoreactivity for IL-17A was statistical significant higher in the subepithelium from distal airways of COPD patients compared with HC subjects ($p<0.0001$) (Figure 2, B). No significant differences were observed for immunoreactivity of IL-17F or IL-17R in subepithelial region from central airways of COPD patients than in HC subjects. A representative images of the immunoreactivity for IL-17A, IL-17F and IL-17R in the epithelial and sub-epithelial regions from central and distal airways of HC and COPD are represented Figure 3.

3.3. Correlations

Immunoreactivity for IL-17A positively correlated with the Total packs year in epithelial cells (cells/mm) from central and distal airways of COPD patients (p=0.006, Rho=-0.7 and p=0.04, Rho=0.6 respectively) (Figure 4A). Furthermore immunoreactivity for IL-17A positively correlated with epithelial cells immunoreactivity for IL-17R (cells /ml) in epithelial cells (cells/mm) from central and distal airways of COPD patients (p=0.05, Rho=-0.5 and p=0.02, Rho=0.6 respectively) (Figure 4B).

3.4. Double immunofluorescence for IL-17A and IL-17R in epithelial cells from surgical specimens.

Double immunofluorescence experiments, performed on tissue sections from surgical specimens, showed that epithelial cells from central and distal airways had higher levels of IL-17A and IL-17R colocalization in COPD patients than in HC subjects (Figure 5). Furthermore, epithelial cells from central and distal airways of COPD had low levels of IL-17F and IL-17R colocalization in COPD patients. These results support the positive correlation obtained between the immunoreactivity of epithelium for IL-17A and IL-17R obtained with the detection of positive cells by immunohistochemistry.

3.5. In vitro, Cigarette smoke extract induces IL-17A, IL-17F and IL-17R expression in 16HBE, A549 and in PBMC

In vitro, we studied the effect of CSE on IL17A, IL17F and IL17R expression in epithelial cell line 16HBE and A549 as well as in PBMC from normal donors. Using flowcytometry, Immunocytochemistry and Immunoflorescence analyses, we showed that CSE used at the percentage of 2.5% statistically significant increase the expression of IL-17F ($p<0.021$) and IL-17R ($p<0.024$) in 16HBE cell line compared with untreated cells (Figure 6). Using the same technique,

we showed that CSE stimulation at the percentage of 5% statistically significant increase the expression of IL-17F ($p<0.015$) and IL-17R ($p<0.002$) in A549 cell line (Figure 7). Finally, we observed that CSE at the percentage of 10% statistical significant increase the IL-17A ($p<0.036$), IL-17F ($p<0.044$) in PBMC compared with untreated cells (Figure 8).

3.5. Effect of CSE and IL-17A on cell proliferation and apoptosis of 16HBE and A549

CSE 2.5% or rhIL-17A did not affect cell proliferation in stimulated 16HBE cell line compared to untreated cells. The combined use of CSE and rhIL-17A significant increase cell proliferation compared with 16HBE cell line stimulated with CSE or untreated ($p<0.022$, $p<0.004$; respectively) (Figure 9, A, B). CSE 5% or rhIL-17A increased cell apoptosis in stimulated A549 cell line compared to untreated cells although without a statistical significant difference. Furthermore, we showed that the combined use of CSE 5% and rhIL-17A statistical significant increase cell apoptosis in A549 cell line than in untreated cells or than in CSE or in rhIL-17A treated cells ($p<0.003$, $p< 0.012$ and $p<0.011$; respectively) (Figure 9, C, D).

4. Discussion

This study compare for the first time the levels of IL-17A, IL-17F and IL-17R in the central and distal airways from smoking COPD patients. Our results showed that the immunoreactivity of IL-17A, IL-17F and IL-17R was increased in both airway epithelial cells from central and distal airways of COPD patients. Furthermore, we observed that the immunoreactivity of IL-17A showed higher levels in submucosa than in epithelium from central airways of COPD patients as well as in submucosa than in epithelium from distal airways of COPD patients. Furthermore, we observed that the lower levels of IL-17A immunoreactivity in epithelial cells positively correlated with IL-17R expression and with smoke habit in both districts. These findings might suggest that in the presence of cigarette smoke, IL-17A produced by infiltrating cells target higher levels of IL-17R observed in the epithelium from COPD, promoting airway epithelial cell activation during the inflammatory process of the disease that might be involved in the tissue renewal and damage. In vitro experiments of this study support these observations.

The airway epithelium is a dynamic tissue capable of self-renewal and proliferation after injury that undergoes slow but constant renewal [26]. Acting as a physical barrier, the lung epithelium regulates lung fluid balance, modulates metabolism and clearance of inhaled agents, and secretes numerous mediators, several of which recruit and activate inflammatory cells in response to injury [27]. Dysregulation of airway epithelial cell function related to environmental triggers, like cigarette smoke, may contribute to the pathogenesis of major lung diseases such as COPD. Th17 immunity orchestrated mechanisms inherent tissue damage in response to microbial infection and perpetuation of an autoimmune response in airway inflammation. Furthermore, Th17 immunity explain these phenomena underlying T cell–mediated damage to tissue. IL-17A was exclusively expressed in inflammatory cells present in the subepithelial cells even if some immunoreactive cells were present in the epithelium and within smooth muscle bundles in patients with asthma as well as IL-17F [28]. Our results showed that IL-17A, IL-17F and the related receptor IL-17R are increased in the epithelial cells from both central and distal airways and that IL-17A and IL-17F were

