Basic Science Investigations

Respiration

Phospho-p38 MAPK Expression in COPD Patients and Asthmatics and in Challenged Bronchial Epithelium

Davide Vallese[a]   Fabio L.M. Ricciardolo[b]   Isabella Gnemmi[a]   Paolo Casolari[c]
Paola Brun[d]   Valentina Sorbello[b]   Armando Capelli[a]   Francesco Cappello[e, f]
Giorgio Narciso Cavallesco[g]   Alberto Papi[c]   Kian Fan Chung[h]   Bruno Balbi[a]
Ian M. Adcock[h]   Gaetano Caramori[c]   Antonino Di Stefano[a]

[a]Divisione di Pneumologia e Laboratorio di Citoimmunopatologia dell'Apparato Cardiorespiratorio, Fondazione Salvatore Maugeri, IRCCS, Veruno, [b]Divisione di Pneumologia, Ospedale San Luigi, Università di Torino, Orbassano, [c]Centro Interdipartimentale per lo Studio delle Malattie Infiammatorie delle Vie Aeree e Patologie Fumo-Correlate, Sezione di Medicina Interna e Cardiorespiratoria, Dipartimento di Scienze Mediche, and [d]Dipartimento di Medicina Molecolare, Università di Padova, Padova, and [e]Sezione di Anatomia Umana, Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche, Università di Palermo, and [f]Euro-Mediterranean Institute of Science and Technology, Palermo, [g]Section of General and Thoracic Surgery, Department of Morphology, Experimental Medicine and Surgery, Sant'Anna Hospital, University of Ferrara, Ferrara, Italy; [h]National Heart and Lung Institute, Imperial College London, London, UK

Abstract
Background: The role of mitogen-activated protein kinases (MAPK) in regulating the inflammatory response in the airways of patients with chronic obstructive pulmonary disease (COPD) and asthmatic patients is unclear. **Objectives:** To investigate the expression of activated MAPK in lungs of COPD patients and in bronchial biopsies of asthmatic patients and to study MAPK expression in bronchial epithelial cells in response to oxidative and inflammatory stimuli. **Methods:** Immunohistochemical expression of phospho (p)-p38 MAPK, p-JNK1 and p-ERK1/2 was measured in bronchial mucosa in patients with mild/moderate (n = 17), severe/very severe (n = 16) stable COPD, control smokers (n = 16), control non-smokers (n = 9), in mild asthma (n = 9) and in peripheral airways from COPD patients (n = 15) and control smokers (n = 15). Interleukin (IL)-8 and MAPK mRNA was measured in stimulated 16HBE cells. **Results:** No significant differences in p-p38 MAPK, p-JNK or p-ERK1/2 expression were seen in bronchial biopsies and peripheral airways between COPD and control subjects. Asthmatics showed increased submucosal p-p38 MAPK expression compared to COPD patients (p < 0.003) and control non-smokers (p < 0.05). Hydrogen peroxide (H_2O_2), cytomix (tumour necrosis factor-α + IL-1β + interferon-γ) and lipopolysaccharide (LPS) upregulated IL-8 mRNA at 1 or 2 h. p38 MAPKα mRNA was significantly increased after H_2O_2 and LPS treatment. JNK1 and ERK1 mRNA were unchanged after H_2O_2, cytomix or LPS treatments. **Conclusion:** p-p38 MAPK expression is similar in stable COPD and control subjects but increased in the bronchi of mild

Antonino Di Stefano, PhD
Fondazione Salvatore Maugeri, IRCCS, Divisione di Pneumologia e Laboratorio di Citoimmunopatologia dell'Apparato Cardiorespiratorio, Via per Revislate, 13
IT–28010 Veruno, NO (Italy)
E-Mail antonino.distefano @ fsm.it

asthmatics compared to stable COPD patients. p38 MAPK mRNA is increased after bronchial epithelial challenges in vitro. These data together suggest a potential role for this MAPK in Th2 inflammation and possibly during COPD exacerbations.

Introduction

The mitogen-activated protein kinase (MAPK) family includes 3 distinct stress-activated protein kinase pathways: p38, c-Jun N-terminal kinase (JNK) and extracellular regulating kinase (ERK) [1]. The ERK pathway is predominantly activated by mitogenic and proliferative stimuli, whereas the JNK and p38 MAPK respond to environmental stresses [1]. A potential role of activated p38 MAPK has been reported for neutrophil and eosinophil lung cell migration, cytokine release from inflammatory cells and airway smooth muscle, release of matrix metalloproteinases, Th1 cell differentiation and interferon (IFN)-γ production and induction of corticosteroid insensitivity [2]. MAPK and the pro-inflammatory transcription factor nuclear factor-κB (NF-κB) are activated in airway epithelial cells and macrophages exposed to different stimuli such as lipopolysaccharide (LPS), cigarette smoke extracts and oxidants [3–8]. The production of mucin by airway epithelial cells is induced by oxidants and is accompanied by p38 MAPK phosphorylation and activation [6]. Cigarette smoke exposure of airway epithelial cells resulted in upregulation of interleukin (IL)-6 and IL-8 mediated by p38 MAPK and NF-κB [9]. Rhinovirus exposure of human macrophages induces p38 MAPK activation and release of CCL1 (monocyte chemoattractant protein-1) [10]. Increased phospho (p)-p38 MAPK immunopositivity was reported in lung alveolar septa of chronic obstructive pulmonary disease (COPD) patients when compared to control smokers and non-smokers [11], whilst p-p38 MAPK expression in lymphocytes within the submucosa of peripheral airways was similar in COPD patients, control smokers and non-smokers [12]. In bronchial biopsies of asthmatics, epithelial staining for p-p38 MAPK and p-ERK1/2 was increased compared to healthy controls, while healthy subjects showed the highest p-JNK intensity [13]. MAPK expression and activation have not been studied to date in bronchial biopsies of COPD patients. Due to the role of p38 MAPK in inducing inflammation, p38 MAPK inhibitors have recently been studied, particularly in severe asthmatics and COPD patients [2]. The results obtained in these clinical trials are not particularly encouraging [14–16].

In COPD, the airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases [17, 18]. Previous studies have emphasized the potential role of p38 MAPK in the pathogenesis of COPD and asthma [2]. However, the mechanisms of inducing p38 MAPK upregulation and its participation in the inflammatory state in the bronchi of COPD patients are not yet fully clarified.

The present study aimed: (1) to quantify by immunohistochemistry the activated MAPK expression in the bronchial biopsies and peripheral airways of patients with COPD and to compare these findings with control smokers, non-smoking subjects and a group of asthmatics, and (2) to investigate MAPK mRNA expression in vitro in bronchial epithelial cells in response to oxidative (H_2O_2) and inflammatory (LPS, cytomix) stimuli which are implicated in COPD and asthma.

Methods

Subjects

All COPD and healthy control subjects who underwent bronchoscopy and bronchial biopsy collection were recruited from the Respiratory Medicine Unit of the Fondazione Salvatore Maugeri (Veruno, Italy). Asthmatics were recruited from the Division of Pneumology, Ospedale San Luigi, Orbassano, University of Torino, and the severity of asthma was classified according to the GINA and ATS criteria [19, 20]. In COPD patients, the severity of the airflow obstruction was staged using current GOLD criteria (www.goldcopd.com). All former smokers had stopped smoking for at least 1 year. COPD and chronic bronchitis were defined, according to international guidelines, as follows: COPD, presence of a postbronchodilator forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) ratio <70%; levels of shortness of breath, chronic cough, sputum and numbers of exacerbations per year were also taken into account as suggested by new GOLD criteria; chronic bronchitis, presence of cough and sputum production for at least 3 months in each of 2 consecutive years [18]. All COPD patients were stable with no previous exacerbation in the 6 months before bronchoscopy. None of the COPD patients was treated with theophylline, antibiotics, antioxidants, mucolytics and/or glucocorticoids in the month prior to the bronchial biopsy. The peripheral lung tissues were collected at the University Hospital of Ferrara, during lung resection for a solitary peripheral neoplasm, and all subjects were not under regular treatment with glucocorticoids and/or bronchodilators. Asthmatics were under therapy following GINA criteria [19]. The study conformed to the Declaration of Helsinki and was approved by the ethics committees of the Fondazione Salvatore Maugeri, Veruno (Novara), San Luigi Hospital, Orbassano (Torino), and the University Hospital of Ferrara, Italy; written informed consent was obtained from each participant, and bronchial biopsies were performed according to the guidelines of the local ethics committee.

Table 1. Primary antibodies and immunohistochemical conditions used for identification of MAPK and inflammatory cells

Primary antibody	Origin	ID	Secondary antibody	Dilution	Positive control
p-p38	Santa Cruz	SC-17852-R	Rabbit	1:150	Nasal polyp, human tonsil
p-JNK1	Abcam	Ab-18680	Rabbit	1:150	Nasal polyp, human tonsil
p-ERK1/2	Epitomics	1481-1	Rabbit	1:100	Nasal polyp, human tonsil
CD4	Dako	M716	Mouse	1:100	Nasal polyp, human tonsil
CD8	Dako	M7103	Mouse	1:200	Nasal polyp, human tonsil
CD68	Dako	M814	Mouse	1:200	Nasal polyp, human tonsil
Neutrophil elastase	Dako	M752	Mouse	1:100	Nasal polyp, human tonsil

Lung Function Tests and Volumes

Pulmonary function tests were performed as previously described [21] according to guideline recommendations [22]. Pulmonary function tests included measurements of FEV_1 and FEV_1/FVC under baseline conditions in all the subjects examined (6200 Autobox Pulmonary Function Laboratory; Sensormedics Corp., Yorba Linda, Calif., USA). In order to assess the reversibility of airflow obstruction and postbronchodilator functional values, the FEV_1 and FEV_1/FVC percent measurements in the groups of subjects with $FEV_1/FVC \leq 70\%$ before bronchodilator use was repeated 20 min after the inhalation of 0.4 mg of salbutamol.

Fibre-Optic Bronchoscopy, Collection and Processing of Bronchial Biopsies

Subjects were at the bronchoscopy suite at 8.30 a.m. after having fasted from midnight and were pretreated with atropine (0.6 mg i.v.) and midazolam (5–10 mg i.v.). Oxygen (3 l/min) was administered via nasal prongs throughout the procedure, and oxygen saturation was monitored with a digital oximeter. Using local anaesthesia with lidocaine (4%) to the upper airways and larynx, a fibre-optic bronchoscope (Olympus BF10 Key-Med, Southend, UK) was passed through the nasal passages into the trachea. Further lidocaine (2%) was sprayed into the lower airways, and 4 bronchial biopsy specimens were taken from segmental and subsegmental airways of the right lower and upper lobes using size 19 cupped forceps. Bronchial biopsies for immunohistochemistry were gently extracted from the forceps and processed for light microscopy as previously described [21]. At least 2 samples were embedded in Tissue Tek II OCT (Miles Scientific, Naperville, Ill., USA), frozen within 15 min in isopentane precooled in liquid nitrogen and stored at –80°C. The best frozen sample was then oriented, and 6-μm-thick cryostat sections were cut for immunohistochemical light microscopy analysis and processed as described below.

Collection and Processing of the Peripheral Lung Tissue

Thirty subjects undergoing lung resection surgery for a solitary peripheral neoplasm were recruited. Fifteen were smokers with normal lung function, and 15 subjects were smokers with COPD (table 3). All former smokers had stopped smoking for more than 1 year. No subject underwent pre-operative chemotherapy and/or radiotherapy and had been treated with bronchodilators, theophylline, antibiotics, antioxidants and/or glucocorticoids in the month prior to surgery. Lung tissue processing was performed as previously described [23, 24]. Two to 4 randomly selected tissue blocks were taken from the subpleural parenchyma of the lobe obtained at surgery, avoiding areas grossly invaded by tumour. Samples were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) at pH 7.2 and, after dehydration, embedded in paraffin wax. Serial sections 4 μm thick were first cut and stained with haematoxylin-eosin in order to visualize the morphology and to exclude the presence of microscopically evident tumour infiltration. Tissue specimens were then cut for immunohistochemical analysis and were placed on charged slides as previously reported [24].

Immunohistochemistry on OCT-Embedded Bronchial Biopsies

One cryostat section from each biopsy was stained applying immunohistochemical methods with a panel of antibodies specific for inflammatory cells (CD4+, CD8+, CD68+, neutrophil elastase+) or p-MAPK (table 1). Briefly, after blocking non-specific binding sites with serum derived from the same animal species as the secondary antibody, primary antibody was applied at optimal dilutions in Tris-buffered saline (0.15 M saline containing 0.05 M Tris-hydrochloric acid at pH 7.6) and incubated for 1 h at room temperature in a humid chamber. Antibody binding was demonstrated with secondary anti-mouse (Vector, BA 2000) or anti-rabbit (Vector, BA 1000) antibodies followed by ABC kit AP AK5000 Vectastain and fast red substrate (red colour) or ABC kit HRP Elite, PK6100, Vectastain and diaminobenzidine substrate (brown colour). Human tonsils or nasal polyps were used as positive controls. For the negative control, normal mouse or rabbit non-specific immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) were used at the same protein concentration as the primary antibody.

Immunohistochemistry in Human Peripheral Lung Tissue

Immunostaining of paraffin-embedded peripheral lung tissue was performed as previously described [23]. After deparaffinization and rehydration to expose the immunoreactive epitopes, the sections to be stained, immersed in retrieval solution citrate, pH 6.0, or EDTA, pH 8.0, were incubated in a microwave oven (model NN S200W; Panasonic, Milano, Italy) on high power for 40 min. Endogenous peroxidase activity was blocked by incubating slides in 3% H_2O_2 in PBS followed by washing in PBS. Cell membranes were permeabilized adding 0.1% saponin to the PBS. Non-specific labelling was blocked by coating with blocking serum (5% normal goat serum) for 20 min at room temperature. After washing in PBS the sections were treated with the following primary antibodies: rabbit monoclonal p-p38 MAPK (p-Thr180/p-Tyr182; code 1229-1) and rabbit p-ERK1/p-ERK2 (p-Thr202/p-Tyr204; code 1229-1), both

obtained from Epitomics, and mouse monoclonal anti-p-SAPK/JNK (Thr183/Tyr185; www.scbt.com; code sc6254) at the dilution of 1.25, 1:50 and 1:300, respectively. Sometimes we have used different primary antibodies for the immunohistochemical and the Western blotting (see below) studies because the cell signalling technology does not provide the concentration of their primary antibody, and this does not allow the use of appropriate negative controls (non-specific IgG at the same concentration of the primary antibody) for immunohistochemical studies. For the negative control slides normal rabbit or mouse non-specific immunoglobulins (Santa Cruz Biotechnology) were used at the same protein concentration as the primary antibody. Control slides were included in each staining run using human normal tonsils (kindly provided by Prof. Stefano Pelucchi, ENT Section at the University of Ferrara, Italy) as a positive control for all the immunostaining performed. After repeated washing steps with PBS, the sections were subsequently incubated with goat anti-rabbit or horse anti-mouse biotinylated antibody (Vector ABC Kit, Vector Laboratories; www.vectorlabs.com) for 30 min at room temperature. After further washing, the sections were subsequently incubated with ABC reagent (Vector ABC kit, Vector Laboratories) for 30 min at room temperature. Slides were then incubated with chromogen-fast diaminobenzidine as chromogenic substance, after which they were counterstained in haematoxylin and mounted on permanent mounting medium.

Scoring System for Immunohistochemistry in the Bronchial Biopsies

Morphometric measurements were performed with a light microscope (Leitz Biomed, Leica, Cambridge, UK) connected to a video recorder linked to a computerized image system (Quantimet 500 Image Processing and Analysis System, Software Qwin V0200B, Leica). Light-microscopic analysis was performed at a magnification of ×630.