increased in the sub epithelium from both central and distal airways of smoking COPD. These findings might suggest that cigarette smoke habit are able to increase the expression of these cytokines and of IL-17R in both district of COPD patients. The data of our study are in agreement with previous results showing that cigarette smoke exposure, upregulated IL-17A/F in human lung tissue explants from both non-COPD and COPD subjects [29]. On the other hand, it was observed that COPD and control smokers with normal lung function have increased numbers of interleukin 17A+ cells in the bronchial submucosa where T-cells might represent an important source of this cytokine in the presence of smoking habit [13]. Furthermore, the up-regulation of Th17 might be clearly associated with cigarette smoke in lung tissue of mice [30]. These observations together with our findings support the implications of Th17 immunity in the pathogenesis of COPD dependent on the risk factor cigarette smoke. Particularly, we found higher levels of cells/mm2 immunoreactive for IL-17A and IL-17F in the sub epithelium of smoking COPD from both central and distal airways than in epithelium. Furthermore, we observed that the cells/mm2 immunoreactive for IL-17F are elevated also in the epithelium. The use of immunofluorescence to study the co-localization clearly demonstrated that both IL-17A and IL-17F are expressed with IL-17R in epithelial cells from both central and distal airways of smoking COPD patients than in HC subjects. These findings might suggest the concept that IL-17A and IL-17F are involved in the activation of epithelium in COPD patients having smoking habit. Moreover, the analysis of the correlations showed a positive relationship between IL-17A expression and smoking habit or between IL-17A and IL-17R expression in both central and distal airway epithelial cells from smoking COPD patients. No positive correlation were found for the cells immunoreactive for IL-17F. These findings leave to suppose a not clear secondary role for IL-17F in the pathogenesis of COPD. On the other hand, although it was observed that IL-17F may also have similar neutrophil-promoting effects in chronic inflammatory and allergic lung disease [31], an animal models of lung inflammation showed that the administration of anti-IL-17F antibodies has no effect on lung neutrophilia [32] a pathogenetic mechanism involved in airway inflammation of COPD patients.

Accordingly, with ambiguous role of IL-17F, Hizawa et al [31] showed that Mutant IL-17F was unable to activate the Raf1-MEK-ERK1/2 pathway, but antagonized wild-type IL-17F activity, suggesting that IL-17F might be able to bind the receptor, but not activate the signaling pathways. IL-17F has at least an order of magnitude lower affinity for IL-17R than IL-17A however, due to similar biological activity, there has been speculation whether both IL-17A and IL-17F signal act via the IL-17R [33]. Given that asthma and COPD are complex diseases involving a number of genetic and environmental factors, the genetic impact of IL-17F H161R with regard to the development of chronic airway inflammation likely varies among individuals with different genetic backgrounds and environmental exposures [34]. These findings suggest that IL-17F present in the airways of smoking COPD might not be involved in the activation of airway epithelial cells during inflammation of COPD patients. However further study might be necessary to clarify the role of IL-17F and IL-17R in the pathogenesis of airway diseases. In this scenario, we suggest that Th17 immunity involved in the activation of airway epithelial cells during the inflammatory actions of cigarette smoke is represented by IL-17A. Particularly we suppose that IL-17A detected in the epithelium from COPD patients, is IL-17A produced in the submucosa of central airways and in the parenchyma of distal airways by infiltrating cells and target epithelial cells by the increased levels of IL-17R. By exploiting, the growing understanding of the epithelium and its interactions with inflammatory cells obtained by our findings might open new pharmacological perspective to treat the epithelial dysregulation associated with Th17 immunity during inflammatory lung conditions.

Chronic cigarette smoke has been shown to induce both Th1 and Th17 cells in mice [35] suggesting parenchymal tissue is an important source of IL-17A/F in individuals who smoke. To study the role of cigarette smoke on IL-17A, IL-17F and IL-17R production in epithelial cells and in inflammatory cells of the lung, we stimulated epithelial cell line 16HBE and A549 as well as PBMC from normal donors with different concentration of CSE (0, 2.5, 5, and 10%). CSE was able to increase the production of IL-17F and IL-17R in 16HBE and in A549 while in PBMC increase only IL-17A and IL-17F. This effect was at different concentration of CSE: 2.5% for 16HBE, 5%

for A549 and 10 % for PBMC. These results further suggest and support that IL-17 immunity involving the activation of epithelial cells in the lung, might be regulated by smoking habit (pack years) in COPD patients.

Th17 cells may play an important role in the pathogenesis of emphysema, their role is not exclusive and Th1 and Th2 cells may have contributory roles in the central rather than distal airways of COPD. Th17 is now credited for causing and sustaining tissue damage in these diverse situations. The Th1 pathway antagonizes the Th17 pathway in an intricate fashion. The evolution of our understanding of the Th17 pathway illuminates a shift in immunologists' perspectives regarding the basis of tissue damage [36]. Much of our knowledge from the interactions between environmental and inflammatory stimuli, and the airway epithelium has been derived extensively from in vitro cell culture models. Taken together, in vitro studies have shown that differentiated cell culture is an invaluable model in understanding the physiological properties of the human airway epithelium. Accordingly using an in vitro model, we studied the effect of rhIL-17A in 16HBE and A549 to understand its potential action in the activation of epithelial cells in central and distal airways involving cell proliferation and apoptosis during the pathogenesis of COPD patients. Particularly, we found that in the presence of CSE promoting higher levels of the IL-17R receptor expression (as observed), hrIL-17A is able to generate proliferative mechanism of bronchial epithelial cell line 16HBE. These findings might suggest that epithelium proliferation, at baseline of the mechanism of squamous metaplasia observed in central airways of COPD patients [37], is a considerably disturb of the innate immune functions of the airway epithelium involving IL-17A. Furthermore, it was observed that IL-17A is essential to the development of emphysema in mice [38] and that cigarette smoke induces apoptosis in A549 [39]. Accordingly, we observed that CSE or hrIL-17A generate the mechanism of apoptosis in type II alveolar epithelial cell-derived A549 cell line with a synergistic effect when used in combination in agreement with the observed increased expression of IL-17R in the presence of CSE stimulation. Accordingly with Duan MC et al showing that Th17 immunity is involved in the lungs of cigarette smoke-induced emphysema