The immunostaining for all antigens studied was scored (range: from 0 = absence of immunostaining to 3 = extensive intense immunostaining) in the intact (columnar and basal epithelial cells) bronchial epithelium, as previously described [21]. The final result was expressed as the average of all scored fields performed in each biopsy. A mean ± SD of 0.700 ± 0.260 mm of epithelium was analysed in COPD patients and control subjects. Immunostained cells in bronchial biopsy lamina propria were quantified 100 μm beneath the epithelial basement membrane in several non-overlapping high-power fields until the whole specimen was examined. The final result, expressed as the number of positive cells per square millimetre, was calculated as the average of all the cellular counts performed in each biopsy.

Scoring System for Immunohistochemistry in the Peripheral Lung Tissue

Staining analysis was performed as previously described [24]. Staining data were interpreted blinded with no prior knowledge of the clinicopathological parameters. A bronchiole was taken to be an airway with no cartilage and glands in its wall. To count the number of positive cells on the sections stained for p-p38 MAPK, p-JNK and p-ERK, the area of bronchiolar epithelium to be studied was selected randomly. Cells with nuclear immunostaining were counted on each of 10 consecutive, non-overlapping, high-power fields (about 300 cells) with 1 count on each of 3, when available, bronchioles for each section stained. Results were expressed as percentages of total bronchiolar epithelial cells counted.

To quantify kinase expression in alveolar macrophages, at least 20 high-power fields of lung parenchyma were randomly selected for each section, and at least 100 macrophages inside alveoli were evaluated. Alveolar macrophages were defined as mononuclear cells with well-represented cytoplasm present in the alveolar spaces and not attached to the alveolar walls using a previously validated method [25]. Results were expressed as percentages of total alveolar macrophages counted.

Western Blot Analysis for p-p38 MAPK, p-JNK and p-ERK in the Peripheral Lung

Whole cell protein extraction from peripheral lung parenchyma, gel electrophoresis and nitrocellulose filter transfer were performed as previously described [21]. After blocking for 45 min at room temperature in Tris-buffered saline, 0.05% Tween-20 and 5% non-fat dry milk, filters were incubated with rabbit anti-p-p38 MAPK (Cell Signalling, monoclonal antibody No. 9215) or rabbit anti-p-JNK1 (Abcam, Ab-18680) or rabbit anti-p-pERK1/2 (Epitomics, 1481-1) for 1 h at room temperature in Tris-buffered saline, 0.05% Tween-20 and 5% non-fat dry milk at a dilution of 1:500 to 1:1,000 (0.1–0.2 mg/ml). HeLa cells were used as positive controls. After washing, filters were incubated with goat anti-rabbit (Dako, UK) antibody conjugated to horseradish peroxidase at a dilution of 1:4,000. Visualization was performed using enhanced chemiluminescence as recommended by the manufacturer (Amersham Pharmacia Biotech). Anti-human actin antibody (Santa Cruz Biotechnology) was used as an internal control. Bands were quantified using densitometry with Grab-It and VisionWorks LS software (UVP, Cambridge, UK) and expressed as a ratio with the corresponding actin optical density value of the same line.

Cell Culture and Treatments

We used the SV40 large T antigen-transformed 16HBE cell line that retains the differentiated morphology and function of normal human bronchial epithelial cells [26]. For experiments 16HBE cells were passaged using Dulbecco's modified minimum essential medium (DMEM), supplemented with 10% v/v fetal bovine serum (FBS), 50 IU/ml penicillin, 50 μg/ml streptomycin, 1× non-essential amino acids, 1 mM sodium pyruvate and 2 mM glutamine (37°C, 5% CO_2). When cells were at 60–70% confluence, the complete medium was replaced with DMEM without FBS for starvation time (24 h), followed by DMEM plus 1% FBS in the absence or presence of H_2O_2 (100 μM), cytomix (TNF-α 10 ng/ml + IL-1β 1 ng/ml + IFN-γ 10 ng/ml; R&D System), LPS from *Pseudomonas aeruginosa* (Sigma, L9143; 10 μg/ml), at 1, 2 and 4 h. Passage numbers of cells used in this study ranged from 22 to 24. All experiments were performed at least in quadruplicate for 4 independent experiments for each type of treatment (H_2O_2, cytomix, LPS) and each time of exposure (1, 2, 4 h).

Extraction and Quantification of RNA and qRT-PCR from 16HBE

Total cellular RNA from exposed and non-exposed cultures was purified and isolated using an RNAspin Mini RNA Isolation kit (GE Healthcare) following the manufacturer's instructions. Total RNA was resuspended in 100 μl nuclease-free water. RNA concentration was determined using a UV/visible spectrophotometer (λ 260/280 nm, Eppendorf BioPhotometer plus) and stored at –80°C.

Table 2. Clinical characteristics of COPD, asthmatics and control subjects studied by bronchial biopsy analysis

Groups	n	Age, years	M/F	Pack-years	Ex/current smokers	FEV$_1$ before BD, % pred.	FEV$_1$ after BD, % pred.	FEV$_1$/FVC, %
Control non-smokers	9	64±10	8/1	0	0	112±15	n.d.	89±12
Control smokers with normal lung function	16	60±9	11/5	43±29	4/12	100±13	n.d.	81±6
COPD stages I and II (mild/moderate)	17	71±8[c]	14/3	50±28	6/11	63±11[a]	67±13	57±9[a]
COPD stages III and IV (severe/very severe)	16	70±16[c]	10/6	59±39	12/4	36±8[a, b]	43±9	43±11[a, b]
Asthmatics	9	66±8	5/4	0	–	85±17	97±17	81±10

Patients were classified according to GOLD (http://www-goldcopd.com) levels of severity for COPD into mild (stage I), moderate (stage II), severe (stage III) and very severe (stage IV). Mild asthmatics were classified according to the GINA and ATS criteria. Data are means ± SD. For COPD and asthmatic patients FEV$_1$/FVC (%) are postbronchodilator values. M = Male; F = female; BD = bronchodilator; n.d. = not determined. Statistics (ANOVA): [a] $p < 0.0001$, significantly different from control smokers with normal lung function and control never-smokers; [b] $p < 0.0001$, significantly different from mild/moderate COPD; [c] $p < 0.05$, significantly different from control smokers with normal lung function.

The expression of genes of interest was measured using Syber green (Qiagen, UK) for qPCR in a Corbett Rotor Gene 6 (Corbett, Cambridge, UK) system. One-step real-time PCR was carried out by amplifying mRNA using the QuantiFast™ Syber green RT-PCR kit (Qiagen, Italy) according to the manufacturer's instructions and the gene specific primers (Qiagen, Italy). We detected the expression of CXCL8 (IL-8; cat. QT00000322, Qiagen), p38α (cat. QT00079345), JNK1 (cat. QT00091056), ERK1 (cat. QT00065933) and NF-κB p65 (cat. QT01007370) mRNA after each stimulation. We performed independent experiments and quantitative PCR measurements in quadruplicate for each type of treatment (H$_2$O$_2$, cytomix, LPS) and each 16HBE time of exposure (1, 2, 4 h). A single qPCR determination was performed for each type of treatment and time of exposure. Briefly, the PCR reaction mix, prepared in a total volume of 25 μl, was run on the Rotor Gene Q (Qiagen, Italy), and the following PCR run protocol was used: 55°C for 10 min (reverse transcription); 95°C for 5 min (PCR initial activation step); 40 amplification cycles of 95°C for 5 s (denaturation) and 60°C for 10 s (combined annealing/extension), followed by melting curve analysis to ensure the specificity of PCR amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; QT01192646, Qiagen) was used as the reference gene for every target gene per sample, and the data were normalized against the respective GAPDH signalling. Cycle threshold values were determined using the Rotor Gene Q software (Rotor-Gene Q Series Software 2.0.2). The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the 2$^{-\Delta C_t}$ method [27].