observed in mice [30], our data might support the relevant contribution of IL-17A in the development of pulmonary inflammation and emphysema of COPD patients.

In conclusion, Th17 immunity associated with IL17A and IL-17F cytokines and the related IL-17 receptor showed a relevant role in the epithelium and subepithelium of both, central and distal airway from smoking COPD patients. Using our ex vivo/in vitro studies we showed that smoke habit might contribute to increase IL-17F and IL-17R production in epithelial cells as well as to increase IL-17A and IL-17F production in infiltrating cells present in the subepithelium from large and small airways. Finally, by in vitro study, we identified a potential role of IL-17A rather than IL-17F in the phenomena of epithelial cell proliferation and apoptosis involved in the tissue damage and repair during the pathogenesis of COPD in the central and distal airways.

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Disclosure statements

The authors of the manuscript have not conflict of interest.

Authors' contributions

The authors MP, AMM and LR conceived the study and designed the experiments. LS, GC, GA, RG, CDS performed the technical procedures. FLMR and VS give us some

suggestions about the data analysis. PV and LP give us the support for surgical specimens. MP revised the final draft of the manuscript. All authors read and approved the final version of the MS.

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Legends for the figures

Figure 1: Immunoreactivity for IL-17A, IL-17F and IL-17R in surgical specimens from central airways of HC (n = 10) and COPD (n = 15) subjects. Cells were stained with an anti-IL-17A, anti-IL-17F and anti-IL-17R antibodies. Negative control was performed using rabbit immunoglobulins negative control (see materials and methods for details). A) Counts for the number of positive epithelial cells/mm basement membrane (Left column); B) counts for the number positive subepithelial cells/mm2 (right column) in central airways (Right column). Results were expressed as box plot representing median and 25-75 percentiles. Statistical analysis was performed by Kruskal Wallis test followed by Fisher's PLSD correction for multiple comparison. Significance was accepted at $p<0.05$.

Figure 2: Immunoreactivity for IL17A, IL17F and IL-17R in surgical specimens from distal airways of HC subjects (n = 10) and COPD patients (n = 15). Cells were stained with an anti-IL-17A, anti IL-17F and anti IL-17R antibodies. Negative control were performed using rabbit immunoglobulins (see materials and methods for details). A) Counts of the number of positive epithelial cells/mm basement membrane (Left column); B) counts of the number positive subepithelial cells/mm2 (right column) in central airways (Right column). Results were expressed as box plot representing median and 25-75 percentiles. Statistical analysis was performed by Kruskal Wallis test followed by Fisher's PLSD correction for multiple comparison. Significance was accepted at $p<0.05$.

Figure 3: Represenative immunostaining of IL17A, IL17F and IL17R in central and in distal airways. **A**) Representative images for IL17A, IL-17F and IL-17R immunostaining (red stain) of epithelium and subepithelium in central (left panel) and distal (right panel) airways of HC, and COPD subjects (original magnification X400).

Figure 4: Spearman's rank correlations in central and distal airways of COPD (n=15) patients. A)

Correlation between the Total Pks year and immunoreactivity for IL-17A in the epithelium of central and distal airways; B) Correlation between the immunoreactivity for IL-17A and immunoreactivity for IL-17R in the epithelium of central and distal airways. Data were reported as individual values. Statistical analysis was performed by Spearman's rank test. Significance was set at $p<0.05$.

Figure 5: Double immunofluorescence for IL-17A or IL-17F and IL-17R in epithelial cells from surgical specimens. Epithelial cells from HC subjects and COPD patients showed single staining for IL-17A (green) (A) or IL-17R (red) (B), or a double positivity (orange) (C) in central airways. Some of the latter are indicated by arrows. Epithelial cells from HC subjects and COPD patients showed single staining for IL-17A (green) (D) or IL-17R (red) (E), or double positivity (orange) (F) in distal airways. Some of the latter are indicated by arrows. Epithelial cells from HC subjects and COPD patients showed single staining for IL-17F (green) (A) or IL-17R (red) (B), or a double positivity (orange) (C) in central airways. Some of the latter are indicated by arrows. Epithelial cells from HC subjects and COPD patients showed single staining for IL-17A (green) (D) or IL-17R (red) (E), or double positivity (orange) (F) in distal airways. Some of the latter are indicated by arrows.

Figure 6: Effect of CSE 2.5% on the expression of IL-17F and IL-17R in 16-HBE cells. Cells were incubated with and without CSE for 24 hrs and tested for IL-17F and IL-17R (cytokines or proteins) expression by A) Flowcytometric analysis. Bars represent mean ± SD of % of positive gated cells of 6 separate experiments. B) Representative flow-cytometry analysis for IL17F and IL17R in 16HBE cell line; C) Representative immunocytochemical and immunofluorescent analysis (original magnification X400). Statistical analysis was performed by ANOVA test with Fisher's correction for multiple comparisons. Significance was set at $p<0.05$.

Figure 7: Effect of CSE 5% on the expression of IL-17F and IL-17R in A549 cell line. Cells were incubated with and without CSE for 24 hrs and tested for IL-17F, and IL-17R (cytokines or proteins) expression by A) Flowcytometric analysis. Bars represent mean ± SD of % of positive gated cells of 6 separate experiments. B) Representative flow-cytometry analysis for IL17F and IL17R in A549 cell line; C) Representative immunocytochemical and immunofluorescent analysis (original magnification X400). Statistical analysis was performed by ANOVA test with Fisher's correction for multiple comparisons. Significance was set at $p<0.05$.

Figure 8: Effect of CSE 10% on the expression of IL-17A and IL-17F in PBMC from normal donors. Cells were incubated with and without CSE for 24 hrs and tested for IL17A and IL17F (cytokines or proteins) expression by A) Flowcytometric analysis. Bars represent mean ± SD of % of positive gated cells of 6 separate experiments. B) Representative flow-cytometry analysis for IL17A, IL17F and IL17R in PBMC. C) Representative immunocytochemical and immunofluorescent analysis (original magnification X400). Statistical analysis was performed by ANOVA test with Fisher's correction for multiple comparisons. Significance was set at $p<0.05$.

Figure 9: Effects of CSE and rhIL-17A on cell proliferation in 16HBE and on apoptosis in A549. A) 16HBE, cultured in the presence and absence of CSE 2.5 % alone or in combination with rhIL-17A 20ng/ml for 192 hrs, were used for evaluating proliferation using a CFSE test (see Material and Methods for details). Bars represent mean ± S. D. fluorescence intensity (MFI) of three separate experiments and were plotted as fold-change compared to untreated cells, which were chosen as the reference sample. B) A549, cultured in the presence and absence of CSE 5 % alone or in combination with rhIL-17A 20ng/ml for 24 hrs were used for evaluating apoptosis using an Annexin V Test (see Material and methods for details). Bars represent mean ± S. D. fluorescence intensity (MFI) of three separate experiments and were plotted as fold-change compared to untreated cells, which were chosen as the reference sample. Statistical analysis was performed by ANOVA test with Fisher's correction for multiple comparisons. Significance was set at $p<0.05$.

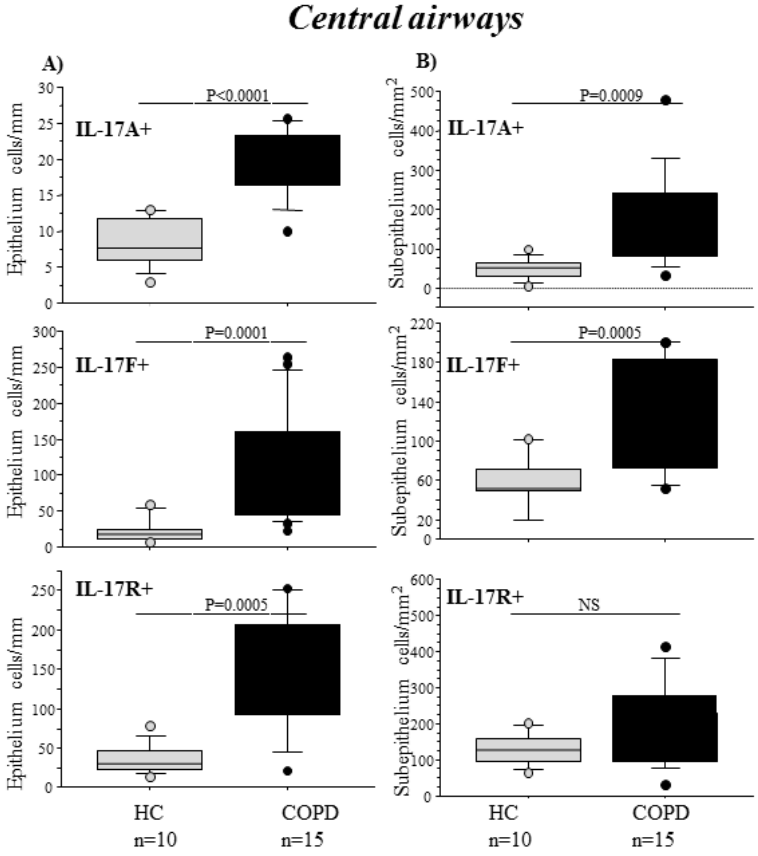
Central airways
A)
B)
IL-17A+
P<0.0001
P=0.0009
IL-17F+
P=0.0001
P=0.0005
IL-17R+
P=0.0005
NS
Epithelium cells/mm
Subepithelium cells/mm2
HC
n=10
COPD
n=15