Statistical Analysis

Group data were expressed as mean (with standard deviation) for functional data and median (with range) or interquartile range for morphological data. Differences between groups were analysed using analysis of variance (ANOVA) for functional data. The ANOVA test was followed by the unpaired t test for comparison between groups. The Kruskal-Wallis test applied for morphological data was followed by the Mann-Whitney U test for comparison between groups. In vitro data were expressed as means ± standard deviation and analysed by the t test. Correlation coefficients were calculated using the Spearman rank method. Probability values of $p < 0.05$ were considered significant. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts Inc., Berkeley, Calif., USA).

Results

Clinical Characteristics of Subjects Studied by Immunohistochemistry

We obtained and studied bronchial biopsies from 58 subjects: 33 with stable COPD, 16 were current or ex-smokers with normal lung function, and 9 were non-smokers with normal lung function (table 2). COPD patients were divided into 2 groups: mild/moderate (GOLD stage I–II, n = 17) and severe/very severe (GOLD stage III–IV, n = 16; www.goldcopd.com). Subjects in all 4 groups were age-matched. The smoking history was similar in the 3 smoking groups. Values of FEV$_1$ (% predicted) and FEV$_1$/FVC (%) were significantly different in the groups with mild/moderate and severe/very severe COPD compared to both control groups (healthy smokers and healthy non-smokers). Severe/very severe COPD patients also differed significantly from mild/moderate COPD patients (for overall groups, ANOVA test: $p < 0.0001$ for FEV$_1$ % predicted and FEV$_1$/FVC % values). Thirty-six percent (n = 12) of the total COPD patients and 25% (n = 4) of healthy smokers with normal lung function also had symptoms of chronic bronchitis. There was no significant difference when COPD patients and healthy smokers were compared for the presence of chronic bronchitis. For comparative purposes related to the expression of p-

Table 3. Characteristics of subjects for the immunohistochemistry and Western blotting studies on the peripheral lung tissue

Subjects	n	Age, years	M/F	Ex/current smokers	Pack-years	Chronic bronchitis (yes/no)	FEV_1, % pred.	FEV_1/FVC, %
Control smokers	15	66±7	12/3	8/7	40±34	7/8	100.3±15.6	77±3.7
COPD patients	15	69±7	13/2	8/7	42±22	7/8	72.4±20*	58±9.7*

Data are expressed as means ± SD. M = Male; F = female. For COPD patients and smokers with normal lung function, FEV_1 and FEV_1/FVC are postbronchodilator values. Statistics (ANOVA): * $p < 0.001$, significantly different from control smokers with normal lung function.

Table 4. Immunohistochemical quantification of inflammatory cells and MAPK related to inflammatory response in bronchial mucosa

	Control non-smokers	Control smokers	Mild/moderate COPD	Severe/very severe COPD	Kruskal-Wallis p value
Epithelium (score 0–3)					
p-p38	0.75 (0.25–1)	0.75 (0.25–1.25)	0.5 (0.25–1.25)	0.5 (0.25–1.5)	0.618
p-JNK1	0.12 (0–0.25)	0.1 (0–1)	0.12 (0–1)	0.25 (0–1)	0.521
p-ERK1/2	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	n.a.
Submucosa, cells/mm^2					
p-p38	89 (27–118)	105 (32–155)	52 (20–106)	49 (27–161)	0.223
p-JNK1	5 (0–20)	7 (0–45)	17 (0–64)	11 (3–51)	0.262
p-ERK1/2	0 (0–6)	0 (0–21)	0 (0–13)	0 (0–2)	0.212
CD4	164 (101–212)	246 (37–500)	258 (107–731)	252 (66–470)	0.206
CD8	147 (76–301)	179 (86–657)	195 (86–523)	244 (111–355)[a]	0.365
CD68	284 (128–516)	275 (97–904)	367 (158–759)	340 (204–1,054)	0.671
Neutrophil elastase	93 (58–166)	97 (45–308)	94 (28–512)	151 (47–470)[a, b]	0.045

n.a. = Not applicable. Data expressed as medians (ranges). Statistics: the Kruskal-Wallis test was used for multiple comparisons followed by the Mann-Whitney U test for comparison between groups; [a] $p < 0.05$, significantly different from control non-smokers; [b] $p < 0.05$, significantly different from control smokers with normal lung function; the exact p values for comparison between groups are given in the Results section.

MAPK in the bronchi of COPD patients and asthmatics, we also studied bronchial biopsies from 9 stable age-matched mildly asthmatic patients. Clinical characteristics of asthmatics are included in table 2. Asthmatics were using short-acting bronchodilators on demand. Two out of 9 asthmatics were using inhaled corticosteroids at low doses.

We studied peripheral lung specimens from 15 stable COPD patients and 15 control smokers with normal lung function matched for their age and smoking history (table 3).

Measurement of Inflammatory Cells in the Bronchial Submucosal Biopsies of COPD Patients

These data, obtained from stable COPD patients by immunohistochemistry, confirm previously reported higher numbers of neutrophils and CD8+ cells in severe/very severe COPD (table 4) [28]. COPD patients with chronic bronchitis had a similar number of neutrophils when compared with COPD patients without chronic bronchitis [21, 28].

p-MAPK Immunohistochemistry in Bronchial Biopsies and Peripheral Airways

Immunohistochemistry in the Bronchial and Bronchiolar Epithelium and Alveolar Macrophages

In COPD and control subjects the most frequently expressed activated MAPK in bronchial epithelium was p-p38 MAPK. p-JNK1 was occasionally expressed in bronchial epithelium whereas p-ERK1/2 was virtually absent. Scored values for p-p38 MAPK and p-JNK1 did not show any significant differences between groups (table 4). No significant differences were observed for p-p38 MAPK between asthmatics (median 0.75, range 0.25–1.5) and
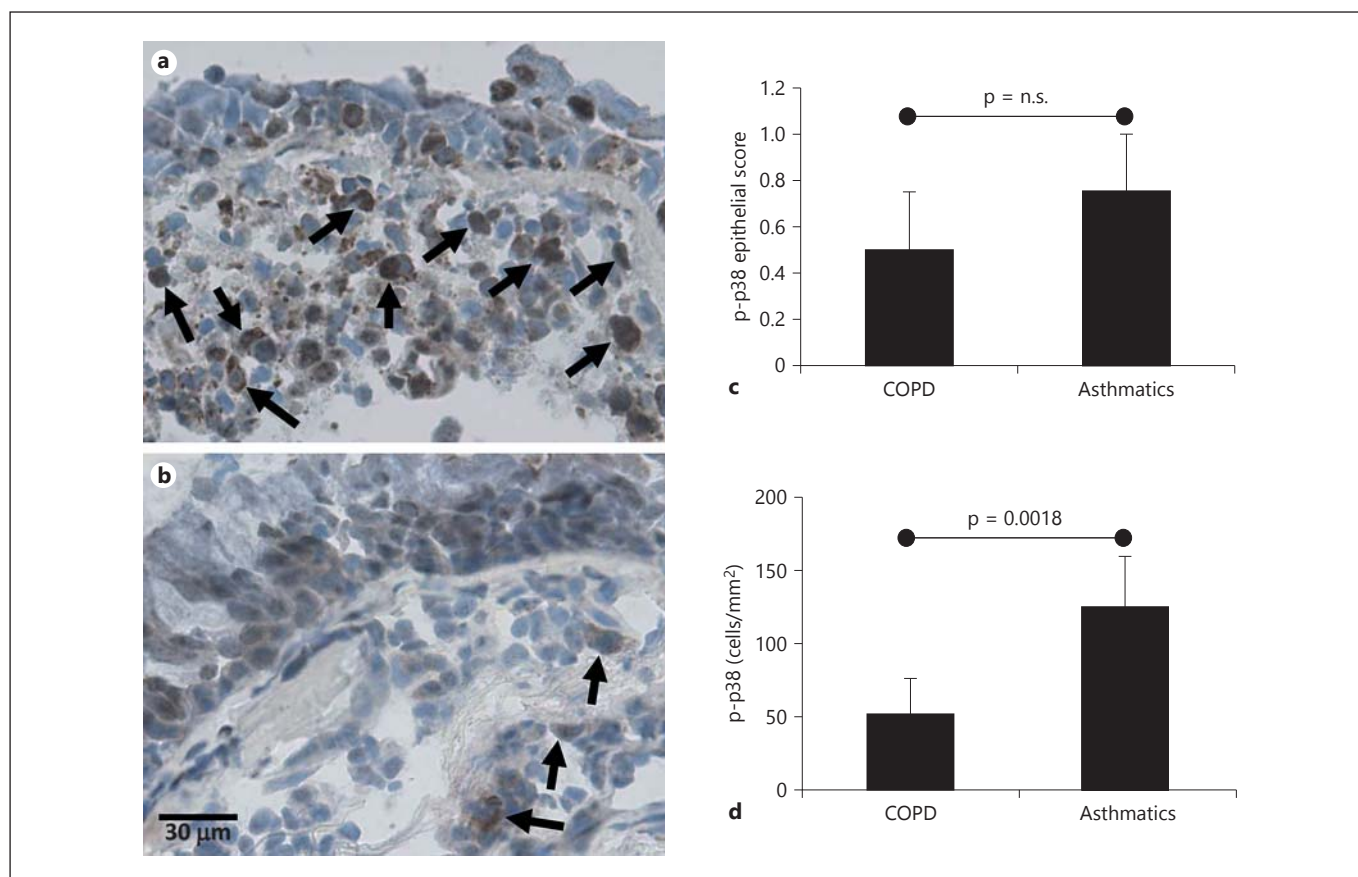
Fig. 1. Photomicrographs showing the bronchial mucosa from an asthmatic subject (**a**) and a patient with COPD (**b**) immunostained for identification of p-p38+ cells (arrows). Results are representative of those from 33 (mild/moderate and severe/very severe) COPD patients and 9 mild asthmatics. Column bar graphs indicate median (and interquartile range) values of p-p38 scored in the epithelium (**c**) and p-p38+ cells quantified in the submucosa (**d**) of all COPD patients (n = 33) and asthmatics (n = 9) studied. p values are based on the Mann-Whitney test for comparison between groups.