Figure 1

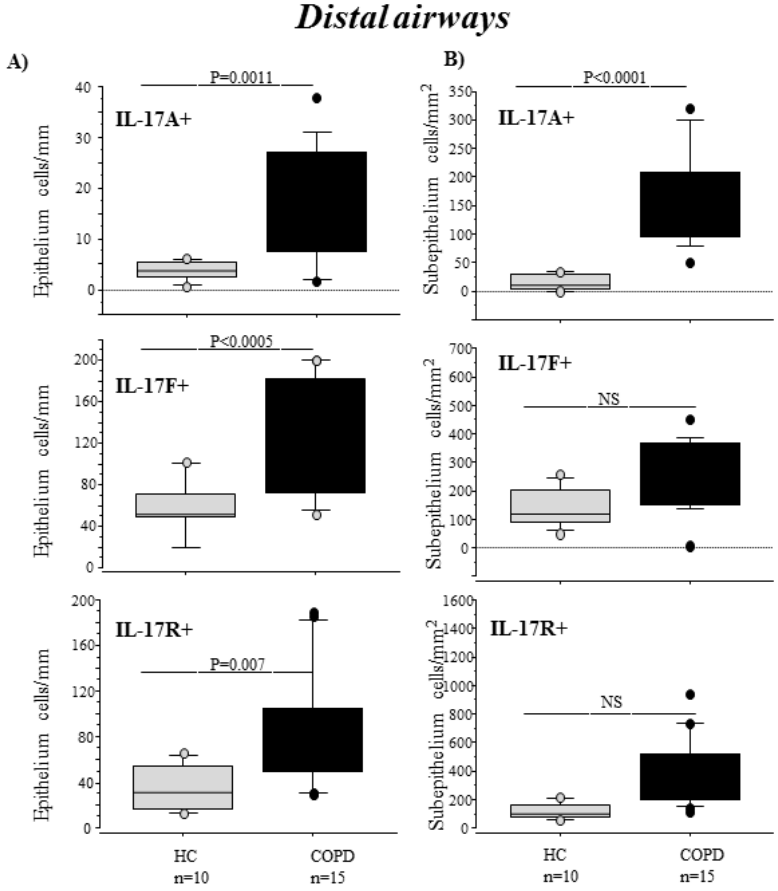
Distal airways
A)
B)
P=0.0011
IL-17A+
Epithelium cells/mm
P<0.0001
IL-17A+
Subepithelium cells/mm2
P<0.0005
IL-17F+
Epithelium cells/mm
IL-17F+
NS
Subepithelium cells/mm2
IL-17R+
P=0.007
Epithelium cells/mm
IL-17R+
NS
Subepithelium cells/mm2
HC
n=10
COPD
n=15
HC
n=10
COPD
n=15

Figure 2

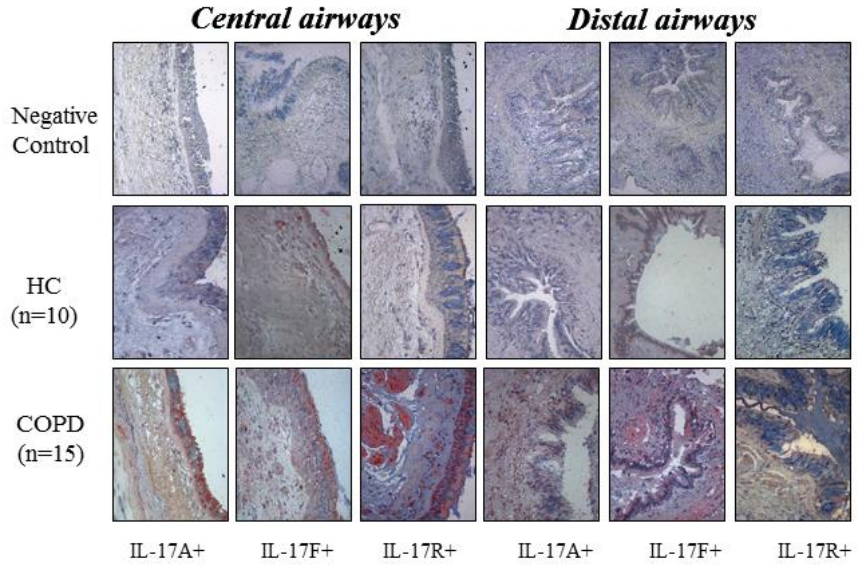
Central airways
Distal airways
Negative Control
HC (n=10)
COPD (n=15)
IL-17A+
IL-17F+
IL-17R+
IL-17A+
IL-17F+
IL-17R+