control non-smokers (p = 0.958), severe/very severe COPD (p = 0.385), mild/moderate COPD (p = 0.247) or all COPD patients (p = 0.249; fig. 1). Similarly, no significant differences were observed for p-JNK1 between asthmatics (median 0.125, range 0–0.25) and control non-smokers (p = 0.957), severe/very severe COPD (p = 0.270), mild/moderate COPD (p = 0.345) or all COPD patients (p = 0.256). No significant differences were observed for p-ERK1/2 between asthmatics (median 0, range 0–0) and control non-smokers (p > 0.999), severe/very severe COPD (p > 0.999), mild/moderate COPD (p > 0.999) or all COPD patients either (p > 0.999).

Immunohistochemistry in the Bronchial Submucosal Biopsies

In COPD and control subjects the most frequently expressed MAPK in bronchial submucosa was, as for epithelium, p-p38 MAPK. It was mainly expressed by mononuclear cells and occasionally by endothelial cells. p-JNK1 was poorly expressed in all groups studied, and p-ERK1/2 was only occasionally found. In COPD patients and control subjects no significant differences between groups were observed for all p-MAPK studied (table 4; fig. 2). Interestingly, asthmatics showed higher levels of p-p38 MAPK protein (median 125, range 54–387) when compared to control non-smokers (p = 0.040), severe/very severe COPD (p = 0.013), mild/moderate COPD (p = 0.0018) or all COPD patients (p = 0.0016; fig. 1). No significant differences were observed for p-JNK1/mm^2 counted in asthmatics (median 13, range 5–161) when compared to control non-smokers (p = 0.112), severe/very severe COPD (p = 0.654), mild/moderate COPD (p = 0.571) or all COPD patients (p = 0.570). No significant differences were observed for p-ERK1/2/mm^2
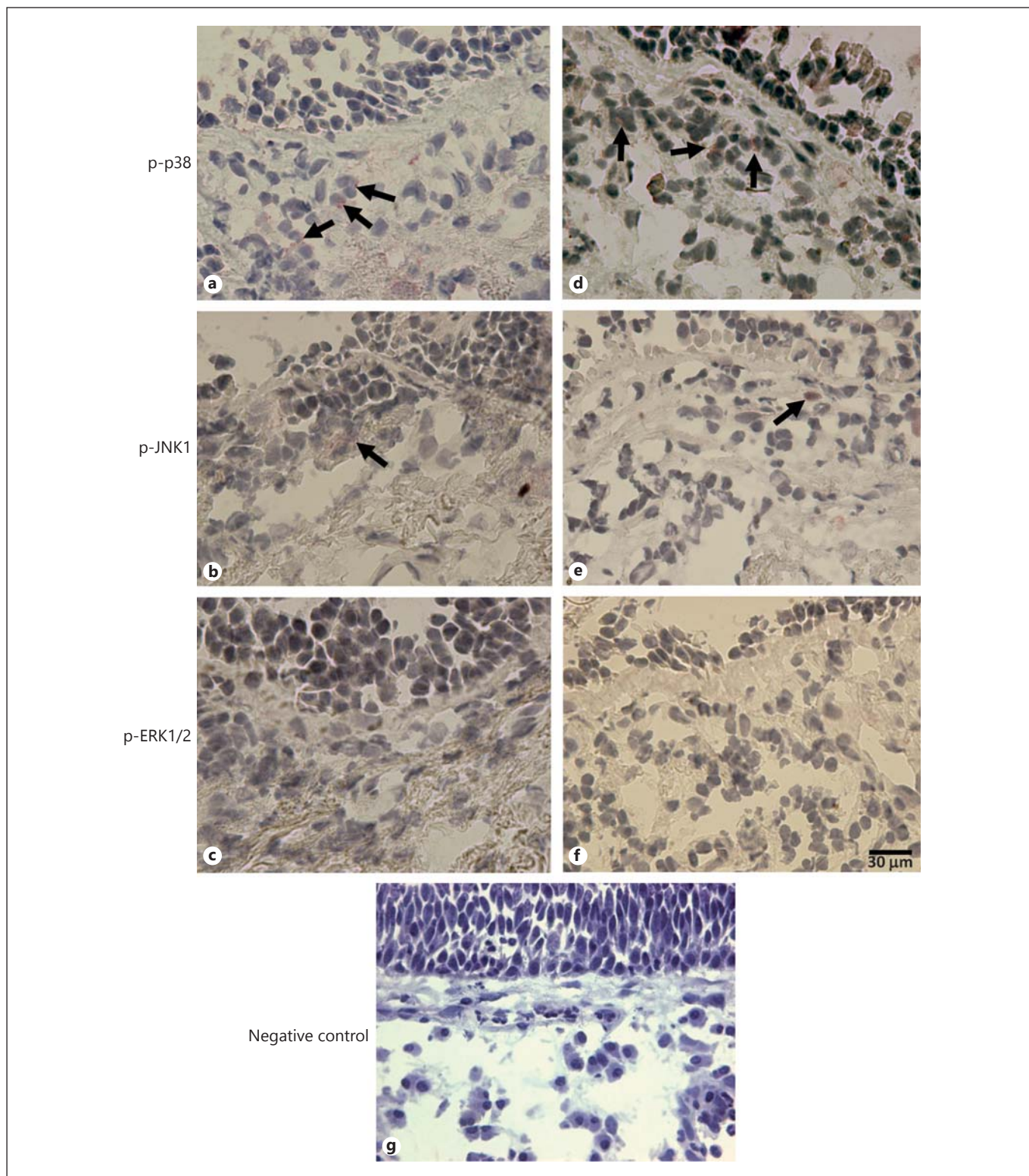
Color version available online

Fig. 2. Photomicrographs showing the bronchial mucosa from control non-smokers (**a–c**) and patients with mild/moderate COPD (**d–f**) immunostained (arrows) for identification of p-p38 (**a**, **d**), p-JNK1 (**b**, **e**) and p-ERK1/2 (**c**, **f**) in the bronchial epithe-lium and submucosa. Results are representative of those from 9 non-smokers and 33 COPD patients. **g** Negative control immu-nostaining, performed in a nasal polyp section, including an irrel-evant rabbit primary antibody.