Figure 3

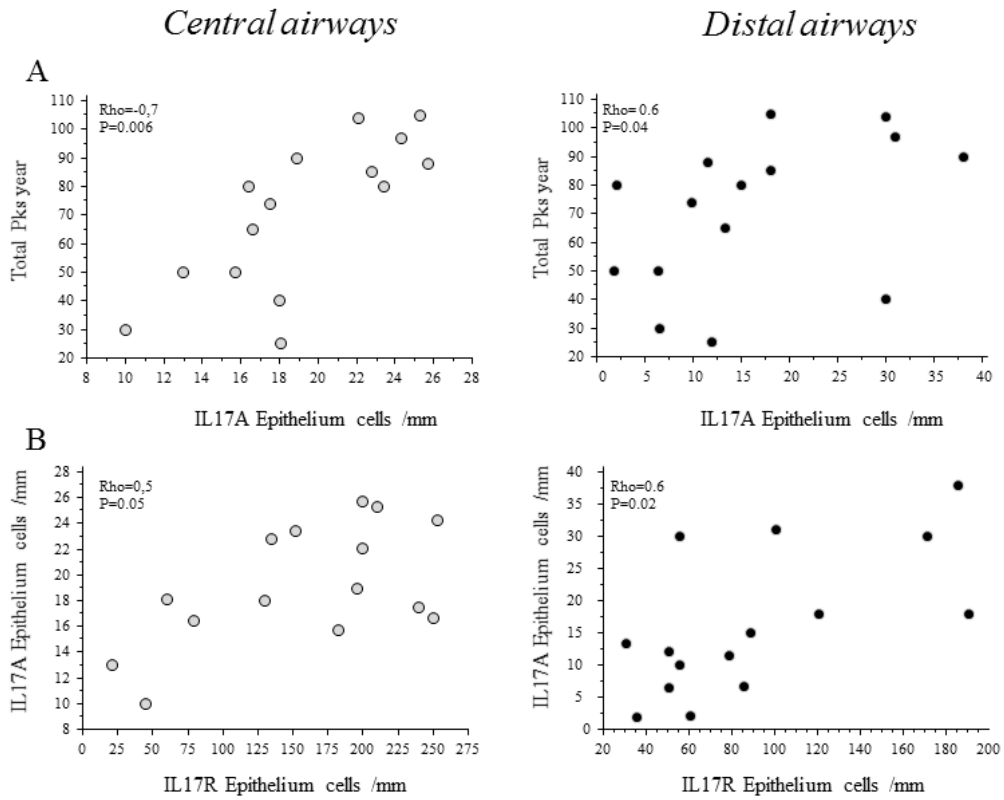
Central airways
Distal airways
A
Rho=-0,7
P=0.006
Total Pks year
IL17A Epithelium cells /mm
Rho= 0.6
P=0.04
Total Pks year
IL17A Epithelium cells /mm
B
Rho=0,5
P=0.05
IL17A Epithelium cells /mm
IL17R Epithelium cells /mm
Rho=0.6
P=0.02
IL17A Epithelium cells /mm
IL17R Epithelium cells /mm

Figure 4

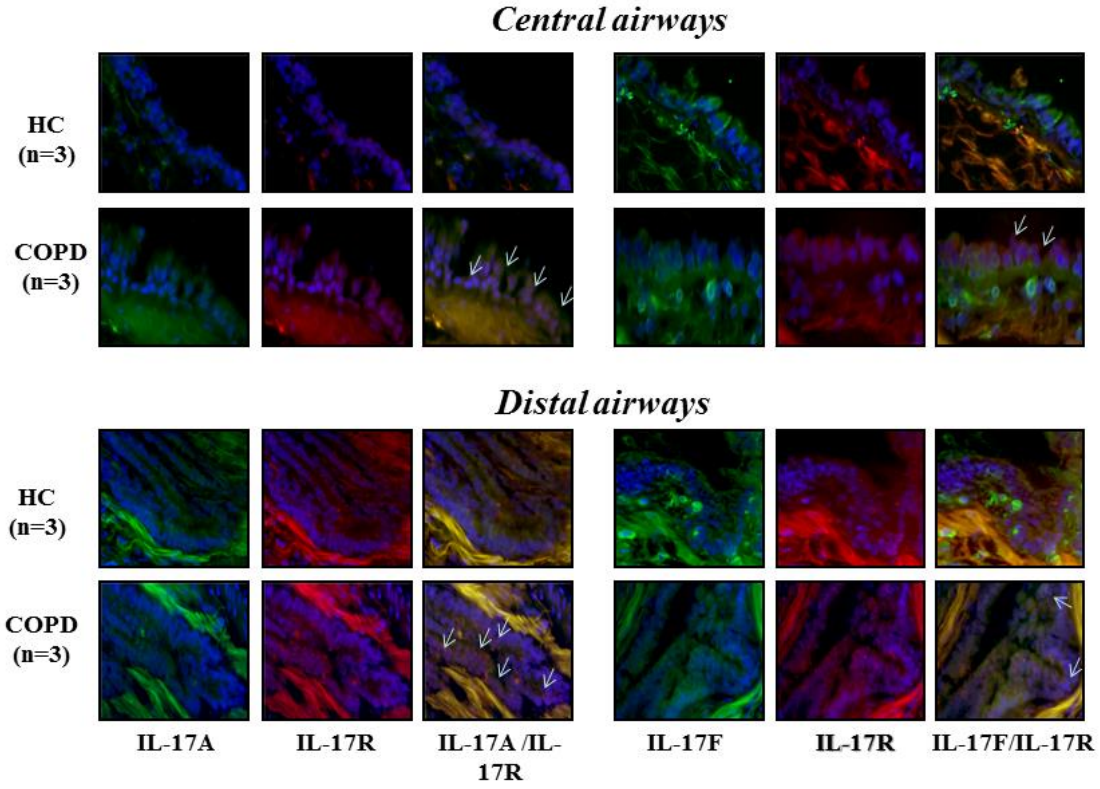
Central airways
HC (n=3)
COPD (n=3)
Distal airways
HC (n=3)
COPD (n=3)
IL-17A
IL-17R
IL-17A /IL-17R
IL-17F
IL-17R
IL-17F/IL-17R