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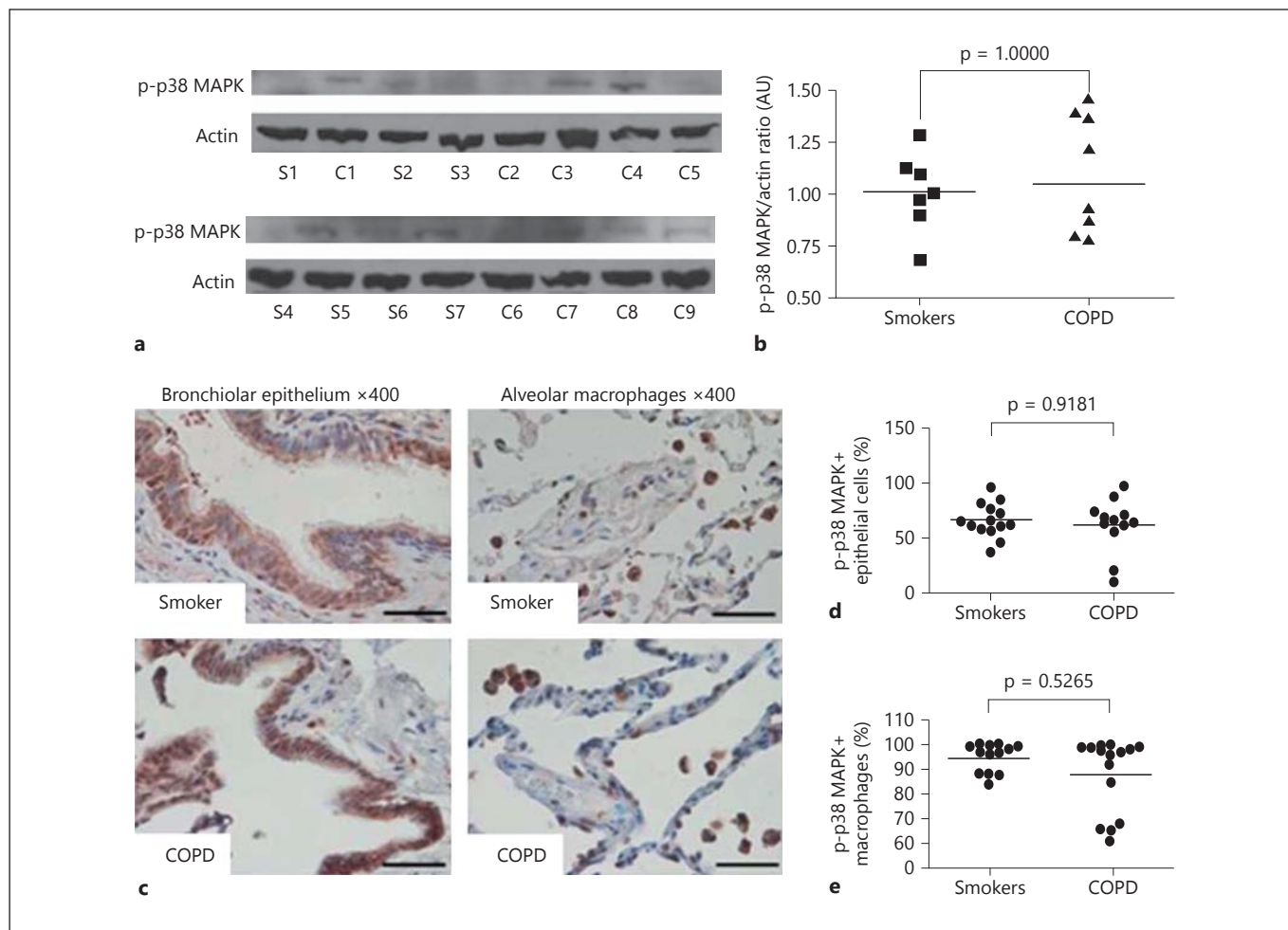
Fig. 3. a Western blot analysis of activated p-p38 MAPK in snap-frozen peripheral lung lysates of stable COPD (n = 9) and age-matched control smokers with normal lung function (n = 7) with actin as the control for loading. **b** Graphical analysis of the densitometric ratio (arbitrary units, AU) of p-p38 MAPK/actin. **c** Im-munohistochemical images of p-p38 MAPK immunostaining in bronchiolar epithelial cells and alveolar macrophages representative of 15 COPD and 15 control smokers. Magnification ×400. Bar = 50 μm. **d**, **e** Graphical presentation of the percentage of epithelial and macrophage staining.

counted between asthmatics (median 5, range 0–17) and control non-smokers (p = 0.216), mild/moderate COPD (p = 0.608) or all COPD patients (p = 0.156). A slight but significant increase was observed in asthmatics in comparison with severe/very severe COPD (p = 0.034).

Immunohistochemistry in the Peripheral Lung Tissue

In the peripheral airways no significant differences were observed for percentages of p-p38 MAPK immunostained bronchiolar epithelial cells between COPD (median 68, range 10–100) and control smokers (70, range 40–100; p = 0.9181). Percentages of p-p38 MAPK+ alveolar macrophages in COPD (median 87, range 60–100) versus control smokers (94, range 82–100; p = 0.5265) were not significantly different either (fig. 3).

No significant differences were observed for percentages of p-JNK immunostained bronchiolar epithelial cells between COPD (median 38, range 2–64) and control smokers (36, range 5–58; p = 0.5439). Percentages of p-JNK+ alveolar macrophages in COPD (median 88, range 60–100) versus control smokers (90, range 40–100; p = 0.3216) were not significantly different (fig. 4).

No significant differences were observed for percentages of p-ERK1/2-immunostained bronchiolar epithelial cells between COPD (median 40, range 0–90) and control smokers (37, range 0–70; p = 0.5495). Percent-

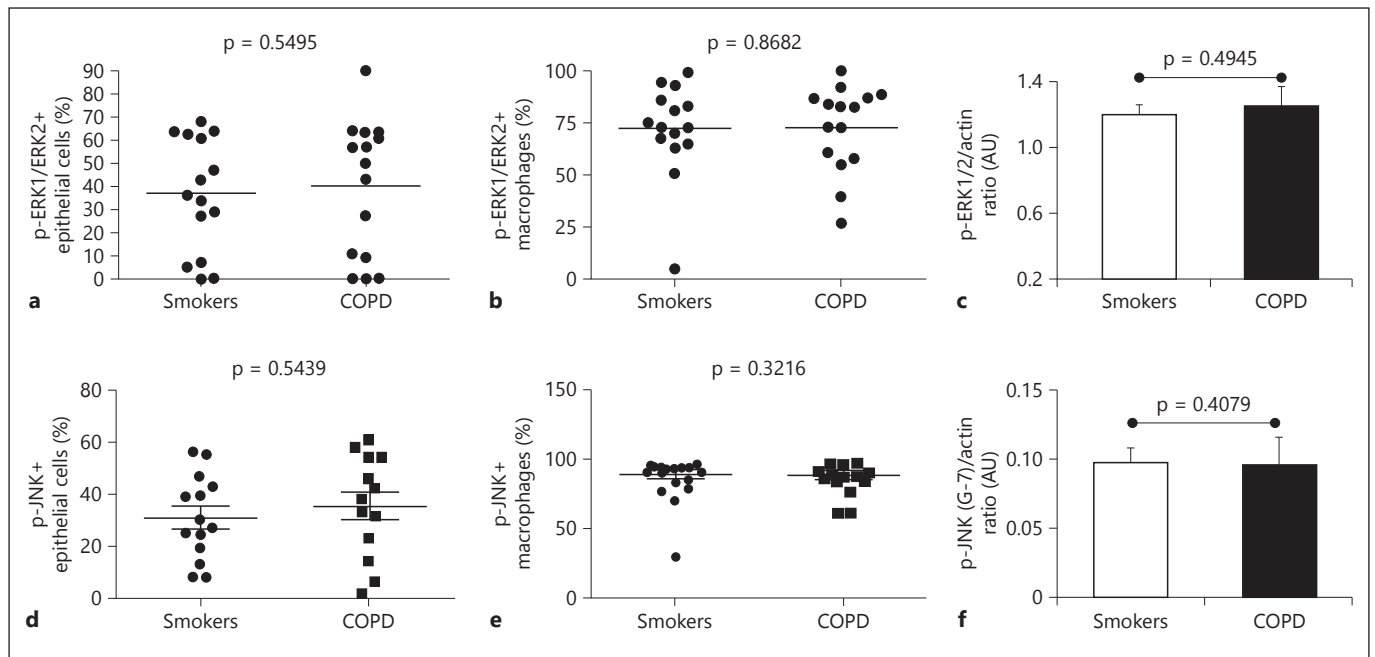**Fig. 4.** Graphical presentation of the percentage of p-ERK1/2-immunostained bronchiolar epithelial cells (**a**), percentage of p-ERK1/2-immunostained positive macrophages (**b**) and p-ERK1/2/actin ratio from peripheral lung lysates (**c**), percentage of p-JNK-immunostained bronchiolar epithelial cells (**d**), percentage of p-JNK-immunostained positive macrophages (**e**) and p-JNK/actin ratio from peripheral lung lysates (**f**) in stable COPD and control smokers. AU = Arbitrary units. Data are representative of 15 stable COPD and 15 control smokers for immunohistochemical data and 9 stable COPD and 7 control smokers for Western blot analysis. The Mann-Whitney test was used for comparison between groups.