Figure 5

Human bronchial epithelial cell line

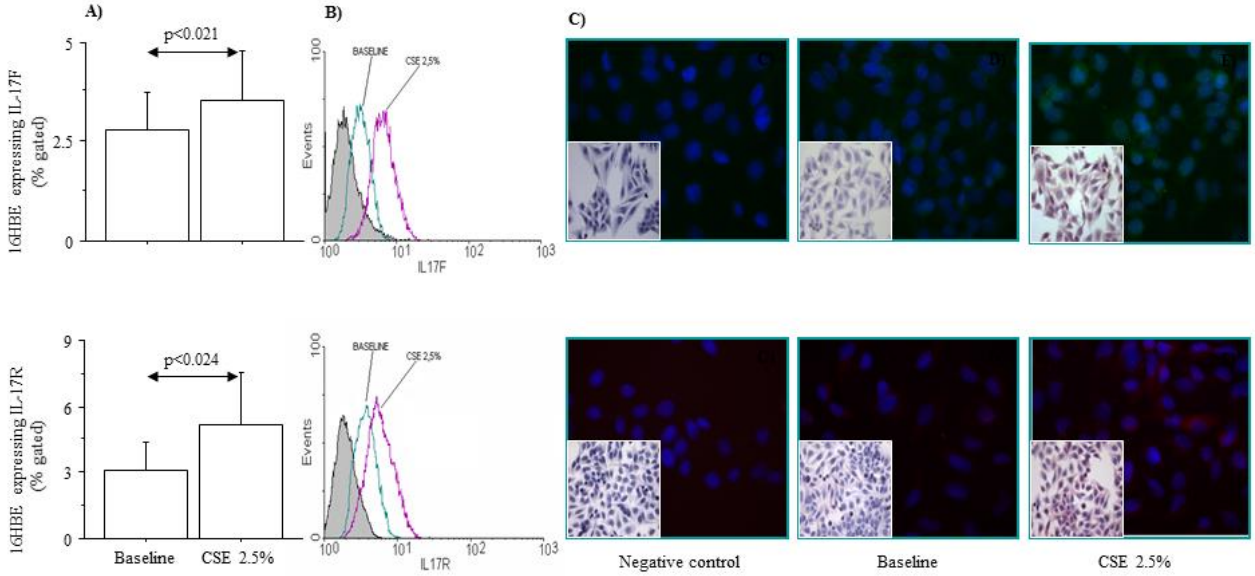

Figure 6

A549 cell line

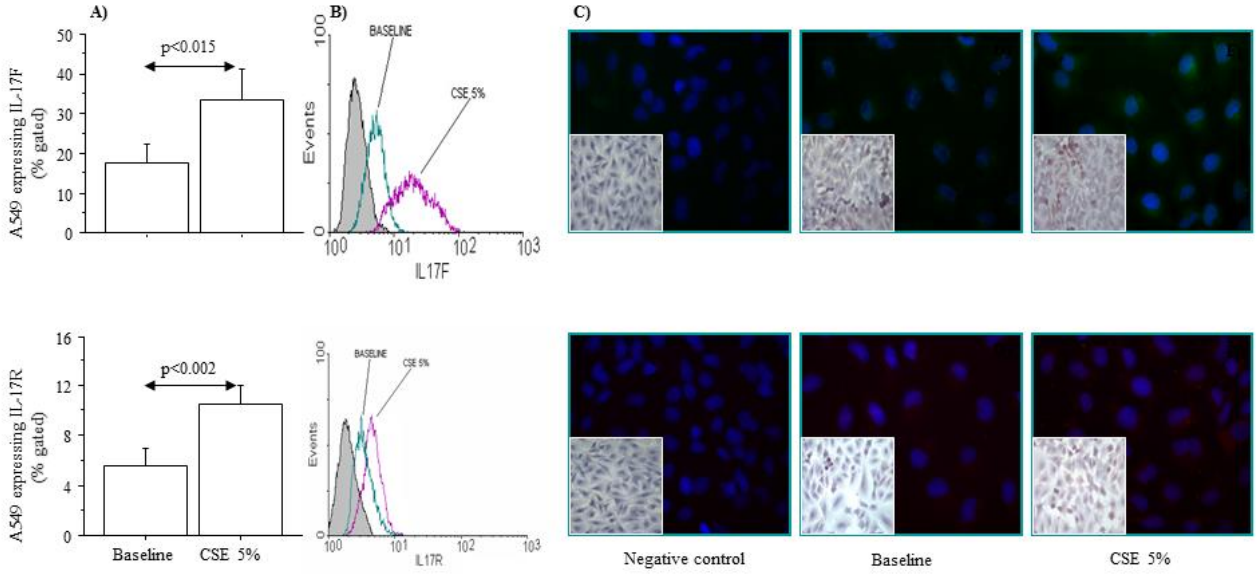

Figure 7

Peripheral blood mononuclear cells (PBMC)