ages of p-ERK1/2+ alveolar macrophages in COPD (median 75, range 25–100) versus control smokers (72, range 8–100; p = 0.8682) were not significantly different either (fig. 4).

Western Blotting for p-p38 MAPK, p-JNK and p-ERK1/2 in Peripheral Lung Tissue

No significant differences were observed for the p-p38 MAPK/actin ratio between COPD (median 1.02, range 0.2–1.40) and control smokers (1.0, range 0.65–1.30; p = 1.0000; fig. 3), the p-JNK/actin ratio between COPD (median 0.10, range 0.05–0.13) and control smokers (0.10, range 0.06–0.12; p = 0.4079; fig. 4) and the p-ERK1/2/actin ratio between COPD (median 0.10, range 0.05–0.13) and control smokers (0.10, range 0.06–0.12; p = 0.4079; fig. 4).

Correlations between p-MAPK Cell Counts, Clinical Parameters and Inflammatory Cells in the Bronchial Biopsies

In all smokers and in patients with COPD alone we did not observe any significant correlation between numbers of p-p38+, p-JNK1+ and p-ERK1/2+ cells per square millimetre and numbers of cigarettes smoked (pack-years), functional (spirometry values and clinical parameters) data or structural (inflammatory cells) data.

MAPK, NF-κB p65 and IL-8 mRNA Expression in 16HBE Cells Induced by Oxidative and Inflammatory Stimuli in vitro

Human bronchial epithelial (16HBE) cells were stimulated with H_2O_2 (100 μM), cytomix (TNF-α, 10 ng/ml, IL-1β, 1 ng/ml, and IFN-γ, 10 ng/ml) and LPS (10 μg/ml), and the expression of IL-8 (fig. 5a), p38 MAPKα (fig. 5b), JNK1 (fig. 5c), ERK1 (fig. 5d) and NF-κB p65 subunit (fig. 5e) mRNA was quantified by qRT-PCR. IL-8 mRNA was significantly increased 2 h after H_2O_2 (p < 0.0001), 1 and 2 h after cytomix (p = 0.001 and p = 0.017, respectively), and 2 h after LPS (p = 0.043; fig. 5a). p38 MAPKα mRNA was significantly increased 2 h after H_2O_2 (p = 0.030) and 1 h after LPS (p = 0.010) but it did not change after cytomix treatment (fig. 5b). JNK1 mRNA was not significantly changed by any of the treatments used (fig. 5c). ERK1 mRNA was not significantly increased after H_2O_2, cytomix or LPS treatments (fig. 5d). NF-κB p65 subunit mRNA was significantly increased
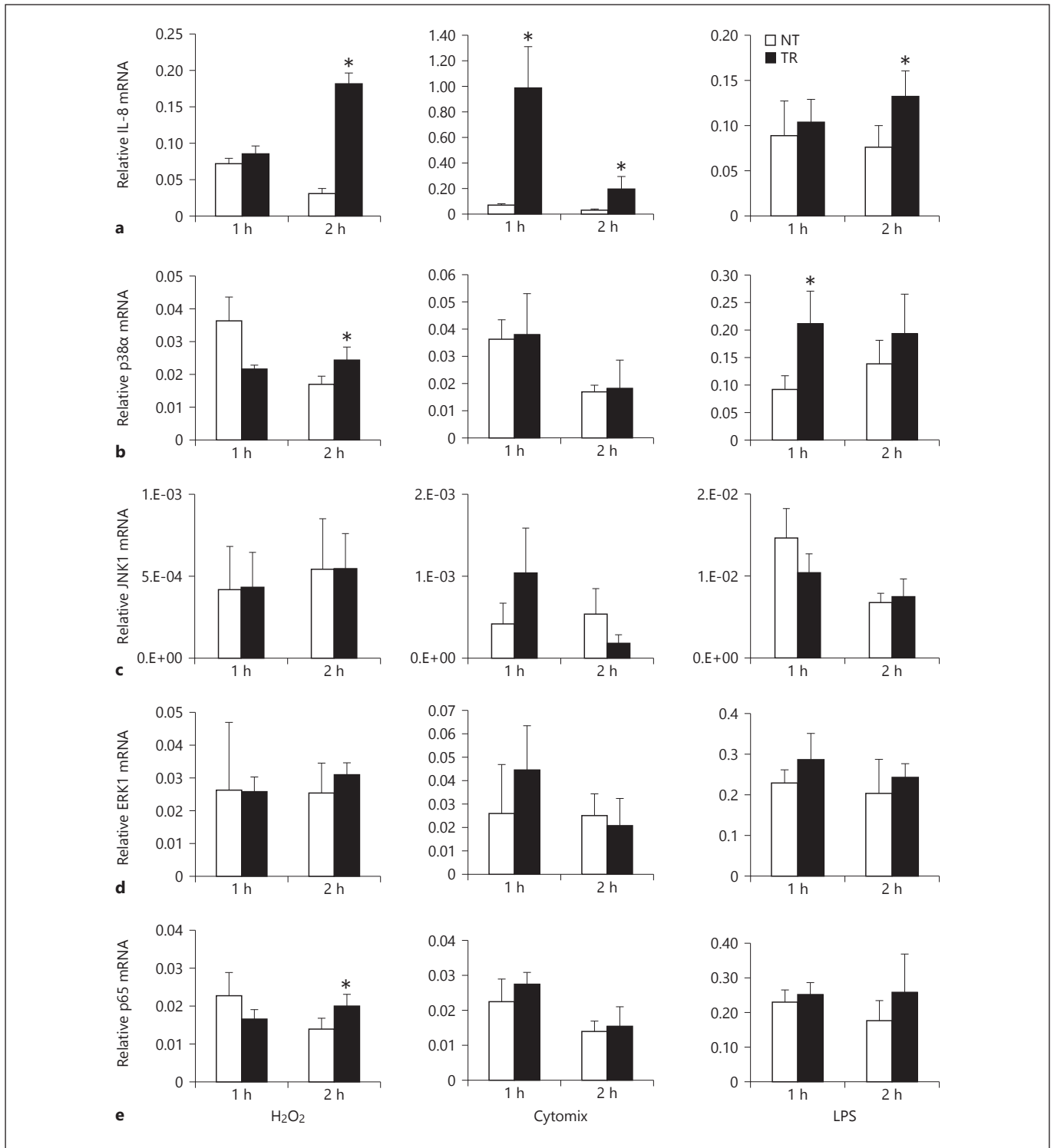
Fig. 5. In vitro expression of CXCL8 (IL-8; **a**), p38α (**b**), JNK1 (**c**), ERK1 (**d**) and NF-κB p65 subunit (**e**) mRNA in 16HBE cells treated with H_2O_2 (100 μM), cytomix (TNF-α, 10 ng/ml, IL-1β, 1 ng/ml, and IFN-γ, 10 ng/ml) and LPS from *P. aeruginosa*. All treatments upregulated IL-8 mRNA expression 1 or 2 h after treatment (**a**). H_2O_2 stimulation upregulated p38 (**b**) and p65 (**e**) mRNA 2 h after stimuli. LPS upregulated p-38 (**b**) mRNA 1 h after stimulus. All experiments were performed in quadruplicate. Data are expressed as means ± standard deviation. Statistical analysis: t test (* $p < 0.05$) for comparison between treated (TR) and non-treated (NT) cells. The exact p values for comparison between groups are given in the Results section.