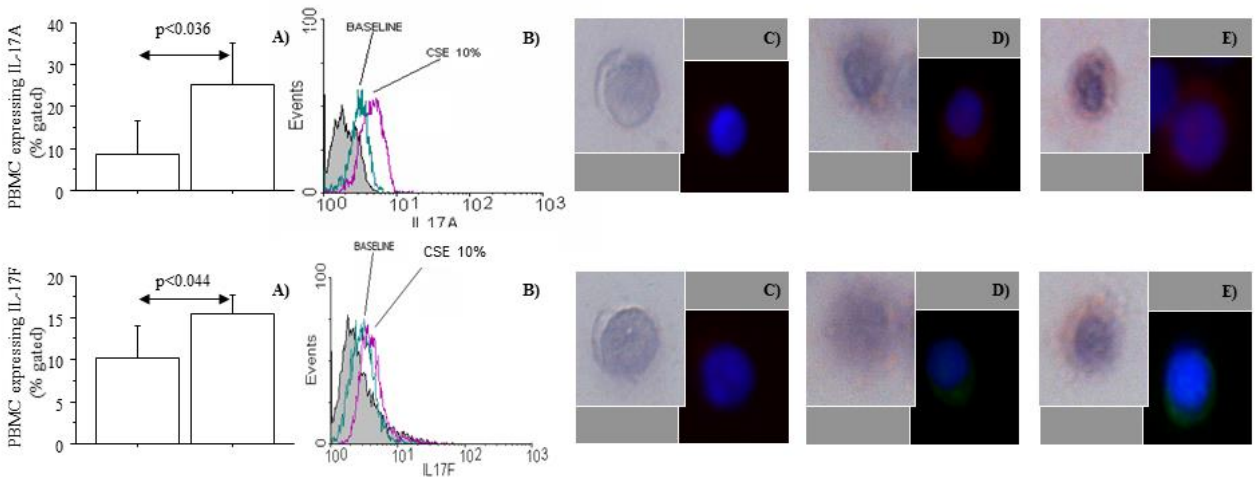

Figure 8

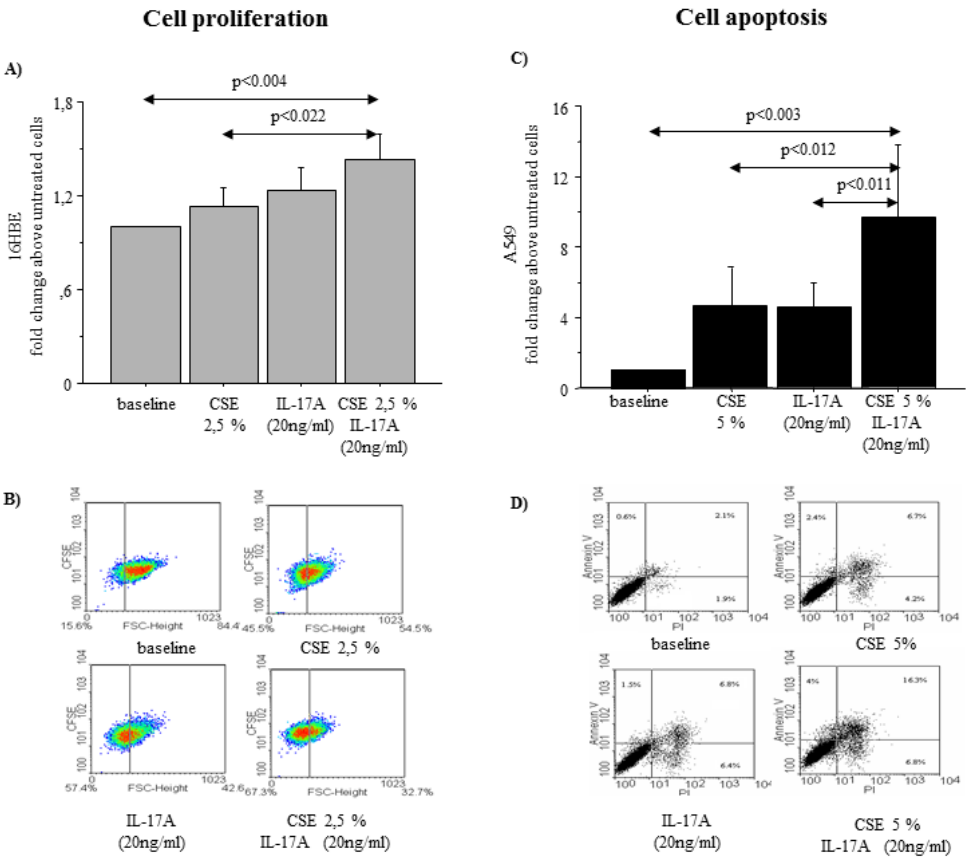
Cell proliferation
Cell apoptosis
A)
C)
B)
D)
p<0.004
p<0.022
p<0.003
p<0.012
p<0.011
16HBE
fold change above untreated cells
A549
fold change above untreated cells
1,8
1,2
,6
0
16
12
8
4
0
baseline
CSE 2,5 %
IL-17A (20ng/ml)
CSE 2,5 % IL-17A (20ng/ml)
baseline
CSE 5 %
IL-17A (20ng/ml)
CSE 5 % IL-17A (20ng/ml)
CFSE
FSC-Height
15.6%
84.4
45.5%
54.5%
57.4%
42.6
67.3%
32.7%
Annexin V
PI
0.6%
2.1%
1.9%
2.4%
6.7%
4.2%
1.5%
6.8%
6.4%
4%
16.3%
6.8%
baseline
CSE 5%
IL-17A (20ng/ml)
CSE 2,5 % IL-17A (20ng/ml)
IL-17A (20ng/ml)
CSE 5 % IL-17A (20ng/ml)

Figure 9

TABLE 1: Demographic characteristics of patients

	Control	COPD	Overall p value
Subject number	n=10	n=15	
Sex, male/female	8/2	12/3	
Age, yr	69.5 ± 5.8	76.3 ± 13.2	N.S.
FEV1, % predicted	106 ± 19.4	68.8 ± 17.5	$p < 0.001$
FEV1/FVC, %	80.3 ± 2.9	60.9 ± 3.5	$p < 0.001$
Smoking, pack/yr	0	50.9±36.2	-

Data are shown as mean±S.D.
Abbreviations: FEV1 = forced expiratory volume in 1 s;
FVC = forced vital capacity.
Statistical analysis for multiple comparisons was performed by Mann-Whitney U-test.