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2 h after H_2O_2 (p = 0.032) but it did not reach statistical significance 2 h after cytomix (p = 0.086) and LPS (p = 0.093; fig. 5e).

Discussion

This study shows similar immune expression of p-p38 MAPK, p-JNK1 and p-ERK1/2 in the bronchial epithelium and submucosa of patients with mild/moderate and severe/very severe stable COPD when compared to control smokers and control non-smokers. Activated MAPK were also similarly expressed in the bronchiolar epithelium of stable COPD patients compared to control smokers with normal lung function. p-p38 MAPK immunopositivity in the bronchial submucosa of asthmatics was significantly increased when compared to stable COPD and control non-smokers. Interestingly, bronchial epithelial cells (16HBE) exposed in vitro to H_2O_2, cytomix and LPS showed increased levels of IL-8 mRNA production which were accompanied by a parallel increase in p38 MAPK mRNA after H_2O_2 and LPS stimulation but not by a parallel increase in JNK1 and ERK1 mRNA. In patients with COPD, p-p38 MAPK immunopositivity was increased in the small airway epithelium [12] when compared to control non-smokers [12] and in alveolar septa [11] when compared to control smokers and non-smokers [11]. p-p38 MAPK immunopositivity in lymphocytes populating the submucosa of peripheral airways of COPD patients was similar to that found in control smokers and non-smokers [12]. These last data are in part in agreement with our present observations of no changes of p-p38 MAPK immunopositivity in the bronchial biopsy submucosa of stable COPD patients compared to control smokers and non-smokers, peripheral airway bronchiolar epithelium of stable COPD patients compared to control smokers, and with our observation of the absence of significant changes in total p-p38 MAPK protein, measured by Western blot, using lung tissue, when comparing stable COPD patients and control smokers with normal lung function. In fact, p-p38 MAPK immunopositivity in our bronchial biopsy specimens was mainly observed in mononuclear cells and occasionally in endothelial cells, and in agreement with Gaffey et al. [12], the mononuclear cell component expressing p-p38 MAPK immunopositivity was similar in COPD patients and control subjects. At variance with previously reported data in peripheral airways [12] of COPD patients and in large airways from asthmatics [13], we did not find a significant increase in this MAPK in bronchial biopsy ep-ithelium in mild/moderate and severe/very severe COPD or in peripheral tissue. This may be due, in part, to the presence and influence of a different inflammatory state of the airways when bronchial biopsies of asthmatics are compared to COPD patients. It is conceivable that a Th2 prevalent inflammatory state, such as in asthma, could better activate MAPK, including p-p38 MAPK. To better understand possible differences between asthma and COPD, we directly compared immune expression of activated MAPK in bronchial biopsies from COPD and asthmatic subjects, matched for age and sex, and we observed a significant increase in p-p38 MAPK in bronchial submucosa of mild asthmatics compared to stable COPD patients. Asthmatics also differed significantly from control non-smokers, confirming, in part, previously reported data [13]. In our asthmatics we found the most significant difference in the submucosal area rather than in epithelium, as previously reported [13]. This indicates that increased p-p38 MAPK levels in our asthmatics are mainly due to an increased infiltration of inflammatory cells. A Th2-type prevalent inflammation, such as in asthma, may also favour the highest p-p38 MAPK expression observed in the bronchi of asthmatics [29].

Inhalation of LPS in human volunteers induced activation of bronchial epithelium by increased expression of p38 MAPK and IL-8 [30]. *P. aeruginosa*-challenged human bronchial epithelial cells showed increased phosphorylation of p38 MAPK and IL-8 gene expression which was reduced by the use of p38 inhibitors [31]. Flagellin from *P. aeruginosa* increased the expression of IL-8 in BEAS-2B cells compared to untreated cells, and addition of p38 MAPK inhibitors reduced IL-8 expression [32]. IL-8 mRNA and protein expression was also increased after LPS stimulation of bronchial epithelial cells [33]. Poly(I:C) costimulation further increased IL-8 production which was reversed by dexamethasone and a p38 MAPK inhibitor [33].

Hydrogen peroxide increased Wnt-4 and IL-8 gene expression in BEAS-2B cells [34]. Wnt-4-stimulated 16HBE cells significantly increased IL-8 secretion and p38 MAPK activation [35]. A lower efficacy of corticosteroids is reported in human bronchial epithelial cells in asthma and COPD after oxidative stress challenge [2, 36]. In paediatric bronchial epithelial cells, Th2 cytokine challenge in the presence of rhinovirus-16 infection augmented IL-8 release [29]. Housedust mite-induced IL-8 release is blocked by an ERK inhibitor in human lung epithelial cells [37]. Our in vitro experiments show that 16HBE cells stimulated with H_2O_2, cytomix and LPS upregulated IL-8 mRNA expression, in agreement with previously report-

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ed observations [30–37]. This was associated with upregulation of p38α MAPK but not JNK1 and ERK1 mRNA after H_2O_2 and LPS exposure. Interestingly, H_2O_2 also increased NF-κB p65 mRNA. These data suggest that in bronchial epithelial cells the p38 MAPK pathway may be more relevant after LPS and oxidative stimulation.

Our bronchial biopsy data, showing a significant increase in p-p38 MAPK immune positivity in mild asthmatics and its prevalence in stable COPD patients, taken together with our in vitro observations of increased p-p38 MAPKα mRNA after bronchial epithelial stimulation suggests that p-p38 MAPK activation may play a significant role in inducing bronchial inflammation in these diseases.

Recently, MacNee et al. [38] described an improvement in FEV$_1$ after 6 weeks of p38 MAPK inhibitor treatment in moderate-to-severe COPD patients. In contrast, a larger study, performed in moderate-to-severe stable COPD patients, treated with the highly selective oral p38 MAPK inhibitor iosmapimod for 6 months, showed no significant changes in exercise tolerance or lung function [16]. This highlights the need to better define the clinical phenotype of patients in order to identify a likely 'responder' population of COPD patients [14, 15]. Our present results may contribute to this scope since p-p38 MAPK was certainly the most expressed MAPK in our bronchial specimens from asthmatics and COPD patients, and specific oxidative and inflammatory challenges, performed in 16HBE cells, showed a significant increase in p-p38 MAPKα mRNA after challenges. Since mononuclear cell infiltration was reported as the prominent cellular type in bronchial biopsies of mild/moderate but not of severe/very severe COPD patients [39], and p-p38 MAPK expression, reported in the submucosa of bronchial biopsies, is mainly due to immunostained mononuclear cells, we can argue, therefore, that selected populations including mild-to-moderate COPD patients with a more inflamed airway (cut-off values for mononuclear cell inflammation should be defined) and COPD patients during an exacerbation or those who frequently exacerbate, or asthmatic patients, could better respond to p38 MAPK inhibitors. The potential for combination therapies, particularly in exacerbated diseased patients, together with a deeper analysis of molecular events and possible activation of redundant inflammatory pathways, developing after p38 MAPK blockade, needs to be further studied.

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